PEA ENATION MOSAIC VIRUS: CHARACTERISTICS OF PURIFIED STRAINS DIFFERENTIALLY TRANSMITTED BY THE VECTOR, ACYRTHOSIPHON PISUM (HARRIS)

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PEA ENATION MOSAIC VIRUS: PURIFICATION AND PURIFIED
STRAINS DIFFERENTIALLY TRANSMITTED BY THE VECTOR,
ACYRTHOSIPHON PISUM (HARR.)

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Experiments were conducted to identify some factors affecting membrane-feeding assay of purified suspensions of a highly transmissible PEMV strain and to contrast UV absorptive spectra and nucleoprotein yields of partially purified suspensions of PEMV strains and variants with widely different aphid transmission characteristics. The pea aphid, Acyrthosiphon pisum (Harris), was used throughout.

PEMV purification procedure, plant host, tissue age and degree of virus purity directly influenced aphid transmission via membrane-feeding assay. Transmissibility of a New York strain (NY-PEMV) varied with the purification procedure and was dependent on aphid feeding behavior as well as concentration of virus in suspension. Greatly improved aphid feeding and subsequent virus transmission was achieved by removal of the solvent and/or chelating agent sorbitol used in certain of the purification procedures from partially purified PEMV suspensions, either through dialysis or dilution.

NY-PEMV partially purified from 5-, 10-, and 15-day infected pea tissue was transmitted by 1st instar pea aphids with 30, 40 and 11% efficiency, respectively, but no transmission was obtained with virus

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ABSTRACT

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from 30-day infected tissues. Highly purified PEMV suspensions were aphid-transmitted with less efficiency than partially purified suspensions, even though virus concentrations were higher in the former than the latter.

Nucleoprotein yields and sedimenting component ratios of PEMV were found to vary with season of the year when inoculations were made and with age of pea source tissues used for partial virus purification. NY-PEMV and CALIF-PEMV strains which vary in aphid-transmissibility could not be differentiated on the basis of yield or component ratios. However, nonaphid-transmissible (CNT-PEMV) and aphid-transmissible (CT-PEMV) variants of the CALIF-PEMV strain were separable on the basis of nucleoprotein yield and component ratio when source peas used for partial purification were grown in controlled environmental chambers. CNT-PEMV produced higher concentrations of top component and greater nucleoprotein yields (often 10 X higher) than did CT-PEMV. The 2 sedimenting components of NY-PEMV were aphid-transmitted with about equal efficiency, 84% vs 89%.

PEMV was successfully purified from pea aphids and in sufficiently high concentrations to be monitored by sucrose density gradient fractionation and UV-spectrophotometric analysis. Electron micrographs showed the particles to be indistinguishable from those isolated from infected plants. Furthermore, virus suspensions were highly transmissible to pea plants by pea aphids or mechanical means.

Virus purified from aphids was established in pea plants by mechanical transmission and compared as to aphid-transmissibility with purified virus from a plant source. The aphid-purified virus source

line was transmitted by 1st instar pea aphids with significantly ($P < 0.05$) higher efficiency than the plant-source virus line after 1- and 4-hour acquisition-access periods and with characteristics superior to those recorded for any PEMV-pea aphid relationships. First instar pea aphids were 95.8% efficient in transmission of the aphid-purified virus source line after only a 4-hour acquisition-access period on infected pea plants and this virus line had a median latent period (LP_{50}) in the 1st instar of only 5.7 hours at 25° C. Other comparable LP_{50} estimates on record are 25.0 hours at 20° C. and 14.0 hours at 30° C.

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By

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GENERAL INTRODUCTION

Most plant viruses are dependent on arthropod or nematode vectors for dissemination and inoculation in nature. But in general only a relatively few viruses are transmitted by a given vector species and only a limited number of vector species transmit a given virus. While the degree of this specificity varies between viruses and vectors, it is most pronounced in the circulative aphid- and leafhopper-borne viruses. Not only is vector-virus specificity present between viruses and vector species but also between virus strains and vector biotypes or races. An understanding of the mechanism which controls vector-virus specificity should enable development of virus disease control procedures that are founded on breaking the compatibility between vector and virus rather than destroying the vector. The literature on vector-virus specificity has been reviewed (Rochow, 1963, 1969).

The aphid-borne (circulative type) pea enation mosaic virus (PEMV) is well suited for studies of vector-virus specificity since its aphid-transmission characteristics are well defined (see review by Harris, 1971); it consists of at least 2 strains that are transmitted with widely different efficiencies by a common pea aphid biotype (Bath and Tsai, 1969); a single strain can be transmitted with markedly different efficiencies by various pea aphid biotypes (Bath and Chapman, 1967; Tsai et al., 1972); and it can be perpetuated by

mechanical, as well as aphid means. The chemical and physical properties of the virus have been extensively characterized (Bozarth and Chow, 1966; Gibbs et al., 1966; Izadpanah and Shepherd, 1966; Shepherd et al., 1968; Musil et al., 1970; Gonsalves and Shepherd, 1972), and some information on the relationship of the virus to pea aphid vector and host plant tissues is available (Shikata, et al., 1966; Shikata and Maramorosch, 1966; de Zoeten, et al., 1972; Harris and Bath, 1972).

To date, research on vector-virus specificity within the pea enation mosaic virus and aphid-vector populations has centered on (a) definition of the transmission (plant-to-plant) characteristics of various strains and isolates by various vector species and biotypes (Bath and Chapman, 1966, 1967; Chapman and Bath, 1968; Bath and Tsai, 1969; Thottappilly et al., 1972; Tsai et al., 1972) and (b) elucidation of the relationship of a highly transmissible PEMV strain (NY) to the tissues of a most efficient pea aphid biotype (Harris and Bath, 1972). Future advances in this mission-oriented research program will undoubtedly be achieved through studies of the fate of various PEMV strains and isolates in various vector biotypes and the relationship of viral and vector composition to transmission efficiencies. Success in both of these approaches is largely dependent on the use of purified virus preparations from both the infected plant and the infested (or infected?) aphid and on systems for assaying infectivity and transmissibility of various experimentally manipulated virus preparations. Recently, the assay problem was alleviated by development of a technique whereby virus suspensions are injected into the vector's hemocoel and an artificial membrane-feeding technique through which virus suspensions are fed to aphids. Both techniques were shown to

be very suitable for PEMV-pea aphid studies (Thottappilly et al., 1972; Clarke and Bath, 1973).

My objectives were to: (a) characterize the nucleoprotein components and yields of PEMV strains and variants of widely different aphid-transmissibilities; (b) determine the suitability of several purification methods for the preparation of virus for membrane-feeding assay; and (c) develop a regime for purification of PEMV from infested (infected?) pea aphids and contrast aphid-transmissibility of virus purified from plant and vector sources.

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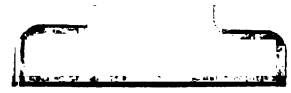

INTRODUCTION

Previously we showed that partially purified suspensions of the aphid-borne (circulative type) pea enation mosaic virus (PEMV) can be assayed for transmissibility by feeding aphids on virus suspensions across an artificial membrane (Thottappilly et al., 1972). Membrane-feeding of virus to test insects has much utility in the study of fundamental vector-virus relationships since the concentration of the virus in the source solution can be controlled and virus acquired during membrane-feeding [illegible] in the vector [illegible]. However, the virus suspension used for membrane-feeding must be relatively free of chemicals which inhibit aphid feeding and thereby virus acquisition.

During our initial study (Thottappilly et al., 1972) in which we used a modification of the method reported by Bozarth and Chow (1966) to obtain partially purified virus that was efficiently transmitted by aphids after membrane-feeding, we occasionally and superficially tested the transmissibility, by membrane-feeding, of virus obtained through other purification procedures. Limited results indicated that virus suspensions yielded by various purification procedures varied considerably in suitability for use in membrane-feeding studies.

In the study described herein, our objectives were to (a) compare directly the suitability of several procedures for preparing

PART I: APHID-TRANSMISSIBILITY OF PEA ENATION MOSAIC VIRUS PREPARED BY VARIOUS PURIFICATION REGIMES

INTRODUCTION

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In the study described herein, our objectives were to (a) compare directly the suitability of several procedures for preparing

partially purified PEMV suspensions that were both relatively free of nonviral contamination and transmissible by membrane-feeding assay; (b) test the transmissibility, by membrane-feeding, of virus purified by rate zonal density gradient centrifugation; and (c) determine the influence of source tissue age on assay of partially purified virus by membrane-feeding.

MATERIALS AND METHODS

Virus purification.--A highly aphid-transmissible isolate of NY PEMV (Bath and Tsai, 1969) was used exclusively; it was maintained in garden pea (Pisum sativum L. 'Midfreezer') and perpetuated by twice-monthly mechanical transfers of expressed sap from diseased plants. Virus source plants were obtained by mechanically inoculating young P. sativum seedlings prior to time of lst-leaf expansion. Unless otherwise noted, tissue for virus purification was harvested 10-12 days after inoculation; only tissue from plants with severe symptoms was used.

Five methods were used to partially purify PEMV from its host tissues; these methods are contrasted in Table 1 and will hereafter be referred to by the designated Roman numeral. Methods I-IV were originally designed for PEMV purification and were followed precisely in our work. Method V was designed for purification of tobacco ringspot virus and was modified as follows: 0.1 M, pH 7.0 potassium phosphate buffer with 5% sucrose was added (1:1 v/v) to the chloroform-butanol extraction solvent; the number of ultracentrifugations was

TABLE 1. Legend to methods used to obtain partially purified pea enation mosaic virus by differential centrifugation for comparative transmission trials

Method no.	Clarification solvent	Buffer used for virus extraction and resuspension	Dialysis used?	Reference for details of method
I	Chloroform	0.05 M, pH 6.0 potassium phosphate	Yes	Bozarth & Chow (1966)
II	Chloroform	0.1 M, pH 6.0-7.0[b] potassium phosphate with 5% sucrose	Yes	Thottappilly et al. (1972)
III	Chloroform +0.05 M EDTA[a]	0.03-0.08 M[c], pH 7.5 potassium phosphate	No	Gibbs, et al. (1966)
IV	None	0.1 M, pH 6.0 sodium acetate	No	Izadapanah & Shepherd (1966)
V	Chloroform & Butanol (1:1)	0.1 M, pH 7.0 potassium phosphate with 5% sucrose	Yes	Steere (1956)[d]

[a] EDTA = ethylenediaminetetraaceticacid.

[b] pH 6.0 in extraction buffer and pH 7.0 in resuspension buffer.

[c] 0.08 M used for extraction, 0.03 M used for resuspension.

[d] Modified from the original by (1) adding 0.1 M, pH 7.0 potassium phosphate buffer with 5% sucrose to the chloroform-butanol extraction solvent (v/v); (2) reducing the number of ultracentrifugations to 2; (3) including a 48-hr dialysis of the 1st lowspeed supernatant against 0.05 M, pH 7.0 potassium phosphate buffer; and (4) resuspending each pellet in 0.1 M, pH 7.0 potassium phosphate buffer with 5% sucrose.

reduced to 2; and a 48-hr dialysis of the lowspeed supernatant against 0.05 M, pH 7.0 potassium phosphate buffer was included.

These methods varied mainly in the type, molarity, and pH of the buffers used in the virus extraction and resuspension phases of purification, and in the types of solvents and chelating agents used for virus extraction (Table 1).

Analysis of purified preparations.--Rate-zonal density gradient analysis was performed by layering one-half to 1 ml of each virus preparation (either undiluted or adjusted to A_{260} nm = 2) on linear 10-40% sucrose (in 0.02 M, pH 7.0 potassium phosphate buffer) columns, centrifuging the columns for 2 hr at 24,000 rpm in a SW25.2 rotor of the model L Beckman ultracentrifuge, and monitoring the columns for UV absorbance (at 254 nm) with an ISCO density gradient fractionator and UV analyzer. Virus concentrations, were determined through planimetry with viral absorbance peaks and conversion of areas to weight of virus per unit volume.

Bioassay of preparations.--The EL biotype (Tsai et al., 1972) of the pea aphid, Acyrthosiphon pisum (Harris), was used to test aphid-transmissibility of the various virus preparations. Aphids were reared on Vicia faba L. First-stage nymphs were used exclusively and were obtained by transferring viviparous adults to V. faba plants for a 12-18 hr nymph-deposition period. Aphids acquired virus by membrane-feeding for specific periods on 0.2 ml of the virus preparation which was adjusted with appropriate buffer to a specific A_{260} nm concentration. The details of membrane-feeding were described earlier (Thottappilly et al., 1972). After completion of membrane-feeding, aphids were

transferred singly to very young P. sativum 'Midfreezer' seedlings for inoculation-access periods of 3-4 days.

Virus preparations also were mechanically inoculated to Midfreezer pea (systemic host) and to Chenopodium amaranticolor Coste and Reyn. (local lesion host) with the aid of glass spatulas and carborundum. The former plants were in the pre-leaf stage and the latter in the 4-5 leaf stage.

RESULTS

Transmissibility of partially purified virus prepared by various differential centrifugation methods.--In the first of 2 experiments, partially purified virus suspensions were obtained through each of 5 procedures (Table 1). Each purification procedure was initiated on the same day with 40-50 g of infected tissue and was completed within 3 days. Most of the virus pellets obtained by each method were resuspended in the same buffer used for virus extraction except for minor changes in pH (Method II) and molarity (Method III); some pellets obtained by Methods I, III and IV were resuspended in 0.1 M, pH 7.0 potassium phosphate buffer with 5% sucrose. Each suspension was held at 4 C until it was bioassayed. On the 4th day, 2 portions of each suspension were fed to aphids during a 4-hr acquisition-access period; one portion was adjusted with the appropriate buffer to A_{260} nm = 7.5, the other portion was not adjusted. Each portion was fed to 30 aphids in a single feeding-chamber (trial 1). Identical portions were mechanically inoculated to 10 pea seedlings and to the terminal 2 fully-expanded leaves on each of 2 C. amaranticolor plants.

