ABSTRACT

CHARACTERISTICS AND SEROLOGICAL ANALYSIS OF APHID-TRANSMISSIBLE AND NONAPHID-TRANSMISSIBLE PEA ENATION MOSAIC VIRUS VARIANTS

By

Richard Gain Clarke

Two variants of pea enation mosaic virus (PEMV) were characterized biologically, physically and serologically. Both variants produced identical symptomatology on and were highly infectious in mechanical sap inoculations to garden pea, _Pisum sativum_ L., and the local lesion host, _Chenopodium amaranticolor_ Coste and Reyn. However, one variant (NT-PEMV) could not be transmitted by the pea aphid, _Acyrthosiphon pisum_ (Harris) regardless of the virus acquisition source or method used. Determination of possible differences between the nonaphid-transmissible (NT-PEMV) variant and its aphid-transmissible counterpart (T-PEMV) was the basic objective of this research.

Both variants were partially purified from ten-day infected pea tissue by a chloroform-extraction and differential centrifugation procedure. Density gradient centrifugation and ultraviolet light scanning profiles of sucrose gradient columns revealed the presence of one major sedimenting component in T-PEMV and two sedimenting components in NT-PEMV. Using a southern bean mosaic virus marker, sedimentation coefficients of 111.5 ± 1.04 (s.d.) for the major component of T-PEMV

and 104.6 ± 2.45 (s.d.) and 117.1 ± 1.73 (s.d.) for the two components of NT-PEMV were determined.

The slower sedimenting (Top) component of NT-PEMV was found to vary greatly in concentration. Fixing NT-PEMV infected pea tissue in 4% gluteraldehyde prior to extraction did not affect the UV absorbency profile on sucrose gradients which indicated that the top component was not an artifact of the purification procedure.

NT-PEMV was found to be unstable in a purified form (after one cycle of density gradient centrifugation) in 0.05 M pH 7.0 potassium phosphate buffer which stabilized T-PEMV. However, a 0.1 M pH 6.0 sodium acetate buffer system was found to stabilize NT-PEMV.

Both variants were found to have typical absorption spectra of nucleoproteins (Max. = 260 nm and Min. = 240 nm). 280 nm/260 nm absorbence ratios of 0.58 (NT-PEMV) and 0.57 (T-PEMV) indicated a relatively high RNA content in both variants.

SDS polyacrylamide gel electrophoresis of both variants indicated the presence of a second higher molecular weight protein in T-PEMV not present in NT-PEMV. Both variants were found to share a common protein with a RF value of about 0.67 for each.

Antisera against T-PEMV and NT-PEMV was produced in rabbits with resulting titers of 1/2560 (NT-PEMV) and 1/1280 (T-PEMV). Reactions in agar gel diffusion tests of antisera against expressed sap from infected pea plants, partially purified or purified virus preparations, produced identical single precipitin band patterns. This indicated that the purification procedure had not altered the serology of the variants. Reactions of both viral antigens simultaneously against

NT-PEMV antiserum produced a confluent band pattern (serological identity) while against T-PEMV antiserum produced a spur formation (serological partial identity). Altering the pH or concentration of the agar did not change the basic serological patterns.

Antiserum absorption experiments indicated the presence of a second specific antibody (B-antibody) in T-PEMV antiserum. This antibody was directed against antigenic sites apparently unique to T-PEMV coat protein. A common cross-reactive antibody (A-antibody) determined in both T- and NT-PEMV antisera indicated the presence of a set of common antigenic sites also to be present on the viral coat protein shell of the variants.

A soluble antigen was detected in the high speed supernatant from T-PEMV infected plants. The antigen was found to be unrelated to NT-PEMV coat protein and only partially related to T-PEMV coat protein.

When tested against a series of PEMV isolates differing in aphid transmissibility, the specific B-antibody was found to react only against aphid-transmissible PEMV isolates. This antiserum appears to represent a valuable tool in determining the aphid transmissibility of PEMV isolates without the necessity of conducting elaborate aphid transmission experiments.

CHARACTERISTICS AND SEROLOGICAL ANALYSIS OF APHID-TRANSMISSIBLE AND NONAPHID-TRANSMISSIBLE PEA ENATION MOSAIC VIRUS VARIANTS

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To Janet and Tricia

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INTRODUCTION

Pea enation mosaic virus is an aphid-borne circulative plant virus containing a single strand of RNA within a protein shell and possessing a polyhedral shape of about 25-30 nm in diameter. Its aphid transmission characteristics have been described from virus-infected source plants (Bath and Chapman, 1968; Chapman and Bath, 1968; Nault et al., 1964; Simons, 1954; Sylvester, 1965; Sylvester and Richardson, 1966), from purified virus preparations fed to aphids across an artificial membrane feeding system (Thottappilly et al., 1972) and from purified virus injected into the hemocoel of the pea aphid vector (Clarke and Bath, 1973).

PEMV has been purified by several authors (Bozarth and Chow, 1966; Bustrillos, 1964; Farro, 1969; Gibbs et al., 1966, Gonsalves and Shepherd, 1972; Hull and Lane, 1973; Izadpanah and Shepherd, 1966; Mahmood and Peters, 1973; Musil et al., 1970; Thottappilly et al., 1972). Except for Musil et al. (1970) and Thottappilly et al. (1972) the virus isolates were mechanically perpetuated during the course of their research with all bioassays completed on local lesion or systematic plant hosts.

Antiserum against pea enation mosaic virus has been produced by several authors (Bustrillos, 1964; de Zoeten and Rettig, 1972; Izadpanah and Shepherd, 1966; Mahmood and Peters, 1973; Musil et al., 1970). In most cases, antisera was produced in conjunction with their purification studies and was used to test the serological activity of their particular virus isolate. PEMV was reported to produce a single precipitin band in agar gel diffusion tests which indicated a serological identity between viral components (Mahmood and Peters, 1973).

Gibbs et al. (1966) and Musil et al. (1970) tested the antiserum of Izadpanah and Shepherd (1966) against their PEMV isolates as did Farro (1969) who also tested antiserum provided by Mahmood and Peters (1973). In all cases, their isolates cross reacted with antiserum against other geographical isolates of PEMV. However, these do not represent detailed serological analysis of the PEMV isolates.

PEMV is unique in the realm of the small spherical plant viruses in that in addition to being transmitted in a persistent manner by aphids it also can be perpetuated by mechanical sap inoculations. PEMV appears to hold an intermediate or transitory position between the mechanically transmitted icosahedral viruses, e.g., southern bean mosaic virus and the non-mechanically transmitted viruses, e.g., barley yellow dwarf and potato leafroll viruses.

It has been reported, however, that strains of several persistently transmitted plant viruses lost their vector-transmissibility after long periods of exclusive propagation within their plant hosts without passage through their insect vectors (Bath et al., 1973; Black, 1953; Black et al., 1958; Liu et al., 1973; Tsai and Bath, 1974; Whitcomb and Black, 1969; Wolcyrz and Black, 1957). This phenomenon also has been reported among the nonpersistently transmitted viruses (Badami, 1958; Evans and Zettler, 1970; Gonzales and Hagadorn, 1971, Hollings, 1955; Kamm, 1969; Swenson, 1957; Swenson _et al_., 1963; Thottappilly _et al_., 1972).

Tsai and Bath (1974) reported that a California isolate of PEMV lost its characteristic of aphid transmissibility following repeated perpetuation by sap inoculation. Although some work has been reported on the characterization of the non-insect transmitted variant of PEMV (French, 1973), detailed research is absent. When French (1973) studied the nucleoprotein components of this nonaphid-transmissible variant and its aphid-transmissible counterpart, he found that they could be distinguished on the basis of yield of their nucleoprotein components. The nonaphid-transmissible variant produced higher concentrations of top component with the total virus yield upon purification always being significantly higher in this variant than in the aphid-transmissible variant.

RESEARCH OBJECTIVE

Research on these biological anomalies or viruses which have lost their vector-transmissibility has generally not proceeded past demonstration of the vectorless quality of the new variant. This has contributed little in the way of new knowledge about the biochemical nature of vector-transmissibility. It was my contention that these anomalies can help to elucidate new physiochemical insights into the vector-virus relationship. It would seem that if one directly compared vectorable and vectorless virus isolates the differences detected may provide circumstantial as well as direct evidence as to what renders one virus suitable for vector transmission and one not.

With the selection of the nonaphid-transmissible (NT-PEMV) isolate of PEMV (Tsai and Bath, 1974), the ideal research tool has become available for the initiation of a comparative study of aphid- and nonaphid-transmissible variants of PEMV. The objective of this research was to directly compare NT-PEMV to its aphid-transmissible mate (T-PEMV) in order to determine (a) some characteristics of and (b) serological differences between the two variants. It was thought that such data could provide a possible explanation as to the biochemical nature of the aphid-transmissibility of PEMV.

MATERIALS AND METHODS

Virus Isolates. An aphid-transmissible (T-PEMV) variant and a nonaphid-transmissible (NT-PEMV) variant of a California isolate of pea enation mosaic virus (Bath and Chapman, 1967) were used in this study. NT-PEMV had been selected by employing a strictly mechanical sap inoculation routine for a period of a few months (Tsai and Bath, 1974). T-PEMV was selected simultaneously through a strictly aphid transmission routine originating from a single PEMV-infected pea plant obtained in the NT-PEMV selection study.

Aphid Colonies and Virus Source Plants. The pea aphid vector, *Acyrthosiphon pisum* (Harris), was reared on broad bean, *Vicia faba* L., in a controlled-environmental chamber as described by Tsai (1967). Garden pea, *Pisum sativum* L., variety Midfreezer served as virus source plants and as transmission test plants for both variants. Pea seedlings were grown to the pre-leaf stage in the manner described by Tsai (1967) and then transplanted to individual 3"-diameter plastic pots prior to inoculation.

Perpetuation of Virus Lines. Throughout this research, T-PEMV variant was perpetuated exclusively by aphid transmission. Ten-day virus-infected pea seedlings were used as virus source plants for perpetuation and purification. Aphid nymphs allotted a 12-hour acquisition access period (AAP) on a virus-infected source plant were then transferred to healthy seedlings (3-4 nymphs per plant) for a 3-day inoculation access period (IAP). The IAP was completed in a controlled-environmental chamber at 22°C, 60-70% RH and a 14-hour photophase. Plants were then fumigated with Dibrom ^{R} insecticide and placed in a greenhouse for symptom expression.

NT-PEMV was perpetuated by mechanical sap inoculations and was purified from 10-day infected pea tissue. Mechanical sap inoculations were made with inoculum prepared by grinding infected leaf tissue in tap water with a sterile mortar and pestle and then rubbing the inoculum on a carborundum-dusted healthy pea seedling with a finger. These seedlings were then rinsed and placed in a greenhouse.

<u>Partial Purification of Virus</u>. The variants were partially purified by the method of Thottappilly <u>et al</u>. (1972). Final virus pellets after high speed centrifugation were resuspended in 0.1 <u>M</u> pH 7.0 potassium phosphate buffer. Virus at this stage of purification will be referred to as partially purified for it had not been through a cycle of sucrose density gradient centrifugation and consequently contained some contaminating plant material. Partially purified virus was stored in 1 ml lots at -20°C until use.

