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SOME PROPERTIES OF POTATO AUCUBA MOSAIC VIRUS-INDICATOR HOSTS, BIOLOGICALAND PHYSICAL PROPERTIES, PURIFICATION, SEROLOGY AND ELECTRON MICROSCOPY

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SOME PROPERTIES OF POTATO AUCUBA MOSAIC VIRUS - INDICATOR HOSTS, BIOLOGICAL AND PHYSICAL PROPERTIES, PURIFICATION, SEROLOGY AND ELECTRON MICROSCOPY

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To My Wife

This dissertation has been examined and approved.

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INTRODUCTION

Potato (Solanum tuberosum L.) is one of the most important crops in the United States. Commercial potato production is important in the New England area where the conditions are favorable for the growth of this crop. Virus infection in an extremely serious problem in the potato industry. Potato crops suffer both in weight and size from the infection of viruses (8, 59). Whitehead (59), in 1953, stated that virus diseases were considered to be the sole cause of progressive degeneration in potato stock. Many virus diseases have been reported (23, 26, 59) for potato, one of which is potato aucuba mosaic.

Potato aucuba mosaic was first described and named by Quanjer (41) in 1921. The term aucuba mosaic was derived from the plant Aucuba japonica L. which is a member of a small genus of shrub of the dogwood family, and is widely cultivated for its handsome yellow mottled evergreen foliage. Quanjer used this term because leaves of diseased potato plants resemble the normal foliage of A. japonica. The disease is characterized by a bright yellow variegation type of symptom which is variable in different potato varieties (14, 21, 32).

This disease is distinct from the aucuba mosaic of tomato which is caused by a virus closely related to tobacco mosaic virus. Potato aucuba mosaic was never considered important enough to devote as much time to its study as has been given to other potato viruses such as potato virus X,

Y, leaf roll, and spindle tuber. This is probably due to the fact that it is not commonly found in the field. However, Rozendaal (44), in 1954 indicated that certain strains of this virus could cause considerable damage to potato crops. Smith (47), in 1957, stated that the consequent reduction in vigor and yield was not significant, but more damage could be done by the resulting tuber necrosis in some potato varieties.

The purpose of this study is to investigate the biological, physical and chemical properties of the virus, including purification, serology, and electron microscopy. This should lead to a better understanding of the nature of the virus, and its biological properties.

LITERATURE REVIEW

History

Potato aucuba mosaic was first described and illustrated in 1921 by Quanjer (41) in Europe and by Murphy (35) in America. It had been shown that potato aucuba mosaic in America is identical to that found in Europe (47). Since Quanjer's first description of this virus disease, numerous isolates or strains have been reported throughout the world under various synonyms. The virus has been shown to occur in Europe (Ireland, Great Britain, Scandinavia, France, USSR, Hungary) as well as North America (United States, Canada), South Africa, Australia, New Zealand, Japan, and even in wild Solanum species from Peru (1, 3, 6, 15, 16, 17, 18, 19, 24, 25, 27, 35, 59).

Quanjer (41), in 1921, also observed another virus disease in several potato varieties which developed pale patches between the veins of potato leaves. He first named it Duke of York mosaic since it was first observed on the potato variety Duke of York, but later (42) he renamed it interveinal mosaic because symptoms were confined to the interveinal areas of the leaves. Smith (45, 46) in 1931, reported that interveinal mosaic was a complex of potato viruses X and Y. Loughnane and Clinch (33), in 1935, reported that interveinal mosaic results from the combined action of potato virus A and tuber blotch virus. In 1936 a latent virus was isolated from the potato variety Monocraat

by Murphy and Loughnane (37) and was designated as Monocraat virus. Clinch, Loughnane and Murphy (14) gave further descriptions of tuber blotch virus, aucuba mosaic and Monocraat virus and concluded that aucuba mosaic virus was closely related to but distinct from tuber blotch and Monocraat virus. They designated tuber blotch as Virus F with Monocraat virus as a synonym and aucuba mosaic as virus G. Koch and Johnson (31), in 1935, isolated a virus from potato plants infected with interveinal mosaic and named it potato streak virus. Later Loughnane and Clinch (33) found it to be identical to tuber blotch virus. Dykstra (21), in 1939, reported a new undescribed disease under the name of Canada streak, and proved it to be a strain of potato aucuba mosaic virus. Kollmer and Larson (32), in 1960, stated that a separation into virus F and virus G is no longer justified, and they classified all the isolates, including aucuba mosaic, collectively as virus F.

