C. 1

PURIFICATION AND PROPERTIES OF POTATO VIRUS M (PVM)

by

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ABSTRACT

Studies on purification and properties of potato virus M (PVM) were carried out using an isolate found in British Columbia. The narrow host range of the virus was confirmed, and no new susceptible species was discovered. Potato cultivars failed to develop symptomps even in plants produced by tubers of inoculated plants, but none was immune. An attempt to demonstrate transmissibility of the virus by plant contact was unsuccessful.

In undiluted potato sap the virus had a thermal inactivation point (TIP) of 65 to 70°C, and a longevity in vitro (LIV) of 2 to 4 days. The dilution end point (DEP) was 10^{-4}. The LIV and DEP of the virus in tomato sap were similar to those in potato sap. Crude sap diluted to 10^{-1} induced more lesions on Red Kidney bean than undiluted sap.

An efficient purification procedure for PVM was developed. The virus was purified from leaves of potato (Solanum tuberosum L.), by extraction with 0.5 M borate buffer, pH 7.8, clarification with ammonium sulfate (20%), and concentration with ammonium sulfate (30%). Further concentration was carried out by high speed centrifugation followed by polyethylene glycol (PEG 6000) precipitation and high speed centrifugation. Final purification was by sucrose density gradient centrifugation. The yield obtained from this procedure was 3.7 to 4.1 mg per Kg of infected leaves.

The purified preparations contained rod-shaped particles 651 nm normal length and 13.4 nm average width. The particles had an A_{260}/A_{280} ratio of 1.23, an A_{max}/A_{min} ratio of 1.24, a

maximum ultraviolet light absorption at 260 nm, a minimum absorption at 245 nm, and a buoyant density in CsCl of 1.304 (suggesting an RNA content of 6.2%). The molecular weight of the protein subunit was about 39,300 daltons.

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INTRODUCTION

Members of the Carlavirus Group occur in characteristically low concentrations of 20 to 100 mg per liter of sap in their respective hosts (Harrison et al., 1971). A large amount of starting material is required to obtain even a small amount of purified virus. Hence it is important to minimize the amount of virus lost during the various steps of any purification procedure.

To study the properties of a virus, especially its biophysical and biochemical properties, an adequate amount of practically purified virus should be available. The difficulty of purification of a virus is often reflected by the paucity of information on these properties. Indeed, most of the Carlaviruses described in the "Description of Plant Viruses" of the Commonwealth Mycological Institute and the Association of Applied Biologists, lack details of their biophysical and biochemical properties (Appendix II).

This thesis describes an efficient purification procedure for potato virus M (PVM), by means of which sufficient amount of the virus was purified to permit a study of some of its biophysical and biochemical properties. Other properties including host range, symptomatology, susceptibility of potato cultivars and properties in crude sap were also investigated.

REVIEW OF LITERATURE

(a). History and Nomenclature.

The name "potato virus M" (PVM) was introduced in 1956 by Bagnall et al. to designate a third virus isolated from 'Irish Cobbler' potatoes (Solanum tuberosum L.), infected with the "interveinal mosaic" disease (McKay and Dysktra, 1932). The other two viruses were potato virus X (PVX) and potato virus S (PVS).

The diseases "leafrolling mosaic" (Schultz and Folsom, 1923) and "paracrinkle" (Salaman and Le Pelley, 1930) were also shown to be caused by PVM in combination with PVX and PVS or with PVS only (Bagnall et al.,1956, 1959). The virus isolates designated as "virus K" (Kohler, 1942) and "D1102" (Kohler, 1953; Wetter and Brandes, 1955 and 1956) were later identified as PVM (Bagnall et al., 1956, 1959).

PVM has had a unique history because it evoked a controversy on the origin of viruses. The apparent observation that the "paracrinkle virus" was only transmitted by grafting (Bawden, 1939), led Darlington (1944) to propose the theory that the "virus" had arisen "de novo" in the cultivar King Edward. This theory assumed that the particle which he called "provirus" could behave like plasmogenes to produce the disease on other cultivars. This proposal gained the support of van der Plank (1948), but was criticized by Corsan et al. (1944) and Smith (1946). The production of virus-free stock of 'King Edward' from virus-infected stock by tissue

culture (Kassanis, 1957), finally put an end to these speculations (Bawden and Kassanis, 1965).

The name "potato virus M" is now commonly accepted and older names are abandoned (Beemster and Rozendaal, 1972) or listed as its synonyms (MacLeod, 1962; Martyn, 1968; Wetter, 1972). The following are considered synomyms of PVM:

Leafrolling mosaic virus	Schultz and Folsom, 1923
Interveinal mosaic virus	McKay and Dysktra, 1932
Paracrinkle virus	Salaman and Le Pelley, 1930
Potato virus E	Bawden, in Smith, 1933
Potato virus 7	Smith, 1937
Potato virus K	Kohler, 1942
Solanum virus 11	Smith, 1937
Kartoffel-Rollmosaic-Virus	Kohler, 1935
Potato virus F,C.	Augier de Montgremier , Devergne, 1958

The term "strain" has been used by various authors to designate PVM isolated from different sources (Rozendaal and Van Slogteren, 1957; Kassanis, 1960, 1961; Bartel and Volk 1966; Bode and Weideman, 1969, 1971; Bagnall et al, 1959). Wetter (1972) listed the following as the strains of PVM:

Leafrolling mosaic isolate

Interveinal mosaic isolate

Paracrinkle isolate

D1102 and Fortuna isolates, and

Dutch isolates.

(b). Distribution and economic importance

PVM has a worldwide distribution in many cultivars of potatoes (Wetter, 1972; Hiruki, 1973). In addition to North America, Germany, Britain and the Netherlands, the virus has been reported from other countries in Europe (Bode, 1958; Pajan, 1967), Asia (Horio et al., 1969) and South America (Beemster, 1969). It has been reported that PVM is the most widespread potato virus in Latvia (Duda, 1969).

The economic significance of PVM has not been assessed in great detail. However, according to Kassanis and Schwabe (1961), and Bawden and Kassanis (1965), the cultivar King Edward gave a higher yield when a virus-free stock derived from tissue culture (Kassanis, 1957) was used for planting. Strains which cause severe symptoms on certain cultivars of potato will severely depress yields of these cultivars (Beemster and Rozendaal, 1972). Tuber necrosis has been found correlated with the foliage symptoms (Loughnane, 1957), thus disproving an earlier suggestion by Smith (1946) that PVM is a virus of no economic significance.

No potato cultivar has been found to be immune to PVM. Zadina (1971) and Hunnius (1972 a) reported susceptibility of 630 and 47 potato cultivars, respectively. The low efficiency of aphid transmission of PVM is evident from the fact that of the 92 cultivars tested by Bode and Weideman (1971) with Myzus persicae Sulz., more than half of them either failed to become infected or were infected in small numbers.

(c). Host range and Symptomatology.

The host range of PVM is relatively narrow. Susceptible species listed by the various authors (MacLeod 1962; Vulcic and Hunnius 1967; Horio et al., 1969; Bagnall et al., 1956) belong to the families Amaranthaceae, Chenopodiaceae, Leguminosae and Solanaceae. USDA (1966) listed 21 susceptible species, but recent investigations in Europe and North America have significantly increased this number (Horvath, 1972; Horvath and de Bokx, 1972; Bagnall, 1972; Dziewonska and Ostrowska, 1975). The majority of these host plants belong to the Solanaceae (Wetter, 1972).

Symptoms vary between plant species. Infected Datura metel L. exhibits local chlorosis, necrosis and systemic necrosis. Nicotiana debneyi Domin. reacts to PVM infection by producing local brownish necrotic lesions (Bagnall et al., 1956, 1959). The virus induces very severe symptoms in Lycopersicon chilense Dun. in the form of epinasty, distortion and leaf abscission (Kowalska and Was, 1976).

In Phaseolus vulgaris L. cv. Red Kidney, minute brown local lesions are observed 3 to 6 days after mechanical inoculations, and this species has been employed as a test plant for assay and detection for PVM (Hiruki, 1970, 1972, 1973; Hiruki et al., 1974). A similar type of local lesion symptoms has been described in Vigna sinensis Savi ex. Hassk (Horvath, 1972).

Variations in symptoms incited by different isolates of the virus have been observed on some host plants (Rozendaal and

van Slogteren, 1957; Bagnall et al., 1959; Ross, 1968; Kowalska and Was, 1976). The appearance of symptoms is affected by temperature, and symptoms are more pronounced at temperatures between 16°C to 20°C (Bagnall et al., 1956; Rozendaal and van Slogteren, 1957; Kowalska and Was, 1976).

In potatoes, symptoms range from none to severe depending on the virus strains and the potato cultivars (Wetter, 1972; Burton, 1966). Cultivars which show no symptoms upon infection by PVM have also been reported (Bawden et al., 1950; Kassanis, 1956; Rozendaal and van Slogteren, 1957). Differences in reactions occur not only between cultivars but also between plants of the same cultivar (Chrzanowska, 1976). In the cultivar Uran, the same isolate of PVM incited symptoms varying from slight leaf-rolling or slight curling of the leaflets to some reduction in growth, and sometimes even to necrosis of leaf petioles and dwarfed growth. Moreover, individual plants with severe symptoms were obtained from tubers of plants with slight symptoms and vice versa.

(d). Serology.

PVM is strongly antigenic (MacLeod, 1962; Beemster and Rozendaal, 1972; Wetter, 1972) and antisera against the virus have been prepared using clarified sap (Bagnall et al., 1956), partially purified virus preparations (Rozendaal and van Slogteren, 1957; Bagnall et al., 1959; Wetter, 1960) and its degraded protein ("D-Protein") (Shepard et al., 1971; Shepard, 1972).

In addition to serological relationships between viruses, PVM antiserum has been utilised for the estimation of virus concentration during purification (Wetter, 1960), serodiagnosis and detection of the virus in potatoes and in the test plants. According to Wetter (1972), serological tests are the best methods for diagnosing the virus in potatoes. For the purpose of diagnosis and detection of PVM, the following serological techniques have been utilised: slide precipitin (Wetter, 1960), bentonite flocculation (Kahn et al., 1967), Ouchterlony double-diffusion and single radial-diffusion (Shepard et al., 1971; Shepard, 1972).

(e). Transmission.

The graft-transmissibility of the paracrinkle isolate was reported as early as 1930 by Salaman and Le Pellay. It appeared then that the virus could not be transmitted by other means. In order to explain this seemingly peculiar phenomenon, Bawden (1939), suggested that the normal method of spread of the virus had been lost, and that it survived and multiplied only because its host was propagated vegetatively. It was not until later that the sap-transmissibility of several isolates was demonstrated (Kohler, 1942, 1953, 1955; Bawden et al., 1950; Bagnall et al., 1956). Sap-transmission is achieved when sap from young leaves is used, but is not achieved with inoculum from older leaves (Wetter, 1972).

Transmission by contact has been reported (Symygla et al., 1973), but tests for seed transmission in potato (Corsan et al.,

1944) or in tomato (Horvath, 1973) were negative.

Transmission of PVM by aphid has been demonstrated. Transmissibility depends on the isolates of the virus and the species of aphid. Transmission by Myzus persicae Sulz. has been shown with K (Kohler, 1942), Bintje (Rozendaal and van Slogteren, 1957), paracrinkle (Kassanis 1961), leaf rolling mosaic, D1102 and Fortuna (Wetter and Volk, 1960) isolates. The Rothamsted paracrinkle isolate (Kassanis, 1960, 1961) and the isolates from Japan (Horio et al., 1969) are not transmitted by this insect species. Aphid transmission is non-persistent, (Bode and Weideman, 1971; Kostiw, 1975a). M. persicae is the most efficient vector, followed by Aphis frangulae Koch, Aphis nasturtii Kalt, and Macrosiphum solanifolii Ashmead (Bode and Weideman, 1971). In contrast, Kostiw (1975a, 1975b) has reported that M. persicae is a poor vector of PVM.

Although a considerable spread might be expected (Zadina, 1971), natural transmission of PVM is less extensive than that of most of other potato viruses (Beemster and Rozendaal, 1972; Rozendaal and van Slogteren, 1957; Kassanis, 1961). This natural spread is mainly by aphids (Beemster and Rozendaal, 1972).

Factors which influence the spread of PVM include the distance of plants from the source of infection (Piechowiak and Gladysiak, 1972), temperature (Piechowiak and Gladysiak, 1972; Chrzanowska, 1973); cultivars of potatoes grown (Bawden and Kassanis, 1965) and the age of plants (Hunnius, 1972b).

(f). Purification

Several methods for the purification of PVM have been described by various authors using potato or tomato as propagation host (Rozendaal and van Slogteren, 1957; Bagnall et al.,1959; Wetter, 1960; Shepard, 1972; Maat, 1972; Hiruki, 1974). Solanum demissum Lindl. has also been used as starting material (Hodrejarv et al., 1971). Leaves of these plants could be used fresh (Bagnall et al.,1959), frozen (Hodrejarv et al.,1971) or freeze-dried (Rozendaal and van Slogteren, 1957). The yields of the PVM obtained from the various methods were not indicated by the various authors.

Purification of PVM has been carried out in various buffers with pH's ranging from 7.0 to 8.2 These buffers include citrate (Hodrejarv et al., 1971), phosphate (Rozendaal and van Slogteren, 1957; Bagnall et al.,1959; Hiruki et al., 1974), borate (Shepard, 1972) and ascorbic acid (Wetter, 1960) buffers. In the steps of purification subsequent to the homogenization of tissues, buffers of the same type, but of lower molarity are used. Thus, Shepard (1972) used o.5 M borate buffer for homogenization, and 0.05 M borate buffer for the subsequent steps. However, in the method of Wetter (1960), ascorbic acid buffer was used for homogenization and phosphate buffer was used for the subsequent steps.

Clarification of crude sap in the purification of PVM has been achieved by shaking with organic solvents, for example

chloroform (Hodrejarv et al., 1971; Shepard, 1972), butanol and carbon tetrachloride (Bagnall et al., 1959), cold chloroform, ethanol and acetone (Rozendaal and van Slogteren, 1957) diethyl ether and carbon tetrachloride (Wetter, 1960; Maat, 1972) and n-butanol (Hiruki et al., 1974). Clarification of PVM-containing sap by stirring it with celite has also been described (Albrechtova and Klir, 1970).

Partially purified PVM preparations have been obtained by subjecting the clarified sap to one or two cycles of differential centrifugation (Wetter, 1960; Rozendaal and van Slogteren, 1957). However, combinations of differential centrifugation and precipitation with polyethylene glycol (PEG) (Albrechtova and Klir, 1970; Shepard, 1972; Hiruki et al., 1974) or precipitation with ammonium sulfate (Maat, 1972) are more commonly practised. According to Albrechtova and Klir (1970), PVM solutions prepared by combining PEG precipitation and differential centrifugation proved to be purer than those prepared by means of gel filtration with Sephadex G-100.

The final step of purification has usually been the density gradient centrifugation (Maat, 1972; Wetter, 1960) or electrophoresis in a sucrose gradient column (Hodrejarv et al., 1971).

(g). Properties

Some physical and biophysical properties of PVM have previously been reported by various authors, and are summarized in Table 1.

Table 1. Biophysical properties and properties in crude sap of PVM as reported in the literature

Properties	values for PVM
1. Length of particle (nm)	644 (i*); 650 (f,j,n,p); 651 (e) 660 (o)
2. Width of particle (nm)	12-13 (e) 15 (f)
3. Longevity in vitro (days)	2 (a,b,g,h,j,)
4. Thermal inactivation point (C^o)	55 (c); 60-65 (d,q); 65-70 (a,b,j) 68-71 (q); 75 (m); 80-85 (k)
5. Dilution end point	10^{-4} (g,h,p); 10^{-3} (j,l)

* (a) Bagnall et al.,1956 (b) Bagnall and Larson, 1957a, 1957b

(c) Bawden et al.,1950 (d) Bode, 1958

(e) Brandes et al.,1959 (f) Brandes, 1960

(g) Hiruki, 1972 (h) Hiruki, 1973

(i) Hitchborn and Hill, 1965 (j) Horio et al.,1969

(k) Kassanis, 1956 (l) Kohler, 1955

(m) Kohler, 1957 (n) Lee, 1971

(o) Rozendaal and van Slogteren, 1957 (p) Tu and Hiruki, 1970

(q) Wetter and Brandes, 1956

Little is known of the biochemical properties of the virus. The only report was made by Nurmiste (1966) who determined that the nucleic acid of the virus is RNA. For the purpose of virus classification, probable similarities of properties of PVM with those of the carnation latent virus have been assumed (Harrison et al., 1970).

From the foregoing review it is evident that gaps exist in our knowledge of this virus. Studies which have been carried out are mainly concerned with its host range, symptomatology, transmission and its properties in crude sap. Evaluation for immunity or susceptibility in European potato cultivars to PVM has been carried out, but there is no corresponding evaluation for the majority of the North American potato cultivars. Moreover, reports on the properties of the virus in purified preparation are scarce. This probably reflects the fact that no efficient purification procedure has been described.

Therefore, the objectives of the present studies are to:

1. Evaluate the host range and symptomatology of a North American isolate of PVM,
2. evaluate the North American potato cultivars for immunity or susceptibility to this virus,
3. investigate the properties in crude sap,
4. attempt to devise a better purification procedure, and
5. determine as many biophysical and biochemical properties of the virus using the purified preparation as possible.