The unused portions of each suspension were frozen. On the 5th day, a portion of each frozen virus suspension was thawed, and the aphid-transmission test was repeated (trial 2). On the 6th day, 0.5 ml of each suspension was thawed, adjusted to $\underline{A}_{260}$ nm = 2 and subjected to rate-zonal density gradient analysis.

The various virus suspensions varied considerably in absorbance at 260 nm, aphid-transmissibility, and weight of virus in suspension (Table 2). Variation in transmission rate between trials and between the adjusted and unadjusted suspensions was extraordinarily low, even though the latter suspensions usually contained much less virus than the former. Transmissibility appeared to be regulated by aphid-feeding behavior and the quantity and quality of the virus in suspension.

Aphids hesitated to feed on the 0.03 M, pH 7.5 potassium phosphate suspension of virus prepared by Method III and on both suspensions obtained from Method IV; but aphids appeared to feed better on the $\underline{A}_{260}$ = 7.5 preparation of each suspension than on the unadjusted portion. Since virus from each of these 3 suspensions was transmitted with poorer efficiency (in relation to virus concentration in test suspension) than virus from all other suspensions, reduced ingestion rate was considered to be primarily responsible for poor transmission.

Virus obtained by Method I and suspended in 0.05 M, pH 6.0 potassium phosphate buffer was transmitted with higher efficiency than all others tested, even though an $\underline{A}_{260}$ = 7.5 suspension contained only 18 µg of virus per ml--an amount exceeded by all other $\underline{A}_{260}$ = 7.5 suspensions tested except that of Method I suspended in 0.1 M, pH 7.0 phosphate buffer with 5% sucrose (Table 2). Method II, while quite similar to Method I, yielded more virus per an $\underline{A}_{260}$ = 7.5 suspension,

TABLE 2. Pea aphid (1st-instar) transmission of pea enation mosaic virus partially purified by various methods

Method no.	Buffer used to resuspend final virus pellet	% Transmission of specified virus suspensions[a]				Concentration of virus (μg/ml) in specified suspensions	
		Unadjusted		A_{260} nm = 7.5			
		Trial 1	Trial 2	Trial 1	Trial 2	Unadjusted[b]	A_{260} = 7.5
I	0.05 M, pH 6.0 potassium phosphate	93.3	93.1	96.7	96.3	155 (62)	18
	0.1 M, pH 7.0 potassium phosphate with 5% sucrose	70.0	60.7	70.0	70.4	144 (85)	13
II	0.1 M, pH 7.0 potassium phosphate with 5% sucrose	80.0	82.1	76.7	75.9	287 (19)	113
III	0.03 M, pH 7.5 potassium phosphate	43.3	44.8	56.7	57.7	1399 (104)	101
	0.1 M, pH 7.0 potassium phosphate with 5% sucrose	66.7	63.0	76.7	67.9	667 (132)	38
IV	0.1 M, pH 6.0 sodium acetate	0	0	0	3.6	370 (11)	252
	0.1 M, pH 7.0 potassium phosphate with 5% sucrose	40.0	24.1	40.0	30.8	543 (19)	214
V	0.1 M, pH 7.0 potassium phosphate with 5% sucrose	26.7	23.3	23.3	36.7	40 (8)	38

[a] Aphids were given a 4-hr acquisition-access feed and transferred singly to healthy test plants; 30 aphids were used per source; trials 1 and 2 were conducted on consecutive days.

[b] No. in parenthesis = A_{260} readings for unadjusted suspensions.

and the suspension contained less nonviral material; however, the virus was transmitted with less efficiency, in relation to concentration, than was virus obtained by Method 1.

While virus obtained from Methods III and IV was inefficiently and poorly transmitted when suspended in the buffers used for extraction, aphid-transmissibility of both viruses was enhanced markedly by suspending the final virus pellet in 0.1 M, pH 7.0 phosphate buffer with 5% sucrose. Method V yielded relatively little virus (40 µg/ml of unadjusted suspension) but it was aphid-transmitted with efficiency intermediate to the others tested, in relation to concentration.

The UV scanning patterns of each virus in density gradient tubes revealed that the virus prepared by Method IV was the most nearly pure even though the suspension was light-green; the meniscus of the gradient was devoid of UV-absorbing material and sharp peaks of top and bottom component were nearly identical to those reported earlier (Izadpanah and Shepherd, 1966). Furthermore, the very low $\underline{A}_{260}$ nm reading for the unadjusted suspension and the relatively high concentration of virus (214-252 µg/ml) in the $\underline{A}_{260}$ nm = 7.5 suspensions of Method IV preparations, indicated relatively little nonviral material was present in the suspensions.

On the other hand, the scanning pattern of Method III virus was characterized by a relatively high and deep absorbance pattern at the meniscus of the gradient; this too is reflected in the high $\underline{A}_{260}$ nm readings for the 2 unadjusted virus suspensions of Method III and the relatively low actual virus concentrations in the $\underline{A}_{260}$ nm = 7.5 preparations. Virus suspensions obtained from Methods I, II and V contained intermediate amounts of nonviral material.

In a second experiment, partially purified virus was obtained by modifications of Methods II, III and IV. Methods III and IV were modified by adding a 48-hr dialysis period to the procedure just prior to the final or only ultracentrifugation step, and the dialysis of Method II was lengthened to 48-hr. In each case, dialysis was against several changes of 0.1 M, pH 7.0 potassium phosphate buffer. Aphid-transmissibility was assayed immediately after an overnight period of virus resuspension and in the same manner as used in the first experiment. Both unadjusted and $\underline{A}_{260}$ nm = 7.5 suspensions of virus prepared by modified Methods II and III were tested, but the virus prepared by modified Method IV was tested only as an unadjusted suspension.

Although the results of the 2 experiments are not directly comparable, it was apparent that dialysis appreciably increased the aphid-transmissibility of virus prepared by Methods III and IV (Table 3). The increase of dialysis time in Method II did not appear to enhance transmission, and in the case of the unadjusted suspension lower transmission resulted from the use of the 48-hr than the 24-hr dialysis. The $\underline{A}_{260}$ nm = 7.5 suspension of the modified Method III partially purified virus contained a relatively high concentration (235 µg/ml) of virus--a concentration exceeded only by the virus prepared by Method IV in the first experiment. On the other hand, an $\underline{A}_{260}$ = 7.5 suspension of virus prepared by modified Method IV contained only 12 µg/ml, in contrast to more than 200 µg/ml produced by Method IV (without the dialysis), even though the unadjusted $\underline{A}_{260}$ nm of the former was more than twice that of the latter preparation.

TABLE 3. Pea aphid (1st-instar) transmission of pea enation mosaic virus partially purified by modified Methods II, III and IV[a]

Modified method no.	% Transmission of specified virus suspensions		Concentration of virus (μg/ml) in specified suspensions	
	Unadjusted	A_{260} nm = 7.5	Unadjusted[b]	A_{260} = 7.5
II	50.0	70.0	160 (37)	35
III	96.6	96.6	2280 (67)	235
IV	33.3	-	73 (42)	12

[a]Methods III and IV were modified by addition of a 48-hr dialysis period prior to the final or only ultracentrifugation step; in Method II the dialysis was lengthened from 24 to 48 hr. All dialyses were against several changes of 0.1 M, pH 7.0 potassium phosphate buffer. See footnote of Table 2 for transmission details.

[b]No. in parenthesis = A_{260} reading for unadjusted suspension.

Transmissibility of virus purified by rate zonal density gradient centrifugation.--Duffus and Gold (1965) found that the aphid-transmissibility of beet western yellows virus purified by rate zonal density gradient centrifugation (DGC) was far superior to that of virus partially purified by differential centrifugation, presumably because the former preparations were in higher concentration than the latter. To characterize the aphid-transmissibility of DGC-purified PEMV suspensions, I thawed an A_{260} nm = 210 partially purified (by Method II) preparation; subjected 1 ml portions of an A_{260} nm = 30 suspension to rate zonal DGC analysis; collected the major portion of the virus band from each gradient tube; combined all fractions of virus; dialyzed the combined suspension for 3 hr against 3 changes of 0.5 M, pH 7.0 potassium phosphate buffer (to remove much of the sucrose from the

preparation); reconcentrated the virus by centrifugation at 45,000 rpm for 2 hr at 4 C; and resuspended the purified virus (overnight) in 0.1 M, pH 7.0 potassium phosphate buffer with 5% sucrose. The aphid-transmissibility of the partially purified virus was monitored soon after it was thawed by feeding portions adjusted to $\underline{A}_{260}$ nm = 1.9, 7.5 and 30 to 40-42 1st-instar aphids for 5 hr. Purified virus infectivity was monitored at each of 3 stages in the final purification procedure. Forty aphids were given 5-hr feeding periods on suspensions immediately (a) after the virus was fractionated from the gradient, (b) after the dialysis period, and (c) after the reconcentrated virus was resuspended and adjusted to $\underline{A}_{260}$ nm = 7.5, or presumably 1.0 mg/ml; the 3rd suspension was fed to aphids for 5 hr and 12 hr. All suspensions also were mechanically inoculated to 7-12 young pea seedlings.

The partially purified virus suspensions were transmitted with high efficiency by aphids and 26.2% transmission was attained at a concentration ($\underline{A}_{260}$ nm = 1.9) that was not mechanically transmissible (Table 4). The $\underline{A}_{260}$ nm = 1.9, 7.5 and 30.0 concentrations were found to actually contain 0.026, 0.104 and 0.416 mg of virus per ml of solvent. With the exception of the virus preparation tested immediately after fractionation, the DGC-purified suspensions were poorly transmitted by aphids even though mechanical transmissibility of the same suspension was high, and virus concentrations were higher than those of the partially purified preparations. Whereas the concentration of the fractionated virus was calculated to be only 0.096 mg/ml of gradient solution, it was transmitted by 95% of the test aphids.

TABLE 4. Comparative transmission of pea enation mosaic virus as partially purified and density gradient purified suspensions

Preparations	% Transmission		Virus concentration (mg/ml)
	Aphids[a]	Mechanical[b]	
Partially Purified			
At A_{260} nm = 1.9	26.2	0	.026
7.5	82.9	71.4	.104
30.0	95.1	100	.416
DGC-Purified			
From gradient	95.0	28.6	.096
After dialysis:			
not reconcentrated	21.4	28.6	ca. .060
reconcentrated (A_{260} nm = 7.5)			
5-hr AAP	33.3	100	ca. 1.0
12-hr AAP	61.9	100	1.0

[a] Forty to 42 1st-instar pea aphids were tested singly after a 5-hr acquisition-access period (AAP) on each virus preparation; a 12-hr period also was tested for the purified, reconcentrated preparation.

[b] Seven to 12 young pea seedlings were mechanically inoculated with each preparation.

In a follow-up experiment, the purification procedure from tissue to a final DGC-purified virus suspension was conducted with rapidity and completed in 24 hr. Fractionated virus was dialyzed 4 hr prior to a 2-hr centrifugation at 45,000 rpm and 4 C, and the pellet re-suspension period was 2 hr. Thirty-nine to 42 1st-instar pea aphids

were fed suspensions of (a) partially purified virus adjusted to $\underline{A}_{260}$ nm = 30, (b) DGC-purified virus after fractionation from a gradient which was layered with 0.5 ml of an $\underline{A}_{260}$ nm = 320 suspension, and DGC-purified virus after reconcentration, resuspension and adjustment to (c) 0.25, (d) 1.0, and (e) 4.0 mg of virus per ml of buffer with 5% sucrose. Because the gradients were overloaded with virus, the UV scanning pattern did not discern virus from other components of the suspension; thus fractionation was done by collecting samples from usual virus-containing positions in tubes, and calculation of actual virus concentration in the partially purified suspensions was not possible.

Transmission results compared closely with those obtained in the earlier experiment. Whereas 87.2% of the aphids transmitted virus from the $\underline{A}_{260}$ nm = 30 partially pure preparation and 100% transmitted the virus fed directly from the gradient, only 23.8, 47.5 and 72.5% transmitted the DGC-purified suspension at concentrations of 0.25, 1.0 and 4.0 mg/ml, respectively. All preparations were 100% mechanically transmissible.

Transmissibility of partially purified virus obtained from source tissue of various ages.--About one hundred pea seedlings were mechanically inoculated with PEMV 6, 10, 20, and 30 days before they were used as source plants for virus purification. Forty to 50 g of leaf tissue with severe symptoms were processed immediately after harvest for each of the 4 tissue-age treatments; partial purification was accomplished by Method II (Table 1). Virus pellets obtained from each purification run were resuspended in 0.1 M, pH 7.0 potassium

phosphate buffer with 5% sucrose. The infectivity of the virus from each purification was determined by (a) aphid-transmission assay in which 1st-instar aphids were fed an A_{260} nm = 6 preparation for 15 min and 4 hr and (b) mechanical-transmission assay involving inoculations of A_{260} nm = 6 preparations to C. amaranticolor and P. sativum.

The virus purified from 6 and 10-day old tissue was considerably more infective by aphid- or mechanical-transmission assay than was virus from 20- and 30-day old sources (Table 5). While virus from 6-day-old tissue was transmitted by 80% of the aphids after a 4-hr acquisition-access period, no transmission of virus from the 30-day-old tissue was obtained. These results are similar to those reported by Izadpanah and Shepherd (1966) except that they found 6-7 day-old tissue to contain very little virus. Apparently the NY PEMV strain that we tested reaches higher titres in 6 days than did the strain used by Izadpanah and Shepherd (1966) but both strains reach a peak in virus titre at or near the 10th day of infection.