<u>Density Gradient Centrifugation</u>. Rate-zonal density gradient centrifugation and analysis was accomplished by layering 1 ml of a partially purified virus preparation on a linear 10-40% sucrose gradient in either 0.05 <u>M</u> pH 7.0 potassium phosphate buffer or 0.1 <u>M</u> pH 6.0 sodium acetate buffer. These were centrifuged for two hours at 24,000 rpm (4°C) in a SW 27.1 rotor of the model L Beckman ultracentrifuge. Tubes were monitored for ultraviolet light absorbence (254 nm) using an ISCO density gradient fractionator coupled to a UA-2 ultraviolet

analyzer. In experiments to determine UV absorbence profiles and sedimentation coefficients of viral components, partially purified virus preparations were adjusted to an optical density of 0.5-0.7 per ml using a Beckman DB spectrophotometer before layering on a gradient column in order to be within the 0.5 O.D. sensitive range of the UA-2 ultraviolet analyzer.

Purified Virus. Virus was further purified by collecting the virus zone from several gradient columns using the ISCO density gradient fractionator and UV analyzer, diluting the sucrose solution 1:1 with the appropriate gradient buffer and centrifuging at 41,000 rpm for ninety minutes (4°C) in a 50 rotor. Virus pellets were resuspended in either 0.05 *M* pH 7.0 potassium phosphate buffer or 0.1 *M* pH 6.0 sodium acetate buffer. These purified virus solutions were then clarified by low speed centrifugation with viral concentrations determined using the optical density at 260 nm and an extinction coefficient of 7.2 OD/mg/ml/cm (Gonsalves and Shepherd, 1972).

Bioassay. Virus-infected plant sap, partially-purified and purified virus preparations were assayed for mechanical inoculativity on either the local lesion host, *Chenopodium amaranticolor* Coste and Reyn, or pea seedlings. Aphid-transmissibility of the variants from infected pea plants was tested as described by Bath and Tsai (1969). Aphid-transmissibility of partially purified and purified virus preparations was assayed by using both an artificial membrane feeding system (Thottappilly *et al*., 1972) and mechanical injection of virus into the body cavity of the vector (Clarke and Bath, 1973).

Polyacyrlamide Gel Electrophoresis in SDS. Stock reagent solutions and gels were prepared following the method of Fairbanks et al. (1971) with modifications of Welton (1974). ConAcBis was prepared with 40 gm of acrylamide (Eastman 5561), 1.5 gm of methylenbis-acyrlamide and distilled water to 100 ml and was filtered before use. A 10X buffer solution consisted of 0.4 M Tris-base, 0.2 M sodium acetate and 0.02 M Ethylenedinitrilotetraacetic Acid (EDTA) in distilled water to one liter adjusted to pH 7.4 with glacial acetic acid. Ten per cent (w/v) sodium dodecyl sulfate (SDS), 1.5% (w/v) ammonium persulfate and 0.5% (v/v) TEMED (Canalco 204) solutions also were prepared.

Glass electrophoresis tubes (100 mm x 7 mm-outer diameter) were cleaned in chromic sulfuric acid (three hours), rinsed in distilled water and oven dried. Each tube was then dipped in a solution of dimethyl dichlorosilane and dried at room temperature. Tubes were finally placed in 1% SDS for one hour, rinsed in distilled water and oven dried.

In preparing the 5.6% polyacrylamide gels, stock solutions were mixed in the following proportions: 5.6 ml ConAcBis, 4.0 ml 10X buffer, 4.0 ml 10% SDS, 20.4 ml distilled water, 4.0 ml 1.5% ammonium persulfate and 2.0 ml TEMED. After allowing the solution to stand fifteen minutes, it was pipetted into the glass tubes to 1 cm from the top (9 cm gel) and gently overlayered using a microliter syringe with a freshly prepared solution of 0.1% SDS, 0.15% ammonium persulfate and 0.05% TEMED. After polymerization (about forty five minutes), the overlayered solution was poured off and replaced with electrophoresis buffer (100 ml of 10X buffer, 50 ml of 10% SDS and 850 ml of distilled water). Gels were left for at least twelve hours before use.

Purified virus preparations used in an electrophoresis sample were prepared by substituting sodium phosphate buffer during purification for potassium phosphate which eliminated the presence of K^+ ions. Stock reagent solutions used in electrophoresis sample preparation were: 10% SDS, 35% sucrose - 50 m$\underline{M}$ tris HCL - 5 m$\underline{M}$ EDTA adjusted to pH 8.0 and 0.4 $\underline{M}$ di-thiothriotol (DTT). An electrophoresis sample was prepared by mixing 0.1 ml 10% SDS, 0.3 ml sucrose-tris-EDTA, 0.1 ml DTT and 0.5 ml of a purified virus solution and then heating at 100°C for fifteen minutes in a water bath. After heating, 20 µl of a tracing dye pyronin B was added to the sample.

Prior to sample application, gels were pre-electrophoresed for thirty minutes. Ten-40 µl of an electrophoresis sample were layered on each gel (1.5 to 6 µg of virus) and run at 3 mA per gel until the tracing dye had traveled about 6-cm into the gel (about three hours).

Gels were fixed for several hours to overnight in a solution of 7.5% acetic acid, 5% methanol and 10% isopropanol and then electrophoresed for two hours in a mixture of 4.5% acetic acid and 5% methanol to remove unbound SDS. Gels were stained in Coomassie Blue for six hours and destained by electrophoresis in a solution of 4.5% acetic acid and 5% methanol.

<u>Preparation of Antisera</u>. Two-3 mg/ml of purified virus was emulsified with an equal volume of Freund's incomplete adjuvant and injected at about weekly intervals into the hind leg muscles (IM) of New Zealand White rabbits. Purified virus solutions in buffer only also

were injected intravenously (IV) in some instances as a booster before bleeding (Table 3). Seven to ten days after the final injection, rabbits were bled from the ear with the serum separated by low speed centrifugation and stored in small lots at -20°C.

Antiserum also was produced against fraction 1 pea protein. Pea protein was concentrated by ammonium sulfate precipitation, adjusted to 5 mg/ml, mixed with an equal volume of Freund's incomplete adjuvant and injected into the hind leg muscle of a New Zealand White rabbit. Three IM injections followed by a single intravenous injection were given at weekly intervals with bleedings done from the ear ten days after the final injection. Serum was separated by low speed centrifugation and stored in small lots at -20°C.

Test Antigens. Sap from virus-infected and healthy non-infected pea plants was collected by grinding 1-2 g of a pea tissue in a sterile mortar and pestle and expressing the sap from the tissue through a cheese cloth. The expressed sap was given a low speed centrifugation to eliminate large particulate material and used immediately in serological tests. Partially purified virus was used in preliminary antiserum testing. Purified virus was adjusted to 0.2 - 0.8 mg/ml for agar gel diffusion tests and to 50-80 μg/ml for precipitin ring tests. Pea protein from non-infected plants was concentrated by ammonium sulfate precipitation and adjusted to 0.5-2.0 mg/ml for agar gel diffusion tests.

Test Procedure. Agar gel diffusion tests were conducted in plastic petri dishes (9-cm in diameter), each containing 20 ml of 1% Ionagar No. 2S (Wilson Diagnostics, Inc.) in PBS (phosphate-buffered saline, 0.1 M pH 6.0 potassium phosphate, 0.15 M NaCl) with about 0.01% sodium azide. Three test patterns were used with the antigen and antiserum well diameter of 8 mm held constant in all three patterns. The three patterns consisted of: a pattern with a central antiserum well and six peripheral antigen wells, a four-well pattern (two antisera and two antigens), and a three-well pattern (one antiserum and two antigens) in a triangular arrangement. During reactions, plates were kept in a moist chamber at room temperature and observed for precipitin band formation daily for a two-to-five-day period. Agar gel plates were washed in PBS for twenty-four hours to remove unprecipitated protein and photographed immediately without staining.

Precipitin ring tests (Whitcomb and Black, 1961) were conducted to determine antiserum titers. Glass tubes (7-mm outer diameter) were cut into about 15-cm pieces pulled over a flame into two tubes with the tapered end sealed by flame. Two-fold antiserum dilutions were made in 10% glycerine saline (0.15 M NaCl), pipetted into the glass tubes and overlayered with virus antigen. Reactions were observed for ninety minutes with the highest dilution forming a visible precipitin line at the antigen-antiserum interface recorded as the antiserum titer.

RESULTS

Symptomatology of Variants. Symptom expression of T- and NT-PEMV on the systemic host garden pea, *Pisum sativum* L., variety Mid-freezer and the local lesion host, *Chenopodium amaranticolor* Coste and Reyn, was identical. French (1973) determined a 4X to 10X yield difference between NT-PEMV and T-PEMV during purification. This was supported by observing that NT-PEMV produced more severe symptoms after ten days and also that the symptoms appeared earlier in NT-PEMV-inoculated plants.

Transmission Characteristics. Both variants were readily transmissible to carborundum-dusted pea seedlings by mechanical inoculation of expressed sap from virus-infected pea plants. Partially purified and purified virus preparations of both variants also were highly infective following mechanical inoculation.

Aphid-transmissibility of the two variants was tested (Table 1) using three virus acquisition methods. (NT-PEMV was not transmitted by the pea aphid by any of the methods.) The green peach aphid, *Myzus persicae* (Sulzer), also was shown (Tsai and Bath, 1974) not to transmit NT-PEMV. In the virus acquisition and transmission experiments utilizing an artificial membrane feeding system (Thottappilly *et al*., 1972) and injection technique (Clarke and Bath, 1973), partially purified virus preparations were used. Even when high concentrations of NT-PEMV were

Table 1. Transmission of T- and NT-PEMV variants by the pea aphid, *Acyrthosiphon pisum* (Harris), using three virus acquisition methods.

PEMV Variant	Virus Acquisition Method	% Transmission in Trial[b] 1	2	3
T-PEMV	Plant[a]	94	100	88
	Membrane[c]	55	45	60
	Injection[c]	95	90	45
NT-PEMV	Plant	0	0	0
	Membrane	0	0	0
	Injection	0	0	0

[a]Second instar nymphs given a four-hour acquisition access period (AAP) on PEMV-infected source plants.

[b]Each trial represents virus acquisition by 20 aphids which were placed singly on 20 test seedlings for a three-day inoculation period.

[c]Partially purified virus was used in both membrane feeding and injection trials. The second instar nymphs were given a six-hour AAP on the partially purified virus solutions.

injected directly into the hemocoel of or fed directly to early instar nymphs, no transmission of NT-PEMV resulted.

Pea aphids transmitted partially purified and, in other experiments, purified T-PEMV with high efficiency (ca. 95%) following virus acquisition by injection and membrane feeding which indicated that in addition to mechanical transmissibility the characteristic of aphid-transmissibility was maintained during purification.

Characteristics of Partially Purified Variants. When T- and NT-PEMV variants were compared by density gradient centrifugation and UV analysis striking differences were revealed. Both visual inspection and UV light scans of density gradient columns consistently indicated two sedimenting components (Figure 1) in fourteen of fourteen purifications over a two-year period in NT-PEMV. The concentration of the top (slower sedimenting) component in NT-PEMV varied greatly between purifications over the period from only a shoulder (bottom profile) to a concentration greater than the bottom (faster sedimenting) component (top profile). The single sedimenting component in T-PEMV (Figure 1) was consistent in fifteen of fifteen purifications over the same two-year period with the two profiles being representative.