MATERIALS AND METHODS

(a) The PVM Isolate

The PVM isolate was kindly provided by Dr. N.S. Wright, Canada Department of Agriculture, Vancouver, British Columbia. It was maintained in potato plants "cultivar Banana", a locally assigned name to designate a selection which has banana-shaped tubers. The isolate was free from contamination by potato viruses A, F, S, X or Y as indicated by the negative results of biological tests on indicator plants including Capsicum annuum L., Nicotiana tabacum L. 'White Burley', Nicotiana clevelandii Grey, Gomphrena globosa L., Chenopodium quinoa L. and Chenopodium amaranticolor Coste and Reyn. The symptoms observed in C. amaranticolor, C. quinoa and G. globosa were typical of PVM infections (Bagnall et al., 1956; Horvath and de Bokx, 1972). Serological tests against potato viruses S, X and Y confirmed the negative results from inoculation tests.

The initial PVM-infected potato plants, cv. Banana, were then propagated by cuttings. Cut shoots were placed under mist for about 10 days for rooting and were then replanted into pots. Leaves from the newly generated plants could be harvested in about 5 weeks. It was also possible to root the cuttings in soil. Watering them twice daily was as effective as misting in a mist chamber.

Throughout the studies the temperature of the greenhouse was kept at 18 to 20°C, and the virus culture was periodically checked for the presence of potato viruses S, X or Y,

by inoculations on test plants and by serology.

(b) Host Range and Symptomatology.

Forty-one species or cultivars of plants belonging to the families Amaranthaceae, Apocynaceae, Carophyllaceae, Chenopodiaceae, Compositae, Cucurbitaceae, Graminae, Leguminosae, Liliaceae, Cruciferae, Solanaceae and Umbelliferae were tested for their susceptibility to PVM. Symptoms induced by the virus in infected plants were observed. Inoculation procedure was as follows: Leaves from PVM-infected tomato plants, 5 weeks after inoculation, were ground in a mortar. The sap was filtered through muslin cloth, then diluted in 5 volumes of 0.05 M borate buffer, pH 7.8, and finally inoculated with the aid of cotton swabs onto leaves or cotyledons which had been dusted with carborundum. Eight to twenty plants were inoculated per species or cultivar at the most susceptible stage or age. After inoculation the excess inoculum was immediately washed off with water.

Symptoms were observed for 3 to 4 weeks, after which time the plants were tested for PVM infection. Both inoculated and uninoculated leaves were tested by rubbing onto Red Kidney bean. The host range and symptomatology studies were done throughout the later half of 1975 and the first half of 1976. Inoculation tests on some species or cultivars of plants were repeated when the results obtained were contrary to those reported in the literature.

In addition, 33 cultivars of potato were inoculated to

determine their immunity or susceptibility to this isolate of PVM. The plants were grown from virus-free tubers in 5-in pots. The inoculum was prepared as described above, but the carborundum was premixed in the inoculum before application with polyurethane sponges onto the leaves of the plants. Three to eight plants per cultivar were inoculated at the two-leaf stage. The inoculations were repeated one week later. Uninoculated plants were maintained as controls. All plants were thinned to a maximum of four shoots. Inoculations were carried out in spring 1975, winter 1975 and spring 1976. In most cases inoculation test on a particular cultivar was done only once.

Detection of infection was carried out serologically from the top four leaves, 5 weeks after first inoculation (Hunnius, 1972b). Three to four shoots were tested from each plant. In cases where infection was not detected, the serological testing was repeated 3 weeks later. Observation for symptoms was carried out until the 8th week postinoculation. Tubers from the primarily infected potato plants, cultivars Banana, Red La Soda and White La Soda were harvested and replanted in Fall 1976. At the same time some cultivars which had failed to become infected with the virus through mechanical inoculation were graft-inoculated with PVM-infected shoots from potato plants cultivar Banana. Testing for virus infection was done 6 weeks postinoculation.

Experiments were also carried out to test transmissibility of the virus through plant contact. In one experiment tomato plants, cultivar Rutgers, were used. Four uninoculated plants

were placed in contact with four mechanically inoculated plants throughout the growing period. Four other plants placed about 2 feet away were maintained as controls. Six weeks after inoculation the plants were serologically tested for PVM infection, and when negative results were obtained, the detection was repeated 2 weeks later. The experiment was replicated three times.

In another experiment, two virus-free potato plants, cv. Arran Victory were placed in contact with PVM-infected 'White La Soda' plants for 8 weeks, after which serological detection for virus infection was carried out. This experiment was also replicated three times.

(c) Serology

Antiserum against PVM was prepared by injecting a young rabbit with the virus purified by a modification of the method of Shepard (1972). Overnight incubation of the crude sap with Triton X-100 added to 1%, and one cycle of sucrose density gradient centrifugation were incorporated into the purification procedure.

The first injection was done intramuscularly with 0.5 mg per ml of virus solution in 0.05 M borate buffer, pH 7.8. Prior to the intramuscular injection, the virus solution was first emulsified with Freund's complete adjuvant. In each of the subsequent injections, 1 mg of virus was used. One intravenous injection was carried out 1 week after the first injection, followed by one intravenous and one intramuscular

injection 2 weeks later. Two "booster" injections, one intravenous and the other intramuscular, were applied 8 weeks after the first injection. The rabbit was bled weekly and the titers of the antiserum were estimated by the tube precipitin test against sap from virus-infected or virus-free plants.

Antiserum against normal plant components was obtained from a young rabbit which had been given two intramuscular and three intravenous injections of normal plant components from virus-free plants. Extraction of the normal plant components followed the same method as above, except that the treatments with chloroform, Triton X-100 and sucrose density gradient centrifugation were omitted.

(d) Properties in Crude Sap

The following properties in crude sap were investigated: Dilution end point (DEP), longevity in vitro (LIV) and thermal inactivation point (TIP).

Sap for the experiment to determine the DEP and LIV was obtained from potato plants (cv. Banana) and tomato plants (cv. Rutgers). For the TIP determination, only sap from potato plants was used. The tomato plants were inoculated with PVM 5 weeks prior to the test. Leaves collected from these plants were macerated in a mortar, and the sap extracted was then filtered through muslin cloth. In all three experiments the treatments were duplicated, and each replicate was rubbed onto three half-leaves of 10 to 12 day old Red Kidney bean plants.

The number of local lesions per half-leaf for each treatment was recorded 6 and 12 days after inoculation.

Determination of DEP was made with sap diluted in 0.05 M borate buffer, pH 7.8, to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} or 10^{-6} of the original concentration. The undiluted sap was inoculated onto one of the half-leaves of each Red Kidney bean plant. Another half-leaf from the opposite primary leaf was inoculated with buffer only. The treated sap was then inoculated randomly onto the remaining two half-leaves.

In the determination of TIP, the sap was incubated in a water bath for 10 min at temperatures of 30°C, 40°C, 50°C, 60°C, 70°C or 80°C, and subsequently with sap incubated at temperatures of 60°C, 65°C, 70°C or 75°C. To facilitate heating and cooling, the sap samples were placed in narrow and thin-walled lyophilizing tubes. They were immediately cooled in ice for 30 sec, then inoculated onto Red Kidney bean half-leaves.

The determination of LIV was done by incubating sap samples at room temperature for 1, 2, 3, 4 or 5 days, after which they were inoculated onto Red Kidney bean plants. Freshly extracted sap from leaves of PVM-infected potato plants was considered as the untreated control.

(e) Purification

The progress of purification during the various steps was followed by several methods. For the early steps of purification the amounts of plant debris in the preparations were estimated by measuring the turbidity values at 540 nm. A preliminary

investigation (Appendix I) showed that the variation in this turbidity value was proportional to the concentration of debris originating from the host plant leaves. In the case of partially purified virus preparation the criteria of purity used were a value of 1.20 to 1.35 for the ratios of absorptions at 260 nm to 280 nm (A_{260}/A_{280}), the lack of reaction with antiserum against normal plant components and the homogeneity of particles when examined by electron microscopy. The majority of viruses belonging to the Carlavirus Group have A_{260}/A_{280} values between the above mentioned range. (Appendix II). The A_{260} values were also used for the estimation of concentration of partially purified and purified virus preparations.

The tube precipitin serological test was used to estimate the relative virus concentrations which were assumed to be proportional to the reciprocals of the respective serological dilution end points. The procedure was as follows: Double dilution series of the virus preparations were made with physiological saline (0.85% NaCl) in test-tubes. To 1 ml of each of these diluted samples was added an equal volume of the antiserum against PVM which had been diluted 200 times in saline. Incubation of the mixtures was carried out for 2 hr at 37^{o}C, but the presence or absence of a positive serological reaction was recorded only after a further incubation at room temperature overnight. Sap from leaves of virus-free plants was also included in the test to detect any unspecific serological reaction.

In experiments involving different buffers, or those involving buffers with different molarities, pH's or additives, the various samples were first dialysed overnight in a common buffer. In the extraction step, the common buffer was 0.5 M borate, pH 7.8, and in the subsequent steps it was 0.05 M borate. Following dialysis, the samples were transferred into a 10 ml measuring cylinder to make sure that there were no volume changes. In experiments involving volumes, examples, homogenization of leaf tissues or resuspension of precipitates, the differently treated samples were first centrifuged, then separated from the pellets, and finally brought to the same volume for serological dilution end point determination and turbidity measurement.

Techniques which showed minimum loss of virus and maximum exclusion of materials of plant origin when employed at a particular step were chosen. Usually, a compromise between retention of virus and clarity or purity of preparations was made.

1. Extraction of virus from host tissues

Experiments were carried out to determine a suitable procedure for extraction of the virus from propagation host plant tissues. For this purpose, the effects of homogenizing leaf tissues in different extraction buffers, in the same buffer but of different pH's or in the same buffer but added in different tissue to buffer ratios, were studied. The effect of incorporating a reducing or chelating agent into the

extraction buffer was also examined. The object was to obtain crude sap with the maximum concentration of virus.

PVM-infected potato leaves were cut into small pieces. Five gram samples were then taken and individually homogenized in a buffer in a 10 ml blender for 2 min. The blender was cooled under running water for a short period, after which tissue homogenization was continued for a further 2 min. The standard procedure was to homogenize the leaves in 0.5 M borate buffer, pH 7.8, at the ratio of 1:3 (w/v), tissue to buffer. No reducing or chelating agent was used in the system. The other treatments were essentially the variations of this system with respect to the pH of buffer, type of buffers, tissue to buffer ratios, or the addition of a reducing or a chelating agent in the buffer. The homogenates were filtered through muslin cloth and the filtrates were then centrifuged at 9,500 rpm (10,800 g) for 10 min in a Servall RC-2 centrifuge. The virus concentrations of the supernatants were then compared.

Experiments were undertaken to determine the most suitable propagation host for use in purification. Potato plants cvs. Banana, Columbia Russet and White La Soda, and tomato plants cvs. Rutgers and Subarctic were tested. In addition, the effect of age of plants on the concentration of virus was investigated by harvesting the leaves of PVM-infected plants cv. Banana 4, 5, 6, 8, 10 or 15 weeks after transplanting. The plants harvested 4, 5 or 6 weeks after transplanting were

allowed to regrow for a further 4 weeks, after which a second harvest was carried out. An experiment was also carried out to determine the possibility of using frozen leaves or leaves which had been stored overnight at $4^{o}C$.

In most experiments the replication of treatments was four but in others the replication was five.

2. Clarification of Crude Sap

Investigations were carried out to determine the suitability of several physical and chemical clarification methods which had been employed in virus purifications. In a preliminary study, 11 methods were "screened" for ones which showed promising results as indicated by their low turbidity values and their high reciprocals of serological dilution end points.

PVM-infected potato leaves were homogenized in a Waring blender with 0.5 M borate buffer, pH 7.8, at the ratio of 1:3, tissue to buffer. Sodium diethyl dithiocarbamate (DIECA) was added as chelating agent at 0.5% (w/v) final concentration. The homogenates were squeezed through muslin cloth and 10 ml aliquots of the resulting filtrates were dispensed into centrifuge tubes. Six of these samples were then individually clarified with one of the following methods:

(1) Emulsification with equal volume of chloroform;

(2) Emulsification with 1.5 ml chloroform;

(3) Incubation for 30 min with 0.8 ml n-butanol;

(4) Emulsification with equal volume of carbon tetrachloride;

(5) Emulsification with equal volume of ether;

(6) Overnight incubation with Triton X-100 made up to 1% (v/v);

(7) Emulsification with 1.5 ml chloroform, broken by centrifugation, followed by treatment with Triton X-100 as in 6;

(8) Incubation at 45°C for 10 min;

(9) Centrifugation at 9,500 rpm for 10 min, then stirring the supernatant with 0.5 gm of celite for 5 min;

(10) Overnight precipitation at 4°C with 2.5 gm ammonium sulfate;

(11) Overnight freezing, then thawing at room temperature.

The emulsions or suspensions were then centrifuged at 9,500 rpm for 10 min. The supernatants were brought to the same volume by adding 0.5 M borate buffer, pH 7.8. Turbidity values at 540 nm were measured immediately, but the serological dilution end point was determined only after an overnight dialysis against the same buffer.

Further investigations were carried out on methods which showed promising results. In addition, methods which resulted in very clear sap, though low in virus content were also considered for further investigation. Methods which caused appreciable loss in virus and which resulted in high turbidity values were not tested further.

Crude sap obtained by homogenizing leaves in 0.5 M borate buffer at the ratio of 1:3 or 1:1.5, tissue to buffer, were

used as test materials. For brevity these ratios were assigned the terms "1:3 sap" or 1:1.5 sap", respectively. When few clarification methods which showed promising results were obtained, direct comparison between them were carried out, and 1:3 and 1:1.5 sap from potato or tomato leaves were tested.

3. Concentration of virus from clarified sap

From this section onward only 1:1.5 sap was tested. Borate buffer, pH 7.8 was used throughout unless otherwise stated. Four methods for the concentration of virus from clarified sap were compared: high speed centrifugation, ammonium sulfate precipitation, acid precipitation and polyethylene glycol (MW=6000) precipitation. Treatments were carried out on 25 ml samples of crude sap.

High speed centrifugation was carried out at 28,000 rpm for 1.5 hr in a Beckman Model L ultracentrifuge with no.30 rotor. Precipitations were carried out by addition of polyethylene glycol (PEG) and sodium chloride added to 5% (w/v) and 4% (w/v) respectively (Shepard, 1972), ammonium sulfate to 30% (Bawden, 1950), or by acidification to pH 4 with HCl. The precipitates were pelleted by centrifugation at 9,500 rpm for 15 min in a Servall RC-2 centrifuge (hereafter called the low speed centrifugation). Preliminary experiments were carried out to determine the effects of molarity and volume of buffer during resuspension of pellets obtained by each of these four methods. Resuspension was carried out overnight in 2.5 ml

(1:10), 5.0 ml (1:5) or 10 ml (1:2.5) of 0.5 or 0.05 M buffer; the figures in parentheses represented the ratios of the original volumes to the final volumes of virus preparations. Following resuspension, the undissolved pellets were removed by a low speed centrifugation, and the supernatants were brought to 12.5 ml (1:2) by addition of the appropriate buffer. After overnight dialysis, the serological dilution end points were determined.

In a separate experiment, PVM particles were precipitated from carbon tetrachloride-clarified sap by various combinations of PEG and sodium chloride. The pellets were then resuspended overnight in 5 ml of 0.05 M borate buffer, and before the serological dilution end points were determined the supernatants were brought to 12.5 ml.

The effect of time of incubation with ammonium sulfate was also investigated. Twenty-five ml samples of ammonium sulfate-clarified sap were individually brought to 30% concentration by addition of more ammonium sulfate. They were then incubated for 2, 6 or 16 hr at $4^{o}C$. The precipitates were pelleted by a low speed centrifugation, and then resuspended overnight in 5 ml of 0.05 M borate buffer. After a low speed centrifugation, the supernatants were diluted to 12.5 ml and their serological dilution end points determined.

Finally, direct comparisons were made between the various methods which showed promising results. In all cases 25 ml

samples of sap were used. The resulting pellets were resuspended overnight in 5 ml of 0.05 M borate buffer. After a low speed centrifugation, the supernatants were diluted to 12.5 ml and their serological dilution end points determined. The pellets were again resuspended overnight in 5 ml of the same buffer. After a low speed centrifugation, the "third supernatants" were diluted to 12.5 ml, and then their serological dilution end points were determined. Corresponding experiments were carried out with sap extracted from tomato leaves.

4. Further concentration of virus preparations

The starting materials for the experiments carried out in this section were: (1) virus preparations clarified by ammonium sulfate, then concentrated by ammonium sulfate precipitation; (2) virus preparations clarified by carbon tetrachloride, then concentrated by PEG precipitation. Resuspension of the ammonium sulfate or PEG precipitates were done twice, each time with one-fifth volume of 0.05 M borate buffer. The two components were then added together and their serological dilution end points determined.

A precipitation experiment with PEG carried out during the second stage of concentration followed the same procedure as previously described. PEG was added at the rate of 2.5% or 5.0 % followed by sodium chloride at the rate of 2% or 4%.

Twenty-five ml samples of the starting materials were further concentrated by high speed centrifugation or by PEG

precipitation. The resulting pellets were then resuspended in one-fifth volume (5 ml) of 0.05 M borate buffer. High speed centrifugation was carried out at 28,000 rpm for 1.5 hr in a Beckman Model L ultracentrifuge. Precipitation was carried out by addition of PEG and sodium chloride at 5% (w/v) and 4% (w,v), respectively. After resuspension, the undissolved pellets were removed by a low speed centrifugation. The supernatants were diluted to a final volume of 12.5 ml, and their serological dilution end points were then determined. Similar experiments were carried out with virus preparations derived from tomato plants cultivar Rutgers.

Table 2. Combinations of methods used for the second and third stages of concentration of PVM preparation originally obtained from ammonium sulfate- or carbon tetrachloride-clarified sap.