DISCUSSION

It is most desirable to use membrane-feeding assay in aphid-transmission studies of purified virus preparations since virus acquired through a membrane follows essentially the same course in the aphid as virus acquired from plants. While aphids can also be made infective through injection of purified PEMV suspensions into the hemocoel (Clarke and Bath, 1973), the virus short-cuts much of the circulative route and presumably bypasses some potential sites of inactivation and/or barriers to circulation, e.g. the membranes of the

TABLE 5. Aphid- and mechanical-transmissibility of pea enation mosaic virus partially purified from source plants of varying ages

Age of source[a]	Percent transmission[b] by aphids after acquisition feeds of:		Mechanical inoculation[c]	
	15 min	4 hr	Percent transmission to P. sativum	Mean number of local lesions on C. amaranticolor[d]
days				
6	27.7	80.0	80.0	14.7
10	50.0	60.7	100.0	19.3
20	0	33.3	40.0	8.4
30	0	0	46.7	6.6

[a] Time in days after mechanical inoculation; all plants were inoculated on the same day with the same inoculum.

[b] Thirty 1st-instar aphids were tested singly per treatment; a 4 day inoculation access period was used.

[c] Fifteen young seedlings of P. sativum and 2 leaves on each of 4 young C. amaranticolor plants were rubbed per treatment.

[d] Mean number of lesions per leaf.

gut epithelium and the enzymes of the digestive tract. Unfortunately, membrane-feeding assay is handicapped by its dependence on aphid feeding behavior which is greatly influenced by feeding inhibitors that often are present in feeding solutions, such as virus suspensions extracted and partially purified from plant or aphid tissues. Since rate of feeding affects rate of virus ingestion and ultimate transmission efficiency, it is to be expected that the same virus in various solutions, or in the same solution at various times, will be transmitted with various efficiencies. Membrane-feeding is therefore

much more reliable as a qualitative than quantitative measure of virus transmissibility (Whitcomb, 1969).

Direct aphid-transmission comparisons of the several partially purified (Methods 1-5) virus preparations indicated that all methods yielded suspensions that contained appreciable amounts of aphid-feeding and/or virus inhibitors. The presence of inhibitors was indicated by transmission data (Table 2) which showed that virus was transmitted from diluted suspensions (A_{260} = 7.5) with nearly equal or greater efficiency than it was from concentrated suspensions. In these trials, it was apparent that reduction in aphid-feeding inhibitors and/or virus inhibitors compensated for the negative transmission effects expected from a reduction in the concentration of virus fed to the aphids. Thus, even though aphids were exposed to less virus either they were able to acquire nearly the same amount of virions and/or the acquired virus was less affected by inhibitors to circulation in, and transmission by the vector.

While virus inhibitors are usually of plant origin, it was apparent in our comparisons that some of the aphid-feeding inhibition originated with the purification procedure. Virus suspensions obtained via Method IV were virtually free of nonviral host material yet aphids were noticeably reluctant to feed on it. Chemicals used for virus extraction, stabilization and resuspension may have adverse effects on membrane-feeding assay. Residues of sodium acetate, chloroform, butanol, and perhaps EDTA appeared to be inhibitory to aphid feeding. The use of dialysis appears to be beneficial in ridding low speed supernatant suspensions of feeding inhibitors, but we experienced an apparent loss of virus stability when a 48-hr

dialysis was used in Methods II and IV. While the sitting and/or dialysis time is useful in dissipating chemical residues, it undoubtedly affects virus stability if it is of too long a duration. A dialysis of about 24-hr appears to be most suitable.

Of the various procedures tested for preparing partially purified PEMV, Method I (Bozarth and Chow, 1966) yielded virus with the highest aphid-transmissibility in relation to virus concentration, regardless of whether the final pellet was resuspended and fed to aphids in 0.05 M, pH 6.0 potassium phosphate or 0.1 M, pH 7.0 potassium phosphate with 5% sucrose. The ratio of percent transmission to µg of virus per ml was 5.4 for both suspensions adjusted to $A_{260} = 7.5$. This ratio was much higher than any others tested. Furthermore, the virus suspension in 0.05 M, pH 6.0 phosphate buffer was probably even more transmissible for aphids possibly did not ingest as much of it as the virus in 0.1 M, pH 7.0 buffer with sucrose suspension. Sucrose, a feeding stimulant was absent and yet virus was transmitted near maximum even at the lowest concentration tested. The superior transmissibility of this preparation even though it still contained appreciable nonviral host material, suggests that the virus is more stable, transmissible and infectious when purified in buffers at pH 6.0 than at higher pH's. We find it noteworthy that partial purification by Methods III (Gibbs, et al., 1966) and IV (Izadpanah and Shephard, 1966), were superior to others for obtaining high yields of virus and virus suspensions nearly devoid of nonviral material.

Our failure to obtain highly aphid-transmissible virus preparations by density gradient centrifugation appears to indicate that the

nonviral material in partially purified preparations enhances transmissibility or that during the lengthy process of DGC, dialysis and reconcentration there occurred some loss of infectivity and subsequent aphid-transmissibility. Even though this loss of infectivity was not apparent from the mechanical assay tests (100% of the peas were infected) it still could be sufficiently reduced to result in lowered transmission by aphids. Also, increasing the rapidity of the purification process from tissue to final DGC-purified preparation didn't improve the aphid-transmissibility significantly. It seems likely that nonviral material in partially purified preparations adds some protection to the virus as it enters the aphid system.

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INTRODUCTION

Several studies on the intrinsic properties of the aphid-borne (circulative-type) pea enation mosaic virus have been reported (Bozarth and Chow, 1966; Gibbs, et al., 1966; Izadpanah and Shepherd, 1966; Shepherd, et al., 1968; Musil, et al., 1970; Gonsalves and Shepherd, 1972). Although these studies have been conducted with various PEMV isolates, no apparent attempt has been made to directly contrast nucleoprotein components and yields of isolates which vary

PART II: CHARACTERIZATION OF NUCLEOPROTEIN COMPONENTS OF STRAINS OF PEA ENATION MOSAIC VIRUS THAT DIFFER IN APHID-TRANSMISSIBILITY

Two strains of PEMV

aphid-transmissibility,

the pea aphid, Acyrthosiphon pisum (Harris), where NY-PEMV was efficiently transmitted (Bath and Tsai, 1969). Furthermore, pea aphid biotypes that vary in capabilities of transmitting either virus strain have been isolated (Tsai, et al., 1972).

The objectives of this study were to directly contrast the nucleoprotein yield and sedimenting component ratios of (a)

and NY-PEMV strains and (b) two CALIF-PEMV variants with aphid-transmissible (CT-PEMV) and nonaphid transmissible

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Two strains of PEMV were separated previously on the basis of aphid-transmissibility; CALIF-PEMV was inefficiently transmitted by the pea aphid, Acyrthosiphon pisum (Harris), where NY-PEMV was efficiently transmitted (Bath and Tsai, 1969). Furthermore, pea aphid biotypes that vary in capabilities of transmitting either virus strain have been isolated (Tsai, et al., 1972).

The objectives of this study were to directly contrast the nucleoprotein yield and sedimenting component ratios of (a) CALIF- and NY-PEMV strains and (b) two CALIF-PEMV variants with aphid-transmissible (CT-PEMV) and nonaphid transmissible (CNT-PEMV) characteristics (Tsai and Bath, unpublished). Data are also presented to show that component ratios are affected by season of source plant incubation and age of source plant tissue.

MATERIALS AND METHODS

Virus strains and variants.--Garden pea (Pisum sativum L. cv. Midfreezer) served throughout as the systemic host for PEMV and tissue source for virus purification. Two strains of PEMV previously referred to as CALIF-PEMV and NY-PEMV (Bath and Chapman, 1966 and 1967), and two additional variants derived from the parent CALIF-PEMV strain were utilized. These variants differ markedly in their transmission characteristics: the first is highly aphid-transmissible (hereafter CT-PEMV) but the second variant is not aphid-transmissible (CNT-PEMV). Both variants are mechanically transmissible. The variants originated by allowing non-viruliferous pea aphids an acquisition-access feeding period on CALIF-PEMV and then transferring single aphids to individual healthy pea seedlings. Because CALIF-PEMV has very low-aphid transmissibility only a limited number of plants became infected and developed symptoms. The process of aphid acquisition and transfer was done again by selecting and using these infected plants as PEMV sources. By repeating this process numerous times, the highly aphid-transmissible CT-PEMV variant was developed. Similarly, CALIF-PEMV was transmitted repeatedly but only by mechanical means, thereby selecting for the highly mechanically inoculable CNT-PEMV variant.

All strains and variants (except CT-PEMV) were maintained by twice-monthly mechanical inoculations of 6-7-day-old pea seedlings with sap expressed from severely infected peas. However, at intermittent intervals, the mechanical transmission sequence of CALIF-PEMV and NY-PEMV was interrupted with an aphid acquisition-transmission cycle. CT-PEMV was maintained by twice-monthly transfers of 1st- to

3rd-stage pea aphid nymphs from infected to healthy peas. Plants were usually held in the greenhouse for symptom development but in certain experiments they were maintained in growth chambers at conditions of 23° C., ca. 50% R.H. and ca. 1500-ft. candles.

Aphid culturing and transmission bioassay.--Pea aphid biotype - East Lansing (Tsai et al., 1972) was used exclusively for all PEMV transmission trials. The handling and maintenance of aphid culture has been previously described (Thottappilly et al., 1972, French et al., 1972). Assay of purified PEMV preparations for aphid-transmissibility was done by use of our artificial membrane aphid-feeding system (Thottappilly, et al., 1972).

Virus purification.--PEMV was partially purified from severely infected pea seedlings by the method of Thottappilly et al. (1972) and unless otherwise specified 10-day-old infected tissues were used. In certain experiments the purification method was slightly modified to include a reextraction of the plant tissue debris which was sedimented by the first low-speed centrifugation. This sediment was again homogenized with a 1:1 mixture of chloroform and 0.1 M, pH 6.0 potassium phosphate buffer with 5% sucrose and allowed to sit in an ice bath for 5-10 minutes. The emulsion was broken by low-speed centrifugation and the aqueous phase removed and combined with the aqueous phase from the first extraction. Combined aqueous phases were then subjected to dialysis and processed through the remainder of the purification schedule.

Virus pellets resulting from final high-speed centrifugation were resuspended in 0.1 M potassium phosphate buffer pH 7.0 with 5%

sucrose. Pellets were usually allowed to resuspend 12 hrs and virus resuspension was aided by the use of a mechanical shaker. Following partial purification virus preparations were subjected to rate-zonal density gradient centrifugation. Linear gradients of 10-40% sucrose in .02 M potassium phosphate buffer (pH 7.0) were prepared and refrigerated 12 hrs at 4 C., after which 0.5-1.0 ml of partially purified PEMV (undiluted or adjusted to varying A_{260} concentrations) was layered on the top of each gradient. Centrifugation was done for 2 hrs at 24,000 rpm in the SW 25.2 or SW 27.1 rotor of the Spinco Model L ultracentrifuge at 4 C. Gradients were first monitored visually for light-scattering virus zones by placing them before a dark background and projecting light down through the gradient from a strong light source above. The zones were noted and their distance measured from the meniscus of the gradient. Gradients were then analyzed for sedimenting components and fractionated with an ISCO model D density gradient fractionator and U-2 ultraviolet analyzer monitoring at 254 nm. By utilizing the scanning profiles from UV analysis and measuring the area under virus peaks with a polar planimeter, the optical density could be computed and converted to micrograms of virus; an extinction coefficient of 7.5/mg/ml/cm at 260 nm (Shephard, et al., 1968) was assumed.

RESULTS

Influence of tissue age on nucleoprotein yields and sedimenting components of NY- and CALIF-PEMV strains.--Several hundred pea seedlings were mechanically inoculated with crude sap extracts from

peas infected with NY-PEMV and CALIF-PEMV. Ten, 15, and 20 days after inoculation, 40-50 grams of tissue showing severe symptoms were collected from each group of seedlings and immediately used as sources for virus purification. After each purification nucleoprotein yields were determined and resulting virus preparations were subjected to sucrose density gradient centrifugation; gradients were monitored for sedimenting components by UV analysis.