Using a southern bean mosaic virus (SBMV) marker having a single sedimenting component of a known sedimentation coefficient (115 S), the sedimentation coefficients of the variants were determined. One or two gradient columns containing SBMV were run simultaneously with T- and NT-PEMV layered columns. The average distance traveled by the SBMV marker was compared directly to the distance traveled by the T- and NT-PEMV components in a proportion to determine their corresponding S-values. With N=9 the two components of NT-PEMV averaged 104.6 ± 2.45 (s.d.) and 117.1 ± 1.73 (s.d.) and with N=5 the single sedimenting component of T-PEMV averaged 111.5 ± 1.04 (s.d.). The sedimenting components of the two variants apparently differ in some factor of size, shape and/or density resulting in different sedimentation rates on the sucrose gradient columns.

Because the concentrations of the two sedimenting components of NT-PEMV varied greatly between purifications, an experiment was performed in which gluteraldehyde-fixed and unfixed pea tissue was used as a starting source in a purification in an attempt to maintain viral integrity during extraction and partial purification. The technique has been used successfully during the purification of unstable viruses such as cucumber mosaic to maintain viral integrity (Francki and Hibili, 1972).

NT-PEMV-infected pea tissue was divided into two equal weight batches with one being fixed (under vacuum) in 4% gluteraldehyde for thirty minutes prior to extraction and the other left unfixed. The source material for purification was ten-day NT-PEMV-infected pea tissue. Both tissue batches were kept separate and purified simultaneously with density gradient centrifugation and UV analysis being performed on the resultant partially purified virus solutions.

UV light scans of sucrose gradient columns of both NT-PEMV gluteraldehyde-fixed and unfixed solutions revealed two sedimenting components of similar concentration, component pattern and S-values. The two sedimenting components of NT-PEMV which differ in their sedimentation rate on sucrose gradient columns appear to represent virus particles produced within the infected plants not artifacts of purification (Figure 2).

Characteristics of Purified Variants. The purification method of Thottappilly *et al*. (1972) was used initially for both variants. However, only the T-variant was stable when resuspended in a purified form in 0.05 *M* pH 7.0 potassium phosphate buffer. When virus pellets of NT-PEMV were resuspended in this buffer, the solution would immediately turn cloudy with some obvious precipitation occurring. Spectrophotometer readings revealed a high 280/260 nm wavelength absorbence ratio (greater than 0.80). A value of 0.58-0.60 is generally accepted for PEMV (Izadpanah and Shepherd, 1966). After low speed centrifugation, the virus concentration of purified NT-PEMV solutions decreased dramatically, which indicated virus aggregation.

Several combinations of pH and molarity of potassium phosphate buffer with and without EDTA were tested without success as stabilizers of the NT-PEMV variant. However, 0.05 *M* pH 6.0 sodium acetate buffer which was used in the PEMV studies of Izadpanah and Shepherd (1966) was found to stabilize the NT-PEMV variant much like the 0.05 *M* pH 7.0 potassium phosphate buffer stabilized T-PEMV.

To demonstrate how acetate buffer stabilized NT-PEMV, one experiment (Table 2) was performed in which an equal number of virus pellets was resuspended in the indicated buffers, given a low speed centrifugation, with the virus concentration determinations made using an extinction coefficient of 7.2 OD/mg/ml/cm. Solutions were subsequently kept at 4°C with concentrations again determined after twelve hours. Another low speed centrifugation was then given with concentration

Table 2. Results of an experiment which demonstrates how sodium acetate buffer stabilized purified NT-PEMV.

Virus Treatment	Buffer[a]	Ratio (absorb) 280 nm/ 260 nm	mg/ml[b]
Resusp + centrif[c]	Phosphate	0.56	2.40
12-hr at 4° C[d]	"	0.82	2.00
Centrifuged[e]	"	0.57	0.61
Pellet[e]	"	0.57	1.55
Resusp + centrif	Acetate	0.56	1.62
12-hr at 4° C	"	0.60	1.33
Centrifuged	"	0.59	1.12
Pellet	"	No pellet	

[a]0.05 M pH 7.0 potassium phosphate and 0.05 M pH 6.0 sodium acetate buffers.

[b]Determined by the optical density at 260 nm and an extinction coefficient of 7.2 OD/mg/ml/cm.

[c]An equal number of virus pellets from a density gradient centrifugation purification were resuspended and clarified by low speed centrifugation. Note that the initial concentrations were not the same after low speed which indicated that the virus pellets resuspended more completely in the phosphate buffer.

[d]Concentration determined after virus solutions were kept for twelve hours at 4° C.

[e]Virus solutions again given low speed centrifugation with concentrations determined. If a visible pellet was present, it was resuspended and its concentration determined.

determinations made on the resultant supernatants and resuspended pellets. The virus concentration in the phosphate buffer dropped from 2.00 mg/ml to 0.61 mg/ml after low speed centrifugation but only from 1.33 mg/ml to 1.12 mg/ml in the acetate buffer. The phosphate buffered virus solution had an observable pellet after the second low speed centrifugation which had a high virus concentration (1.55 mg/ml). The 280/260 nm absorbence ratio was relatively constant during the various treatments of the acetate buffered NT-PEMV solution but increased in the phosphate buffer to 0.82 in this experiment.

The NT-PEMV variant was stable in the partially purified form in 0.1 M pH 7.0 potassium phosphate buffer. This was likely to be due to the presence of plant contaminating material which helped to maintain virus stability. A purification procedure was subsequently adapted in which both variants were extracted and partially purified by the same procedure (Thottappilly et al., 1972). However, during density gradient centrifugation and resuspension of the final virus pellet, NT-PEMV was maintained in 0.1 M pH 6.0 sodium acetate buffer. The sodium acetate buffer was increased from 0.05 M to 0.1 M because at the higher ionic strength, it appeared to better stabilize NT-PEMV.

Ultraviolet Absorbence Spectra. The two variants (Figure 3) had absorbence spectra typical of nucleoproteins. Maximum and minimum absorbence was at 260 nm and 240 nm, respectively. The ratio of absorbence at 280 nm to 260 nm (280/260) was 0.58 (NT-PEMV) and 0.57 (T-PEMV) in this experiment, which suggested a relatively high nucleic acid content in both variants.

SDS Polyacrylamide Gel Electrophoresis of Variants. Virus from two different purifications was used in this phase of the research in order to verify the protein patterns in the gels. Preliminary runs of the two variants on 5.6% gels using several virus concentrations indicated that 1 to 6 μg of virus per gel was a suitable concentration range.

Plant protein used for electrophoresis was purified by an ammonium sulfate precipitation method. Healthy ten-day (non-infected) pea tissue was processed in the same manner as PEMV-infected tissue. After high speed centrifugation, an equal volume of saturated ammonium sulfate solution was added to the supernatant during stirring (thirty minutes) over ice. The precipitated protein was pelleted by high speed centrifugation in the 30 rotor at 25,000 rpm for fifteen minutes at 4°C. Pellets were resuspended in phosphate buffered saline pH 7.0 (PBS), freeze-dried and stored at -20°C. Prior to an electrophoresis run, a 1 mg/ml solution was made of the freeze-dried protein in 0.15 M saline. Layering 20 μg of the 1 mg/ml solution per gel produced a suitable band formation.

Electrophoresis runs with NT-PEMV, T-PEMV and plant protein were made simultaneously. Both PEMV variants (Figure 4) produced one major protein (I) band with the T-PEMV variant consistently producing a second higher molecular weight protein (II) band. In six electrophoresis runs, RF values for the apparently common protein (band I) were 0.677 ± 0.0260 (T-PEMV) and 0.676 ± 0.0179 (NT-PEMV) and for the second protein band (II) in T-PEMV 0.389 ± 0.0529.

Four to five protein bands were revealed in the plant protein solution. Other work has shown that these proteins were not serologically related to viral coat protein. The presence of only a single protein in preparations of the NT-PEMV variant and two protein bands in the T-PEMV variant without other minor bands indicated that the purified virus samples were relatively free of plant protein contaminants.

Preliminary Antisera Testing. NT-PEMV in the phosphate buffer system failed to induce an antibody titer of the same level as did T-PEMV; of three rabbits injected a titer of only 1/64 was achieved with two rabbits producing only 1/32 (Table 3). NT-PEMV in acetate buffer, however, induced a high antibody titer (1/2560), similar to that of T-PEMV in phosphate buffer. Antiserum against T-PEMV in acetate buffer also was produced (1/1280) to confirm the serological results obtained by T-PEMV antiserum produced in potassium phosphate buffer.

Fraction 1 plant proteins are known to be the major contaminants which hinder plant virus purification and antiserum production (van Regenmortel, 1966). These low molecular weight (18 S) proteins tend to aggregate and often contaminate plant virus pellets after high speed centrifugation. Antiserum was produced against this protein fraction, which reacted specifically (1/64) against pea protein from non-infected plants concentrated by ammonium sulfate precipitation. No precipitin line formation was observed in an agar gel diffusion test with this antiserum against purified T- and NT-PEMV preparations (Figure 5a). This indicated that the purified virus used in both the antiserum production and the subsequent serological tests was relatively free of detectable fraction 1 pea proteins.

Table 3. Titers of antisera produced against aphid-transmissible (T-PEMV) and nonaphid-transmissible (NT-PEMV) pea enation mosaic virus variants in rabbits.

PEMV Variant[a]	Buffer System	Number of Rabbits[b]	Number and Type of Injection[c]	Injection Period (Months)	Antisera Titers[d]
T-PEMV	Phosplate	3	8-IM	5	640,1280,2048
NT-PEMV	"	3	8-IM	5	32, 32, 64
T-PEMV	Acetate	1	3-IM, 1-IV	1	1280
NT-PEMV	"	2	4-IM, 2-IV	2	2560

[a]Purified virus was resuspended in the indicated buffer system with 2-3 mg/ml given per injection.

[b]New Zealand whites.

[c]Intramuscular injections (IM) were given in the hind leg muscles with the purified virus samples emulsified in Freund's incomplete adjuvant. Intravenous (IV) injections were given through marginal ear vein with the virus suspended in the indicated buffer only.

[d]Titers expressed as reciprocals of the highest dilution of antiserum producing a visible precipitin line at the antiserum-antigen interface in a precipitin ring test (Whitcomb and Black, 1961).

Serological Relationship and Reactions of Variants. In all agar gel diffusion tests in which both viral antigens were reacted simultaneously with one of the antisera (Figure 6a-f) there appeared a coalescent (confluent) precipitin band pattern with NT-PEMV antiserum and a spur pattern with T-PEMV antiserum. Precipitin band formations were observed in twelve to twenty-four hours depending upon the antigen-antibody concentrations. The precipitin band formations were identical when testing expressed sap, partially purified and purified viral antigens against either antisera. The pattern of precipitin band confluence indicated that antibodies produced against antigenic sites (determinants) on NT-PEMV coat protein reacted identically against heterologous antigenic sites on T-PEMV coat protein (serological identity). The spur pattern with T-PEMV (serological partial identity) antiserum indicated either incomplete binding of cross-reactive antibodies to the heterologous antigen (NT-PEMV) which results in the formation of a spur or the presence of, in addition to cross-reactive antibodies, a second set of antibodies specific for T-PEMV which do not react with NT-PEMV antigen.