Source of virus preparations	Combination of methods of concentration		
	Designation	Second stage	Third stage
Ammonium sulfate-clarified sap	I	PEG	high speed
	II	high speed	PEG
Carbon tetrachloride-clarified sap	III	PEG	high speed
	IV	high speed	PEG

In another experiment the PEG precipitation and high speed centrifugation methods of concentration were combined in different sequences I to IV (Table 2). The sample size was 25 ml and 10 ml for the second stage and third stage of concentration, respectively. Resuspension of the pellets obtained from the second stage of concentration was done overnight.

In the third stage, high speed centrifugation was carried out in a no. 65 rotor at 35,000 rpm for 1.5 hr. The pellets were resuspended in 0.3 ml or 2.0 ml of borate buffer, and after a low speed centrifugation the supernatants were brought to 10 ml for serological dilution end point determination.

Finally when tentative procedures for PVM purification were obtained, 40 gm samples of PVM-infected potato leaves were individually homogenized in 60 ml of 0.5 borate buffer, with 0.5 gm of DIECA added to each of the samples as a chelating agent. Three of the samples were subjected to clarification with ammonium sulfate and then to the various concentration methods arranged in the following sequence: Ammonium sulfate precipitation, high speed centrifugation, PEG precipitation, and high speed centrifugation (hereafter called the ammonium sulfate method). The others were subjected to clarification with carbon tetrachloride and then to the various methods of concentration arranged in the following sequence: PEG precipitation, high speed centrifugation, PEG precipitation, and high speed centrifugation (hereafter called the carbon tetrachloride method). At the end of each step the virus preparations were examined with the electron microscope by the negative staining method. The final concentrated virus preparations were then centrifuged through a sucrose density gradient in SW 41 rotor.

5. Density gradient centrifugation

Sucrose density gradient centrifugation was carried out in an SW 41 or SW 25 rotors at 27,000 rpm or 23,000 rpm, respectively, for

1.5 hr. Usually 5 to 35% or 10 to 40% sucrose gradients were prepared. The amounts of samples layered onto the gradients were 0.3 ml and 2.0 ml for the SW 41 and SW 25, respectively. The density gradients were scanned with an ISCO density gradient scanner. The virus peak was collected and then dialysed overnight in 0.05 M borate buffer. Virus preparations from the SW 41 gradients were diluted to 2.0 ml before their A_{260} and A_{280} values were measured. Virus band in the SW 25 gradient was sometimes removed with a syringe.

6. Electron microscopy

A drop of purified PVM preparation in 0.05 M borate buffer was allowed to stand on a grid for 5 min and then drained off by touching the edge of the grid with the edge of filter paper. The grid was then negatively stained with 2% phosphotungstate solution, pH 7.4 for 1 min. Excess solution was drained off and the grid was then examined with a Philips EM 300 electron microscope. Measurement of length of the virus particles was done with the aid of a magnifying glass from electron micrographs at a magnification of 39,960X. Measurement of the particle width was obtained directly from the negatives at a magnification of 23,197X with the aid of a measuring Gaertner Comparator. TMV particles were included for comparison.

(f) Determination of the Biophysical and Biochemical Properties

In this section, purified virus preparation of PVM obtained from a second sucrose density gradient was used.

1. Absorption spectra

The absorption spectra of the purified preparation of PVM in 0.05 M borate buffer, pH 7.8 were measured in a Beckman model Du-spectrophotometer between 230 and 310 nm. Correction for light scattering was achieved by extrapolation of the "absorption" at wavelengths between 315 and 600 nm as described by Noordam (1973).

Measurements for correction for light scattering were obtained from concentrated solutions of PVM. From each of them were taken 3 samples and diluted 10-fold in 0.05 M borate buffer for measurements at wavelengths between 230 nm and 310 nm. The intervals of measurement varied from 1 nm to 10 nm units on the ultraviolet region, and from 15 nm to 50 nm units in the visible region.

2. Estimation of sedimentation coefficient

Purified virus preparations in 0.05 M borate buffer, pH 7.8, were centrifuged in a Spinco Model E analytical ultracentrifuge at 21,740 rpm and at 20^{o}C. Immediately after reaching the intended speed, the Schlieren patterns were photographed at 4 min intervals for about 20 min. The concentration of virus used as estimated by assuming the extinction coefficient to be 3.0 (Shepard, 1972), ranged from 0.25 mg per ml to 0.8 mg per ml. The sedimentation coefficient was then estimated graphically from the negatives by the method of Markham (1960).

3. Estimation of buoyant density

The buoyant density of PVM was estimated by the equilibrium banding method in a cesium chloride solution as described by Chervenka (1973). Centrifugation was done in a Spinco Model E

analytical ultracentrifuge, with a mixture of PVM and TMV in one cell and PVM alone in the other.

Test samples were prepared in two volumetric tubes by dissolving 436.9 mg of cesium chloride in 0.5 ml of 0.005 M borate buffer containing 30 to 50 µg of PVM. To one tube was then added 0.005 M borate buffer while to the other was added 10 to 35 µg of TMV diluted in the same buffer. The samples were centrifuged at 44,770 rpm and at a temperature of 25°C. Under these conditions the bands indicating the positions of the virus particles were formed at about the middle of the equilibrium density gradient profile.

The distance of each band from the center of the rotor was measured by the method of Markham (1960), accurate to 0.001 cm. The true densities of the cesium chloride solutions were estimated from their respective refractive indices determined in an Abbe Model 60 Refractometer. Calculations of the buoyant densities of PVM and TMV standard were carried out as described by Chervenka (1973).

(g) Estimation of molecular weight of protein subunit

The molecular weight of protein subunit was estimated by polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate (SDS). The protein subunit of PVM was prepared by dissociating the virus in 0.1 M phosphate buffer, pH 7.2, containing 4 M urea, 1% SDS and 1% mercaptoethanol (Dunker and Rueckert, 1969). The sample was placed in boiling water for 90 sec to aid dissociation. The protein standards were dissolved in the same buffer

at a concentration of 1 mg per ml and were placed in boiling water for 90 sec. Five proteins of known molecular weights, as indicated in the parentheses, were used as standards. These were bovine serum albumin (62,000), ovalbumin (43,000), alcohol dehydrogenese (37,000), carbonic anhydrase (29,000) and myoglobin (17,200).

The gels were prepared according to Weber and Osborne (1969) and protein subunit of PVM was run in combination with BSA and Mg or alone, while the standards were run in groups of 2 or 3. In all cases at least 2 measurements were available for each protein per run. To all test samples was added a drop of glycine containing a "marker dye" Bromophenol blue, after which 10 μl were individually layered on top of the gels.

Electrophoresis was performed with gel concentrations of 4% through 9% at 50V and 7.5 to 8.3 mA/gel for 3 to 4.5 hours in 0.1 phosphate buffer, pH 7.2 containing 0.1% SDS (Weber and Osborne, 1969). Fixation and staining were done by overnight soaking of the gels in 12.5% trichloroacetic acid solution (TCA) and 12.5% TCA solution containing 0.01% (w/v) Coomasie brilliant blue dye respectively (Chambrach et al., 1967). Destaining was done overnight in 7.5% acetic acid. Calculation of the relative mobilities of each protein with respect to the dye, was achieved by the method of Weber and Osborne (1969), and the molecular weight of PVM-protein subunit was estimated graphically from the plot of proteins' relative mobilities against their molecular weights (Shapiro et al., 1967).

RESULTS

(a) Host Range and Symptomatology

Inoculation tests confirmed previous reports that the host range of the virus is narrow (Bagnall et al., 1956; MacLeod, 1962; Vulcic and Hunnius, 1967; Horio et al., 1969). Of the 41 plant species and cultivars tested, only 12 representing four families, showed susceptibility to PVM: Amaranthaceae - G. globosa; Chenopodiaceae - C. amaranticolor, C. quinoa; Leguminosae - P. vulgaris 'Red Kidney' and Bountiful', V. sinensis 'Black Eye'; Solanaceae - L. esculentum 'Rutgers', 'Subarctic' and 'Tiny Tim', Lycopersicon hirsutum M.B. Eth., L. peruvianum Mill. and L. pimpinellifolium Mill.

All the susceptible plants belonging to the genus Lycopersicon were systemically infected but without symptoms. Others showed non-systemic infections with symptoms of various kinds (Table 3).

PVM was not recovered from the following dicotyledonous species or cultivars belonging to eight families: Apocynaceae - Vinca minor L.; Caryophyllaceae - Dianthus barbatus L., D. caryophylus L.; Chenopodiaeceae - Beta vulgaris L., Chenopodium capitatum L., Spinacea oleraceae L.; Compositae - Zinnia elegans Jacq.; Cucurbitaceae - Cucumis sativus L. 'Straight 8'; Cruciferae - Brassica campestris L. 'Rapa'; Leguminosae - P. vulgaris 'Pinto' and 'Stringless Green Pod', Pisum sativum L.; Solanaceae - C. annum, D. metel, D. stramonium L., N. debneyi, N. clevelandii, N. glutinosa L., N. rustica, N. tabacum L. 'Havana 425', 'Samsun NN', 'White Burley' and 'Xanthii', Physalis floridana Rybd., Petunia hybrida Vilm.;

Table 3 Local lesion symptoms on various species or cultivars of plants incited by PVM

Plant species or cultivars	Symptoms		
	Appearance	Size	Number of days after inoculation
C. amaranticolor	Irregular yellowish green lesions turning slightly reddish in older infection	2 to 3 mm	15 to 18
C. quinoa	Irregular yellowish green lesions turning dark brown in older infection	2 to 3 mm	15 to 18
G. globosa	Diffused brownish lesions turning dark brown in older infection	2 mm	18 to 20
P. vulgaris 'Red Kidney'	Faint greyish lesions turning dark brown in colour in older infection	minute	3 to 6
P. vulgaris 'Bountiful'	Same as in 'Red Kidney'	minute	3 to 6
V. sinensis 'Black Eye'	Reddish-brown lesions turning brown in older infection	minute	8 to 12

Table 4. Mechanical and graft-transmissibility of PVM into the various potaro cultivars carried out at four different times

Cultivars	Number of plants infected over the number of plants inoculated			
	Spring 75	Winter 75	Spring 76	Fall 76
Katahdin	0/4			
Kennebec	0/4		0/3	
Netted Gem	0/4	0/4		
Norchip	0/3		1/3	
Red La Soda	3/3		3/3	
Red Pontiac	0/4		2/3	4/10
Sebago	0/4			
Warba	0/4			
White Rose	0/3		0/3	7/10*
Avon		0/4		6/10*
Columbia Russet		4/8		
White La Soda		7/8		
Abnaki			2/3	
Alma (Tbique)			0/3	8/8*
Arran Victory			2/3	2/2*; 4/12
Banana			3/3	
Batoche			1/3	
Canus			0/3	8/8*
Cariboo			3/3	
Chinook			2/3	
Epicure			0/3	8/8*
Fundy			1/3	
Green Mountain			2/3	
Huron			3/3	
Keswick			0/3	
La Chipper			0/3	
Nooksak			0/3	
Peconic			1/3	6/10*
Raritan			0/3	6/10*
Sable			0/3	1/10
Snowchip			0/3	8/8*
Waseca			0/3	
Norland			1/3	

*Results of graft-inoculation experiment

Umbelliferae - Apium graveolens L.

All the three monocotyledonous plants tested were not susceptible to PVM: Graminae - Hordeum vulgarae L., Zea mays L.; Liliaceae - Allium porrum L.

PVM was recovered in only 17 of the 33 potato cultivars which had been mechanically inoculated with the virus (Table 4). However, all the nine potato cultivars used in the graft-inoculation experiment were infected with the virus, and the infection success was 60 to 100%. None of the infected plants showed symptoms. Likewise, secondary plants grown from tubers derived from PVM-infected plants of the cultivars Banana, Red La Soda and White La Soda did not exhibit any symptoms. When serological detection was carried out, all of them were found to be infected with PVM.

In the experiments to determine the transmissibility of PVM by contact, it was found that all the six 'Arran Victory' plants placed in contact with the PVM-infected 'White La Soda' plants remained free from the virus. A similar observation was obtained with the 12 tomato plants placed in contact with those mechanically inoculated with the virus. Thus under the condition of the experiment PVM was not transmissible through plant contact.

(b) Serology

The highest titer obtained for antiserum against PVM was 2560. The titer, however, was decreased to 640 two weeks later and remained so for about four weeks. The progress of the antibody production was followed weekly, and is shown in Fig. 1.

The same rabbit was reinjected intravenously 4 months after the last injection with the virus prepared by the purification procedure developed in the present studies. The antiserum titer rose to 1280, but 2 months later it had decreased to 640. At the lowest dilution tested, no reaction was detected against healthy plant sap.

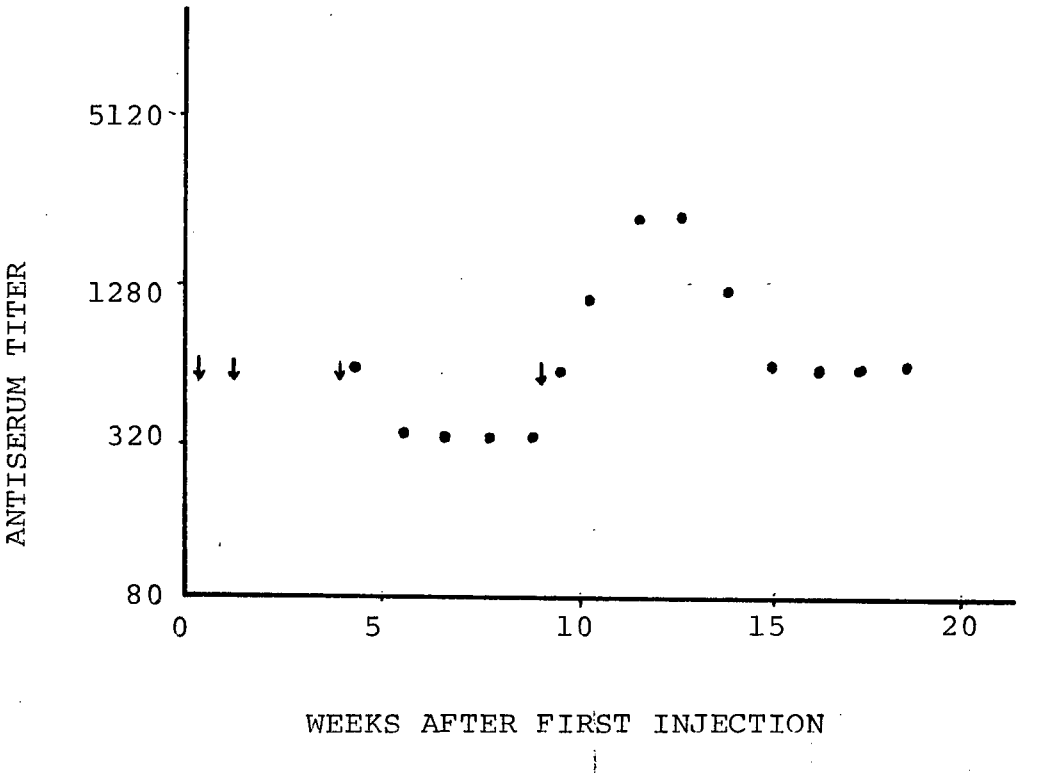

Fig. 1. Antibody production in rabbit against PVM antigen. Arrows indicate the time of virus administration into the immunized rabbit.

(c) Properties in Crude Sap

Twelve days after inoculations, the number of local lesions on each of the inoculated half-leaves was recorded. The presence

or the absence of local lesions determined the physical properties of the virus in crude sap.

1. Dilution end point (DEP)

Local lesions were observed on half-leaves which had been inoculated with sap diluted to 10^{-1}, 10^{-2}, 10^{-3} or 10^{-4} but not with sap diluted to 10^{-5} or 10^{-6}, suggesting that the DEP of PVM was 10^{-4}. Sap extracted from potato or tomato leaves gave similar results. It was interesting to note that the average number of local lesions per half-leaf of Red Kidney bean plants was higher when the inoculation was carried out with sap diluted to 10^{-1} than the undiluted sap (Fig. 2A).

2. Longevity in vitro (LIV)

One of the two replicates incited local lesions on Red Kidney bean half-leaves after 3 days, and the other after 2 days of preincubation at room temperature, suggesting that the LIV of PVM was between 2 to 4 days. Sap extracted from potato or tomato leaves gave similar results. The average number of local lesions per half-leaf decreased sharply from 12 on day 0 to 2 on day 1 (Fig. 2B).

3. Thermal inactivation point (TIP)

Local lesions were observed on Red Kidney bean half-leaves which had been inoculated with sap preincubated for 10 min at room temperatures of 20°C through 65°C, but not at temperatures of 70°C or higher, thus suggesting a TIP between 65°C and 70°C. It was observed that the average number of local lesions increased when the sap was heated to 30°C or to 40°C (Fig. 2C).