Both visual inspection and UV light scans of density gradient columns showed the presence of 2 sedimenting components for both NY- and CALIF-PEMV at each tissue sampling date. The intensity of the light-absorbing virus zones and the amplitude of the UV absorbance peaks of these virus zones varied between sampling dates and virus strains (Figure 1). The bottom component appeared as a very opalescent zone 17-18 millimeters from the meniscus. The top component zone was 15-16 millimeters from the meniscus; however, it varied from a very faint virus zone in those gradients layered with preparations from 10-day-infected tissues to a very discrete zone (almost as pronounced as that for the bottom component) in gradients layered and centrifuged with either NY- or CALIF-PEMV preparations from 20-day-infected tissues. Furthermore, UV scanning profiles showed that the top component peak was very intense in both NY- and CALIF-PEMV partially purified preparations from 20-day-infected tissues (Figure 1). Thus, the amount of top component increased with virus purified from increasing age tissues. This was particularly evident with the NY-PEMV strain, where the top component peaks in scanning profiles often approached the height of the bottom component peaks; however, the top component of both strains

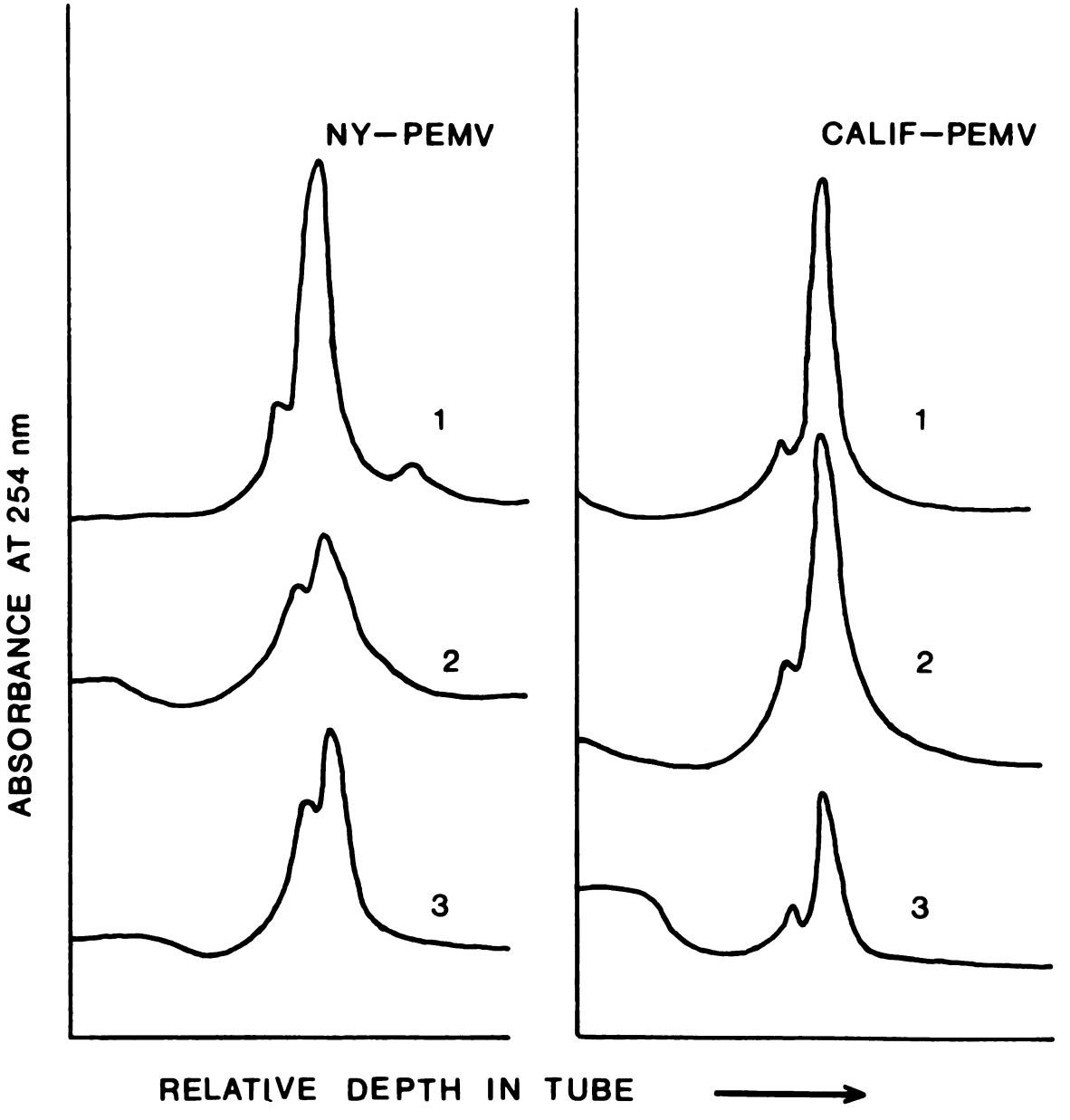

FIGURE 1. UV scanning profiles at 254 nm of sucrose gradients (10-40%) layered with NY- or CALIF-PEMV preparations partially purified from infected peas 10, 15, and 20 days (1, 2, 3, respectively) after mechanical inoculation. Density gradient centrifugation was for 2 hrs at 24,000 rpm in the Spinco SW 25.2 rotor.

purified from 15-day-infected tissues was not discrete on the scanning profiles and appeared only as a shoulder on the ascending slope of the bottom component peak.

The nucleoprotein yield of NY- and CALIF-PEMV decreased with increasing age of infected tissue used for partial purification (Table 6). Yield of NY-PEMV decreased approximately five-fold between tissues used for purification 10 and 15 days after inoculation, but yield was essentially the same from both 15- and 20-day-infected tissues. Yields of CALIF-PEMV from 10- and 20-day-infected tissues were lower than the same age tissues infected with NY-PEMV; however, CALIF-PEMV yield was higher than that of NY-PEMV when both were partially purified from 15-day-infected tissues. Apparently the titre of NY-PEMV in plants declines with age of tissue at a faster rate than does the CALIF-PEMV strain.

TABLE 6. Influence of tissue age on nucleoprotein yield of two strains of pea enation mosaic virus

PEMV Strain	Yield (μg/g) of virus obtained from infected pea tissue at specified days after inoculation[a]		
	10 days	15 days	20 days
NY	274.33	54.02	53.07
CALIF	183.11	87.22	12.29

[a]Virus was purified by the method of Thottappilly et al. (1972). Peas used for purification were mechanically inoculated and grown under greenhouse conditions during November, 1970.

Seasonal influence on nucleoprotein yield and sedimenting components of NY-PEMV.--Beginning in September of 1970, 10-day NY-PEMV-infected pea tissues were harvested at about 2-month intervals for about 1 year. These tissues were sources for partial purification of virus and only tissues showing very severe symptoms were used. At each purification date nucleoprotein yield was computed, and UV scanning profiles were made of density gradient columns containing ultracentrifuged virus.

Ultraviolet scanning profiles of centrifuged density gradient columns layered with partially purified NY-PEMV varied considerably with different seasons of the year (Figure 2). All profiles clearly exhibited the predominant 113S[1] bottom component, but the presence (in varying proportions) or complete absence of the 94S[1] top component was evidenced at certain seasonal periods of the year. No top component was recovered in purifications made in September of 1970, or in June and July of 1971, and only minimal but detectable amounts of top component were obtained in a purification made in August, 1971 (Figure 2).

In those purifications of NY-PEMV made during the fall, winter and spring seasonal periods, the top component was readily apparent both as a visible zone in gradient columns and on UV scanning profiles. The highest level of top component was attained in virus preparations from tissues harvested and processed in March of 1971 (Figure 2), and occurred during a period when the total yield of extracted NY-PEMV

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[1]Sedimentation coefficients for top and bottom nucleoprotein components of PEMV as derived by Bozarth and Chow (1966) and Musil et al. (1970).

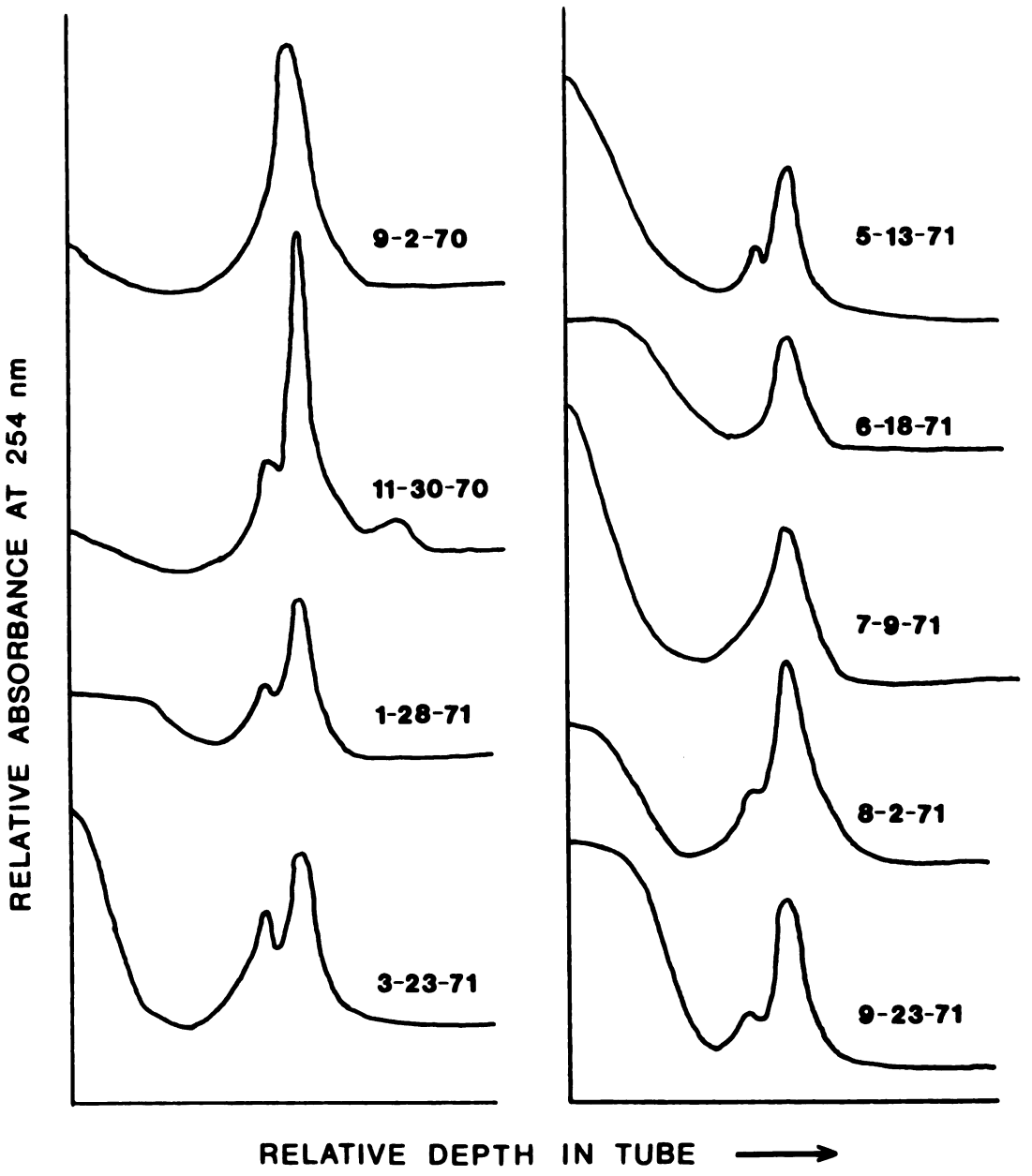

FIGURE 2. UV scanning at 254 nm of sucrose gradients (10-40%) layered with NY-PEMV partially purified at various seasonal periods from 10-day infected peas. Density gradient centrifugation was for 2 hrs at 24,000 rpm in the Spinco SW 25.2 rotor.

was at or near its lowest level - 9.64 micrograms virus/gram of infected tissue (Table 7).

TABLE 7. Influence of seasonal variation on yield of NY-PEMV from P. sativum L. grown under greenhouse conditions

Purification date	Mean temperature[a]	Yield (µg/g) of virus[b]
9-2-70	82.6	36.83
11-30-70	39.7	274.33
1-28-71	28.5	56.60
3-23-71	41.3	9.61
5-13-71	68.9	8.19
6-18-71	81.3	36.28
7-9-71	82.4	39.78
8-2-71	79.5	22.85
9-23-71	71.4	160.06

[a]Mean temperatures in East Lansing were computed for that period from time pea seedlings were inoculated and held in greenhouse to date of purification, or total of 10 days. Data obtained from U.S. Department of Commerce.

[b]Virus was purified by the method of Thottappilly et al. (1972) from infected pea 10 days after mechanical inoculation.

Virus symptoms on pea seedlings infected by NY-PEMV between June to September and December to March were not as severe as during other periods of the year. During those periods of mild symptoms, infected seedlings were not severely stunted and the active-growing portions of these plants often did not show PEMV symptoms; furthermore, very few enations (neoplasms) were evident. The most severe symptoms

were observed on seedlings inoculated and used as tissue sources for purification in the spring and fall. These seedlings, while showing the usual symptoms of PEMV infection were severely stunted and all leaves showed chlorotic flecking (windows). Symptom severity could not be used as a reliable visual means for estimating NY-PEMV yield, since the highest and lowest recorded yields were obtained at different periods of the year (Table 7), but the infected pea seedlings used for partial purification in each case showed similar severity of symptoms.

Failure to recover NY-PEMV top component appeared to coincide with mild symptom expression on infected pea seedlings and occurred at periods in which higher light intensities and elevated temperatures were most likely to occur in the greenhouse. Although internal greenhouse temperatures were not monitored during plant incubation periods, outside temperatures (available for East Lansing from U.S. Dept. of Commerce) have a direct bearing on the internal greenhouse temperatures. Greenhouse coolers and shading compound on the glass are inadequate to hold temperatures much below 80° F when outside temperatures reach into the 80's on sunny days. Often greenhouse temperatures reach 90-100°, even though the outside temperature is at or near 80°.

Through the use of climatological data, it was possible to compute the mean temperatures for the 10 days prior to each purification date (Table 7). It is of particular interest to note that in each of those purifications in which no top component was recovered (September 1970, and June, July, 1971, Figure 2), the mean temperature exceeded 80° F. This was opposed to an average temperature of 41.3° F recorded in March, 1971, when the highest level of top component was

recorded in any one purification. While an average temperature was computed for each 10-day period, temperatures often deviated 10 or more degrees from the mean. In each of those purification periods with a mean above 80° F, there were specific days in which temperatures approached or exceeded 90° F. Since greenhouse cooling systems and supplementary shading are often not adequate to control high light intensities and high temperature fluctuations, they could be particularly detrimental to pea seedling growth and, therefore, a limiting factor in virus production.