Symptomatology

As mentioned previously, the disease was characterized by a bright yellow variegation on the leaves of infected potato plants. The symptoms always appeared on the lower leaves of the diseased plants and were confined there, but in the potato variety Irish Chieftain, a pronounced brilliant yellow mottle developed all over the plants. Clinch et al (14) suggested that the exaggeration of mottle was due to the occurrence of potato virus A which was usually present in this variety. In the European variety, British Queen, (14, 47)

foliar necrosis and wilting of the leaves developed in addition to pronounced yellow mottle. The reaction of American potato varieties to aucuba mosaic virus or strains of potato virus F was variable. The reaction of Johnson's streak virus (31) on potato varieties Green Mountain and Bliss Triumph was a distinct necrotic or streak-like symptom on leaf veins, lamina and stems of potato, followed by downward necrosis which killed the entire plants. Kollmer and Larson (32), in 1960, indicated that American potato varieties inoculated with seven different isolates of potato virus F, including aucuba mosaic isolates, developed two principal types of foliar symptoms. Those in the first group (Seedling 41956, Chippewa, Dazoc, Houma, Russet Rural, Warba, Green Mountain) developed local necrotic lesions on the inoculated leaves, followed by leaf necrosis and leaf drop. The newly formed leaves of the systemically infected plants showed small necrotic lesions and leaf tip necrosis. Those in the second group (Saco, Burbank, Irish Cobbler, Kennebec, La Soda, Pontiac, Sequoia, Sebago, White Rose) reacted with severe top and stem necrosis a few days after inoculation. The second type of reaction was so severe in some varieties that the infected plants were completely killed.

In 1926 Atanasoff (1) stated that net necrosis was a tuber symptom of aucuba mosaic, but later Quanjer and his associate (43) indicated that tuber net necrosis did not result from infection with potato aucuba mosaic but from another necrosis virus which they called "pseudo net-necrosis"

instead of net necrosis because net necrosis was known to result from infection with the leaf roll virus. However, tuber necrosis did occur in some potato varieties (14, 32) which were infected with aucuba mosaic virus or isolates of virus F. Clinch et al (14), in 1936, indicated that tuber necrosis was found in seven of the 14 varieties tested. They also reported that tuber necrosis began first toward the heel end of the tuber and was favored by darkness and high temperature. Kollmer and Larson (32), in 1960, stated that potato plants which reacted with severe top and stem necrosis were usually dead before the formation of tubers. However, tubers from plants which developed mild foliar symptoms after inoculation with different isolates of the virus, showed internal necrosis in some varieties.

Tomato (Lycoperisicon esculentum Mill.) infected with potato aucuba mosaic virus or its related strains developed small rounded yellow spots on the lower leaves of the infected plants (14, 47). Pepper (Capsicum annuum L.) inoculated with this virus or tuber blotch virus developed necrotic local lesions, followed by systemic symptoms on the next higher leaves, consisting of vein-clearing, developing into pale or necrotic interveinal mottle, puckering, distortion, and leaf-drop. However, Kollmer and Larson (32), in 1960, stated that the systemic reaction of pepper plants to inoculation with aucuba mosaic or virus F was characterized by the production of numerous small necrotic lesions, followed by severe top necrosis, resulting in death of the infected plants. Clinch et al (14), in 1936, indicated that potato aucuba mosaic and

potato virus F (tuber blotch and Monocraat) produced identical symptoms on Solanum nodiflorum L., consisting of spots with brown or purple borders, followed by purple rust and mosaic-type symptoms.

The reaction of other plant species to aucuba mosaic virus, potato virus F, or its related strains was helpful in the differentiation and identification of different virus isolates or strains. The specific reactions of those plant species will be mentioned in the sections concerned.

Isolation and Transmission of the Virus

Quanjer (41), in 1921, demonstrated that aucuba mosaic virus could be transmitted by grafting from potato to potato, tomato, Solanum nigrum L., and S. Dulcamara L., all of which showed similar symptoms, and to tobacco, Atropa belladonna L., Datura Stramonium L. and Hyoscyamus niger L. which showed no symptom. Back grafting from these symptomless hosts to potato indicated that they were virus carriers. At about the same time, Quanjer (41) also reported that Duke of York mosaic was transmitted by grafting from potato to potato, tomato, tobacco and S. nigrum. Smith (45), in 1930, indicated that interveinal mosaic could be transmitted from diseased potato plants to healthy potato plants by means of needle inoculation. Clinch and Loughnane (13), in 1933, reported that interveinal mosaic, isolated from the potato variety President, produced a pronounced interveinal mottle, necrotic blotching of the foliage, and internal necrotic brown lesions in tubers of