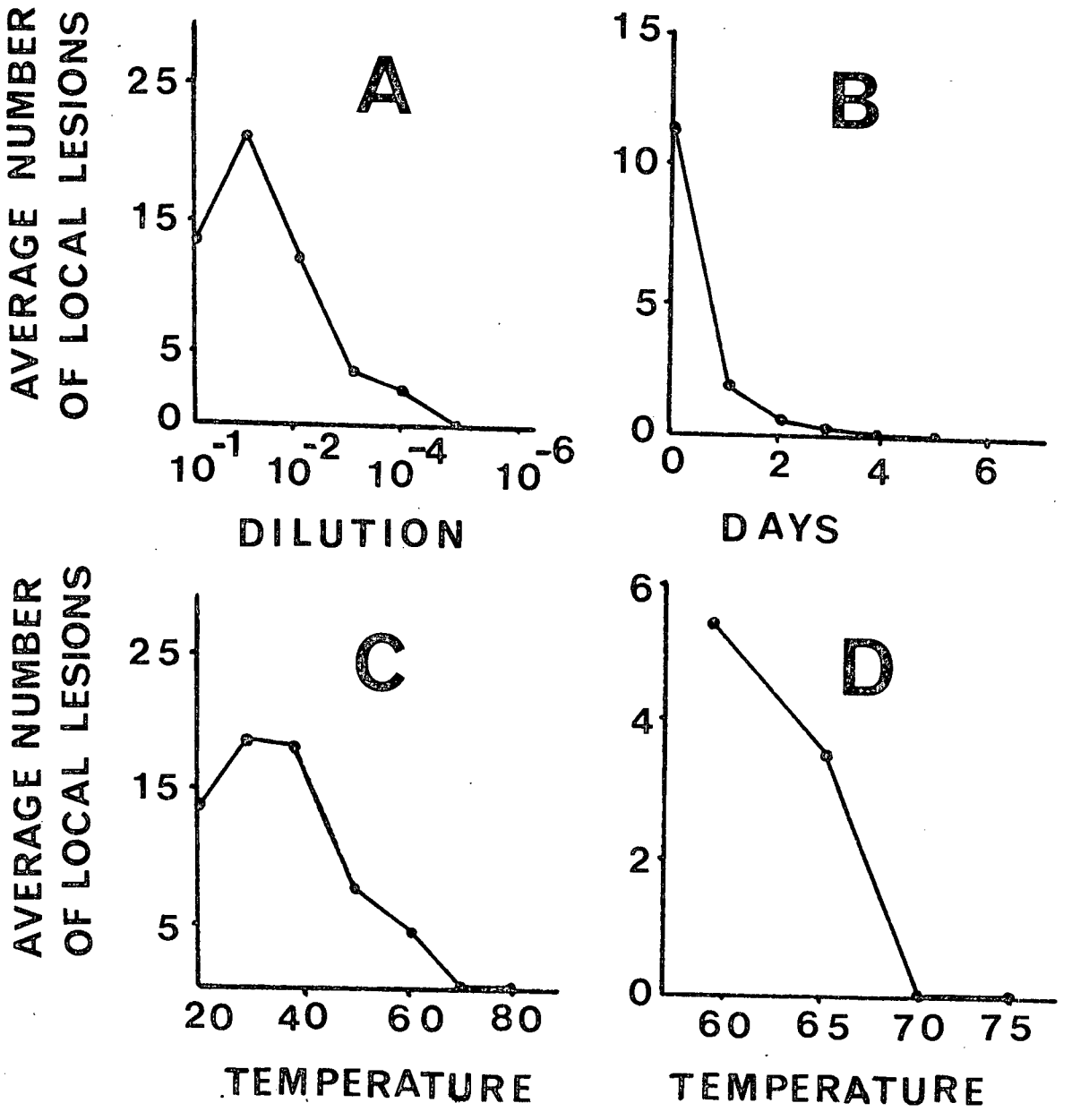

Fig. 2. The relationships between the average number of local lesions per half-leaf of Red Kidney bean with (A) dilutions of sap, (B) days of preincubation of sap at room temperature, and (C,D) preincubation temperature (^{o}C) of sap for 10 min.

Table 5 Variations in the relative concentration of PVM in, and the final pH of, 1:3 sap as a result of using different extraction buffers, at pH 7.8, for homogenizing potato leaves

Buffer*	Reciprocal of serological dilution end point	Final pH
Borate, 0.5 M	32	7.8
Borate, 0.2 M	32	7.8
Phosphate, 0.2 M	16 - 32	7.7
Diethyl barbiturate, 0.1 M	16	7.4
Tris-acetate, 0.2 M	16 - 32	7.3
Citrate-phosphate, 0.2 M	16	7.5
Water	8	6.1

*Compositions as in Appendix III.

(d) Purification

1. Extraction of virus from leaf tissues

PVM-infected potato leaves were homogenized in a buffer at the ratio of 1:3, tissue to buffer. When homogenizations were carried out in 0.5 M borate buffer at pH's 6.0, 7.0, 7.8, 8.0 or 8.5, the serological dilution end points were 1:8, 1:16, 1:32, 1:32, and 1:32, respectively. Thus, borate buffer with a pH range of between 7.8 and 8.5 was suitable for extraction of PVM from potato leaves. It was also shown that, when used at 0.5 M or 0.2 M, borate buffer performed better than the other buffers tested (Table 5). Varying the tissue to buffer ratio to 1:1.5, 1:2.0, or 1:2.5 did not reduce the efficiency of the 0.5 M borate buffer.

Incorporation of DIECA, mercaptoethenol or sodium sulfite to final concentrations of 0.5%, 1.0% or 0.5% respectively, did not improve the efficiency of extraction. However, when the sap was left for 2 weeks, then clarified with chloroform, the untreated sap exhibited a brownish coloration, whereas the others remained as clear yellowish-green solutions. Following a cycle of differential centrifugation, PVM was serologically detected in the pellets derived from the treated sap but was not detected in the pellets derived from the untreated sap. Crude sap extracted from the primary or the secondary shoots of the potato plants, cv. Banana, gave a serological dilution end point of 1:32. Thus, harvesting of young leaves for purification of the virus could be made from plants 4 to 15 weeks after transplanting, or from plants 4 weeks after the first harvest. Potato plants cvs.

Columbia Russet and White La Soda, and tomato plants cvs. Rutgers and Subarctic, were equally suitable propagation hosts for the purification of PVM. Freezing of leaves overnight resulted in a loss of virus. The serological dilution end point was 1:16 compared to 1:32 for leaves stored for the same duration at $4^{o}C$.

2. Clarification of crude sap

The physical and chemical methods of clarification tested significantly lowered the turbidity values of the sap samples compared to the control ($P<0.01$) (Table 6). The methods of clarification employed determined the extent of virus lost. Among those clarification methods which lowered the serological dilution end point of the sap to only 1:16, treatment with an equal volume of chloroform gave the lowest turbidity value. In Duncan's multiple range test, this turbidity value was not significantly different from the turbidity of sap which had been clarified with an equal volume of carbon tetrachloride. Reducing the chloroform to sap ratio to 1:6, did not improve the serological dilution end point. On the other hand, the turbidity value was significantly increased ($P<0.01$) in comparison to that of sap which had been clarified with an equal volume of chloroform. When 1:1.5 sap was clarified with an equal volume of chloroform or with chloroform added to one-sixth volume of sap, the serological dilution end point was 1:32.

Very clear preparations were obtained when 1:3 sap samples were

Table 6. Relative virus concentration and turbidity of 1:3 sap after clarification by various methods

Clarification methods	Virus Concentration*	Turbidity at 540 nm**
1. Ammonium sulfate (25%)	4	0.063 a
2. Freezing	0	0.072 a
3. Chloroform (1:6)+, then Triton X - 100 (1%)	2	0.141 a
4. Chloroform (1:1)	16	0.307 b
5. Triton X - 100 (1%)	8	0.329 bc
6. Carbon tetrachloride (1:1)	16	0.361 bcd
7. n-butanol (8%)	16	0.400 cd
8. Chloroform (1:6)	16	0.424 d
9. Heating at 45°C, 10rmin	8	0.930 e
10. Centrifuge, Celite	8	1.075 f
11. Ether (1:1)	16	1.155 f
12. Control	32	1.340 g

* All the six replicates gave similar serological dilution end points except in 1 and 9 where one replicate differed.

** Level of significance for Duncan's multiple range test. Treatment means (six replications) not followed by the same letters were significantly different ($P < 0.01$).

+Ratio of volume of organic solvent to volume of sap.

subjected to overnight freezing, but the concentration of virus was low. With 1:1.5 sap it was found that removal of the freeze-coagulated plant residues by filtration through No. 54 Whatman filter paper or by centrifugation at low speed resulted in supernatants with a similar serological dilution end point of 1:2. The fact that PVM was not serologically detected from the resuspended residues or pellets, suggested that the reduction in virus concentration in the supernatants was due to virus degradation, and not due to aggregation of the virus particles or to their adsorption onto the plant residues.

Though n-butanol-clarified sap had a turbidity value comparable to that of carbon tetrachloride-clarified sap, it was greenish in colour. An attempt to remove this coloration by lengthening the incubation period from 30 min to overnight, was unsuccessful. Instead, the serological dilution end point of the sap was further reduced from 1:16 to 1:8 in 1:3 sap, and from 1:32 to 1:16 in 1:1.5 sap.

Clarification with an equal volume of carbon tetrachloride resulted in sap with a relatively high serological dilution end point and a relatively low turbidity value. Later experiments revealed that the optimum concentration of the organic solvent for clarification of 1:3 or 1:1.5 sap was 1.25 ml per 10 ml sap. Increasing the concentration to 2.5 ml or to 5.0 ml per 10 ml sap, resulted in a slight reduction of the serological dilution end point (Fig. 3). The turbidity values were, however, not significantly reduced ($P > 0.05$) when carbon tetrachloride was added in excess of 1.25 ml. This concentration was therefore adopted for use in

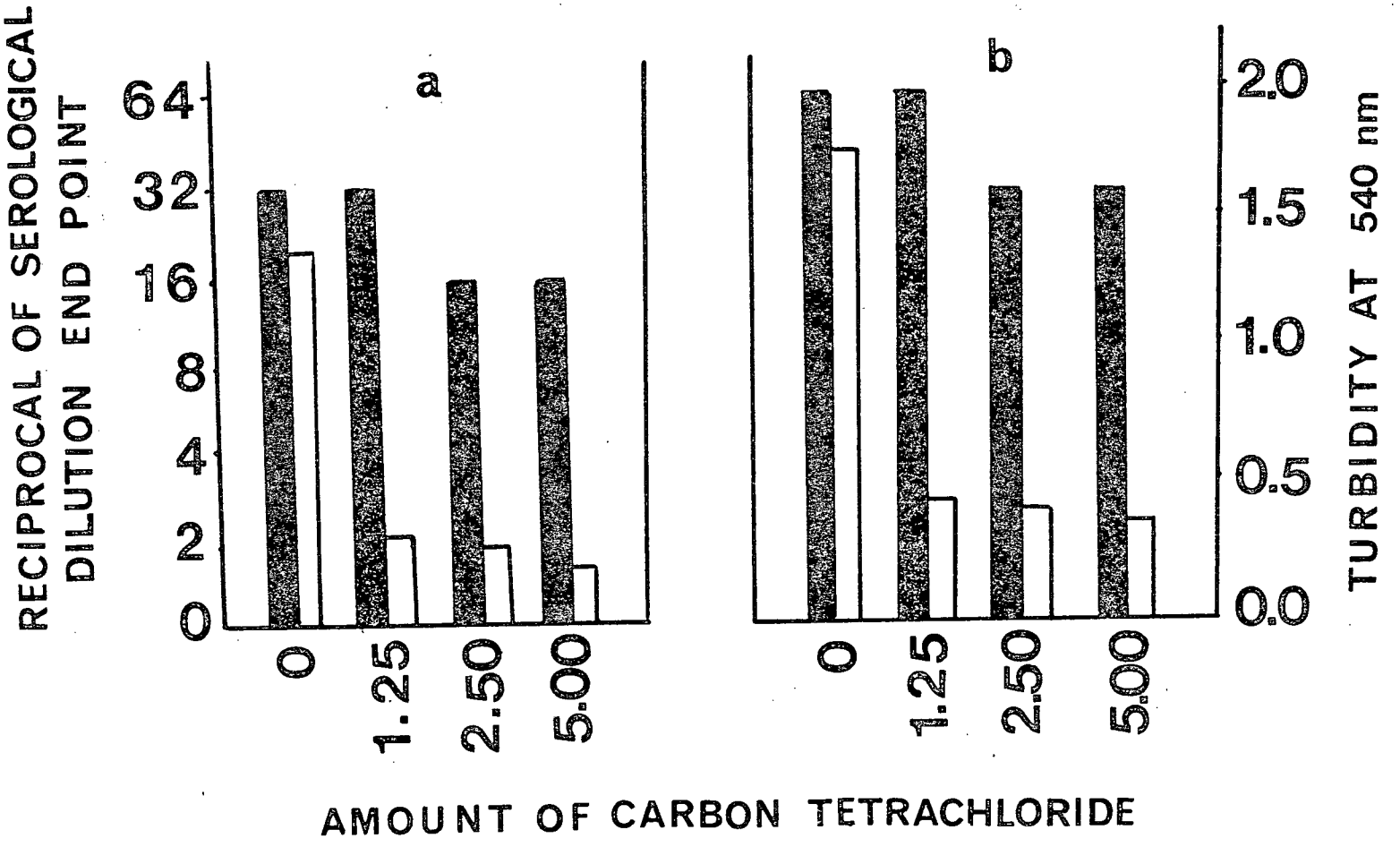

Fig. 3. Relative concentration of PVM[■]in, and turbidity at 540 nm[□]bf, (a) 1:3 sap and (b) 1:1.5 sap, after clarification with various amounts (ml) of carbon tetrachloride per 10 ml of sample.

subsequent experiments.

When carbon tetrachloride-emulsified 1:1.5 sap was centrifuged at 5,000, 6,500 or 9,500 rpm for 5, 10 or 15 min, an identical serological dilution end point of 1:64 was obtained from all the resulting supernatants. Their turbidity values at 540 nm were significantly affected ($P < 0.01$) by the combination of time and speed of centrifugation. Centrifugation at 9,500 rpm for 15 min resulted in the lowest turbidity value (Table 7). Hence, this combination of speed and time was used throughout the present studies. When 1:3 sap was centrifuged at this combination of speed and time, the serological dilution end point of 1:32 was similar to that of sap centrifuged at 5,000 rpm for 5 min.

Encouraging results were obtained when ammonium sulfate was added to the crude sap at lower concentrations than the 25% used in the preliminary investigation (Fig. 4). Although the turbidity value of the sap was significantly increased ($P < 0.01$) as the concentration of the ammonium sulfate was reduced, this was more than compensated by the increase in virus concentration. There was no detectable loss of virus from the clarified sap when the amounts added were 1.5 gm or 1.75 gm per 10 ml of 1:3 or 1:1.5 sap, respectively. The serological dilution end point of 1:1.5 sap which had been clarified with 2.0 gm ammonium sulfate ranged from 1:32 to 1:64. When incubation was carried out for 6 hours instead of overnight, the serological dilution end points were not extended, nor were the turbidity values increased. Overnight incubation with ammonium sulfate at final concentrations of 15% and 20% were therefore adopted for clarification of 1:3 sap and

Table 7. Turbidity values of carbon tetrachloride-clarified sap measured at 540 nm, after centrifugation at different combinations of speed and time

Speed of Centrifugation (rpm)	Time of centrifugation (min)		
	5	10	15
5,000	0.940 f*	0.658 d	0.600 cd
6,500	0.809 e	0.591 cd	0.509 bc
9,500	0.629 cd	0.408 ab	0.341 a

*Level of significance in Duncan's multiple range test. Treatment means (two replications, and two samples per replicate) when followed by the same letter were significantly different ($P < 0.05$).

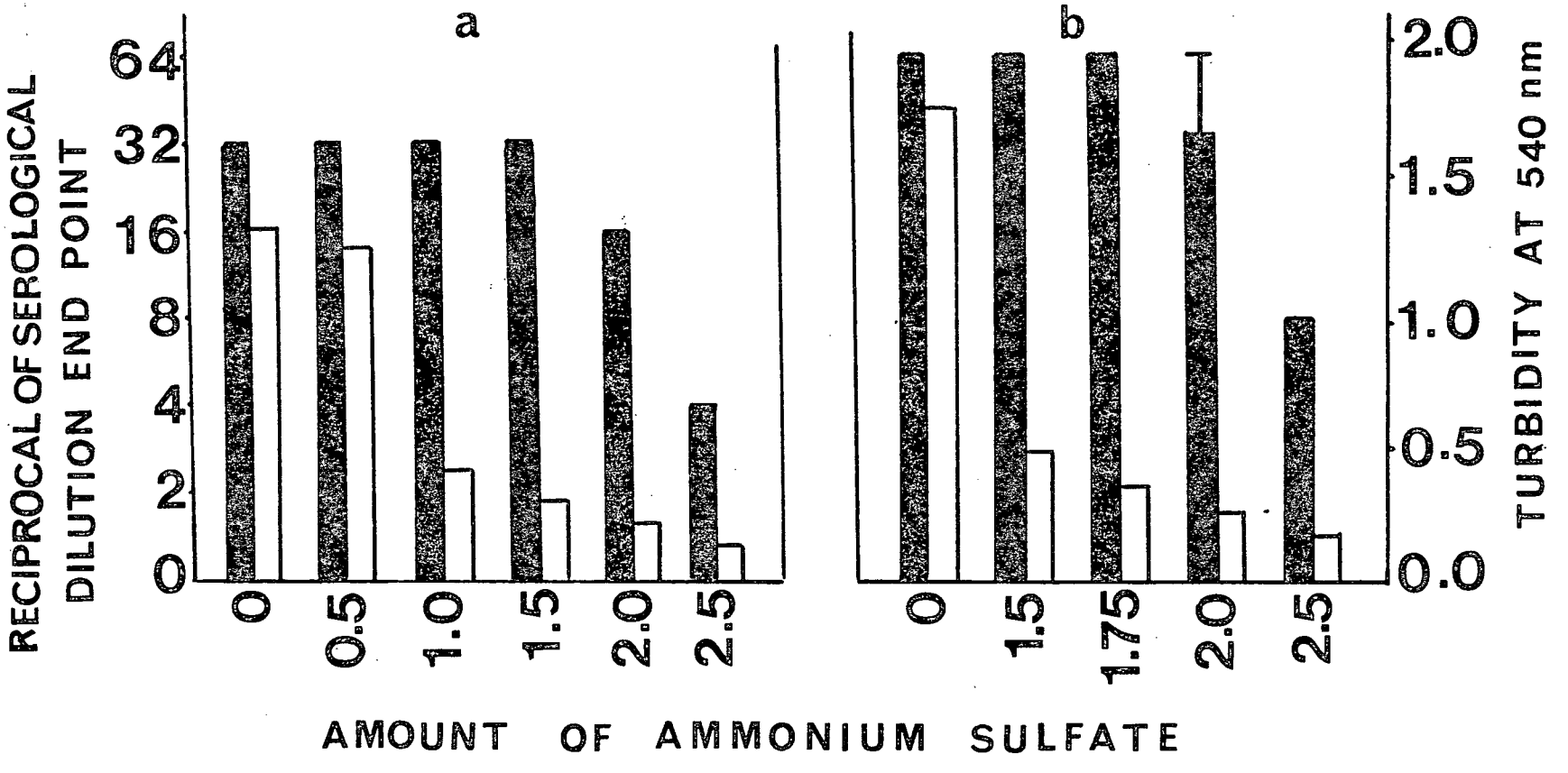

Fig. 4. Relative concentration of PVM in [▪] , and turbiduty at 540 nm [□] of , (a) 1:3 sap and (b) 1:1.5 sap, after clarification with various amounts (gm) of ammonium sulfate per 10 ml of sample.