Several tests were performed to confirm the repeatability of these two basic patterns under different experimental conditions. Spur and confluent patterns were produced in agar gels prepared at both pH 6.0 and 7.0 while at pH 8.0 no visible precipitin bands formed. Similarly, the above precipitin band patterns were duplicated in agar concentrations of 1, 1.5 and 2%. The single precipitin band patterns also were constant (no multiple band formation) over a wide range of purified virus concentrations tested (0.2 to 1.8 mg/ml). Antibodies in the serum from a rabbit injected with T-PEMV virus resuspended in sodium acetate buffer instead of potassium phosphate buffer also produced the spur pattern in agar gel diffusion tests (Figure 1d) with purified T- and NT-PEMV.

Age of plant tissue from which expressed sap was taken did not alter the precipitin band patterns. In one experiment, band formations were identical from 8, 10, 12 and 14-day T- and NT-PEMV infected pea tissue. It was noted also that in pea tissue less than six days virus-infected, the virus concentration was apparently too low to elicit an observable antibody-antigen reaction from T-PEMV infected plants but precipitin bands did form from NT-PEMV infection plants. This confirmed results reported by French (1973) that the virus concentration in NT-PEMV infected plants was significantly higher than in T-PEMV infected plants.

Virus purification procedure did not affect precipitin band patterns. Patterns (Figure 1a,b,e,f) for both expressed sap and purified virus were identical.

Antiserum Absorption. In order to determine the origin of the spur patterns, T-PEMV antiserum was absorbed in three experiments with NT-PEMV and then tested against purified T-PEMV antigen. T-PEMV antiserum (1:5) was mixed with an equal volume of NT-PEMV (0.8 mg/ml) and incubated at 35°C for thirty minutes. After low speed centrifugation the resultant supernatant (1:10) was again mixed with an equal volume of NT-PEMV (800 μg/ml) with the above procedure repeated. The resultant absorbed T-PEMV antiserum (1:20) was then reacted against both viral antigens in agar gel diffusion tests. T-PEMV antigen reacted positively with the absorbed T-PEMV antiserum (Figure 3a) but the NT-PEMV antigen did not, as would be expected if the antibody absorption was complete. This indicated that a second antibody population was indeed present which had not reacted with NT-PEMV antigen during the absorption procedure but instead reacts specifically against antigenic sites apparently unique to T-PEMV coat protein.

In one experiment in which NT-PEMV antiserum was absorbed with purified T-PEMV antigen, no visible precipitin bands formed when the absorbed NT-PEMV antiserum was reacted against either viral antigen (Figure 3b). Antibodies against NT-PEMV were completely absorbed by the T-PEMV antigen during the absorption procedure which confirmed the presence of a single antibody population against NT-PEMV coat protein which completely cross-reacted with heterologous antigenic sites on T-PEMV coat protein.

Homologous, heterologous and absorbed PEMV antisera titers of several antisera lots were determined by a precipitin ring test experiment (Table 4). One way of visualizing these results is to designate the heterologous titer as representing a cross-reactive A-antibody while the absorbed antiserum titer represents a specific B-antibody against T-PEMV. The titers of B-antibody (1/320 - 1/1280) and A-antibody (1/40 - 1/1280) varied between the four rabbits injected with T-PEMV. Homologous and heterologous titers from the NT-PEMV injected rabbit were equal as would be expected with the single A-antibody population.

Because PEMV is a two-component plant virus (French, 1973; Gonsalves and Shepherd, 1972; Izadpanah and Shepherd, 1966), it was postulated that the B-antibody may be specific for one of the viral components (possibly an aphid-transmissible component). To test this

Table 4. Homologous, heterologous and absorbed titers of antisera produced against aphid-transmissible (T-PEMV) and nonaphid-transmissible (NT-PEMV) pea enation mosaic virus variants in rabbits as determined by the precipitin ring test.

Variant Antiserum[a]	Buffer System[b]	Antiserum titer[c]		
		Homologous	Heterologous	Absorbed
NT-PEMV	Acetate	2560[d]	2560[d]	0[d]
T-PEMV R1[e]	Phosphate	640	160	320
T-PEMV R2	Phosphate	1280	1280	1280
T-PEMV R3	Phosphate	2048	80	-
T-PEMV R4	Acetate	640	40	320

[a]Produced as indicated in Table 1.

[b]0.1 *M* pH 6.0 sodium acetate and 0.05 *M* pH 7.0 potassium phosphate. Purified virus injected to the New Zealand white rabbits was suspended in these buffers where indicated.

[c]Expressed as reciprocal of highest antiserum dilution which reacted positively with the viral antigen in the precipitin ring test.

[d]Homologous = NT-PEMV antiserum vs. NT-PEMV purified virus (50-80 μg/ml). Heterologous = NT-PEMV antiserum vs. T-PEMV purified virus. Absorbed = NT-PEMV antiserum reacted (absorbed) with T-PEMV purified virus and then the absorbed antiserum being reacted with its homologous antigen (NT-PEMV).

[e]Rabbit number 1 (Table 1).

hypothesis, two neutralization of infectivity assays were completed with T-PEMV. Samples of purified T-PEMV (0.8 mg/ml) were reacted with an equal volume of either: pre-immune rabbit serum, phosphate buffer, absorbed T-PEMV antiserum (B-antibody) or NT-PEMV antiserum (A-antibody). Each solution was incubated for thirty minutes at 35°C and given a low speed centrifugation. Each of the four resulting supernatants was injected into twelve-second instar pea aphids to determine the aphid transmissibility of the above treated T-PEMV solutions. When these aphids were placed on pea seedlings, in one experiment, no transmissions resulted from those injected with T-PEMV incubated with either A-antibody or B-antibody but twelve of twelve (pre-immune serum) and six of twelve (buffer treated) aphids transmitted in the controls. Apparently the antigenic determinants against which the two antibody populations were developed are both contained on the same T-PEMV coat protein shell.

Detection of a Soluble Antigen. Izadpanah and Shepherd (1966) and Mahmood and Peters (1973) reported the presence of a low molecular weight antigen in expressed sap from PEMV-infected pea plants that was not present in partially purified virus preparations. No such antigen was detected when T- and NT-PEMV-infected pea sap and the two antisera were reacted (Figure 6a,b).

A low molecular weight antigen, however, was detected in six or six soluble protein fractions isolated from the high speed supernatant during the partial purification of T-PEMV from infected pea plants. Ten-day infected T- and NT-PEMV pea plants were processed by the purification procedure of Thottappilly et al. (1972). Supernatants from the high speed centrifugation step were collected and given a second high speed for ninety minutes (4°C) at 41,000 rpm in the 50 rotor of the Model L Beckman ultracentrifuge. An additional small virus pellet was observed after this second centrifugation. The supernatant was again carefully collected, added to an equal volume of saturated ammonium sulfate and left to react for thirty minutes over ice. The precipitated protein was pelleted by centrifugation at 25,000 rpm in the 30 rotor for fifteen minutes (4°C). The protein pellets were resuspended in PBS and dialyzed overnight against PBS to remove any remaining ammonium sulfate. The precipitated protein solutions were tested in agar gel diffusion tests against T- and NT-PEMV antisera (1:40). The T-PEMV protein fraction from six of six extractions reacted positively against T-PEMV antiserum but not with NT-PEMV antiserum (Figure 7c). This indicated that a low molecular weight soluble antigen was present in T-PEMV-infected plants which reacted specifically with B-antibody. No reaction was observable when the same concentrated protein fractions from NT-PEMV-infected plants were reacted with either antisera.

The soluble antigen from the T-PEMV could have originated from two sources. It could either be unassembled coat protein present in the plant which was extracted with the virus, or it could be T-PEMV coat protein subunits which result from virus particle disruption during extraction and high speed centrifugation.

In two agar gel tests where T-PEMV antiserum was reacted with the T-PEMV soluble protein fraction and expressed sap from T-PEMV and NT-PEMV-infected pea plants interesting precipitin band patterns developed (Figure 7d). The precipitin band from the T-PEMV soluble protein fraction joined with the T-PEMV virus band with the virus band forming a spur. The precipitin band from the same soluble protein fraction, however, intersected the virus band from NT-PEMV-infected plants. These patterns indicated that the soluble protein was unrelated to NT-PEMV coat protein and only partially related to T-PEMV coat protein.

Antiserum Detection of Aphid Transmissible PEMV. The specific B-antibody in T-PEMV antiserum was tested against several other geographical isolates of PEMV of known aphid transmissibility. This was in order to determine its usefulness as a diagnostic tool for quick testing of PEMV isolates as to their aphid transmissibility without the use of the aphid vector. Such an antiserum could quickly distinguish greenhouse anomalies like NT-PEMV from biologically active isolates which represent more closely those in nature.

Five virus isolates were tested (Table 5). To determine the aphid transmission characteristics of the isolates, sixteen pea aphid nymphs were given a sixteen-hour acquisition access period on a virus-infected source plant from each isolate. These were transferred singly to sixteen pea seedlings for a three-day inoculation access period. Transmission occurred only in the case of the New York (NY-T) aphid-transmissible isolate (Bath and Tsai, 1969).

With the nonaphid-transmissible characteristics of the other four lines established each line was then tested in agar gel diffusion tests against T-PEMV, NT-PEMV and absorbed T-PEMV (B-antibody) antiserum. Expressed sap and also partially purified virus from each virus line was tested. The four virus lines which were not aphid-transmissible (Table 5) did not react with absorbed T-PEMV antiserum. They did react,

Table 5. Testing the reliability of absorbed (antibody-B) T-PEMV antiserum in distinguishing aphid- and nonaphid-transmissible pea enation mosaic virus isolates.

PEMV Isolate designation[a]	Geographical Origin	% Transmission by pea aphids[b]	Reactivity with Antisera[c]		
			T-PEMV	NT-PEMV	Absorb[d] T-PEMV
NY-T	New York	87	+	+	+
NY-NT	New York	0	+	+	-
NT-C	New York	0	+	+	-
NT-B	California	0	+	+	-
NT-A	California	0	+	+	-

[a]NY-T = New York isolate referred to in (Bath and Tsai, 1969); NY-NT = a nonaphid counterpart of NY-T; NT-C = isolate selected (Bath et al., 1973); NT-B = original NT-PEMV line three years prior to this present research; NT-A = originally selected NT-PEMV variant (Tsai and Bath, 1974).

[b]Percentage of sixteen pea aphids transmitting the isolate after a sixteen-hour acquisition access period on a virus-infected source plant.

[c]+ = precipitin band formed in an agar gel diffusion test (Figure 4a-e); - = no visible precipitin band formed in an agar gel diffusion test.

[d]T-PEMV antiserum absorbed with NT-PEMV purified virus which eliminated cross-reactive antibody-A.

however, with the unabsorbed T- and NT-PEMV antisera which both contain cross-reactive A-antibody (Figure 8a-e). The New York (NY-T) aphid-transmissible isolate reacted with all three antisera.