this variety. They proved that the virus was transmitted mechanically to several potato varieties and other plant species such as tobacco, Datura and Petunia and concluded that interveinal mosaic resulted from the combined action of viruses of different types, one of which was X-type without an insect vector involved, and the other of which was selectively transmitted by aphids (Myzus persicae Sulz.) under certain condition, and was named tuber blotch virus. They isolated tuber blotch virus from potato plants infected with interveinal mosaic by the following two ways: The first one was passage of interveinal mosaic through the potato variety Arran Crest, in which the X virus could not survive. The second one was dependent on insect vectors and a situation in which interveinal mosaic virus was combined with potato virus A. The previous finding was that M. persicae was incapable of transmission of interveinal mosaic, but under this combined condition, M. persicae transmitted to healthy potato not only virus A but also a tuber blotch fraction of interveinal mosaic, causing a conspicuous yellow mottle in lower leaves of virus-infected plants as well as necrosis in tubers. Tuber necrosis resulting from this transmission was identical with that occurring in the interveinal mosaic. Potato virus A was easily eliminated from tuber blotch virus by passage through D. Stramonium which is immune to virus A. When the tuber blotch fraction of interveinal mosaic was graft transmitted to the potato variety President, conspicuous necrosis developed in the

tubers of this variety, but there were no foliage symptoms except occasional slight yellowish spotting at the tip of the lower leaves. Later, it was confirmed that the conspicuous yellow mottle referred to above was due to the combined action of virus A and tuber blotch virus. The above experiments proved that tuber blotch virus was transmitted by aphids in the presence of virus A.

In 1936, Clinch et al (14) reported that the aucuba mosaic which had been regarded as being non-inoculable could be readily sap-transmissible to potato by rubbing infective sap on lower leaves with a ground glass spatula. The inoculated plants developed aucuba mosaic symptoms on foliage and necrosis in tubers.

Early attempts to transmit aucuba mosaic by means of aphids were unsuccessful (14). Clinch et al (14) even failed to transmit the aucuba mosaic virus in the presence of virus A. In view of the effect of virus A facilitating the transmission of the closely related tuber blotch virus, Kassanis (29), in 1961, conducted a comprehensive study to evaluate the nature of insect transmission and its relationship to strains of the virus in the presence of an auxiliary virus. He fed aphids on tobacco plants which were systemically infected with 12 different strains of aucuba mosaic and auxiliary virus A, and then the aphids were colonized on pepper (C. annuum) plants to test for transmissibility. He failed to transmit three strains of potato aucuba mosaic from plants which were also infected with virus A. None of the 12 strains was transmitted by aphids from plants infected

with aucuba mosaic alone. He concluded that transmission of potato aucuba masaic by aphids in the presence of virus A varied with strains. In addition to the insect transmissible relationship between aucuba mosaic and virus A, he also tested the correlation between aucuba mosaic and potato virus Y. His transmission experiments indicated that aphids frequently transmitted potato aucuba mosaic from tobacco plants infected also with potato virus Y. All the evidence indicated that different strains behaved differently in transmissibility. Kassanis (29) classified the 12 strains into four main groups according to their transmissibility. Those in group 1 (Germany No. 6), were transmitted equally readily from plants infected with either virus A or virus Y. Those in group 2 (severe tuber blotch virus from Scotland, mild tuber blotch virus from Scotland, tuber blotch virus from Ireland, potato virus F from England) were transmitted more readily from plants infected with virus Y than virus A. Those in group 3 (Germany PH, potato aucuba mosaic virus Scotland) were transmitted only from plants infected with Y. Those in group 4 (severe tuber blotch virus from Ireland, Germany AH, potato virus G from England, potato virus G from Scotland, potato aucuba mosaic virus from Ireland) were transmitted only occasionally from plants infected with either of the auxiliary viruses (including strains which were not aphid-transmissible). The concentration of potato aucuba mosaic in tobacco plants was increased by the presence of potato virus A and more so by potato virus Y (29), but the increased concentration was not

correlated with its ability to be transmitted by aphids. The amount of potato virus Y in tobacco was not affected by the co-existence of aucuba mosaic virus, while the amount of virus A was increased appreciably in the presence of aucuba mosaic. However, potato aucuba mosaic virus ceased to be aphid transmissible as soon as it was freed from either virus A or virus Y (29).