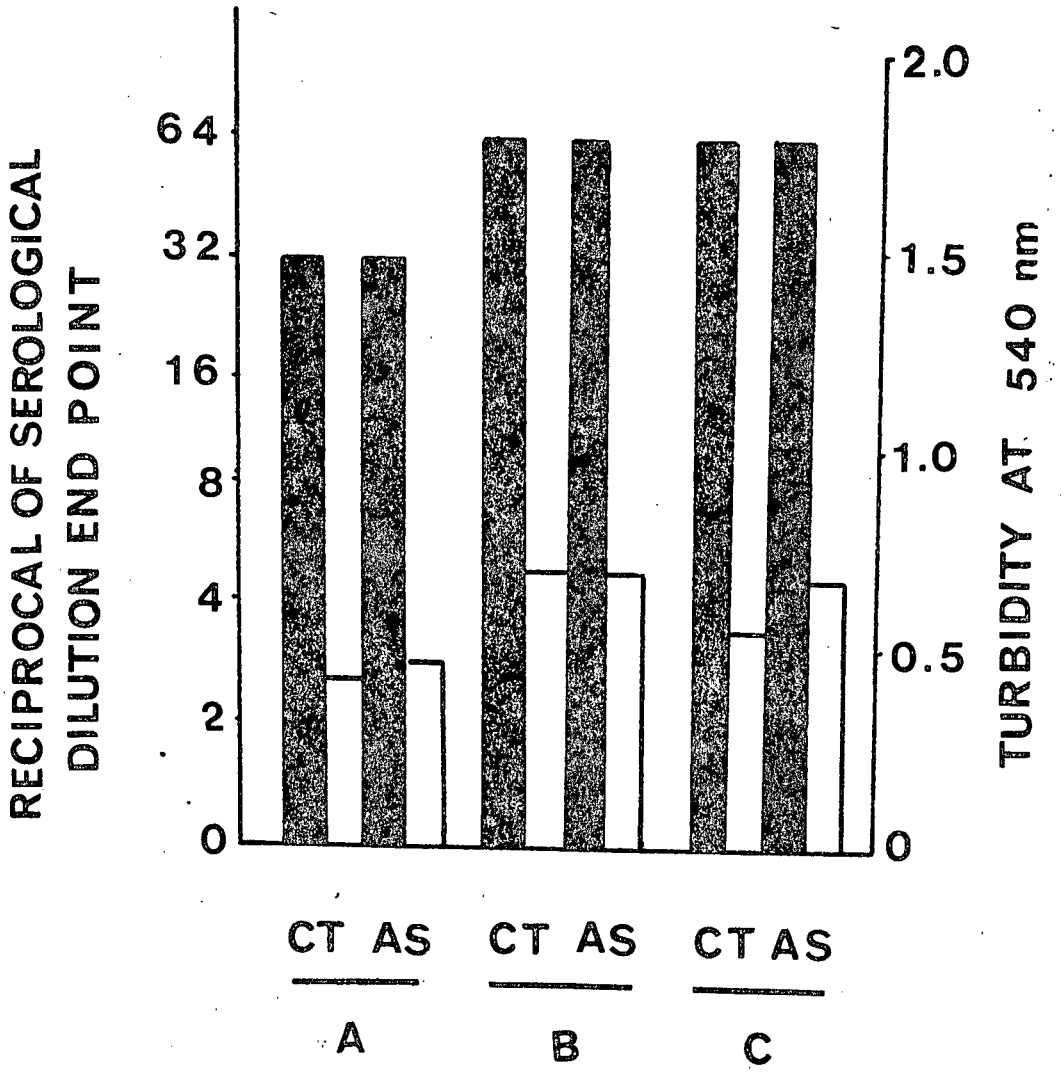

Fig. 5. Relative concentration of PVM [■] in, and turbidity at 540 nm [□] of, (A) 1:3 sap from potato, (B) 1:1.5 sap from potato and (C) 1:1.5 sap from tomato, after clarification with carbon tetrachloride (CT) or ammonium sulfate (AS).

1:1.5 sap, respectively. The serological dilution end point of clarified sap after centrifugation at 9,500 rpm for 15 min was similar to that of clarified sap which had been centrifuged at 5,000 rpm for 5 min.

In direct comparison tests between clarification methods utilising the precipitating or coagulating properties of ammonium sulfate or carbon tetrachloride, a larger sample size of 100 ml of sap was used. Some samples were homogenised for 10 sec with carbon tetrachloride in a Waring blender. Others were treated with the appropriate amount of ammonium sulfate. Stirring was carried out with a magnetic stirrer until all the ammonium sulfate crystals were dissolved, Both these methods produced clarified sap with similar serological dilution end points (Fig. 5). It was significant that all the replicates of 1:1.5 sap which had been clarified with ammonium sulfate added to a final concentration of 20% showed a similar serological dilution end point of 1:64.

3. Concentration of virus from clarified sap

The ratio of the volume of a virus preparation before concentration to its volume after concentration was important. It was found that dissolving the pellets in one-tenth volume of either 0.5 M or 0.05 M borate buffer, invariably resulted in lower serological dilution end points compared to those dissolved in two-tenths volumes of buffer (Fig. 6). Borate buffer at 0.05 M was as good as at 0.5 M when used for dissolving the pellets

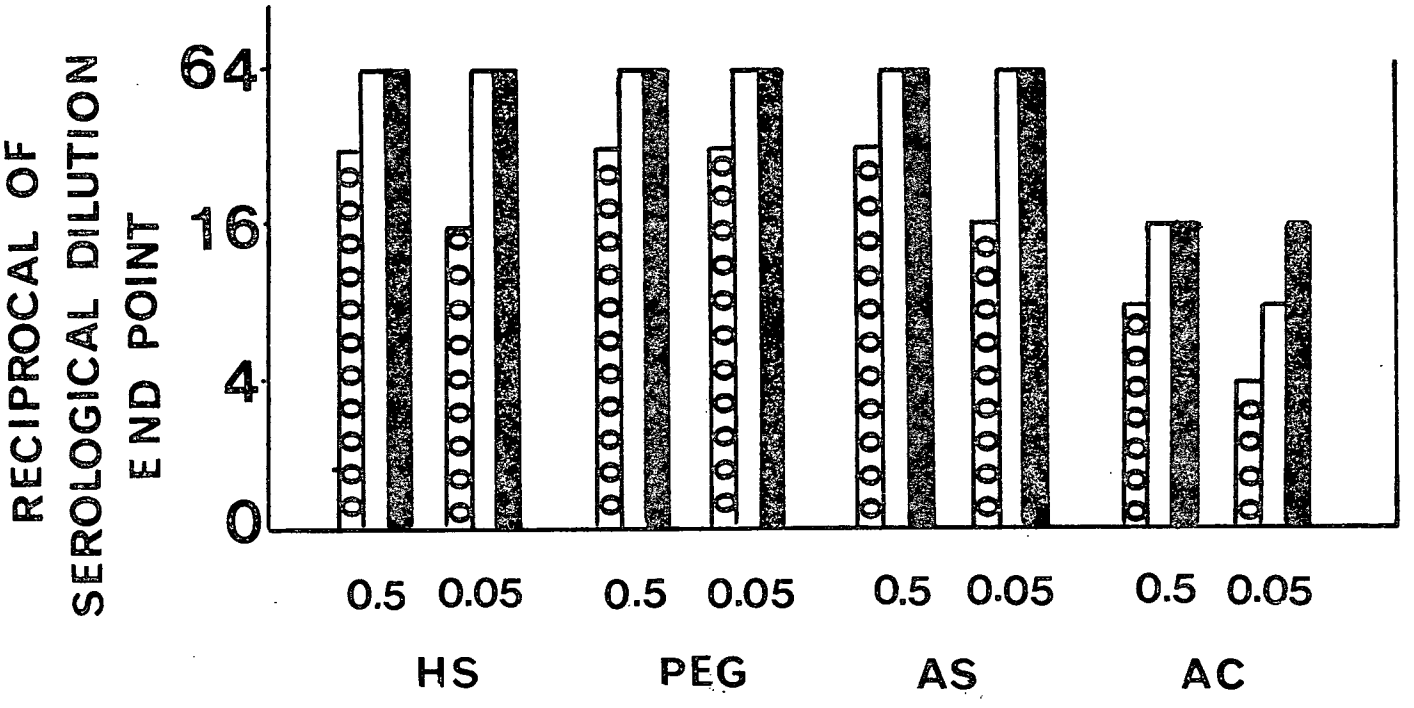

Fig. 6. Relative concentration of PVM in preparations precipitated by the various methods: High speed centrifugation (HC), PEG precipitation (PEG), Ammonium sulfate precipitation (AS) and acid precipitation (AC). The pellets were resuspended in either 0.5 M or 0.05 M borate buffer at one-tenth [▣] , two-tenths [□], or four-tenths [■] of the original volume.

obtained by the various concentration methods, and at the same time reducing the volume of the virus preparation to two-tenths of the original. It was also found that resuspension of ammonium sulfate- or PEG-precipitated pellets in two-tenths volume of 0.05 M phosphate or citrate-phosphate buffer gave similar results as 0.05 M borate buffer.

In the experiment to determine the best combination of PEG 6000 and sodium chloride for precipitation of PVM, it was found that the addition of the former followed by the latter at concentrations of 5% and 4% respectively, was sufficient to precipitate the virus from the sap (Table 8). Increasing the concentrations of either one or both the chemicals did not improve the serological dilution end point of the concentrated virus beyond 1:64.

Previously, ammonium sulfate-treated sap had been allowed to stand overnight before centrifugation to pellet the precipitate was carried out. It was later shown that even when the treated sap was allowed to stand for only 2 or 6 hours, complete precipitation of the virus was achieved. This was indicated by the observation that a similar serological dilution end point of 1:64 was obtained in all the concentrated preparations. In addition, PVM was not serologically detected in the "first supernatants".

Direct comparison tests were carried out between concentration methods which showed promising results. For carbon tetrachloride-clarified sap a comparison was made between high speed centrifugation and PEG precipitation in which PEG 6000 and sodium chloride

Table 8. Relative concentration of virus in preparations obtained by precipitations with different combinations of PEG 6000 and sodium chloride.

Concentration of PEG 6000 (%)	Concentration of sodium chloride (%)		
	2	4	6
2.5	8	8	8
5.0	32 - 64	64	64
7.5	64	64	64

were added to final concentrations of 5% and 4%, respectively. For ammonium sulfate-clarified sap, a comparison was made between high speed centrifugation and ammonium sulfate precipitation. The ammonium sulfate was added to a final concentration of 30%, and the treated sap was then allowed to stand for 2 hours before being centrifuged. In all cases the serological dilution end point of the concentrated preparations was 1:64. Likewise, a similar serological dilution end point of 1:4 was obtained in the supernatants from a second resuspension. Hence the apparent loss of virus could partly be attributed to the difficulty of resuspending the virus from the pellets following precipitation. In a later experiment it was found that the virus was not serologically detectable during the third resuspension of the pellets, regardless of whether the two preceeding resuspensions were carried out in two-tenth or four-tenth volumes of 0.05 M borate buffer. Subsequently, resuspension of pellets obtained from the first stage of concentration was done twice, each time with two-tenth volume of buffer.

4. Further concentration and final purification

During the second stage of concentration, either by a high speed centrifugation or by PEG precipitation, the virus preparations obtained had a similar dilution end point of 1:128, regardless of the type or source of the starting materials. Hence there appeared to be no detectable loss in virus, as opposed to the results of the first stage of concentration.

Table 9. Relative concentration of virus preparations after the third stage of concentrtion using four different procedures

Procedure*	Volume of buffer in which pellets were resuspended (ml)	
	0.3	2.0
I	32 - 64	128
II	32	128
III	32	128
IV	32	64 - 128

*I - ammonium sulfate, PEG, high speed.

II - ammonium sulfate, high speed, PEG.

III- PEG, PEG, high speed.

IV - PEG, high speed, PEG.

In the third stage of concentration, a significant reduction in dilution end point was observed when the pellets were resuspended in 0.3 ml of buffer (Table 9). Moreover, separation of the undissolved pellets from the supernatants following a low speed centrifugation was difficult. As a result, the virus preparations were yellowish in colour. This was more obvious in the case of polyethylene glycol pellets from procedures II (ammonium sulfate, high speed, PEG) or IV (PEG 6000, high speed, PEG 6000) than the high speed pellets from procedures I (ammonium sulfate, PEG, high speed) or III (PEG 6000, PEG 6000, high speed). Thus, high speed centrifugation was more suitable to be employed in the final stage of concentration than PEG precipitation.

However, when the pellets were resuspended in 2.0 ml of buffer, there was no detectable loss in virus. Moreover, partially purified virus preparations could be obtained from the preparations which had been concentrated by the procedures I and III, and subsequently centrifuged through 5 to 35% sucrose density gradient in SW 25 rotor for 1.5 hours at 23,000 rpm (Fig. 7).

In order to obtain purer preparations of the virus, a fourth stage of concentration was carried out. Another cycle of differential centrifugation was added to the procedure. The serological dilution end point of these preparations was 1:64, showing that the loss of virus was less than when only three stages of clarification were employed.

Plant materials were gradually removed from the virus preparations as purification progressed. The virus particles appeared to be normal

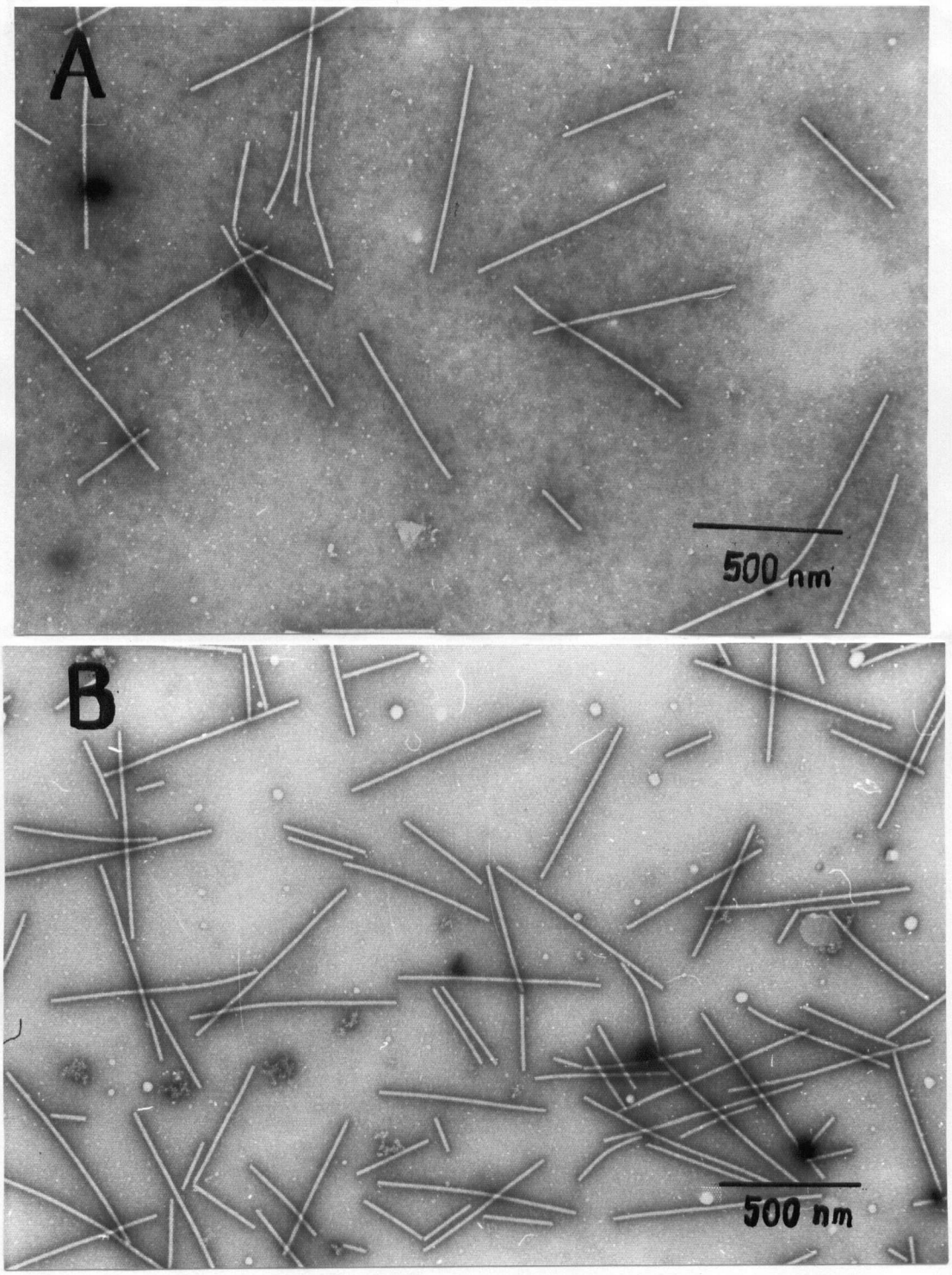

Fig. 7 Electron micrigraphs of virus preparations after three stages of concentration followed by a sucrose density gradient centrifugation in SW 25 rotor; A. ammonium sulfate method, B. carbon tetrachloride method.