Characterization of nucleoprotein yield and sedimenting component ratios of aphid-transmissible and nonaphid-transmissible PEMV variants.-- The aphid-transmissible (CT-PEMV) and nonaphid-transmissible (CNT-PEMV) variants of CALIF-PEMV were partially purified on several occasions from infected peas which were incubated in the greenhouse and compared by rate-zonal density gradient analysis. In each case source plants of CT-PEMV were obtained through aphid transfers of virus and CNT-PEMV by mechanical transfers of sap from infected tissue; each purification trial included both variants. Analyses were inconsistent between purification trials; however, top/bottom component ratio data tended to show CNT-PEMV to contain higher ratios of top component than did CT-PEMV.

Three additional purification comparisons were made of CNT- and CT-PEMV, but to restrict inter-trial variability, source plants (inoculated as before) of each variant were incubated in a controlled environmental chamber at 23 ± 1 C, ca 50% R.H., and ca 1500-ft-candles, with a 12-hr photophase. Furthermore, the purification procedure was

modified to insure maximum nucleoprotein recovery by the addition of a reextraction of the sedimenting plant debris pellet from the first low-speed centrifugation.

Since Bozarth and Chow (1968) showed that sucrose in buffer solutions, used for resuspension of the final high speed PEMV pellets, affected the yield and component ratios, I also tested its effect on both variants by resuspending pellets in 0.1 M potassium phosphate buffer (pH 7.0) with and without 5% sucrose. Component ratios were computed from planimetry measurements of the UV-scanning profiles of top and bottom components of the 2 variants.

Comparative UV-scanning profiles of gradient columns, layered with PEMV preparations from repeated purifications of the 2 variants consistently demonstrated CNT-PEMV to contain much greater proportions of top component than did CT-PEMV. When CNT-PEMV pelleted by ultracentrifugation was resuspended in potassium phosphate buffer containing no sucrose the resultant preparation had a higher level of top than bottom component (Figure 3). The predominance of top component was also shown by a top/bottom ratio of less than 1 (Table 8). However, when the resuspending phosphate buffer contained 5% sucrose significantly higher proportions of bottom component were obtained and top/bottom ratios varied from 2.8 to 4.4 for the 3 purification trials. If final high speed pellets of CT-PEMV were treated in a similar manner the level of top component was not appreciably changed by the presence or absence of sucrose in the resuspending buffer (Figure 3). Bottom component was always extracted in much higher proportions than top component; this was shown by top/bottom component ratios often exceeding 5.0 (Table 8). Like CNT-PEMV the amount of CT-PEMV bottom

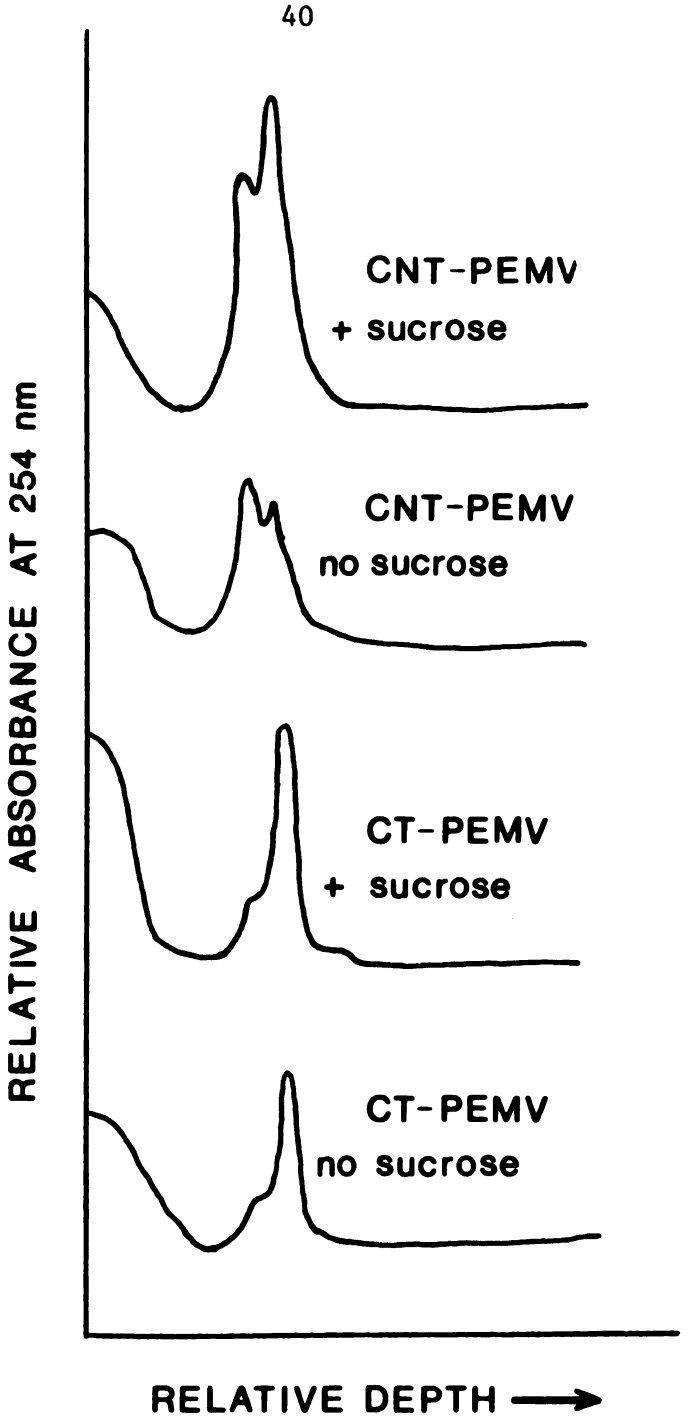

FIGURE 3. UV scanning profiles at 254 nm of sucrose gradients (10-40%) layered with CNT- or CT-PEMV partially purified and resuspended in potassium phosphate buffer either with or without 5% sucrose. Centrifugation was for 2 hrs at 24,000 rpm in the Spinco SW 27.1 rotor.

component could be further increased by resuspension of virus pellets in phosphate buffer containing 5% sucrose.

TABLE 8. Ratio of top/bottom sedimenting components of CNT- and CT-PEMV variants differentially resuspended in potassium phosphate buffer with and without 5% sucrose[a]

Variant	Virus pellets resuspended in	Component ratios for purification trials[c]		
		1	2	3
CNT	buffer[b]	0.60	----[d]	0.59
CNT	buffer + sucrose	2.81	4.40	3.00
CT	buffer	5.83	----[d]	5.00
CT	buffer + sucrose	3.33	----[d]	5.33

[a]CNT-PEMV was mechanically inoculated to pea, and CT-PEMV was aphid inoculated to pea. Tissues used 10 days later for partial purification by method of Thottappilly et al. (1972).

[b]0.1 M potassium phosphate buffer, pH 7.0.

[c]Component ratios determined from planimetry measurements of UV-scanning profiles of sucrose gradients layered with partially purified CNT- or CT-PEMV preparations from 3 separate purification trials.

[d]Component ratios could not be determined because top component was unresolved from bottom component on UV-scanning profiles.

Comparative nucleoprotein yield data from the 3 trials showed that consistently higher yields were obtained from plants infected with CNT-PEMV than from those infected with CT-PEMV (Table 9). In some cases the yield of CNT-PEMV was 10-fold higher than CT-PEMV. Nucleoprotein yields of both CT- and CNT-PEMV at the time of each purification was also substantially increased by resuspending final high speed virus pellets in potassium phosphate buffer containing

5% sucrose; in some instances the yield was 3 times greater than when the virus was resuspended in phosphate buffer having no sucrose (Table 9).

TABLE 9. Comparative yields of CNT-PEMV and CT-PEMV purified at 3 different times from infected tissues of P. sativum L. grown in a controlled environmental growth chamber[a]

Purification trial	PEMV variant	Yields (μg/g) when partially purified virus is resuspended in[b]	
		Buffer[b]	Buffer[b] + 5% sucrose
1	CNT	84.4	244.4
	CT	31.2	42.8
2	CNT	168.0	284.7
	CT	14.4	44.8
3	CNT	122.2	180.6
	CT	32.4	54.0

[a]CNT-PEMV was mechanically inoculated to pea, and CT-PEMV was aphid inoculated to pea. Tissues used 10 days later for partial purification by method of Thottappilly et al. (1972).

[b]0.1 M potassium phosphate buffer, pH 7.0.

While the nucleoprotein yield from pea tissues infected with CNT-PEMV was substantially higher than from CT-PEMV-infected tissues, there was not a corresponding difference observed in symptom severity between the two variants. Independent of the variant used, symptoms on infected pea seedlings grown under the regulated environmental conditions of the growth chamber were always extremely severe.

Aphid-transmissibility of separated sedimenting components of NY-PEMV.--Since there has been some controversy over the infectivity of PEMV top component (Bozarth and Chow, 1966; and Izadpanah and

Shephard, 1966), it was decided to test both components (separate and mixed) for their aphid-transmissibility. In one experiment partially purified NY-PEMV was layered on each of 6 gradients and after centrifugation 4 fractions were collected from each tube. These fractions as shown by the UV scanning profile (Figure 4A) correspond to top, top and bottom mixture, bottom and bottom aggregate component mixture. Care was taken to collect individual fractions from the gradient in the indicated areas of the scanning profile. Like PEMV fractions from each tube were combined, dialyzed 48 hours against 0.02 M potassium phosphate buffer pH 7.0 and virus pelleted from solution by ultra-centrifugation. Viral pellets were resuspended in the same dialysis buffer but with 5% sucrose added. The 4 PEMV suspensions were then adjusted to equal concentrations of $\underline{A}_{260}$ = 2.0 and fed to first-stage pea aphids across an artificial membrane. After a 24-hr acquisition-access feeding period (AAP) aphids were transferred singly to healthy pea seedlings for a 3-day inoculation-access feeding period (IAP). Twenty-three to 30 aphids were used per treatment. The 4 suspensions also were each mechanically rubbed on 4-6 pea seedlings for systemic infectivity bioassay.

In a second experiment NY-PEMV was again partially purified and subjected to sucrose density gradient centrifugation. However, this aphid feeding trial differed from the first in that fractions of 10 drops each were collected from the sucrose gradient corresponding to top, bottom and aggregated bottom components (Figure 4B). These fractions were fed to the aphids directly from the gradient without the recycling steps of dialysis, ultracentrifugation and concentration determination and adjustment. First-stage aphids were given only a 4-hr AAP on the component fractions followed by a 3-day IAP on healthy

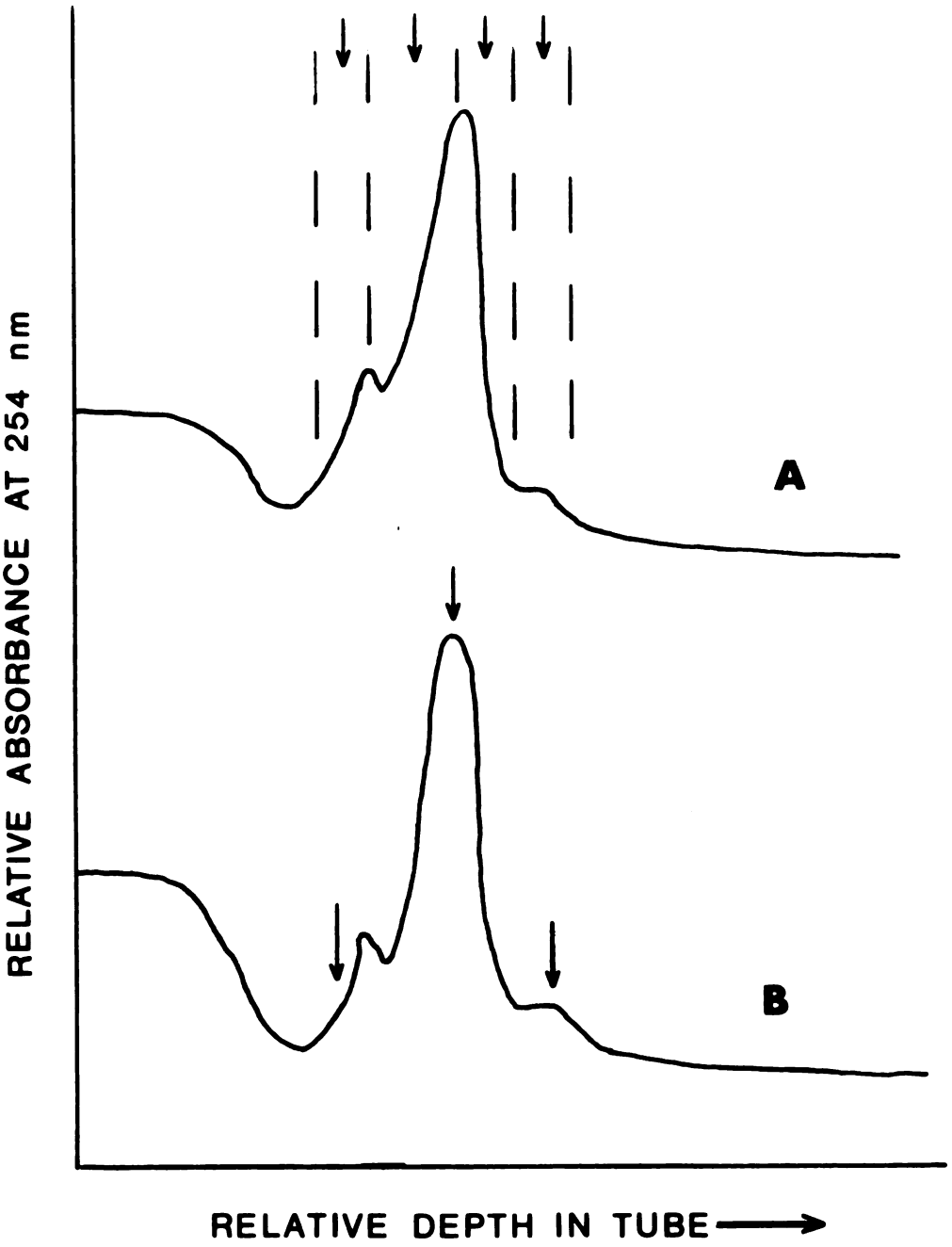

FIGURE 4. UV scanning profiles at 254 nm of sucrose gradients layered with NY-PEMV. Arrows in (A) correspond to fractions containing virus components which were collected from the gradients, dialyzed and reconcentrated by ultra-centrifugation and membrane-fed to 1st-instar aphids. Arrows in (B) correspond to 3 virus component fractions collected from the gradient and without reconcentration membrane-fed directly to 1st-instar aphids.

peas. Thirty-five to 45 aphids were used per treatment. Component fractions were each mechanically rubbed on 5 pea seedlings for infectivity bioassay.