Kassanis (29) agreed with Bradley's (19) suggestion that particles of aucuba mosaic virus were carried mechanically by aphids near the tip of their stylets and the presence of auxiliary virus might cause the particles of this virus to aggregate with each other or with those of auxiliary viruses and thus form large virus units which could be attached to the stylets, making the virus insect-transmissible.

Identification and Nomenclature of the Virus

Symptomatology and host range have been the most widely used criteria for identification of plant viruses, but their inadequacy as a basis for identification has long been recognized. It has been shown that a given virus can cause different symptoms in different host species, while different viruses may induce similar symptoms in the same plant species. On the other hand, different strains or isolates of the same virus can cause entirely different diseases. Therefore, an accurate identification must be achieved by a combination of all available diagnostic characters such as cross protection and serology.

Clinch et al (14), in 1936, conducted an extensive,

comparative investigation among potato aucuba, tuber blotch and Monocraat viruses and made the following statement :

> The three viruses produce identical symptoms, consisting of spots with brown or purple borders, followed by purple rust and mosaic in Solanum nodiflorum and Capsicum annuum, which are diagnostic. The three viruses are readily inoculable to tobacco, Datura Stramonium, and Petunia, and react similarly, producing no symptoms. The aucuba mosaic virus is differentiated from the others, however, by causing symptoms in tomato and Solanum Dulcamara. The three viruses are similar in their physical properties, including filterability, thermal death-point, and longevity in vitro. The tuber blotch and Monocraat viruses are identical, and they probably correspond to the virus underlying pseudo net necrosis. The aucuba virus is a related but distinct form, and the calico virus is probably related also.

They concluded that aucuba mosaic was closely related to tuber blotch and Monocraat virus, but it was a distinct form, and they designated aucuba mosaic as virus G while tuber blotch was named as virus F with Monocraat as a synonym.

Muro (34), in 1959, stated that Solanum miniatum Bernh. was a reliable, specific indicator plant for potato virus F. He differentiated potato virus G and F on the basis of symptoms produced on this plant. Accordingly, S. miniatum infected with potato virus G (aucuba mosaic) developed yellowish green blotches and mottle on the leaves, while infection with F resulted in half rings and blotches and brown necrotic blotches on younger upper leaves of the infected plants.

The first application of cross protection technique in aucuba mosaic was made by Clinch (12) who isolated a

virulent tuber blotch virus which differed from that of original tuber blotch by producing a lethal top necrosis in the potato variety Irish Chieftain. She demonstrated that if the potato variety Irish Chieftain was infected with a strain of potato virus F, it was immune from virulent tuber blotch which caused lethal top necrosis of this variety. She also indicated that potato aucuba mosaic (virus G) and virulent tuber blotch were also mutually protective. Based on mutual cross protection, she identified the virulent tuber blotch as a strain of potato virus F.

Kollmer and Larson (32), in 1960, made a comprehensive investigation of virus F, aucuba mosaic and its related strains or isolates. They dealt with seven isolates (necrotic F from Holland, aucuba F from Holland, severe F from Ireland, virus F from Ireland, virus G from Ireland, virus F from USA, virus G from England) of virus F or aucuba mosaic virus and reported that young Saco potato plants developed typical mottle symptoms three weeks after inoculation with mild isolates, while plants infected with virulent isolates resulted in severe top and stem necrosis. Their immunological studies indicated that Saco potato plants artificially inoculated with mild isolates protected themselves against further infection by severe isolates. They also grafted scions of Saco potato plants which were infected with mild isolates onto Nicotiana glutinosa plants which were artificially inoculated with virulent isolates. The results of this interspecific grafting disclosed that healthy Saco scions which

had been top grafted to N. glutinosa stock infected with virulent isolates showed severe top and stem necrosis, while Saco scions previously infected with mild isolates developed no top or stem necrosis. On the basis of the above mutual protection results they stated that separation into virus F and virus G is no longer justified. They termed all the isolates used in their experiments collectively as potato virus F, including aucuba mosaic virus. The mutual immunological relationship of potato virus F with alfalfa mosaic, cucumber mosaic and potato virus Y was also reported. They indicated that pepper plants could be infected artificially with these viruses and developed mild systemic infection, while pepper plants infected with any isolate of virus F or aucuba mosaic developed severe top necrosis. The infection of pepper with algalfa mosaic, cucumber mosaic, or potato virus Y offered no mutual protection against severe top and stem necrosis caused by virulent isolates of potato virus F.