(Fig. 8). After the sucrose density gradient centrifugation, fairly pure preparations were obtained from both the ammonium sulfate and the carbon tetrachloride methods (Fig. 9). These preparations failed to react with antiserum against healthy plant components. The A_{260}/A_{280} ratios of 1.23 and 1.25 (Table 10) supported the electron microscopic and serological evidence that the virus preparations derived from the two purification procedures were fairly pure.

Data from the serological dilution end point and the A_{260} determinations suggested that the ammonium sulfate method is slightly superior to the carbon tetrachloride method (Table 10). In addition, it could be seen that the density gradient profile of the former showed a slightly higher peak than the latter (Fig. 10). In addition to being fairly pure, the virus preparations obtained by the two methods were also infectious. In a later experiment, it was shown that the peak of infectivity determined by assay on Red Kidney bean coincided with the peak of the density gradient profile (Fig. 11).

PVM particles purified by the ammonium sulfate method had a normal length of 651 nm. Of the 200 particles measured, 39% had this length. Sixty-seven percent of the particles had lengths between 644 to 660 nm which is the range of values reported as the normal length of PVM particles (Hitchborn and Hill, 1965; Rozendaal and van Slogteren, 1957). Only 12% of the particles had lengths less than 600 nm, and only 2.5% had lengths greater than 700 nm.

Dialysis of the virus preparations after sucrose density gradient could be deleted from the purification procedure. When 5 ml samples of these preparations were diluted with 0.05 M borate buffer and the

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Fig. 8. Electron micrographs of PVM particles after clarification and concentration of the virus preparations by the following methods:

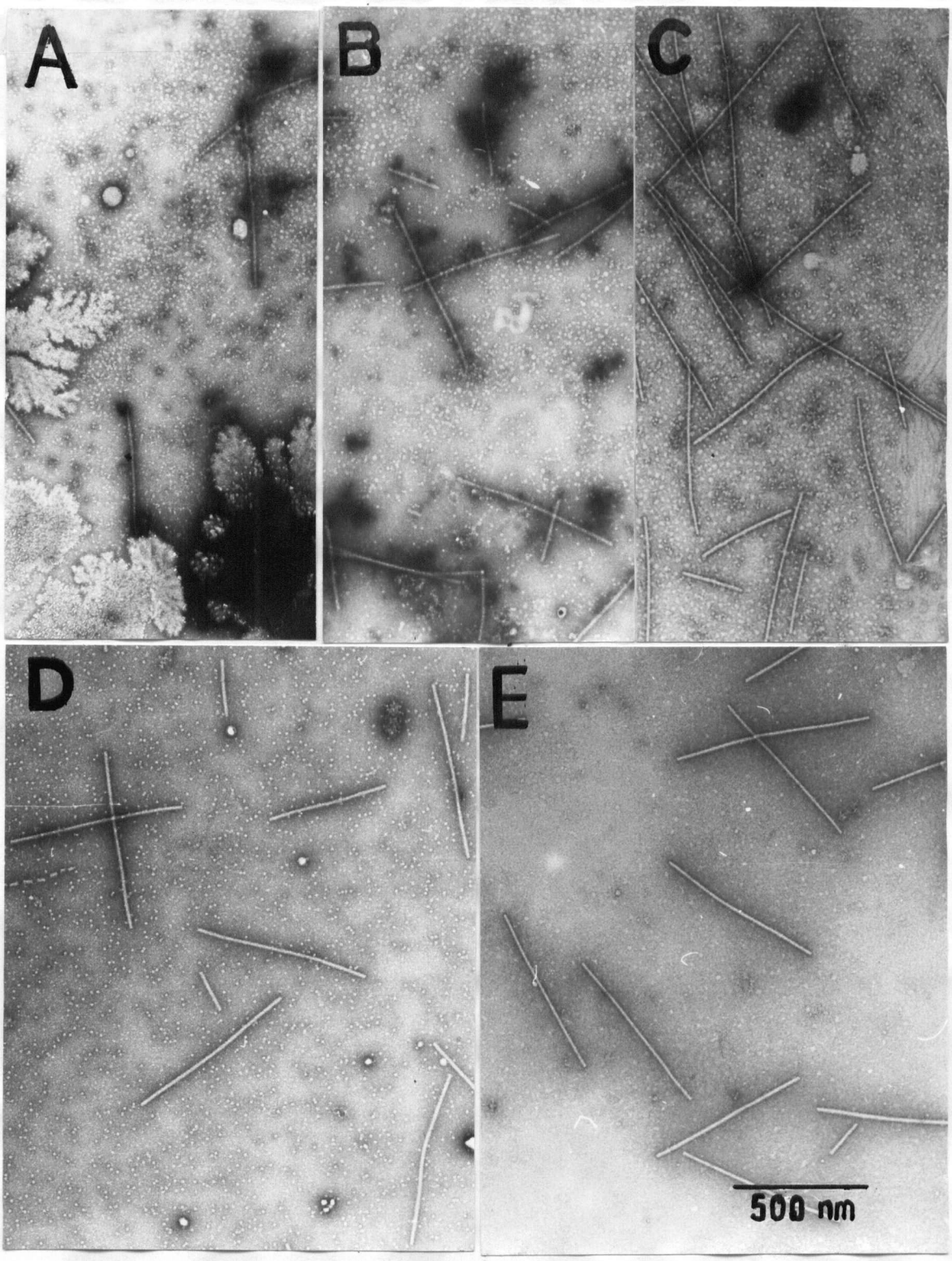
A. Clarification with ammonium sulfate;

B. First stage of concentration by precipitation with ammonium sulfate;

C. Second stage of concentration by high speed centrifugation;

D. Third stage of concentration by precipitation with PEG 6000;

E. Fourth stage of concentration by high speed centrifugation.


A
B
C
D
E
500 nm

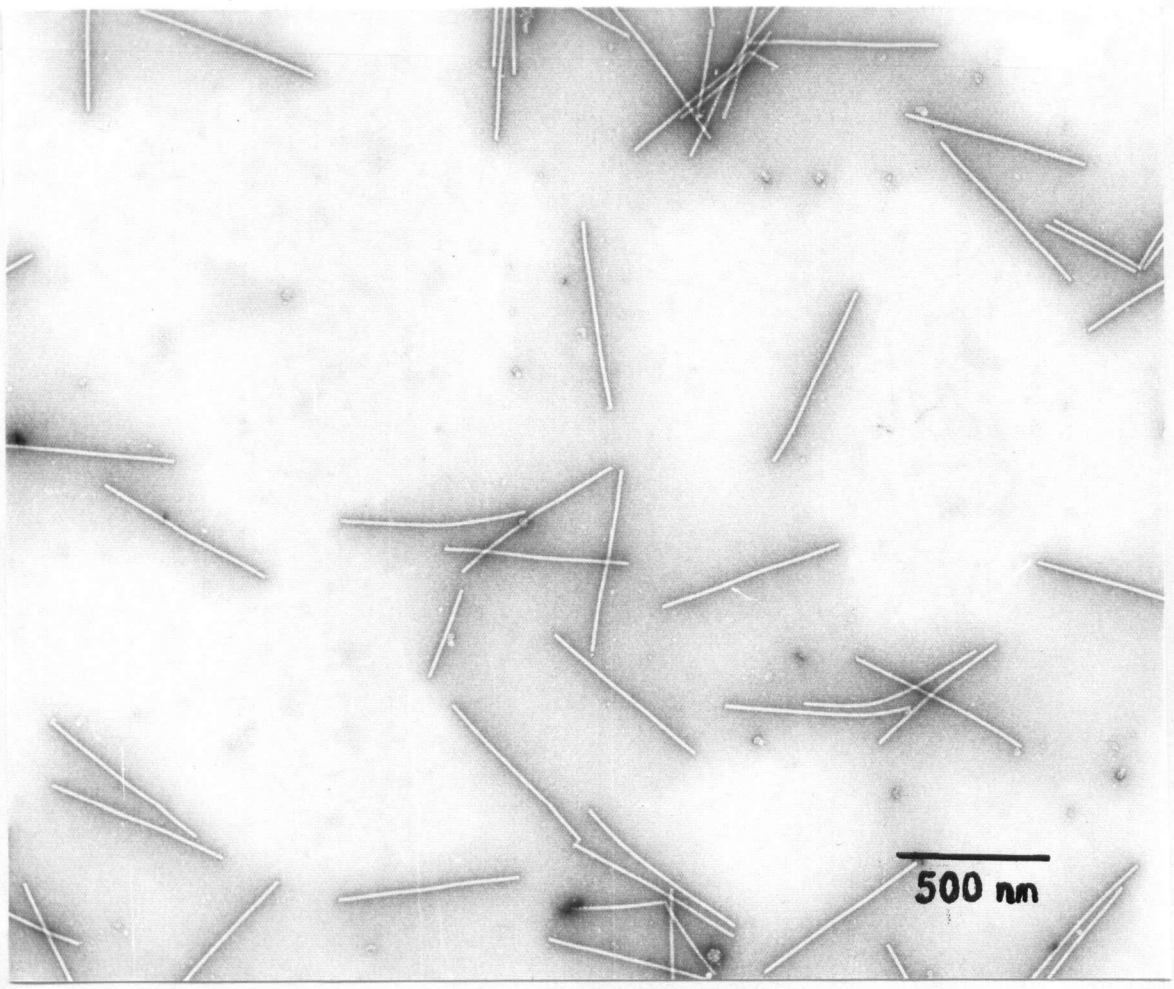


Fig. 9 Electron micrograph of PVM particles after purification by the ammonium sulfate method.

Table 10. Range of concentration, average A_{260}/A_{280} ratios and infectivity of PVM preparations purified from 40 gm of PVM-infected potato leaves

Method of purification	Reciprocal of serological dilution end point	A_{260}	A_{260}/A_{280}	Infectivity
Ammonium sulfate	128	0.100	1.25	Positive
Carbon tetrachloride	64 - 128	0.081	1.23	Positive

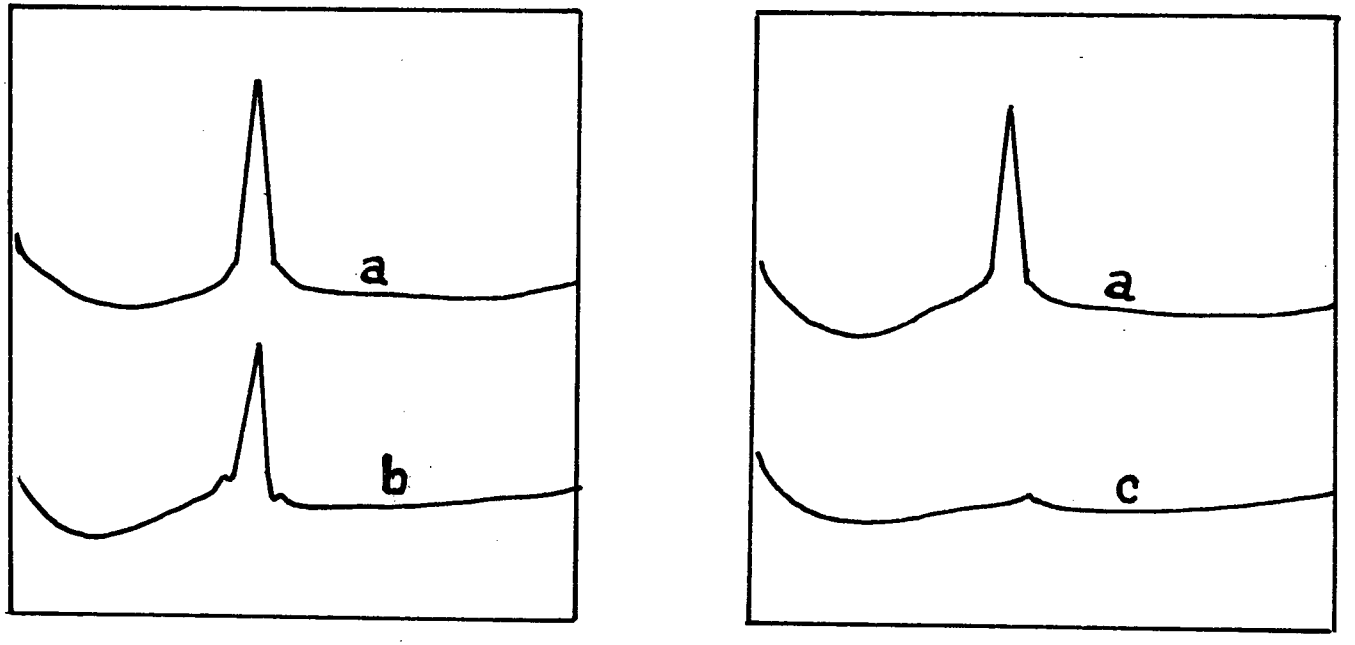


Fig. 10. Sucrose density gradient profiles of PVM (254 nm) obtained in two different experiments. The virus preparations were derived from ammonium sulfate-clarified sap (a), carbon tetrachloride-clarified sap (b), and freeze-clarified sap (c).

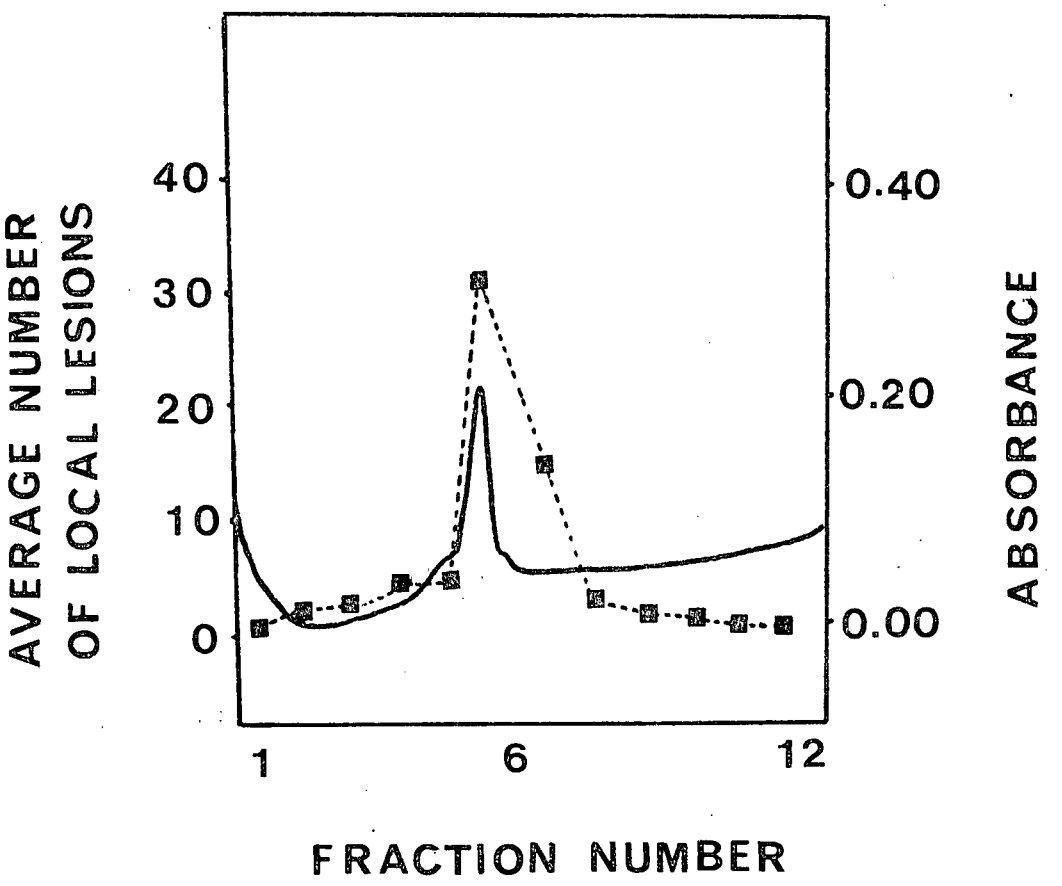


Fig. 11. Average number of local lesions per six half-leaves of Red Kidney bean plants recorded in infectivity assay of 1 ml fractions from SW 41 sucrose density gradient; _____, absorbance at 254 nm; .■----■., number of local lesions.

centriguged in no. 65 rotor at 35,000 rpm for 1.5 hr, there was no loss in virus in comparison to those samples which had been dialysed overnight.

From the foregoing experiments, a procedure for purification of PVM was developed. This procedure was tested with three 400 gm samples of potato leaves as described in the flow-chart in Fig. 12.

The A_{260} values for the three 2 ml samples obtained at the end of the procedure was 2.45, 2.20 and 2.27. The corresponding yields, taking an extinction coefficient of 3.0, were 4.1, 3.7 and 3.8 mg of virus per Kg of leaves, respectively.

(e) Biophysical and Biochemical Properties

The absorption spectra of PVM solution before and after correction for light scattering is shown in Fig. 13. Ultraviolet absorption characteristics of the virus before and after correction for light scattering differed slightly with the corresponding corrected values (Table 11).

The individual values of the sedimentation coefficient of PVM obtained from six analytical runs ranged from 159s to 166s with a mean value of 162 ± 1s. The Schlieren pattern showed that the virus particles sedimented as one band (Fig. 14).