DISCUSSION OF RESULTS

The aphid transmissibility of most PEMV isolates previously purified was not reported except for occasional mention that the original isolate was collected from the field. For this reason direct comparisons of the characteristics determined for T- and NT-PEMV variants to those reported in the literature are difficult to make.

Viral stability is affected by many factors including: degree of purity, salt concentration, pH and type of buffer (Brakke, 1956; Brakke, 1962). Brakke (1962) noted that the effect of any one of these factors will often depend on the levels of the others. In this work, degree of purity, type of buffer and its concentration were quite important. In the partially purified form both variants were apparently stable in 0.1 <u>M</u> pH 7.0 potassium phosphate buffer. In the purified form, however, NT-PEMV was physically unstable in the phosphate buffer system which was adequate for stabilizing T-PEMV. Changing to a 0.1 <u>M</u> pH 6.0 sodium acetate buffer system eliminated the problem of aggregation.

The major consideration in the initial purification of PEMV (Thottappilly <u>et al</u>., 1972) was to maintain in addition to mechanical infectivity the characteristic of aphid transmissibility. The phosphate buffer system proved to be best for these purposes when working with an aphid-transmissible PEMV strain. NT-PEMV is not aphid-transmissible so changing to the sodium acetate buffer system did not hamper research objectives.

The nature of the components of PEMV has been investigated by several authors (Bozarth and Chow, 1966; French, 1973; Gibbs et al., 1966; Hull and Lane, 1973; Izadpanah and Shepherd, 1966; Musil et al., 1970). The sedimentation coefficients they determined are in general agreement with the values determined using a SBMV marker and our two variants. Generally, they report 95-99 S for the top (slower sedimenting) component and 112-115 S for the bottom (faster sedimenting) component. These are in agreement with that determined for T-PEMV (111.5 S). Generally, only a single component was determined in our T-PEMV variant but occasionally a slight shoulder at the 95-98 S position occurred.

Izadpanah and Shepherd (1966) reported values of 106 and 122 S which are similar to the values determined for NT-PEMV (104.6 and 117.1 S). They also used a SBMV marker to determine their sedimentation coefficients. This would seem to indicate that they may have been working with a nonaphid-transmissible PEMV strain. They indicated in their paper that the virus line had been maintained for several years by mechanical perpetuation. Whether they had selected a NT-PEMV variant must remain a point of speculation.

The selection of a NT-PEMV variant has resulted in an apparent change in the sedimentation coefficients of the viral components. One explanation proposed by French (1973) as to how the selection of the NT-PEMV variant occurred was that one of the PEMV components carries the NT-genome and that the other carries the T-PEMV genome. This hypothesis negates the possibility of an induced mutation and supports the split genome results of Hull and Lane (1973). Presumably, the aphid vector selects for the aphid-transmissible component while the process of mechanical inoculation somehow favors the nonaphid-transmissible component. Since the 112-115 S component predominates in most PEMV strains, it may be assumed to represent the aphid-transmissible component. If this is the case, during selection the 95-99 S component increased and became the NT-PEMV variant (104 and 117 S) with the resultant variant components having higher sedimentation rates than the original PEMV isolate.

Components of plant viruses are known to vary with age of plant and growing conditions (French, 1973). The concentration of the components of our two PEMV variants was found to vary. One comparison which can be drawn between T- and NT-PEMV is that the bottom component is apparently stable in the variants while the top component varies in concentration. The top or slower sedimenting component could represent incompletely assembled virus particles, degradation of bottom component either in the plant before purification or as a result of the purification process or a true component within the virus population of a different RNA content which indicates a split genome (Hull and Lane, 1973). Whatever the case, NT-PEMV is more sensitive to a factor or factors which influence production of this top component.

The structural proteins of PEMV have been studied by several authors (Hill and Shepherd, 1972; Hull and Lane, 1973; Shepherd <u>et al</u>., 1968) with all except Shepherd <u>et al</u>. (1968) using the SDS technique. They reported a single protein from their PEMV strains having a molecular weight of about 22,000 (Hull and Lane, 1973). A single protein also was determined for the NT-PEMV variant.

Revealing the presence of the second higher molecular weight protein in the aphid-transmissible T-PEMV is not the first report of such a phenomenon. Using the SDS polyacrylamide gel electrophoresis technique two structural proteins have been reported from several other small spherical plant viruses including: tomato bushy stunt and turnip crinkle (Ziegler et al., 1974), cauliflower mosaic (Tezuka and Taniguchi, 1972) and cowpea mosaic (Wu and Bruening, 1971).

The presence of two structural proteins in the aphid-transmissible PEMV variant may account for some of the differences noted between it and the nonaphid-transmissible variant. The addition of the second protein to T-PEMV would suggest a significant difference in the architectural structure of the protein coat of the virus which undoubtedly result in different physiochemical and possible biological properties.

Kao (1974) noted two size classes of particles when a purified virus preparation of each variant was negatively stained with neutral phosphotungstic acid and scanned under the electron microscope. T-PEMV contained predominately hexagonal particles (24.4 ± 1.6 nm) with some spherical particles (29.8 ± 2.1 nm) also present. NT-PEMV possessed only a few hexagonal particles (27.4 ± 1.7 nm) with the vast majority being spherical particles (28.8 ± 1.6 nm). The 24 nm hexagonal particles which predominate in the T-PEMV preparations likely represent the bottom component (112 S) with the larger spherical particles possibly representing the top component. This situation of size class and components may also hold true for the NT-PEMV variant. The larger and generally spherical particles found in the NT-PEMV variant may physically reflect the architectural change that has occurred when only a single structural protein is present in the PEMV protein coat. It is possible that the incorporation of the second structural protein is responsible for the isometric (hexagonal) shape of virus particles which predominate T-PEMV preparations.

The instability of NT-PEMV in the phosphate buffer system also may be accounted for by this coat protein difference. The addition of the second protein could give T- and NT-PEMV different isoelectric points resulting from different amino acid compositions and surface configurations. The homogeneity of the protein coat may make protein-protein interactions more prevalent in NT-PEMV resulting in virus aggregation under unfavorable conditions of buffer type, pH, and ionic strength while the heterogeneity of the T-PEMV protein coat making it less subject to these protein-protein interactions.

The detection of the second structural protein in T-PEMV coat protein was confirmed serologically. T- and NT-PEMV were found to be serologically related but no identical. They share a common cross-reactive antibody just as they were found to share a common structural protein. However, T-PEMV was found to possess a second higher molecular weight protein which may be represented by the specific B-antibody population determined in T-PEMV antiserum by adsorption experiments.

Previous serological work on other PEMV isolates (Bustrillos, 1964; Farro, 1969; Gibbs et al., 1966; Izadpanah and Shepherd, 1966; Musil et al., 1970) was inconclusive as to the serological relationship of the isolates because none of these authors had both the homologous and heterologous antigen-antibody system present for direct comparisons. They found their isolates of PEMV to cross-react with antisera from other geographical isolates just as T- and NT-PEMV cross-reacted. If there were serological differences between isolates they could not be detected unless both antigens were reacted simultaneously with an antiserum.

The detection of a soluble antigen from T-PEMV infected plants not present in NT-PEMV raises an interesting possibility. It was found to be unrelated to NT-PEMV coat protein and only partially related to T-PEMV coat protein. It may be that the soluble antigen represents the second coat protein which became dissociated from the coat protein shell during purification or had not been assembled to T-PEMV particles at the time of extraction and partial purification.

The protein situation with PEMV is very similar to that described for simple RNA bacteriophages by Heisenberg (1967) and reviewed by Holn and Holn (1970). Normal phages such as FII, R17 and QB contain coat protein, A-protein and RNA. However, it has been reported that FII containing an amber mutation in the A-cistron will produce noninfectious phage-like particles. These have been referred to as defective particles (Heisenberg, 1967) and are characterized as lacking the A-protein component. Although these defective particles cannot be distinguished from viable phage in the electron microscope, they do differ in a number of respects. Namely, they lack the ability to adsorb to their bacterial host and are RNase sensitive. The A-protein (representing one polypeptide per phage particle) was thought to initiate adsorption of a phage particle to a bacterium which ultimately results in infection when the phage RNA is injected into the bacterial cell. The RNase sensitivity was thought to indicate that a portion of their RNA is exposed outside of the protective protein shell. Defective R17 phage, however, are not RNase sensitive and it was suggested that host protein or extra coat protein may have replaced the missing A-protein in the phage protein shell. RNase digested defective particles of FII were referred to as light defective particles which created a two component phage population. The RNA bacteriophages are thus analogous in many ways to T-PEMV and NT-PEMV which differ in relation to the number of structural proteins and also biologically.

Another example of coat protein structure being implicated in biological transmission was reported by Partridge et al. (1974). They determined the presence of a glycoprotein in barley stripe mosaic (BSMV) and cowpea mosaic (CPMV) viruses which are seed transmitted. No glycoprotein was detected in several related non-seed transmitted viruses including bean pod mottle virus (BPMV) which is similar in many respects to CPMV except it is not seed transmitted. They hypothesized that the presence of the carbohydrates allowed CPMV and BSMV to invade the gametophytic tissue of an infected host while its absence (BPMV) in the protein coat prevented invasion.

Anomalies such as NT-PEMV and defective bacteriophage particles which lack a known biochemical component can be very useful in providing essential biological information. When these anomalies are compared directly to the natural system, many fundamental questions can be answered which may otherwise remain a point of speculation.

The presence of the second structural protein may well be the key to the characteristic of aphid transmissibility of T-PEMV. Because of the circulative nature of PEMV within the body of the insect vector, protein coat compatibility with respect to membrane systems through which the virus must presumably pass and protein coat resistance to proteolytic forces in the harsh chemical environment of the food channel, hemolymph and salivary gland would be important. Rochow (1969) has discussed the possibility that protein coat-vector membrane complementarity could play a role in vector specificity and virus transmission. Catalytic forces within the digestive system, the hemolymph and salivary system of the vector may also play a significant role. The single protein structure of the NT-PEMV variant may be more subject to enzyme disruption than T-PEMV.

GENERAL DISCUSSION

The loss of vector transmissibility by plant viruses is not an infrequent occurrence; it has occurred in both the nonpersistently and persistently transmitted plant viruses. Whether it results from spontaneous or induced mutations or selection of a non-insect transmitted portion of an already existing virus genome is not at issue here. But what is important is that it results in a biological anomaly which can lead to new biological insights into virus-vector relationships.

Except for wound-tumor virus (Black, 1953), all viruses which have lost their vector transmissibility have a common characteristic of being mechanically as well as vector transmissible. In all cases, loss of vector-transmissibility has occurred when the "natural" transmission cycle was disrupted by elimination of its vector component. This one fact demonstrates quite adequately the importance of the three component biological cycle, plant, virus, and vector.

The selection of NT-PEMV could have involved a chance mutation or selection of an already existing NT-PEMV genome in the PEMV population. Hitchborn and Thompson (1960) indicated that it would be difficult to separate either a spontaneous or induced mutation from an earlier mutation already present in the population which was subsequently selected. Swenson et al. (1963) indicated that they believed their nonaphid-transmissible bean yellow mosaic virus variant originated from a spontaneous mutation because it was selected in one sap inoculation.