Chester (10), in 1935, prepared aucuba-mosaic-immune serum by injecting experimental animals with freshly-expressed crude sap from plants infected with aucuba mosaic virus and reported that there was no relationship between aucuba mosaic virus and other well known plant viruses, namely tobacco mosaic, tomato severe etch, potato latent mosaic, potato mottle, potato ringspot, British Queen streak, potato rugose mosaic, potato mild mosaic, potato veinbanding virus, Valleau's tobacco virus, tobacco ringspot virus, and Osborn's pea virus.

Bagnall (2), in 1960, isolated a latent virus from the potato variety Albion, and indicated that this latent virus was related to potato virus F. He reported that antiserum against the virus isolate reacted equally well with typical strains of potato aucuba mosaic and potato virus F.

Although the aucuba mosaic virus was renamed according to the alphabetical system as potato virus G (14), the original name is still widely accepted. Smith (47), in 1957, classified potato aucuba mosaic virus as Solanum virus 9, while the potato F or tuber blotch virus was indicated as Solanum virus 8. Holmes (26), in 1939, designated both aucuba mosaic virus and potato virus F or tuber blotch virus as Marmor aucuba under his binomial system.

Morphology of the Virus

Paul and Bode (38), in 1956, employed Johnson's (28) water pressure exudate technique to obtain clear exudate from plants infected with potato aucuba mosaic virus for morphological examination under the electron microscope. They examined three different strains of the virus and found that all of them were composed of flexible particles with a normal length of 586 mu and a diameter of about 11 mu.

Pathological Effects

Clinch (11), in 1932, made a cytological study of potato leaves infected with aucuba mosaic virus and found that the chlorosis was due to the loss of chlorophyll and disinte-

gration of chloroplasts which resulted in release of quantities of oil droplets within the cells. Later, Clinch et al (14), in 1936, showed that starch translocation in plants infected with aucuba mosaic was impeded. They reported that starch accumulated in great quantity in the young mottled leaves. The recently developed chlorotic spots stained more deeply with iodine than the remaining parts of the leaf. They concluded that the inhibition of carbohydrate translocation was one of the initial pathological effects of the aucuba mosaic on potato plants.

Tuber necrosis, caused by aucuba mosaic virus, was described by Clinch et al (14) in 1936. They reported that tuber necrosis occurred in the parenchymatous cells of both cortex and pith, as a rule, and was usually visible externally as irregularly shaped brown patches, which later developed into sunken dry brown areas. It began first toward the heel end of the tuber, and did not attack the vascular tissue or eyes. The necrotic patches consisted of groups of misshapen dead cells with brown granular contents and swollen brown and disintegrating walls, surrounded by a zone of translucent tissue containing little or no starch, in which incipient cork formation was observed. The center of the spot was occupied by a small group of cells filled with large starch grains. The necrosis usually developed during storage and was favored by darkness and high temperatures, and particularly by a turgid condition.

Resistance of Virus to Chemical Treatment

Kollmer and Larson (32) treated potato aucuba mosaic virus sap with 8.5% butanol and found that high speed pellets resulting from ultracentrifugation were highly infectious. In the past, all immune sera dealing with serological investigations of potato aucuba mosaic virus were obtained from injection of crude virus extract into the experimental animal without prior purification.

Synergistic Effect

As mentioned before (c.f.p. 8) interveinal mosaic in potato resulted from combined action of potato viruses X and F or related strains of F. The synergistic effect of potato virus F or its related strains with other viruses was also demonstrated by Kollmer and Larson (32). They indicated that Samsun tobacco inoculated with a mixture of virus F and alfalfa mosaic, produced a more severe reaction than either one alone, but they failed to demonstrate the synergistic reaction by combination of virus F and cucumber mosaic or combination of virus F with Y.

Varietal Resistance

Bagnall (2), in 1960, reported that an interspecific potato hybrid P. I. 197772 (Solanum tuberosum var Epicure 4n x S. chacoense) was found to be highly resistant to potato virus F on sap inoculation. This finding was confirmed by Kollmer and Larson (32). Meanwhile, they reported that potato plants

of WRF 161.1, WRF 33.1 and WRF 19.2 were also highly resistant, while potato plants of WRF 265.3, WRF 158.2 and WRF 169.2 were extremely sensitive to infection with virus F or its related strains.