The Schlieren pattern of the equilibrium banding in cesium chloride showed that the PVM particles formed a band closer to the center of the rotor than did the TMV particles (Fig. 15). The range of values obtained from four individual estimations was from 1.301 to 1.309 with a mean value of 1.304 ± 0.001. This

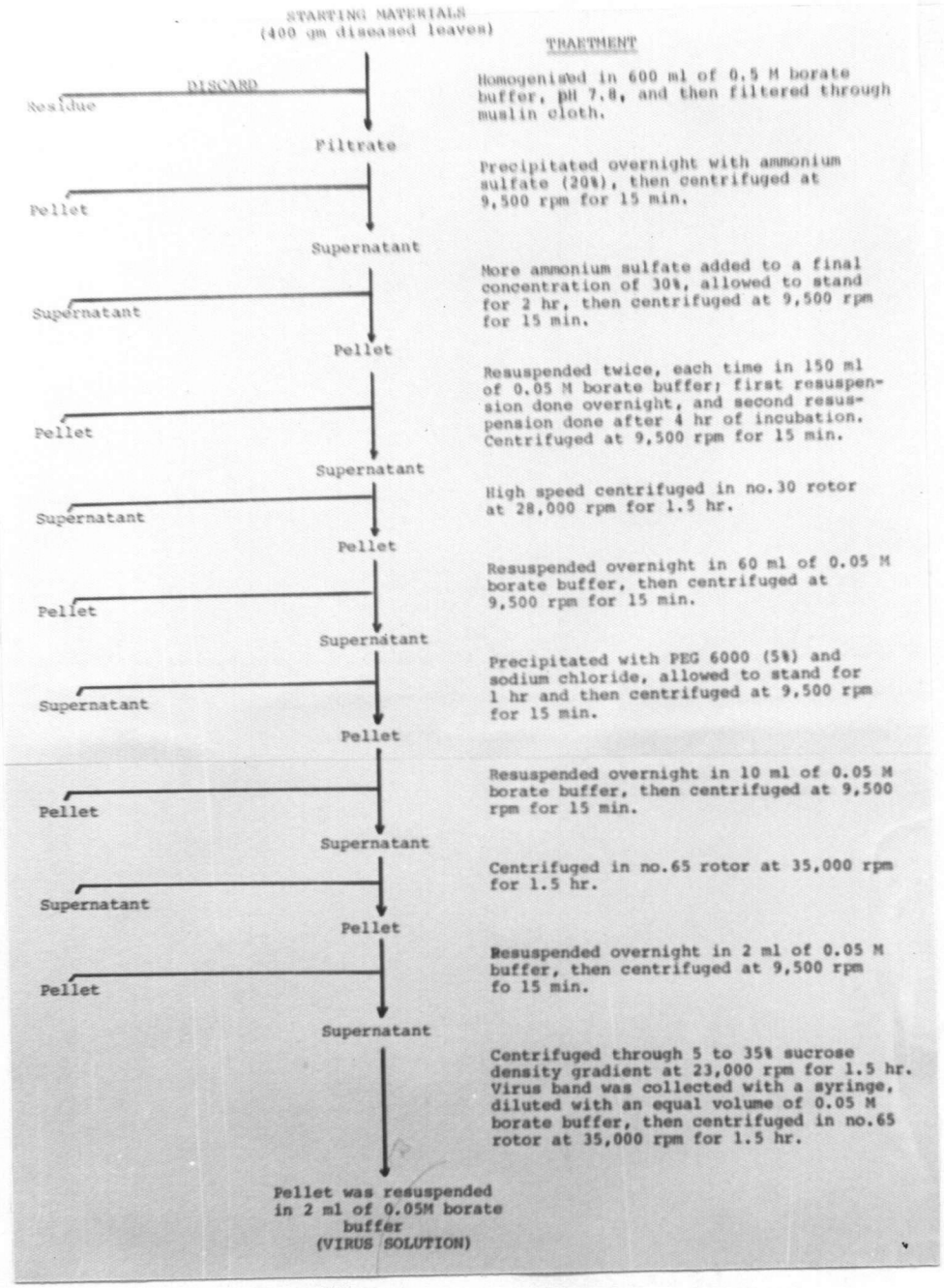


Fig. 12. Flow-chart of the ammonium sulfate method for the purification of PVM.

Table 11. Ultraviolet absorption characteristics of potato virus M before and after correction for light scattering.

Properties	Characteristics of PVM	
	Uncorrected	Corrected
A_{max}	259 nm	260 nm
A_{min}	246 nm	245 nm
A_{260}/A_{280}	1.25 ± 0.04	1.23 ± 0.01
A_{max}/A_{min}	1.08 ± 0.01	1.24 ± 0.01

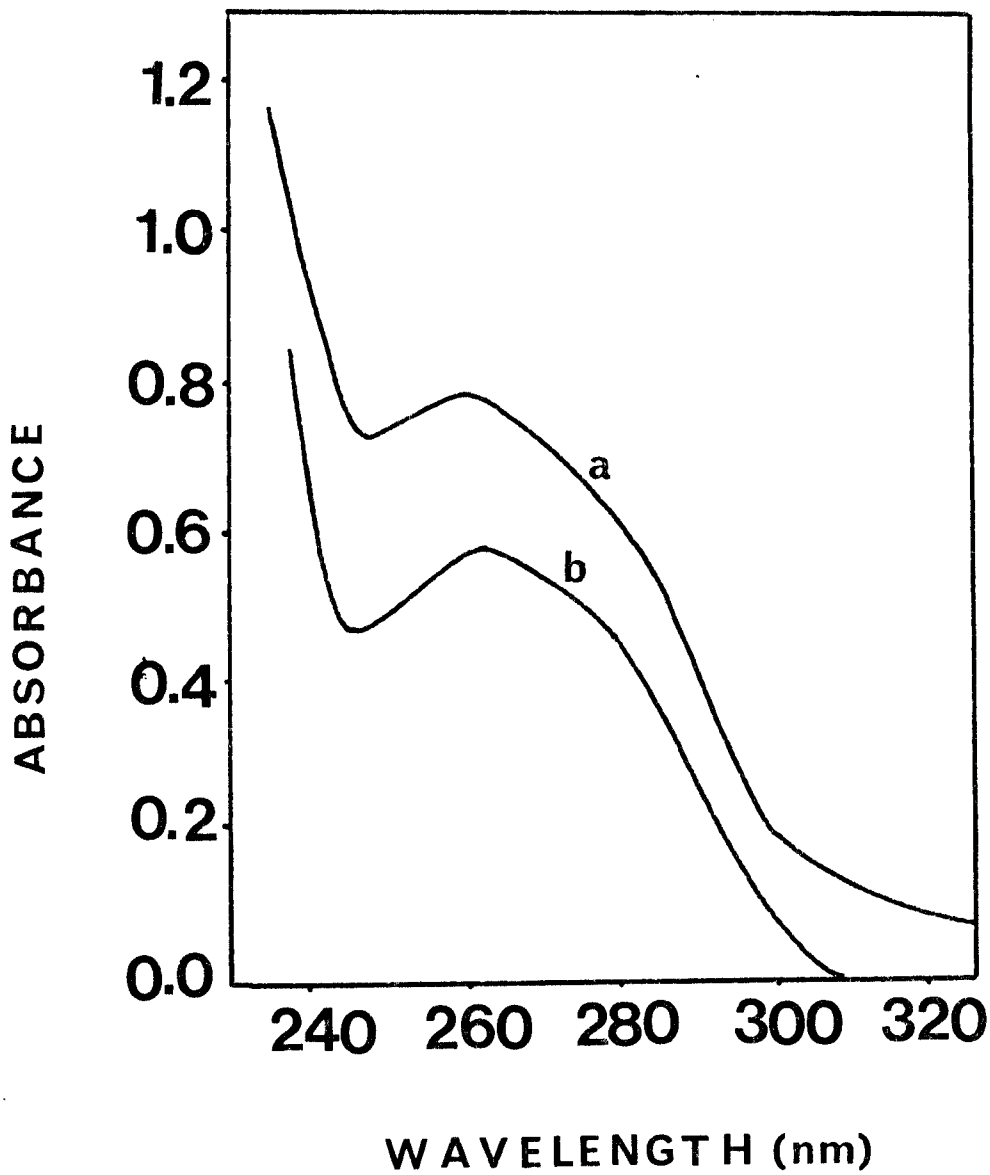


Fig. 13. Absorption spectrum of purified PVM solution:
a. uncorrected for light scattering, b. corrected for light scattering.

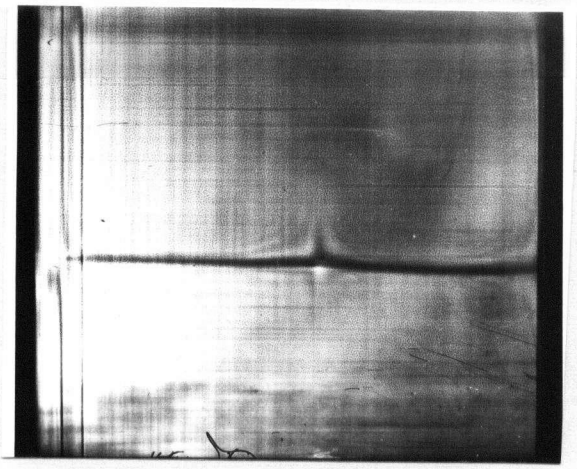


Fig. 14. Schlieren pattern of sedimenting PVM particles in 0.05 M borate buffer, pH 7.8, 16 min after reaching speed of 21,740 rpm

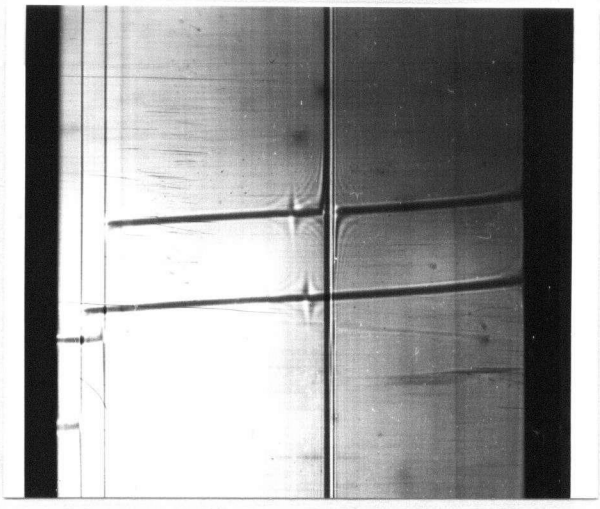


Fig. 15. Schlieren pattern of PVM particles resulting from equilibrium banding in cesium chloride; PVM bands appeared to the left of the TMV band.

suggested that the PVM particles had an RNA content of 6.2% (Gibbs and Harrison, 1976). The TMV standard had a buoyant density of 1.324.

PVM protein subunit moved through the polyacrylamide gel as one band. Its molecular weight lay between those of carbonic anhydrase and ovalbumin (Fig. 16), and ranged from 37,600 to 40,300 daltons. The means of five determinations for the individual gel concentrations were 38,900, 39,100, 39,200, 39,600, 39,700 and 39,800 daltons for 4, 5, 6, 7, 8, and 9% gels respectively. The overall mean was 39.3 $\pm$ 0.6 thousand daltons.

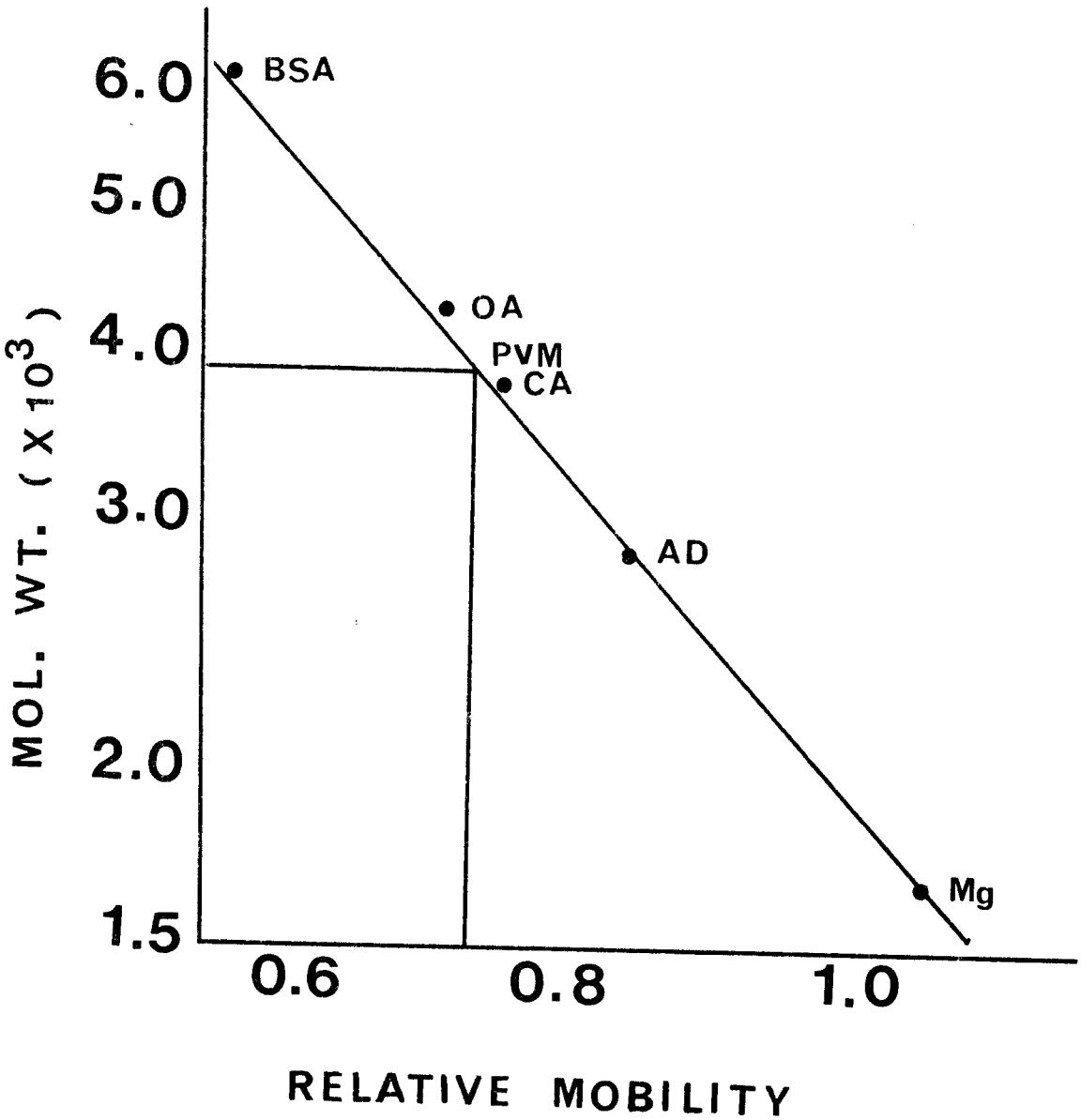


Fig. 16. Plot of molecular weights of proteins versus their relative mobilities in 5% polyacrylamide gels containing 0.1% SDS. The molecular weight of PVM protein subunit was graphically estimated from the plot. The protein standards used were: Bovine serum albumin (BSA), ovalbumin (OA), carbonic anhydrase (CA), alcohol dehydrogenase (AD) and myoglobin (Mg).

DISCUSSION

The host range of PVM is narrow. Only plants belonging to the families Amaranthaceae, Chenopodiaceae, Leguminosae and Solanaceae could be mechanically infected by the virus. Symptoms observed on G. globosa, C. amaranticolor, C. quinoa, P. vulgaris cvs. Red Kidney and Bountiful and V. sinensis were generally similar to the previous descriptions by several authors (Bagnall et al., 1959; Hiruki, 1970; Horvath, 1972; Kowalska and Was, 1976). MacLeod (1962) and USDA (1966) listed the species B. vulgaris, D. metel, D. stramonium and N. debneyi as hosts of PVM. Inoculation tests on these species of plants were unsuccessful. Rozendaal and van Slogteren (1957) noted that the "Bintje" isolate did not infect N. debneyi which had earlier been found to react with various types of local lesions to five other strains of the virus (Bagnall et al., 1956). The reason given to explain this phenomenon was that the temperature of the greenhouses used by the Dutch workers were unsuitable for PVM infection. In the present studies however, inoculation tests with these four species of plants were conducted during winter, spring and summer, indicating that the failure to achieve infection in these plants was not due to the influence of seasonal temperature variations. Moreover, Kowalska and Was (1976) reported that only one of the twelve isolates tested produced local lesions in N. debneyi irrespective of whether the plants were maintained at 16, 22 or 28^{o}C.

Immunity to PVM was not found in North American potato cultivars. Seven of the 16 cultivars which were not infected through mechanical inoculation showed 60 to 100% infection when graft-inoculated with the virus (Table 4). A similar graft-inoculation experiment was not carried out on the other nine cultivars because virus-free tubers were not available. The observation that one of the ten potato plants, cv. Sable, was not infected through mechanical inoculation in fall 1976, but none of the three in spring 1976, reflected the necessity of inoculating more plants from each cultivar. Had this been followed, the mechanical inoculation experiments could have revealed the susceptibility of more than the 17 found in the present studies. Nonetheless, it was demonstrated that variations did exist between cultivars with regards to the ratio of the number of infected plants to the number of inoculated plants. These were exemplified by the fact that all or most of the mechanically inoculated potato plants cvs. Banana, Red La Soda and White La Soda, and that only one of the ten potato plants, cv. Sable, were infected. This phenomenon was probably due to the existence of differential susceptibility among potato cultivars to PVM, as has been suggested by Zadina (1971).