Sedimenting components of PEMV either separate or mixed were aphid-transmitted but with varying efficiency. When fractions from density gradient columns containing PEMV components were reconcentrated by ultracentrifugation and adjusted to equal $\underline{A}_{260}$ = 2.0 concentrations before feeding, aphids transmitted the top and bottom components with approximately equal efficiency (Table 10). However, that fraction which contained a mixture of the 2 components was transmitted with a higher efficiency by aphids than were either the top or bottom components separately. While the ratio of top/bottom components in this mixture was not known it could be assumed that the bottom component predominated since collection of this fraction was restricted to that part of the gradient column between the 2 major component bands, and relative to the UV scanning profile collection included only the descending slope of the top component peak while including all of the ascending slope of the bottom component peak (Figure 4A). The fourth fraction contained an aggregate of the bottom component and was transmitted with the least efficiency - 17.4% (Table 10). All pea seedlings (100%) developed systemic PEMV symptoms that were mechanically inoculated with reconcentrated components from the 4 DGC fractions.

Aphid transmission efficiency was vastly improved when PEMV components were fractioned from the gradient column and fed directly to first-stage pea aphids without any interceding dialysis and reconcentration steps. With only a 4-hr AAP (as opposed to a 24-hr AAP in Exp. 1) aphids transmitted top, bottom and aggregated components

TABLE 10. Transmission of sedimenting components of PEMV separated by sucrose density gradient centrifugation (DGC) and acquired through an artificial membrane by first-stage Acyrthosiphon pisum

	DGC fraction number	PEMV component	Peas infected (percentage)
Experiment 1[a]			
	1	top	14/30 (46.7)[b]
	2	top and bottom	17/24 (70.8)
	3	bottom	12/29 (41.4)
	4	bottom aggregated	4/23 (17.4)
Experiment 2[c]			
	1	top	36/43 (83.7)
	2	bottom	31/35 (88.6)
	3	bottom aggregated	17/40 (42.5)

[a]After separation PEMV component fractions were dialyzed 48 hours against 0.02 M potassium phosphate buffer, pH 7.0, reconcentrated by ultracentrifugation and components resuspended in the dialysis buffer containing 5% sucrose. Component preparations were adjusted to equal A_{260} = 2.0 concentrations and provided to aphids for a 24-hr acquisition-access feeding period (AAP) followed by a single aphid transfer to healthy pea seedlings for a 3-day inoculative-access feeding period (IAP).

[b]Each component fraction was also mechanically inoculated on 4 to 5 healthy pea seedlings with a resultant 100% of the peas infected, except those inoculated with fraction 3 (of Exp. 2) where only 40% were infected.

[c]After separation PEMV component fractions were fed directly to pea aphids (without dialysis and reconcentration) for a 4-hr AAP and single aphid transfer to pea seedlings for a 3-day IAP.

with about 2-fold greater efficiency than comparable component transmission in experiment 1 (Table 10). Top and bottom components were aphid-transmitted with approximately equal efficiency (84% vs 89%). In the mechanical infectivity tests of the top, bottom and aggregated components, -100, 100 and 40% respectively, of the inoculated pea seedlings developed systemic symptoms.

In attempting to explain the difference in component transmission efficiency by aphids these data would appear to indicate that: either the component concentration in those fractions fed directly from the gradient column (Exp. 2) was much higher than the $\underline{A}_{260} = 2.0$ concentration used in the first experiment; or the lengthy procedure of dialysis and reconcentration of component fractions (Exp. 1) resulted in some loss of infectivity of the components (and mixtures) prior to aphid-membrane feeding.

DISCUSSION

Our investigations indicated that the nucleoprotein yield and sedimenting component ratios of PEMV varies with season and age of source tissue. Non-aphid-transmissible and aphid-transmissible variants of CALIF-PEMV were separable on bases of nucleoprotein yield and component ratio when source plants used for partial purification were grown in controlled environmental chambers. The CALIF- and NY-PEMB strains, which vary in transmissibility but to a lesser extent than the CALIF-PEMV variants (CT- and CNT-PEMV), could not be differentiated on basis of yield or component ratios. However, these strains were only tested under greenhouse growing conditions and

apparently unfavorable growing conditions, such as excessively high temperatures, may mask any differences between strains. Furthermore, the top nucleoprotein component of NY-PEMV was not recoverable from peas grown and used for purification during these periods of excessive temperature fluctuations. It is of interest to note that Lapido and de Zoeten (1972) recently published results on a study of host and seasonal variation on the sedimenting components of tobacco ringspot virus (TRSV). They found that with a single TRSV strain the type and amount of components were determined by the host that was used to increase the virus; moreover, with the same host the type and amount of components present were determined by the period of the year in which the host was inoculated.

While it is likely that strains, and variants of those strains, differ in nucleic acid base sequence, it is also probable that coat protein differences are present. Both could account for the differences in nucleoprotein yield which occur between strains and variants, but the coat protein is likely to play a prominent role in aphid transmission efficiency determination. Because of the circulative nature of viruses such as PEMV, protein compatibility (complementarity) is almost certainly involved at the membrane level of one or more tissues within the vector (Rochow, 1969). If protein-membrane complementarity is essential or required before virus enters a cell, e.g., epithelial cells of the aphid mid-gut, it is reasonable to expect that 2 strains with identically infective nucleic acid could be transmissible or nontransmissible by vectors if the protein coat was complementary or noncomplementary, respectively. Just as the coat protein is important in determining plant host range (due to differential

absorption of virus to membranes of some plant species and not others) (Atabekov, 1971; Novikev and Atabekov, 1969) it is likely to be similarly important in determining which if any aphid membranes the virus can attach to and penetrate, either intact or in the form of viral RNA.

If CNT-PEMV and CT-PEMV do indeed differ in their coat protein composition, it would be reasonable that such an altered coat protein in either of the variants could determine vector-virus compatibility and subsequent aphid transmissibility. Thus, when there is a mixed infection with 2 or more variants (as in the case of CALIF-PEMV-infected peas) aphids would select and perpetuate that variant which is compatible with their internal system. Other variants of a mixed infection are undoubtedly acquired by the aphid but fail to be transmitted due to membrane incompatibility or variant susceptibility to degradative or inhibitory enzymes within the aphid. Differences in coat protein could again be the dominant factor in determining variant susceptibility or nonsusceptibility to these enzymes.

Why aphids select a PEMV variant with low levels of top component while repeated mechanical inoculation selects a variant with high levels of top component remains unresolved and will require further investigation. However, Gonsalves and Shepherd (1972) utilizing a different PEMV strain than ours, provided experimental evidence to show that the top component is slightly more mechanically infective than the bottom component. Furthermore, they have found that when nucleic acid preparations from unfractionated PEMV are subjected to electrophoresis in polyacrylamide gels or to sucrose density gradient centrifugation, there occurred three separate RNA species of

34, 30 and 12S. Infectivity was associated only with the 30S viral RNA and was the only RNA species found in the top nucleoprotein component, whereas the bottom nucleoprotein component contained approximately equal amounts of 30S and 12S RNA and larger amounts of 34S RNA. Thus, in light of their findings it would not be unreasonable to expect that continually repeated mechanical inoculation might gradually select a variant with more top component because of its slightly greater infectivity. The role of 12S and 34S RNA species are unknown but it could be theorized that they might have some function in aphid transmission.

CT- and CNT-PEMV variants should be extremely useful tools in further studies on vector-virus relationships particularly since they are at opposite ends of the aphid transmission spectrum, i.e., CT-PEMV being highly aphid-transmissible and CNT-PEMV not transmissible even by our best transmitter-pea aphid biotype. Characterizational studies on coat proteins of the variants and PEMV strains are in progress to attempt to elucidate specific differences. Electrophoretic heterogeneity between variants as resolved by polyacyrlamide gel electrophoresis may provide preliminary evidence of protein dissimilarities. Furthermore, amino acid sequencing of coat protein will undoubtedly be required, and although detection of specific sequence differences would be an important contribution the ultimate objective will be to correlate these differences with aphid transmissibility or nontransmissibility. de Zoeten and Rettig (1972) working with a single PEMV strain have already demonstrated differences in protein patterns between infected and noninfected pea seedlings and between viruliferous and nonviruliferous pea aphids.

Possibly one of the best methods to establish such a correlation is based on the concept of "heterologous encapsidation" as proposed by Rochow (1972) this includes both phenotypic mixing and transcapsidation. Isolated capsids and nucleic acids of certain viruses and virus strains have been combined in vitro to form a wide spectrum of combinations with varying infectivities. Since PEMV has been successfully separated into its constituent protein coat and RNA (without the latter losing its infectivity), as shown by Shephard, et al. (1968) and Gonsalves and Shephard (1972) it seems probable that under the right conditions in vitro reconstitution by transcapsidation or phenotypic mixing or both, could be successfully effected. This coupled with PEMV transmission bioassay by our aphid-membrane feeding system (Thottappilly, et al., 1972), would provide a unique method in determining the role of the protein capsid in vector-virus specificity. In other words, would successful transmission be effected with the coat protein of CT-PEMV on the RNA of CNT-PEMV or through phenotypic mixing would the addition of capsomeres of the coat protein of CT-PEMV in combination with capsomeres of the coat protein of CNT-PEMV result in degrees of aphid transmissibility and plant infectivity. Also, an intriguing question arises as to whether PEMV RNA free of the coat protein could be aphid transmitted. There is some support for this hypothesis since the salivary glands are the final steps in the postulated circulative route of the virus through the aphid vector and to date PEMV particles have not been observed through electron microscopy in these glands (Harris and Bath, 1972; Shikata et al., 1965, 1966). It has further been postulated that PEMV may exist in the salivary glands as free RNA and may be introduced with the salivary

fluid into the plant host by the feeding aphid in this form (free RNA). This is a speculative issue and has a major drawback in the fact that RNA free of the protective coat protein is undoubtedly very sensitive to the enzymes of the aphid's digestive system.

To what extent heterologous encapsidation takes place in plants with mixed virus infections is not known. But this phenomenon could be an explanation for the continuing emergence and isolation of new PEMV virus strains or variants (and for strains of other viruses as well), each with a differing capacity to be transmitted or non-transmitted by insects. Also, very little is known about the effect strains of a given virus, or differing viruses, have upon each other in determining their transmissibility. There is some evidence, particularly in the case of barley yellow dwarf virus (Rochow, 1969), that the capsids or nucleoprotein of a particular transmissible strain may serve as "helper viruses" in mixed infections to aid in the transmissions of other normally non-transmissible strains. It is thought that the major barrier controlling transmission specificity is determined by whether a particular strain can gain entrance into the salivary glands of the aphid vector. Certain strains, because of their complementarity with the membranes of this structure, readily pass through (probably by pinocytosis) while others are unable to penetrate and are not transmitted. But through a mixed infection, the nontransmissible strain is carried through the membrane with the transmissible strain, or its capsid, and transmission is effected. These types of studies, in combination with radioactive labeling or florescent antibody labeling should be able to show specific strain and aphid-membrane relationships.

If such a membrane barrier exists for certain PEMV strains and variants, it is likely at some site other than the salivary glands - perhaps the epithelial cells of the lining of the aphid midgut. Since as before noted, no PEMV particles have been found in the salivary glands of the pea aphid and furthermore, viral particles of NY-PEMV and CT-PEMV (but not CNT-PEMV particles) have been found in the cells of the mid-gut epithelium and in the hemocoel surrounding the gut. Whether this type of "helper phenomenon" can be used for strains and variants to effect aphid transmission, particularly of CNT-PEMV, has yet to be shown.

Investigations on the intrinsic properties of PEMV strains and variants and their relationship to the pea aphid vector are just beginning to yield fundamental insights into the vector-virus specificity phenomenon. In the future the most profitable and beneficial research from PEMV and other circulative aphid-borne viruses will likely result from studies conducted to determine the association and fate of the virus at the histological and cytological levels within the vector.

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PART III: PURIFICATION OF PEA ENATION MOSAIC VIRUS FROM ITS VECTOR, ACYRTHOSIPHON PISUM (HARRIS) AND APHID-TRANSMISSION CHARACTERISTICS

INTRODUCTION

Among the persistent or circulative aphid-transmitted viruses only potato leafroll virus (Peters, 1967a,b; Peters and Van Loon, 1968) and barley yellow dwarf virus (Rochow and Brakke, 1964) have been successfully purified from their vectors. Another aphid-borne circulative virus, pea enation mosaic virus, has been purified on numerous occasions from plant tissue but it has not been purified from its pea aphid vector, Acyrthosiphon pisum (Harris).

This paper describes the successful purification of PEMV from fresh or frozen aphid tissue. In addition the virus purified from aphids was established in plants and compared as to aphid transmissibility with virus purified from infected plants.