Four NT-PEMV variants have been selected in a manner similar to the original NT-variants reported by Tsai and Bath (1974). This phenomenon for PEMV is obviously reproducible.

The characteristic of nonaphid transmissibility was not produced after a single inoculation as in the case of BYMV and some isolates were found to lose their aphid transmissibility faster than others (Tsai and Bath, 1974). Aphid transmissibility slowly declined after repeated sap inoculations and once the decline began it usually involved four or five inoculations before aphid transmissibility was lost. However, this still does not exclude the possibility that the initiation of the sap inoculation perpetuation regime triggered a mutation which was eventually selected for in subsequent inoculations.

It was found that if a new NT-PEMV variant was partially purified after the initial loss of aphid transmissibility and injected into the aphid vector, the new NT-virus isolate was still transmissible but not following virus acquisition from infected pea plants or across an artificial membrane feeding system. This indicated that the aphid transmissible genome was still present at a low concentration and if a high concentration of virus was injected into the body cavity of the pea aphid vector transmission resulted. If these PEMV-infected plants were used as virus source plants in a subsequent aphid transmission experiment, virus was transmitted at a high rate (over 90% of the insects becoming transmitters). This indicated that the aphid re-selected the aphid-transmissible genome following injection resulting in the transmission of an isolate much like the initial isolate before the selection process. If the mechanical perpetuation of the new NT-line was continued, however, without the above aphid interruption, transmission even by injection was lost in two subsequent mechanical inoculations.

This research has shown in direct comparative studies of T- and NT-PEMV that they are indeed different biochemically. T-PEMV was shown to possess a second structural polypeptide (much like the infective RNA bacteriophages) which was lacking in NT-PEMV. This was confirmed serologically and was thought to explain other differences noted such as morphological differences observed under the electron microscope and viral stability in different buffer systems.

The limited knowledge gained to date of differences between these variants can provide the starting point for further investigations elucidating the molecular biology of these variants. Further analysis of the protein coats to determine their characteristic differences and their amino acid composition is essential. Analysis of the viral RNA to determine content, species and distribution among the components is an obvious research area. When this information is then related to a more thorough study of the NT-PEMV selection process, it will provide needed information as to the biochemical nature of vector transmission, vector specificity and to the possible origin of vector-virus relationships.

<u>The Status of PEMV in the Spherical Plant Virus Group and a Model for the Vector Transmissibility of these Spherical Viruses</u>. Within the realm of the plant viruses is a group of small (25-30 nm) spherical viruses superficially similar to PEMV. Within this group are present a wide range of biological relationships including: vector transmitted,

vector and mechanically transmitted and mechanically transmitted viruses (Table 6).

The 30 nm plant viruses are more restricted genetically than their larger cousins in the amount of genetic coding available for coat protein(s) and replicase enzymes. The larger viruses have the genetic potential for more complexity both structurally and biologically. For example, cauliflower mosaic virus, a 50 nm DNA virus, has two coat proteins (Tezuka and Taniguchi, 1974). Rhabdoform viruses such as tomato spotted wilt virus (Mohamed et al., 1973) and potato yellow dwarf virus (Knudson and MacLeod, 1972) have three and four structural proteins, respectively. Larger viruses such as wound-tumor (60 nm) have also been shown to multiply within their insect vectors as well as within their plant host cells. But, within the genetic limitations of these 30 nm viruses, there is still variation in biological activity and structural complexity (Table 6).

Of those viruses analyzed within this group, seven have been shown to have one structural protein and four to have two structural proteins. Hill and Shepherd (1971) determined one structural protein in their PEMV isolate. Whether they were dealing with an aphid-transmissible PEMV isolate is not known, but their results are the same as those obtained for NT-PEMV. Although it is difficult to draw a firm relationship between number of coat proteins and complexity of the vector-virus relationship the trend is toward a more intimate relationship with increased protein coat complexity. It would be very interesting to test this hypothesis by analyzing the protein coats of PLRV and BYDV which are exclusively vector transmitted.

Table 6. Summary of several 30 nm or less spherical plant viruses as to their protein coat structure and vector transmissibility.

Virus	Transmission Mechanical	Transmission Vector	Number of Structural proteins[a]
Cowpea chlorotic mottle virus	+	Unknown	1[b]
Broad bean mottle virus	+	Unknown	1[b]
Brome mosaic virus	+	Unknown	1[b]
Cucumber mosaic virus	+	Aphid (NP)[i]	1[c]
Sowbane mosaic virus	+	Leafhopper, aphid fleahopper	1[d]
Tobacco necrosis virus	+	Fungus	1[e]
Southern bean mosaic virus	+	Beetle	1[f]
Tomato bushy stunt virus	+	"Soil borne"	2[g]
Turnip crinkle virus	+	Flea beetles	2[g]
Cowpea mosaic virus	+	Beetle	2[h]
Pea enation mosaic virus	+	Aphid (P)[j]	1[d], 2
Potato leafroll virus	-	Aphid (P)	?
Barley yellow dwarf virus	-	Aphid (P)	?

[a]Determined by SDS polyacrylamide gel electrophoresis technique.

[b]Agrawal and Tremaine (1972)
[c]van Regenmortel *et al*. (1972)
[d]Hill and Shepherd (1972)
[e]Jones and Reichmann (1973)
[f]Hill and Shepherd (1971)
[g]Ziegler *et al*. (1974)
[h]Wu and Bruening (1971)
[i]Non-persistently transmitted
[j]Persistently transmitted

PEMV with its intimate vector-virus relationship with aphids appears to represent a transitional phase in the relationship between the mechanically and non-mechanically transmissible spherical viruses. Yet, with the apparent simple loss of the second structural protein (NT-PEMV), it assumes characteristics much like the less biologically complex CCMV, BBMV and BMV; namely, being highly infectious, mechanically transmitted, possessing a single coat protein and having no known vector. Whether NT-PEMV is completely a vectorless variant of PRMV is an academic question at this point. Badami (1958) reported the loss of transmissibility of a cucumber mosaic virus isolate by the green peach aphid, Myzus persicae (Sulzer). However, the new variant was still transmissible by two other aphid vectors Aphis gossypii and Myzus ascalonicus.

It appears that the structural configuration and chemical composition of the coat protein of these viruses may hold the key to their vector-transmissibility. Admittedly, even the single coat proteins of these viruses probably differ biochemically which will undoutedly affect their vector-transmissibility, vector specificity and/or mechanism of transmission. Nevertheless, the complex cycle of a circulative plant virus such as PEMV apparently necessitates the addition of structural complexity to the protective coat protein shell. With the development of the SDS polyacrylamide gel electrophoresis technique the coat protein of these viruses have only recently been investigated with any reliability and reproducibility. Those spherical viruses which have not been analyzed should be in order to complete the structural classification of these viruses. Also, the vector transmissibility of the viruses should be established at the time of the protein analysis. This would eliminate the possibility of analyzing a NT-form and representing it as a natural virus isolate.

Virus-vector relationships cannot be explained in totality by simply the physiochemical make-up of the coat protein. The role of virus-host plant and plant-vector interactions must be such that a virus structurally suitable for passage through a vector indeed becomes linked to a vector-plant host cycle.

What triggered the selection of NT-PEMV was the disruption of this three-phase cycle (elimination of the vector phase). Mechanical transmission of PEMV results in the possible early infection of surface tissues with the eventual spread to underlying tissues. This is opposed to vector transmission in which the vascular tissue is probably initially infected. A detailed electron microscopy study of aphid versus mechanically inoculated pea tissue as to the course of infection and tissues infected is lacking. Studies to date (deZoeten et al., 1972; Kao, 1974; Shikata et al., 1966; Shikata and Maramorosch, 1966) have used mechanical plant inoculations or, in the case of Shikata et al. (1966) and Shikata and Maramorosch (1966), used both aphid and mechanically inoculated tissue but did not differentiate their results as to method of inoculation. What effect initial infection site and type of tissue has on viral mutation, selection and propagation is not known.

Perhaps the chemical environment of the cell is important. It is known that if bacteriophage FII is grown in histidine free bacterial cultures, defective particles are produced because the A-protein requires this amino acid for synthesis. Histidine is not a constituent of regular coat protein. Analysis of the two structural proteins of T-PEMV and NT-PEMV may reveal interesting differences.

This model for vector specificity based on the supposition that a virus must possess a specific coat protein structure has been previously proposed (Rochow, 1969). However, my analysis of T- and NT-PEMV represents the first direct evidence linking coat protein structure and vector transmission. But, this certainly cannot explain the total vector-virus relationship for such a biochemical reality must necessarily be linked in time with an established plant-insect relationship. When such a compatible situation exists, a new vector-virus-plant relationship may be established.

Mutations which affect the basic coat protein structure of these small spherical viruses and subsequent selection pressures which favor this portion of the virus population could result in what we observe as the wide range of virus-vector-plant relationships in the field. Whether we can manipulate these pressures for use in virus control remains to be seen. But for now, plant virologists must remain acutely aware of the significance and necessary interaction of all three phases of the biological cycle and the possible consequences when an artificial plant to plant cycle is routinely used in virus research. Such practices by their very nature create selection pressures which could result in greenhouse anomalies. Research results may then be representing a biological anomaly such as NT-PEMV which makes them of dubious applicability to the real world. But on the contrary, if we recognize such anomalies and use them to our advantage, they can lead to biological insights which otherwise may elude us.

FIGURES

Figure 1. Representative ultraviolet absorbency profiles of sucrose gradient columns layered with partially purified T- and NT-PEMV variants. Centrifugation was for two hours at 24,000 rpm in a SW 27.1 rotor. The sucrose columns were monitored with an ISCO density gradient fractionator coupled to an UA-2 ultraviolet analyzer monitoring at 254 nm.