In all the infected potato plants no symptoms developed. With regards to the cultivar Arran Victory, this was not an exceptional case because Bagnall et al. (1959) reported that,

of the five isolates tested, only the paracrinkle and the leafrolling mosaic isolates incited symptoms. Similarly, the progeny plants cvs. Banana, Red La Soda and White La Soda did not exhibit any symptoms. It has been reported that PVM-infected potato plants which did not exhibit any symptoms could produce progeny plants showing various degrees of severity of symptoms (Chrzanowska, 1976; Bagnall et al., 1959). It appeared therefore that the virus isolate used in the present studies is a mild one.

Transmission of the virus by plant contact had been reported by Symygla et al. (1973). Attempts to confirm this observation were unsuccessful even when tomato plants which were easily infected through mechanical inoculation were used. This was probably due to strain differences.

The present studies showed that the physical properties *in vitro* of PVM extracted from potato leaves were similar to those extracted from tomato leaves. The dilution end point of the virus was 10^{-4}, and this was similar to the value reported by Hiruki (1972) and Tu and Hiruki (1970). The thermal inactivation point of between 65 and 70^{o}C for PVM as found in the present studies had previously been reported by Bagnall et al. (1956), Bagnall and Larson (1957a, 1957b) and Horio et al. (1969). The observation that the average number of local lesions increased when the crude sap was heated to 40^{o}C (Fig. 2C) confirmed the finding of Bagnall et al. (1956), who suggested that heating the

crude sap to this temperature resulted in the deinactivation of inhibitory substances. Dilution of the crude sap in 0.05 M borate buffer to 10^{-1} caused an increase in the number of local lesions incited by the virus on Red Kidney bean (Fig. 2A). This is the first report on this phenomenon for PVM. A similar observation has been reported by Saksena and Mink (1969) who noted that the apple chlorotic leafspot virus in crude sap diluted to 2×10^{-1} or 10^{-1}, produced more local lesions on C. quinoa or on Red Kidney bean than the virus in undiluted crude sap. This could be due to the influence of inhibitory substances in the crude sap, as suggested by the relatively short longevity in vitro of between 2 and 4 days. Moreover, the virus particles remained stable for at least 2 weeks in crude sap extracted with 0.5 M borate buffer containing a reducing or chelating agent, but not so in crude sap extracted with the same buffer to which a reducing or chelating agent had not been added.

A suitable buffer for the extraction and resuspension of the virus-containing materials is a prerequisite for an efficient purification procedure. In this connection it was found that the borate buffer with a pH range between 7.8 to 8.5, was suitable for use in the purification of PVM. Borate buffer, pH 8.2 had previously been used by Shepard (1972) for the purification of this virus. The relatively lower virus concentration in the sap extracted in diethyl barbiturate buffer, citrate-phosphate buffer or water could be due to the poor buffering capacities of these buffers, as indicated by the change in their pH (Table 5).

Albrechtova and Klir (1970) had previously described a purification procedure for PVM, in which the diethyl barbiturate buffer was used for the extraction and resuspension of the virus. They did not however, carry out a comparison test between this buffer and other buffers, as has been done in the present studies. The incorporation of a reducing or a chelating agent into the buffer during homogenization of leaves was not essential, but appeared to stabilize the virus particles present in the crude sap which had been incubated for longer times. Purification of the virus should be carried out from freshly harvested leaves irrespective of whether potato or tomato plants were used as propagation hosts. The ratio of 1:1.5, tissue to buffer, should be used for economic reason and for ease of handling of volume.

The aim of clarification is to minimize the amount of plant material in the sap before it is concentrated into a smaller volume. This is necessary because of the difficulty in resuspending the virus from the resulting pellets. In TMV and some other viruses, resuspension can be aided by the addition of Triton X-100 (Nozu and Yamura, 1971). In the present studies it has been shown that the detergent was harmful to the virus. As a result, the virus content of the preparation was reduced considerably (Table 4). Ammonium sulfate precipitation and carbon tetrachloride emulsification were equally suitable for the clarification of PVM-containing sap. Both methods appeared to be superior to the methods of clarification with chloroform

(Shepard, 1972), butanol (Hiruki et al., 1974) or celite (Albrechtova and Klir, 1970). The addition of ammonium sulfate should be made slowly so that it would not occur in a high concentration at the area surrounding the dissolving crystals. In this way precipitation and loss of virus was avoided.

Shepard (1972) recommended that the low speed centrifugation in a purification procedure for PVM should be carried out at 5,000 g for 10 min. Centrifugation at a higher speed for a longer time presumably resulted in a severe loss of virus because of the severe aggregation of the virus particles. However, the results obtained from the present studies revealed that no detectable loss of virus was observed when centrifugation was carried out at 10,800 g (9,500 rpm) for 15 min. At this combination of speed and time, a larger quantity of plant material could be discarded at each step of the purification procedure. This was significant because the concentration of PVM in the propagation host was low and a large quantity of leaves had to be used at the start of the purification procedure.

The loss of virus during the first concentration step was mainly due to the difficulty in resuspending the precipitated virus particles from the pellets. This problem was encountered irrespective of whether the precipitation of the virus was carried out with ammonium sulfate, PEG 6000 or by a high speed centrifugation. Albrechtova and Klir (1970) reported that PVM could still be detected after the third resuspension of PEG pellets.

Similar results were obtained when 0.5 M or 0.05 M borate buffer were used for resuspending these pellets at two-tenths or four-tenths of the original volume. Resuspension of pellets in four-tenths of the original volume of 0.05 M phosphate or 0.05 M citrate-phosphate buffer yielded similar results as 0.05 M borate buffer. Hence, it was decided to use 0.05 M borate buffer throughout the subsequent steps of purification. With this buffer the virus was still detected after the second resuspension of pellets in either two-tenths or four-tenths of the original volume, but not so after the third resuspension. Hence, the resuspension of the pellets obtained from the first stage of concentration was carried out twice, each time with two-tenths of the original volume.

In theory, incorporation of more than one concentration method into the purification procedure should give a good final product. Since the ammonium sulfate precipitation, PEG precipitation and the high speed centrifugation methods gave equally good results, their incorporation into the four stages of concentration was advantageous. In the last stage, high speed centrifugation was prefered to PEG precipitation because in small volumes, separation of the supernatants from the undissolved pellets during a low speed centrifugation was easier in the former than in the latter. Hence, precipitation with PEG should only be carried out during the first, the second or the third stage of concentration. The minimum amounts of PEG 6000 and sodium chloride to be used for the precipitation of virus during the first stage or the second stage of concentration

were 5% and 4%, respectively. It was then assumed that these concentrations were also the most suitable combination for the third stage of concentration. This combination was recommended by Shepard (1972) for the purification of PVM.

Following the sucrose density gradient centrifugation, virus preparations derived from the ammonium-sulfate-clarified sap formed only one peak when scanned by the ISCo sucrose density gradient scanner (Fig. 10). Similarly, the preparations derived from carbon tetrachloride-clarified sap had one peak, but had "shoulders" which were probably caused by particle breakage or aggregation. The breakage of particles could have occurred during emulsification of crude sap with the organic solvent in the Waring blender. This method of treatment could have been too severe for the virus.

The A_{260}/A_{280} ratio of 1.23 for the purified preparations obtained by the carbon tetrachloride method, and the ratio of 1.25 for the purified preparations obtained by the ammonium sulfate method, indicated that both methods could be used for purification of PVM. The evidence from the A_{260}/A_{280} ratios were substantiated by the electron microscopic appearence of these virus preparations (Fig. 9), which were more homogenous compared to the virus preparations shown in Fig. 7. Assuming that the extinction coefficient of PVM is 3.0, then the yields of virus in the preparations purified by the carbon tetrachloride and the ammonium sulfate methods were 1.4 mg and 1.8 mg per Kg of potato leaves, respectively. When yields of virus were estimated from their serological dilution end points at the beginning and at the

end of the purification procedures, the purified virus preparations represented about one-thirtyfifth of the amount of virus in the crude sap. The corresponding value for the method of Wetter (1960) was one-eigtieth. Hence the present methods are superior than the method of Wetter mentioned above.

In the ammonium sulfate method, the particles of PVM appeared to be normal throughout the purification procedure, and after the sucrose density gradient centrifugation the particles were fairly homogenous in length. The normal length was 651 nm. The particle width of 13.4 nm was not different from the value reported by Brandes et al. (1959). The TMV particles introduced into the PVM preparation had width of 18.4 nm, this estimation is comparable to the measurement of between 17 to 18 nm reported by Brenner and Horne (1959).

In bulk purification using the ammonium sulfate method, the yields of three replicates were 4.1, 3.7 and 3.8 mg virus per Kg leaves. These figures represented more than twice the yields previously obtained. This arised from the fact that the final concentrated virus preparations were resuspended in 2.0 ml of buffer and then placed onto sucrose density gradients in the SW 25 rotor, instead of in 0.3 ml of buffer for centrifugation in SW 41 rotor. Resuspension of pellets in 0.3 ml of buffer had resulted in a reduction of the serological dilution end point of the virus preparations.

The biochemical and the biophysical properties of PVM have not been reported previously. The present studies were successful in determining several of these properties using purified virus preparations. The A_{260}/A_{280} ratio of 1.23 for PVM determined in the present studies was within the range of 1.14 to 1.55 reported for the Carlaviruses (Appendix II). The value of 1.24 for the A_{max}/A_{min} ratio was very similar to the value of 1.23 reported for the carnation latent virus (Paul and Wetter, 1964), which is the typespecies of the Carlavirus group (Harrison et al., 1971). The sedimentation coefficient of 162 was lower than most viruses in the same group, for which the sedimentation coefficient of between 160 to 168 have been reported.

One of the main contributions of the present studies is the determination of the buoyant density of PVM, which is 1.304. This is the first report made, not only in PVM, but in the Carlavirus group as well. This value is lower than that of barley stripe mosaic virus (1.309), but higher than that of the empty capsid of tobacco ringspot virus (1.290) or proteins (1.29) (Sehgal et al., 1970). The TMV standard used in the present studies had a buoyant density of 1.324. Siegel and Hudson (1959) reported the values of 1.325 and 1.322 for the buoyant densities of the U_1 and U_2 strains of TMV, respectively. Similarly, Sehgal et al., (1970) found that the U_1 strain had a buoyant density of 1.324.

Although the molecular weight of the protein subunit of PVM appeared to increase with the gel concentration, the differences were not significant ($P > 0.05$). The average value of 39,000 daltons was higher compared to those of the protein subunits of cowpea mild mottle virus (Brunt and Kenten, 1973), alfalfa latent virus, pea streak virus and red clover vein mosaic virus (Venkateswarlu, 1976). The molecular weights of the respective protein subunits were 32,000 to 33,000, 27,000, 27,000 and 33,000 to 34,000 daltons. The estimated RNA content of 6.2% for PVM particles closely agrees with the expected value of 6% for the Carlaviruses (Harrison et al., 1971).

The present studies, therefore, were successful in obtaining a purification method for PVM by which a fairly pure preparation of the virus was obtained. The yield of the virus was relatively high considering that the concentration of the virus in the host tissues is low. The possibility of obtaining a sufficient quantity of the purified virus preparation was a prerequisite for the success in the determination of the various physical, biophysical and biochemical properties of the virus. Studies on this virus along this line have been eschewed probably because of the difficulty of purification.

It was further concluded that the present studies confirmed the narrow host range of the virus, as no new hosts were discovered. In addition, it was revealed that the isolate used in the present studies is a mild one. Even so, no North American potato cultivar was immune to it.

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APPENDIX I

The relationship between turbidity at 540 nm and the relative concentration of crude sap from leaves of virus-free potato plants

Crude sap from leaves of virus free potato plants was extracted with 0.5 M borate buffer at the ratio of 1:3, tissue to buffer. After a low speed centrifugation, the supernatant was diluted with the same buffer to 0.8, 0.6, 0.4 and 0.2 of the original concentration. The turbidity of the undiluted and diluted sap was measured at 540 nm in a Beckman model DU-spectrophotometer.

At the same time, 1 ml of the undiluted sap was added to an equal volume of partially purified PVM preparation which had been diluted to 0.8, 0.6, 0.4 and 0.2 of the original concentration. The turbidity at 540 nm was determined as above.

The regressions of the dilution of sap or dilution of virus (x) on the turbidity value at 540 nm (y) was calculated with the aid of a computer. The regression coefficients of 0.999 and 0.00996 for the dulution of sap and dilution of virus, respectively, demonstrated that the 540 nm value was proportional to the relative concentration of plant debris, and not to the relative concentration of virus contained in the crude sap.

Appendix I (cont'd)

Regression of the dilution of crude sap from potato leaves (X) on the turbidity value at 540 (Y).

```
.....................................................................

                NEW RUN ? (ANS. Y OR N)Y

NO. OF VALUES? 15
(  1)   X .2        Y .294
(  2)   X .2        Y .3
(  3)   X .2        Y .285
(  4)   X .4        Y .593
(  5)   X .4        Y .59
(  6)   X .4        Y .55
(  7)   X .6        Y .89
(  8)   X .6        Y .595
(  9)   X .6        Y .89
( 10)   X .8        Y 1.14
( 11)   X .8        Y 1.15
( 12)   X .8        Y 1.14
( 13)   X 1.        Y 1.4
( 14)   X 1.        Y 1.42
( 15)   X 1.        Y 1.45

                Y=A+BX

SP  0.16696000002E+01
SSX  0.12000000002E+01          SSY=TOTAL SS  0.23834852411E+01
B=SLOPE  0.14056166666E+01      A  0.22900000056E-01
R2  0.99633752 3E+00            R  0.99816644 4E+00
REGRESSION SS  0.23795200 4E+01
RESIDUAL SS=SSY-REGRESSION SS  0.39623673 7E-02

ESTIMATED VARIANCE=RESIDUAL SS / N-2  0.34417485 2E-03
F FOR TEST B=0,  7606.5557,  F HAS (1,  13) DF


DO YOU WANT X AND Y VALUES INTERCHANGED ?
(ANSWER Y OR N)N

.....................................................................
```

Appendix I (cont'd)

Regression of the dilution of partially purified virus preparation (after further dilution with an equal volume of 1:3 potato sap) (X) on the turbidity value at 540 nm (Y).

```
NEW RUN ? (ANS. Y OR N)YY

?0
NO. OF VALUES?5
(  1)   X .2        Y .67
(  2)   X .2        Y .652
(  3)   X .2        Y .68
(  4)   X .4        Y .666
(  5)   X .4        Y .676
(  6)   X .4        Y .677
(  7)   X .6        Y .679
(  8)   X .6        Y .659
(  9)   X .6        Y .663
( 10)   X .8        Y .673
( 11)   X .8        Y .67
( 12)   X .8        Y .68
( 13)   X 1.        Y .683
( 14)   X 1.        Y .664
( 15)   X 1.        Y .655

Y=A+BX

SP  0.400002114E-03
SSA  0.120000000E+01          SST=TOTAL SS  0.134293595E-02
B=SLOPE  0.333335094E-03      A  0.669733332E+00
R2  0.992860027E-04           R  0.996423618E-02
REGRESSION SS  0.133334742E-06
RESIDUAL SS=SST-REGRESSION SS  0.134293261E-02

ESTIMATED VARIANCE=RESIDUAL SS / N-2  0.133292501E-03
F FOR TEST B=0  0.0013,  F HAS (1,  13) DF

DO YOU WANT X AND Y VALUES INTERCHANGED ?
(ANSWER Y OR N)N
```

Appendix II

Some properties of members of the Carlaviruses

Viruses	Properties								
	A**	B	C	D	E	F	G	H	I
Carnation latent (61)*	650X12	60-65	10^{-3}	2-3	167s	1.37	1.23	2.1	
Chrysanthmum B (110)	685X12	75-80	10^{-2}	2-6	168s	1.55	1.20		
Cowpea mild mottle (140)	650X13	65-70	10^{-3}		165s	1.14	1.21		
Lily symptomless (96)	640X17				167s	1.20-1.43			
Pea streak (112)	619-630 X?	78-80	10^{-6}	2-7	160s	1.33-1.35			
Red Clover vein mosaic (22)	645-12	60-65	10^{-3}	2	160s	1.14			
Poplar mosaic (75)	675X?	74	10^{-5}	2	165s				
Potato S (60)	650X12	55-60	10^{-2}	3-4					
Potato M (87)	650X12	65-71	10^{-2}						
Potato M (Present study)	651X13	65-70	10^{-4}	3-4	162s	1.23	1.24		1.304

( )*: Publication number of CMI/AAB Description of Plant Viruses.

** : A-size (length X width in nm); B-TIP (^{o}C); C- DEP; D- LIV (days); E- $S_{20,w}$ F- A_{260}/A_{280}

G- A_{max}/A_{min}; H- Absorbance at 260A^{o}; I- buoyant density.

Appendix III

Compositions of buffers

0.5 M Borate buffer, pH 7.8

Boric acid	24.6 gm
Sodium borate	20.6 gm
Distilled water	1000 ml

0.2 M Phosphate buffer, pH 7.8

Potassium phosphate (monobasic)	2.7 gm
Sodium phosphate (dibasic)	65.4 gm
Distilled water	1000 ml

0.1 M Sodium diethyl barbiturate buffer, pH 7.8

Sodium diethyl barbiturate	20.6 gm
Cysteine hydrochloride	12.1 gm
Sodium ethylenediaminetetraacetate (EDTA)	2.6 gm
Distilled water	1000 ml

Appendix III (cont'd)

0.2 M Citrate-phosphate buffer, pH 7.8

Sodium citrate	9.8 gm
Potassium phosphate (dibasic)	27.2 gm
Distilled water	1000 ml

0.2 M Tris-acetate buffer, pH 7.8

Tris	24.3 gm
Sodium acetate	27.2 gm
EDTA	1.85 gm
Distilled water	1000 ml