MATERIALS AND METHODS

Stock aphid colonies were reared under controlled conditions of light (12-hr photoperiod) and temperature (25°) on broadbean (Vicia faba L.) or garden pea (Pisum sativum L. cv. Midfreezer). Pea aphid Biotype EL (Tsai et al., 1972) and the NY strain of PEMV (Bath and Tsai, 1969) were used exclusively. Non-viruliferous 1st- to 3rd-instar aphids were transferred to PEMV-infected pea plants for acquisition-access periods of 2-7 days. PEMV was partially purified

from viruliferous aphids either freshly-collected or frozen. In any one purification trial 2 to 10 grams (g) of aphids were used.

Virus purification.--Aphids were first homogenized in a Sorvall Omni-mixer together with 0.1 M potassium phosphate buffer (pH 6.0) and chloroform-butanol (1:1) at a ratio of: 1 g/10 ml/10 ml. The homogenate was allowed to stand 15 to 90 min (time varied with experiments) in ice; phase separation was completed by centrifugation at 9,000 rpm for 10 min on a Sorvall SS-1 centrifuge. The upper aqueous phase was decanted and saved, the bottom chloroform-butanol phase was discarded and the interface of insect debris was saved for reextraction. The aphid debris from the interface was homogenized with chloroform-butanol (1 g/5 ml/5 ml), allowed to stand for 15 to 90 min, centrifuged for 10 min at 9,000 rpm and decanted as before. This reextraction of the interface residue was repeated 3-4 times to obtain maximum virus extraction. All aqueous phase collections were combined, unless otherwise specified, and dialyzed against several changes of 0.05 M potassium phosphate buffer (pH 7.0) for 24 hr. Following dialysis the preparation was centrifuged in the No. 30 rotor with a Spinco model L ultracentrifuge at 29,000 rpm for 2 hr at 4°. Virus pellets were resuspended in 0.1 M potassium phosphate buffer (pH 7.0) with 5 or 30% sucrose with the intention of later use in density gradient centrifugation and aphid transmission experiments.

Density gradient centrifugation.--Sucrose density gradient columns were prepared using 4, 7, 7, and 7 ml of 10, 20, 30 and 40% sucrose, respectively, in 0.02 M, pH 7.0 potassium phosphate buffer. After standing over night at 4°, 0.5-1.0 ml of partially purified

virus suspension was layered on each gradient. Centrifugation was done for 2 hr at 24,000 rpm in the SW 25.1 rotor of the Spinco Model L ultracentrifuge, refrigerated at 4°. Tubes were analyzed for sedimenting components and fractionated with an ISCO model D density gradient fractionator and UA-2 ultraviolet analyzer monitoring at 254 nm.

Electron microscopy.--Electron microscopic investigations were made on partially and density gradient-purified virus from viruliferous aphid tissues. In DGC material, fractions collected from above and below the sedimentation zone were examined without reconcentration; the fraction within the zone was recycled, pelleted by differential centrifugation and resuspended in deionized water before examination. Each preparation was negatively stained with 2% phosphotungstic acid (pH 7.0) and examined with a Philips 300 EM. Extracts from non-viruliferous aphids were subjected to identical purification procedures and examined under the electron microscope in the same manner as extracts from viruliferous aphids.

Infectivity assay.--Assays of virus infectivity were made on Midfreezer pea seedlings by mechanical inoculations or aphid-transmissions of partially and density gradient-purified virus. In aphid-transmission assay, virus solutions were fed to 1st-stage pea aphid nymphs across an artificial membrane in a manner identical to that described by Thottappilly et al., (1972). While partially purified virus was fed in phosphate buffer containing 5 or 30% sucrose, virus purified by density gradient centrifugation was fractionated and fed to aphids just as it came from the sucrose gradient column. After

completing the virus acquisition period, aphids were placed singly on test plants for 5-day inoculation periods.

RESULTS

Preliminary tests.--Peters' (1967a,b) chloroform extraction technique for partial purification of potato leafroll virus from Myzus persicae (Sulzer) was initially employed for purification of PEMV from A. pisum. Three grams of young adult aphids were processed through 5 cycles of chloroform emulsification immediately after completing a 5-day feeding period on PEMV infected peas. The aqueous phase from each emulsification was saved and mechanically inoculated to 60 young pea seedlings. No infectivity was associated with aqueous phases from the 1st, 3rd, 4th and 5th cycles and only 3 plants became infected when inoculated with the aqueous phase from cycle 2. This low level of infectivity prompted attempts to improve the purification procedure.

Infectivity of PEMV partially purified by chloroform-butanol technique.--Because the chloroform-butanol method (Steere, 1956) has been used successfully to free virus from plant host material from which viruses are ordinarily difficult to purify, we substituted a chloroform-butanol mixture for the chloroform of Peters' (1967a,b) technique. The technique was modified further by permitting the emulsion to stand for periods much longer than the 5 min used by Peters (see Materials and Methods).

In an initial trial, 2 g of young pea aphids served as sources of virus immediately after completing a 5-day period on PEMV-infected peas. Following the 1st solvent treatment in which the emulsion stood for 15 min, the interface material (aphid residue) was reclaimed and processed through 3 cycles of re-extraction; thus, 4 aqueous phase fractions were obtained. After high speed centrifugation and virus resuspension each fraction was mechanically inoculated to 30 pea seedlings. Aqueous fractions 1, 2, 3 and 4 infected 23.3, 80.0, 70.0 and 73.3%, respectively, of the plants inoculated.

In an additional trial, 9.5 g of aphids that fed on virus source plants for 5 days immediately preceeding extraction and 10 g of aphids reared on healthy plants were used. The interface material of both non-viruliferous and viruliferous aphid preparations was recycled 4 times employing an emulsion standing time of 1 hr. Aqueous phases 2-4 were pooled and compared with phases 1 and 5 in transmissibility by aphids after the virus in the 3 fractions was reconcentrated and resuspended. These fractions were artificially fed in phosphate buffer containing either 5 or 30% sucrose to 1st-instar aphids during a 12-hr acquisition-access period. Thirty-five to 63 aphids were tested per treatment.

Aqueous phase 1 of the viruliferous aphid purification was more infectious than either pooled phases 2-4 or phase 5 as judged by resultant aphid-transmission efficiencies (Table 11). Transmission was not appreciably affected by the concentration of sucrose in the buffer medium. Transmission percentages indicated that most of the recoverable virus was extracted from the aphid homogenate during the

1st or 2nd cycle of solvent treatment. No transmissions of virus resulted from aphids fed on preparations from non-viruliferous aphids.

TABLE 11. Infectivity of successive aqueous fractions obtained by repeated chloroform-butanol emulsification of viruliferous pea aphids

Aqueous fraction[a]	% Transmission by pea aphids[b]	
	Trial 1	Trial 2
1	77.1	80.0
2-4	11.9	36.5
5	7.1	16.7

[a]Fraction 1 was obtained from the initial chloroform-butanol emulsification of aphid extract. Aphid debris was reclaimed from the interface of the aqueous and chloroform-butanol phases and processed through 4 additional cycles of emulsification to produce aqueous fractions 2-5.

[b]1st-stage nymphs were given a 12-hr acquisition-access period on artificial membrane source containing concentrated virus in 0.2 ml of 0.1 M, pH 6.0 phosphate buffer containing 5% sucrose (trial 1) and 30% sucrose (trial 2); 35-63 aphids were tested singly per treatment.

Frozen viruliferous aphids as a virus source.--Because it was difficult to obtain more than 5 g of viruliferous aphids at one time, it was desirable to preserve aphids by freezing. We tested the efficacy of this procedure by purifying virus from two 5-gram batches of aphids; one was frozen at -10° for 2 weeks after a 5-day acquisition-access period and the other was used for purification immediately after an identical exposure to source plants. As a control, 10 g of aphids (freshly-collected) that were reared on healthy plants were processed concomitantly with the viruliferous samples. The emulsion stood for

1 hr in each case before phase separation was completed by centrifugation. The interface material of each preparation was recycled twice and all 3 aqueous phase collections were pooled for high speed centrifugation. Virus pellets were obtained from both viruliferous aphid preparations and when resuspended in phosphate buffer containing 30% sucrose were found to be identical in $\underline{A}_{260}$ nm concentration. These suspensions were fed to 1st-instar aphids during 4- and 17-hr acquisition-access periods to compare infectivity when transmitted by aphids. While no visible pellets were obtained from non-viruliferous aphid preparations, the preparation was treated identically to that of the viruliferous aphids and fed to test aphids. An average of 41 aphids were tested per treatment.

The fresh preparation from viruliferous aphids was transmitted with greater efficiency after either acquisition period than was the frozen preparation (Table 12); however, the difference was small and both preparations were highly infective. No infectivity was associated with the preparation of non-viruliferous aphids when assayed by mechanical and aphid-transmission tests.

Infectivity and UV analysis of density gradient-purified virus.--The virus preparation obtained from fresh aphids in the previous tests was further purified by density gradient centrifugation and monitored with UV light at wavelength 254 nm. A partially purified preparation from non-viruliferous aphids was treated identically. The preparation from viruliferous aphids had an absorbance pattern very similar to that obtained from PEMV preparations purified from plants and was characterized by a virus absorbance peak which consisted of a low intensity

top component and a high intensity bottom component (Figure 5). Both preparations contained a non-viral component near the meniscus of the tube; a virus peak was not detected in the healthy preparation. The virus absorbance peak of the viruliferous aphid preparation was collected by gradient column fractionation and artificially fed to 1st-instar pea aphids during a 21-hr acquisition period. A fraction was collected from the density gradient column of the non-viruliferous aphid preparation at the same depth in the column as the UV-absorbing zone occurred in the column containing the viruliferous aphid preparation and fed to aphids in a manner identical to that used for the viruliferous preparation. Twenty-two of 32 test aphids transmitted the density gradient-purified virus from viruliferous aphids. No transmission resulted from the non-viruliferous aphid preparation.

TABLE 12. Aphid-transmission of partially purified PEMV prepared from fresh or frozen viruliferous pea aphids[a]

Condition of aphids	% Transmission after acquisition-access period of:	
	4 hr	17 hr
Fresh	85.7	95.1
Frozen	70.7	87.5

[a]Artificially fed to 1st-instar pea aphids at a relative concentration of A_{260} = 7.5 in phosphate buffer containing 30% sucrose; 40-42 aphids were tested singly per treatment.

Electron microscopy.--Partially and density gradient-purified preparations of both non-viruliferous and viruliferous aphids were negatively-stained and examined with the electron microscope. Virus

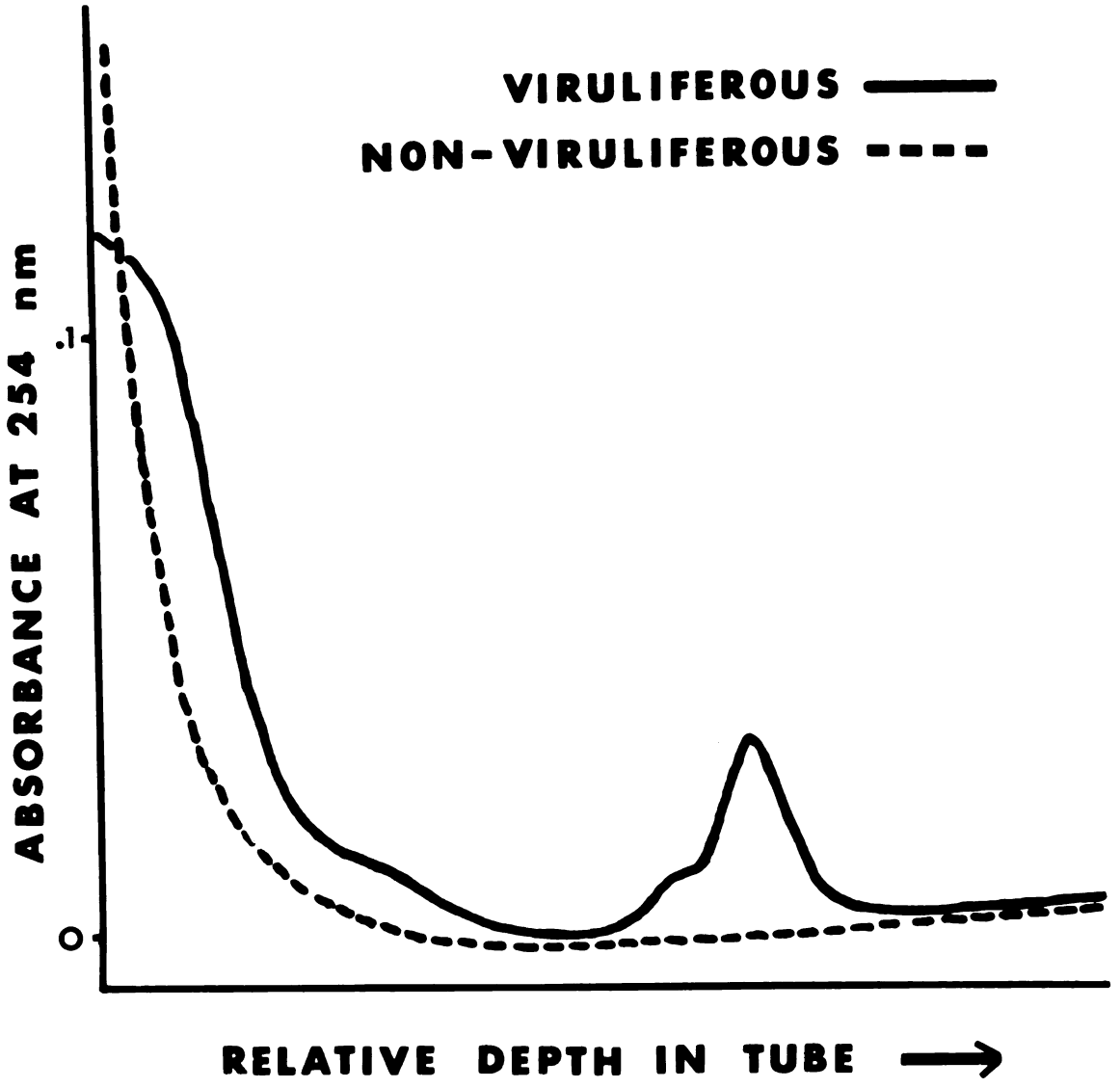

FIGURE 5. UV scanning profile at 254 nm of preparations of non-viruliferous and pea enation mosaic virus-infested pea aphids after centrifugation into sucrose density gradients (10-40%) for 2 hrs at 24,000 rpm in the Spinco SW 25.1 rotor.

particles were abundant in both preparations from viruliferous aphids, but aphid tissue debris obscured virus particles in the partially purified preparation. The density gradient preparation contained very little cellular debris and virus was easily detected in samples from the sedimentation zone (Figure 6A and B). Virus particles were of uniform size and shape, and were ca. 27 nm in diameter. Although each grid opening (300) mesh contained numerous distinct virus particles, the real concentration might have been much higher as the distinct negatively-stained particles were apparently underlain with thousands of other particles which were only faintly stained (Figure 6A). Only a few scattered virus particles were found in samples from above and below the sedimentation zone. No virus-like particles were found in either preparation of non-viruliferous aphids.