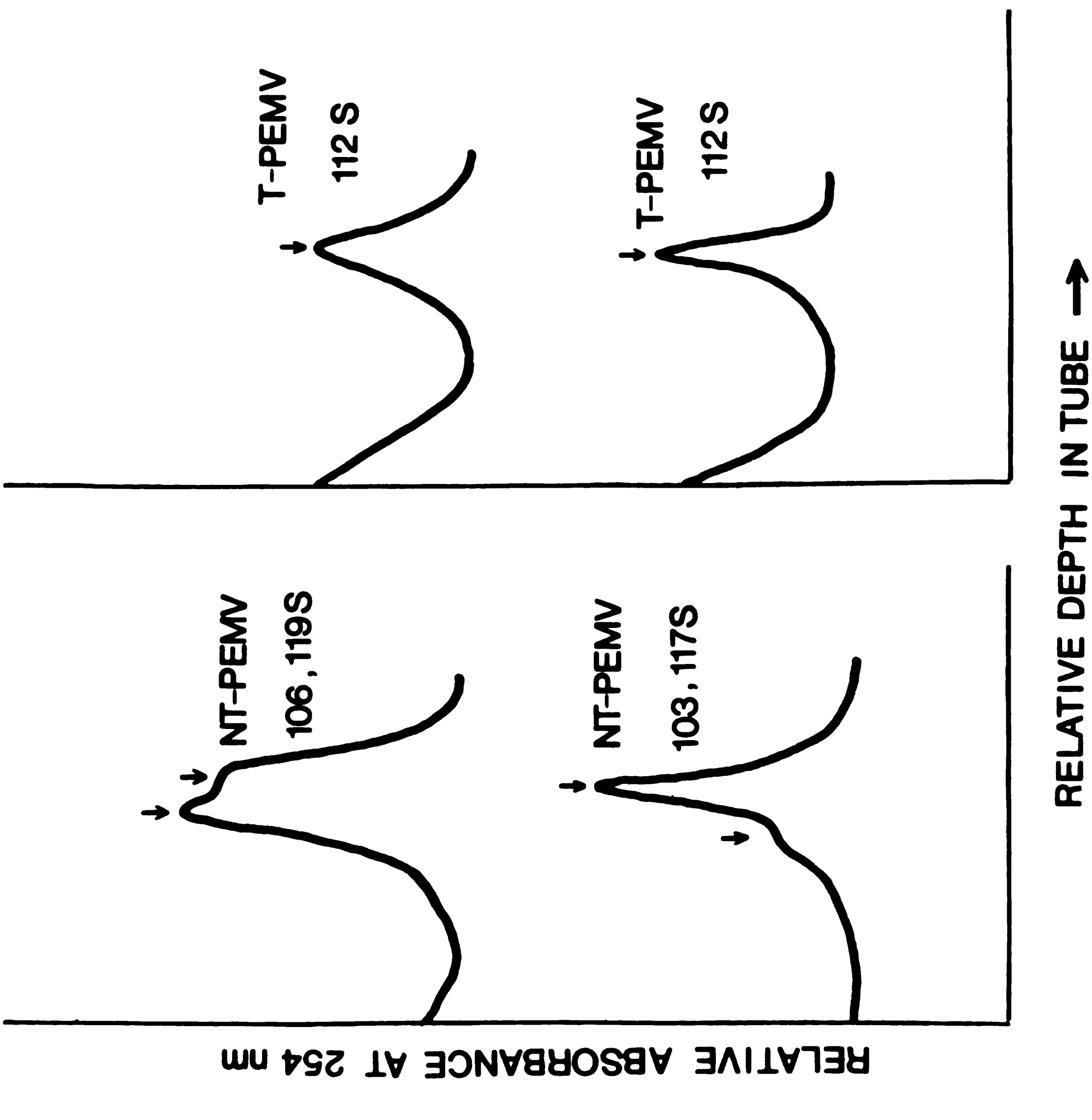

T-PEMV
112 S
T-PEMV
112 S
NT-PEMV
106, 119S
NT-PEMV
103, 117S
RELATIVE DEPTH IN TUBE →
RELATIVE ABSORBANCE AT 254 nm

Figure 1

Figure 2. Ultraviolet absorbency profiles of sucrose gradient columns layered with partially purified NT-PEMV which had been fixed with 4% gluteraldehyde prior to extraction or left unfixed and processed normally. Centrifugation was for two hours at 24,000 rpm in a SW 27.1 rotor. The sucrose columns were monitored with an ISCO density gradient fractionator coupled to a UA-2 ultraviolet analyzer monitoring at 254 nm.

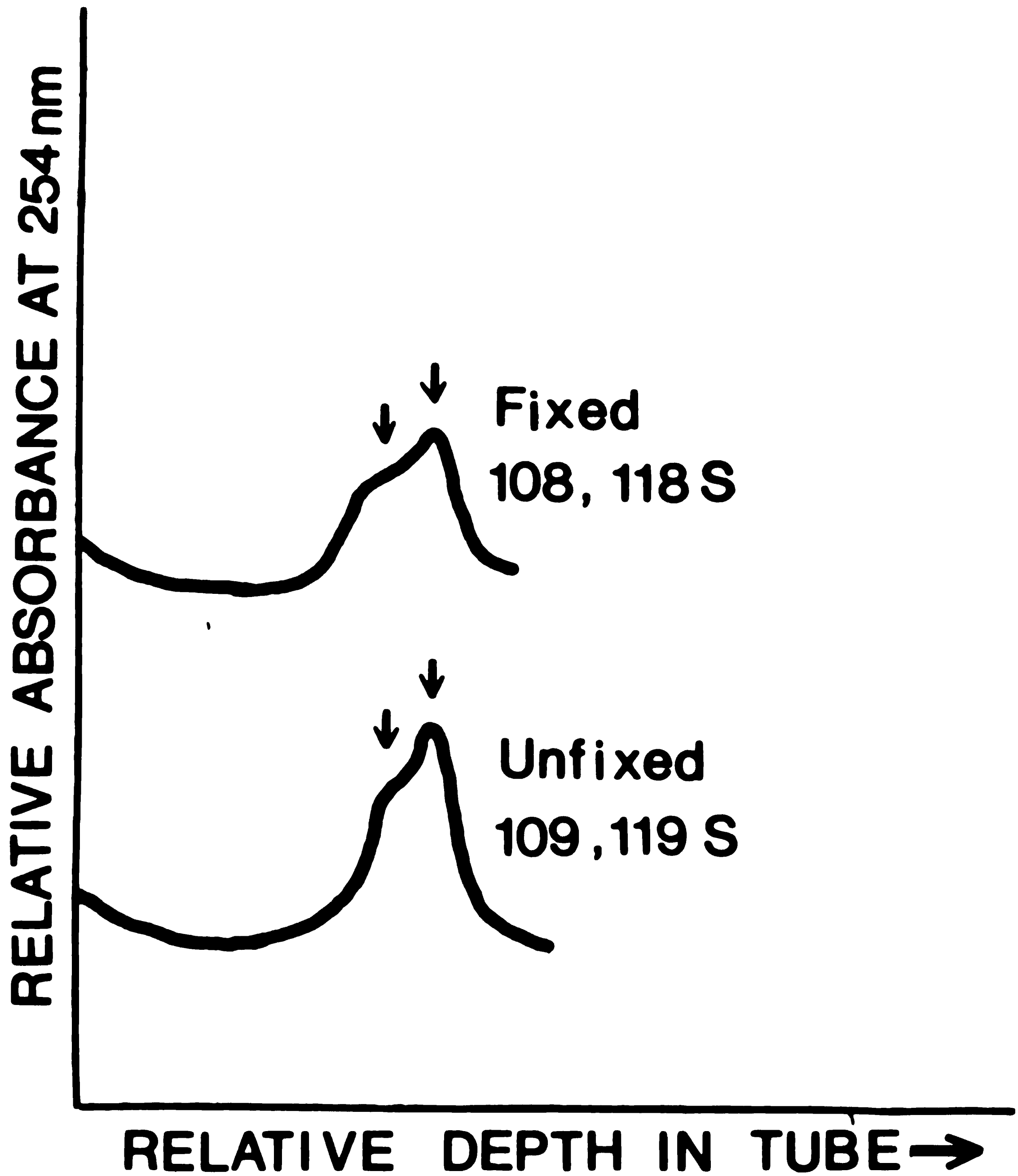

RELATIVE ABSORBANCE AT 254 nm
Fixed
108, 118 S
Unfixed
109, 119 S
RELATIVE DEPTH IN TUBE

Figure 2

Figure 3. Ultraviolet absorption of purified T- and NT-PEMV. Variants were purified by chloroform extraction, differential centrigugation and one cycle of density gradient centrifugation. The virus zone from the sucrose columns was collected with the virus isolated from the sucrose solution by centrifugation at 41,000 rpm for ninety minutes (4°C) in a 50 rotor of the model L Beckman ultracentrifuge. Final pellets were resuspended in either 0.1 M pH 6.0 sodium acetate buffer (NT-PEMV) or 0.05 M pH 7.0 potassium phosphate buffer (T-PEMV).

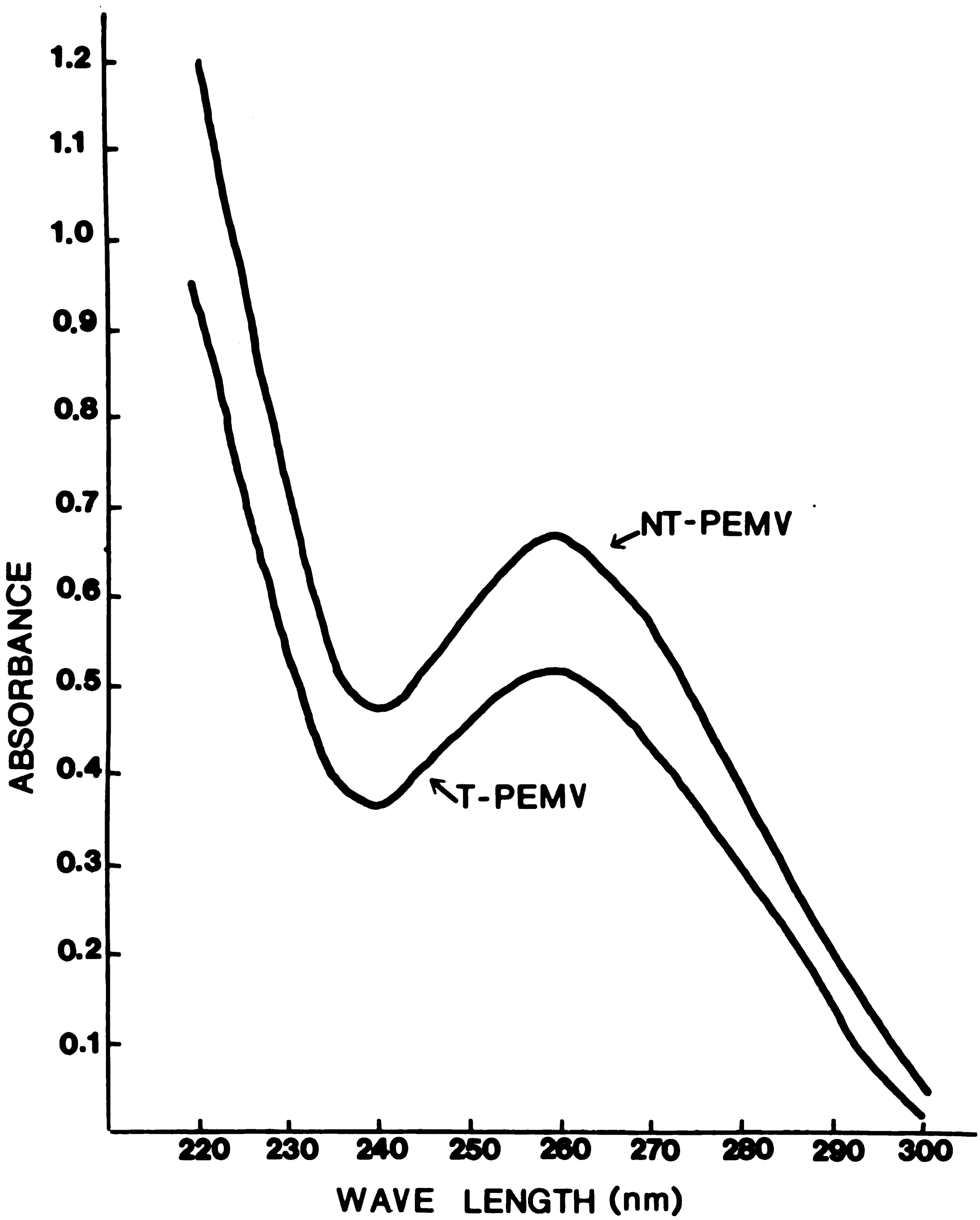

1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
ABSORBANCE
NT-PEMV
T-PEMV
220
230
240
250
260
270
280
290
300
WAVE LENGTH (nm)

Figure 3

Figure 4. Protein patterns of T-PEMV (A) and NT-PEMV (B) following polyacrylamide gel electrophoresis in SDS. 6 μg of purified virus was layered per gel and run at 3 mA per gel with the front traveling about 6 cm (three hours). Protein Band I had a RF = 0.67 and protein Band II had a RF = 0.39.

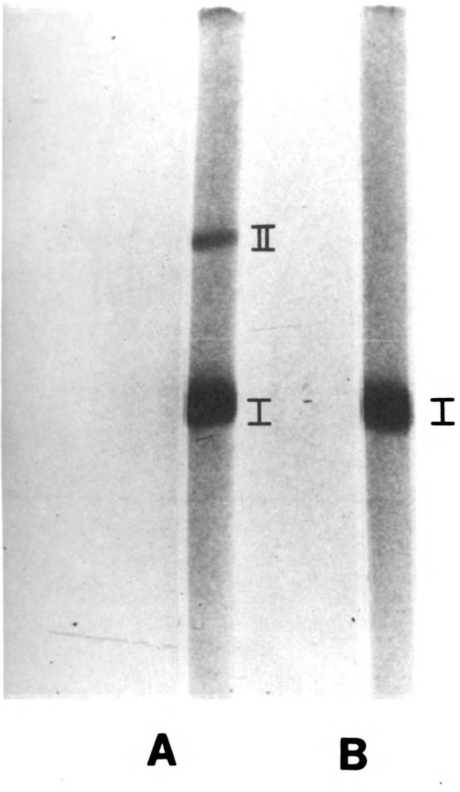


Figure 4

Figure 5. Precipitin band patterns in agar gel duffision tests of T- and NT-PEMV antisera (1:5 and 1:10, respectively) and anti-pea protein antiserum (undiluted) against various antigens. Antisera were: Anti-pea protein (pp as), T-PEMV antiserum (t as) and NT-PEMV antiserum (nt as).

(A) Reactions of: (1) = purified T-PEMV 0.2 mg/ml; (2) = purified NT-PEMV 0.2 mg/ml; (3) = pea protein 4 mg/ml; (4) = pea protein 2 mg/ml; (5) = not loaded; (6) = pea protein 1 mg/ml.

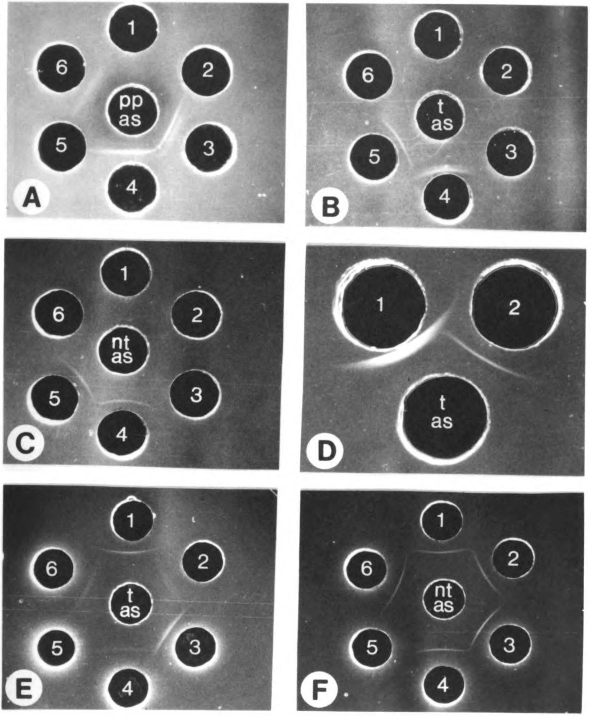
(B) Reactions of: (1) = pea protein 10 mg/ml; (2) = pea protein 2 mg/ml; (3) = pea protein 2 mg/ml; (4) = purified T-PEMV 0.2 mg/ml; (5) = purified NT-PEMV 0.2 mg/ml; (6) = pea protein 4 mg/ml.

(C) Outer wells same as (B).

(D) Reactions of T-PEMV antiserum (t as) produced by injecting T-PEMV suspended in 0.1 M pH 6.0 sodium acetate buffer against: (1) = partially purified T-PEMV; (2) = partially purified NT-PEMV.

(E) Reactions of: (1) = purified T-PEMV 0.8 mg/ml; (2) = purified NT-PEMV 0.8 mg/ml; (3) = T-PEMV expressed sap; (4) = NT-PEMV expressed sap; (5) = expressed sap from healthy (non-infected) pea plants; (6) = NT-PEMV expressed sap.

(F) Outer wells same as (E).


1
6
2
pp
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as
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B
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6
2
nt
as
5
3
4
C
1
2
t
as
D
1
6
2
t
as
5
3
4
E
1
6
2
nt
as
5
3
4
F

Figure 5

Figure 6. Reactions of T- and NT-PEMV antisera (1:5 and 1:10, respectively) against various purified states of T- and NT-PEMV antigens.

(A) and (B) Reactions of: (1) = T-PEMV expressed sap; (2) = NT-PEMV expressed sap.

(C) and (D) Reactions of: (1) = partially purified T-PEMV; (2) = partially purified NT-PEMV.

(E) and (F) Reactions of: (1) = purified NT-PEMV 0.8 mg/ml; (2) = purified T-PEMV 0.8 mg/ml.

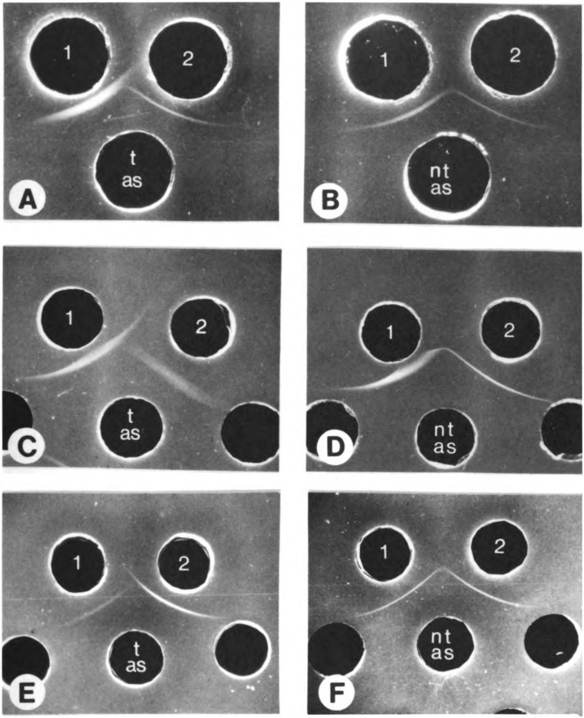

Figure 6

Figure 7. Reactions of absorbed T- and NT-PEMV antisera and viral antigens; T- and NT-PEMV antisera and soluble protein fractions.

(A) Reactions of: (1) = T-PEMV antiserum absorbed with NT-PEMV antigen; (2) = purified T-PEMV 0.2 mg/ml; (3) = purified NT-PEMV 0.2 mg/ml; (4) = T-PEMV antiserum unabsorbed.

(B) Same as (A) except NT-PEMV antiserum absorbed with purified T-PEMV antigen is in well (1).

(C) Reactions of: (1) = T-PEMV antiserum (1:40); (2) = T-PEMV soluble protein fraction; (3) = NT-PEMV soluble protein fraction; (4) = NT-PEMV antiserum (1:80).

(D) Diagramatic representation of: (1) = T-PEMV antiserum (1:40); (2) = T-PEMV soluble protein fraction; (3a) = T-PEMV expressed sap; (3b) = NT-PEMV expressed sap; (4) = NT-PEMV antiserum (1:80).

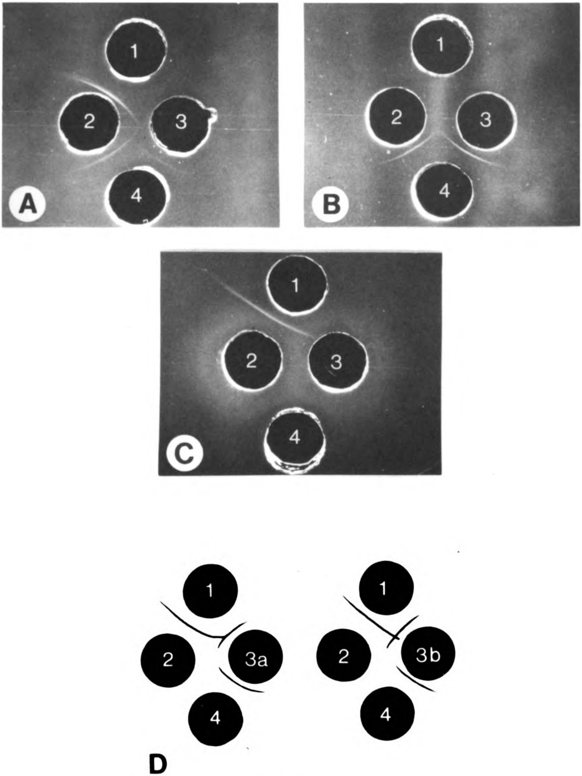

Figure 7

Figure 8. Reactions to test the specificity of absorbed T-PEMV antiserum (1:12) for aphid-transmissible PEMV isolates. (1) = absorbed T-PEMV antiserum; (2) = NT-PEMV antiserum (1:20); (3) = T-PEMV antiserum (1:10); (4) = expressed sap from a ten-day virus infected pea plant of each virus isolate. See Table 5 for explanation of isolates.

(A) NT-C

(B) NT-B

(C) NT-C

(D) NY-NT

(E) NT-T

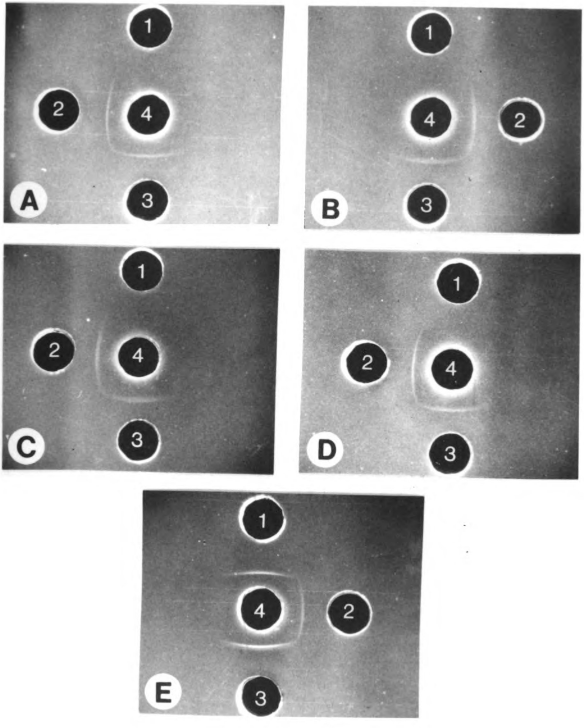

Figure 8

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