Comparative transmissibility of virus after consecutive aphid-to-plant or plant-to-plant transfer.--Virus partially purified from aphids was mechanically inoculated to pea seedlings to establish an aphid-source PEMV line for subsequent aphid-transmission comparisons with a PEMV line that was established after purification from a plant source. Both lines originated from the same isolate of NY-PEMV. The plant-source line was initiated 2 months previous to the aphid-source line through use of the purification technique previously reported (Thottappilly et al., 1972) and was maintained by thrice-monthly mechanical transfers of crude sap after the initial purification. Prior to the following experiment the aphid-source line had been mechanically transferred on 1 occasion and the plant-source line mechanically transferred on 6 occasions.

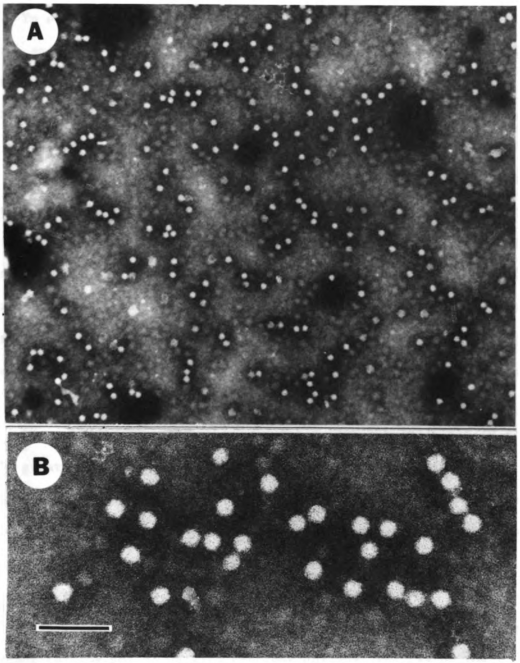

FIGURE 6. Electron micrographs of negatively-stained preparations of DGC-purified suspensions obtained from PEMV-carrying pea aphids. A. Virus particles at magnification of 69,000 X. B. Particles at 198,000 X. Bar represents 100 nm.

To obtain source plants for comparative transmission tests, the plant- and aphid-source lines were inoculated to young pea seedlings by mechanical and aphid means, respectively. Ten days later plants of both lines were used as virus sources for aphid-transmission trials and for virus purification.

First-stage A. pisum nymphs were starved 2 hr and given 1- and 4-hr acquisition-access periods on source plants of both lines; source plants of the 2 lines were indistinguishable and showed severe symptoms. Thirteen to 16 aphids were tested per acquisition period per each of 3 source plants (replicates) per virus line. The 4-hr treatment aphids were serially transferred at short intervals to healthy pea seedlings to enable detection of latent period differences. The 1-hr treatment was allowed a 5-day inoculation period.

The aphid-source line was transmitted with significantly ($P < 0.05$) greater efficiency after either the 1- or 4-hr acquisition periods than was the plant-source line (Table 13). The median latent period (LP_{50})[1] of the aphid-source line was significantly shorter than the plant-source line, and the former virus line completed latency in 50% of the test insects in less than 6 hr.

Each virus line was partially purified from plants, assayed by UV scans (at 254 nm) of density gradient columns, and tested for

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[1]The median latent period (LP_{50}) was calculated by transforming the time, in hours, at the midpoint of the transfer interval to logarithms and the cumulative percent of first transmissions to probits, calculating a least squares linear regression and solving for value of time when probit value of cumulative first transmission was equal to 5. Virus acquisition was assumed to have occurred at the start of an acquisition-access period and inoculation at the midpoint of a transfer interval.

TABLE 13. Aphid-transmission characteristics of PEMV lines after consecutive aphid-to-plant or plant-to-plant transfers

Virus line	Virus acquired from source plants[a]: Mean % transmission after AAP of: 1 hr	4 hr	Mean LP_{50} (hr)	% Aphids with latent period of < 6 hr	Virus artificially acquired from partially purified preparations[b]: % Transmission after 4-hr AAP on virus solution at $\underline{A}_{260}$ nm: 1.9	7.5	LP_{50} (hr)	% Aphids with latent period of < 6 hr
Aphid-source	45.9	95.8	5.7	50.0	50.0	100	11.4	22.7
Plant-source	31.3	78.6	10.4	21.9	20.8	66.7	12.5	7.2

[a]Three source plants (replicates) were used per AAP (acquisition-access period); 13-16 first-stage pea aphid nymphs were tested/source/treatment; each transmission and latent period mean is significantly different ($\underline{P} < 0.05$) from every other mean; LP_{50} = that elapsed time when virus completed latent period in 50% of test population; latent periods were determined from aphids of the 4-hr AAP.

[b]Virus was purified from plants of the same lot as those used for source plants in other phase of the experiment; 21-24 first-stage pea aphid nymphs were tested singly per treatment; the $\underline{A}_{260}$ = 7.5 concentration was used for latent period determinations.

aphid-transmissibility. Tissue used from the aphid-source line totalled 42.1 g; plant-source line 39.1 g. Partially purified virus preparations were adjusted to an absorbance at 260 nm of 7.5; this solution and a 1:3 dilution ($\underline{A}_{260}$ = 1.9) were fed to 1st-instar pea aphids via artificial feeding for 4 hr. The $\underline{A}_{260}$ = 7.5 treatment was serially transferred at short intervals to enable determination of latent periods; an overall 5-day inoculation period was provided both treatments. The purified aphid-source line at either concentration was transmitted with much greater efficiency after the 4-hr acquisition period than was the plant-source line (Table 13). Both lines showed similar LP_{50}'s (aphid: 11.4 hr; plant: 12.5 hr), but the plant-source line completed latency in only 7.2% of the test plants within 6 hr whereas the aphid-source line exhibited completion of latency in 22.7% of the aphids in the same interval.

When 0.5 ml of each partially purified virus suspension (adjusted to $\underline{A}_{260}$ = 8) was layered on density gradient tubes and centrifuged for 1 1/2 hr at 24,000 rpm, similar and typical UV-absorbance patterns were obtained for both virus lines (Figure 7). However, the aphid-source line preparation contained only 40 µg of virus per milliliter whereas the plant-source line contained 67 µg, as determined through planimeter measurements of virus absorbance peaks and assuming an extinction coefficient of 7.5/mg/ml/cm at 260 nm (Shepherd et al., 1968). Furthermore, plants infected with the aphid-source line yielded less virus than did plants infected with the plant-source line (56 vs 93 µg/g of tissue).

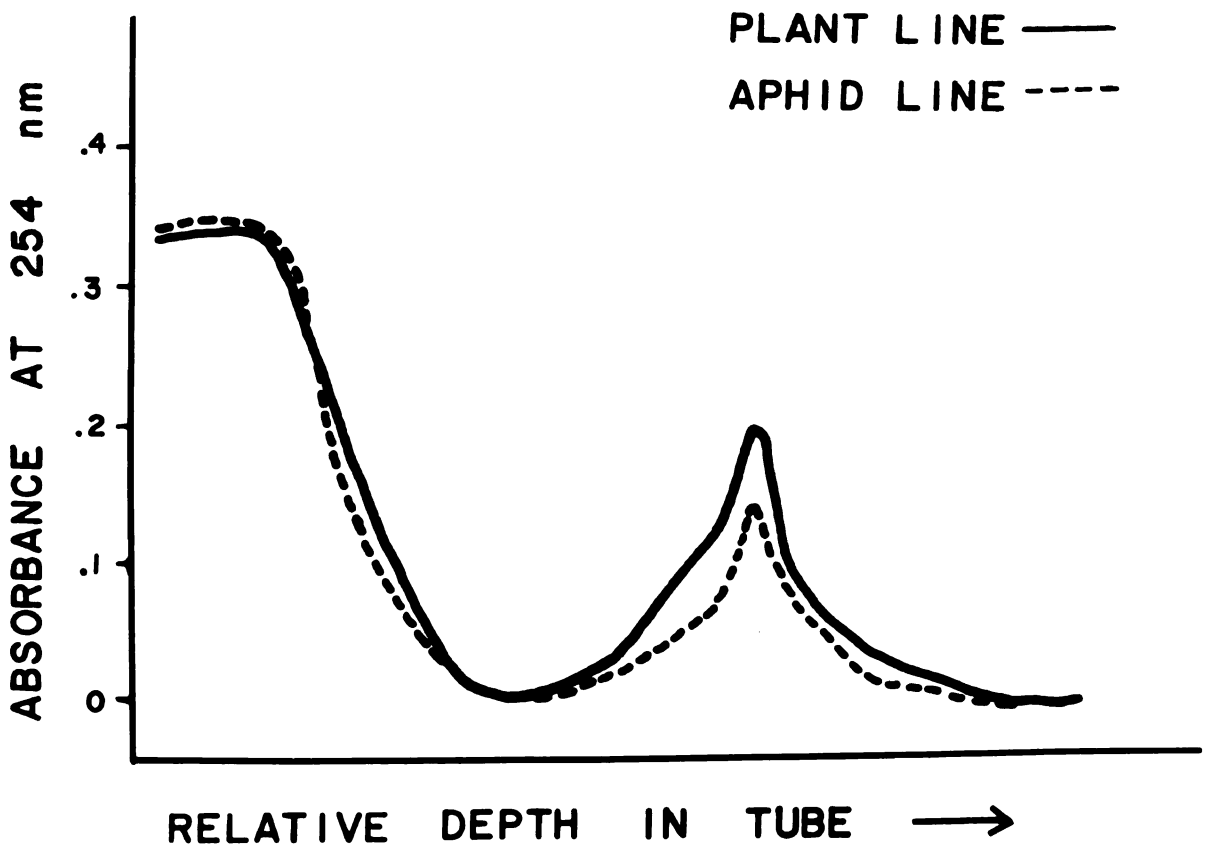

FIGURE 7. Scanning pattern at 254 nm of fractions from preparations of pea infected with an aphid line (initiated with partially purified virus from pea aphids) and a plant line (initiated with partially purified virus from pea plants) of pea enation mosaic virus. Sedimentation occurred in sucrose density gradients (10-40%) centrifuged for 1-1/2 hr at 24,000 rpm and 4° in the Spinco SW 25.2 rotor.

DISCUSSION

The size and shape of the PEMV particles that we purified from viruliferous pea aphids were generally the same as particles purified from infected plants (Bozarth and Chow, 1966; Gibbs et al., 1966; Musil et al., 1970) and observed in ultra-thin sections of plants and aphids (Shikata et al., 1966; Harris and Bath, 1972), and the UV scanning pattern closely approximated those we have obtained with preparations from plant sources. However, after the purified aphid-source virus was established in plants it was transmitted by aphids with remarkably high efficiency. The median latent period (LP_{50}) of the aphid-source virus line (5.7 hr at 25°) was not only significantly shorter than that obtained for the comparable virus line from a plant source, but it was by far, the shortest LP_{50} recorded for any PEMV isolate in any pea aphid biotype. Even if inoculation was assumed to have occurred at the end of inoculation access periods, the LP_{50} was only 7.8 hr. Other comparable LP_{50} estimates on record are: 25.0 and 14.0 hr at 20° and 30°, respectively (Sylvester and Richardson, 1966), 20.6 hr at 24° (Chapman and Bath, 1968), and 19.5 hr at 22° (Bath and Tsai, 1969). In addition the efficiency with which 1st instars transmitted the aphid-source virus line after 1- and 4-hr acquisition-access periods was approximately equal to the highest transmission efficiency (51.7 and 92.9% after 1- and 4-hr AAPs, respectively) known for a PEMV and pea aphid relationship (Bath and Chapman, 1968). Furthermore, after the aphid- and plant-source virus lines were purified from pea plants of the same lot as those used for virus source plants in the plant-to-plant transmission

trials, virus of the aphid line was much more efficiently transmitted by aphids after membrane feeding than that of the plant line.

The enhanced aphid-transmissibility of the aphid-source line over the plant-source line apparently was not a function of virus concentration in the source plants or membrane-feeding system; for the source plants of the aphid line yielded less virus than those of the plant line and the UV scans of density gradient columns showed that less virus was present in the partially purified aphid-source preparation that was fed to test aphids than was present in the plant-source preparation.

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