EXPERIMENTAL PROCEDURES AND RESULTS

The Primary Virus Culture

The potato aucuba mosaic virus (PAMV) used in the present investigation was originally obtained from the U.S. D.A. Plant Industry Station, Beltsville, Maryland, and from the Canada Department of Agriculture Research Station, New Brunswick, Canada, respectively, in the form of infected tubers. Tubers infected with PAMV were planted in a 3:1:1 mixture of composted soil, peat and sand in 8-inch pots and kept in insect proof cages. Plants grown from these tubers developed a typical yellow mottle symptom on foliage (Fig. 1). Leaves from a plant showing a yellow mottle symptom were removed and ground in a mortar with 0.05 M phosphate buffer at pH 7. The virus was extracted from pulp by squeezing through a double layer of cheesecloth and was used as primary virus culture or inoculum.

The primary virus culture or inoculum was first checked for the presence of potato virus X by inoculating leaves of Gomphrena globosa L. The inoculation was made simply by rubbing leaves of the test plants which were previously dusted lightly with powdered carborundum of 500 mesh grade with a cheesecloth pad which had been dipped in the virus solution. The inoculated plants were placed on the greenhouse bench at a temperature of 25-30 C. All the inoculations in subsequent experiments were performed in the

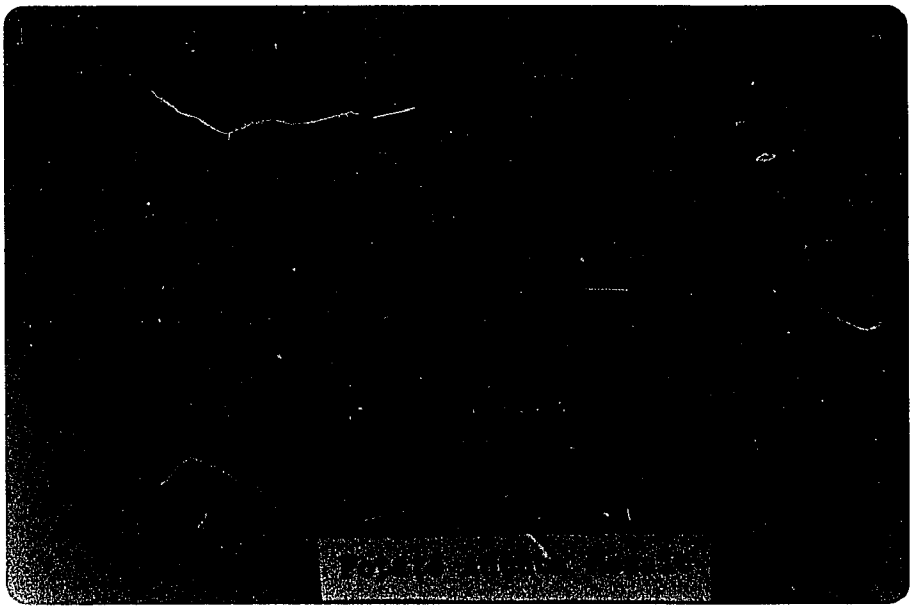

Fig. 1. Symptom on leaf of Saco potato plant infected with potato aucuba mosaic virus.

same manner unless otherwise indicated. The results of inoculation on G. globosa revealed that there was no indication of contamination by virus X in the tubers received from Canada, but tubers obtained from Maryland were doubly infected with virus X, since inoculated leaves of G. globosa developed typical local lesions induced by virus X. This contamination was eliminated by passage of the culture through Saco potato plants which are immune to virus X. The same type of inoculation was made on Physalis floridana Rydb. to determine the presence or absence of potato virus Y, but no such contamination could be detected.

Virus cultures from Maryland and from Canada were designated as PAMV-M and PAMV-C, respectively, and were maintained in tomato plants or other hosts by serial passage.

Reactions of Different Plant Species to PAMV-M and PAMV-C

In order to determine the identity of PAMV-M and PAMV-C, a number of plant species were inoculated with inocula which were prepared from potato plants infected with PAMV-M and PAMV-C, respectively. The infection of various plants was judged on the basis of symptoms which developed and recovery of virus from inoculated plants.

Symptoms in pepper (C. annuum L.) : Pepper plants inoculated with either PAMV-M or PAMV-C developed severe top and stem necrosis a few days after inoculation, usually resulting in complete death of the infected plants (Fig. 2).

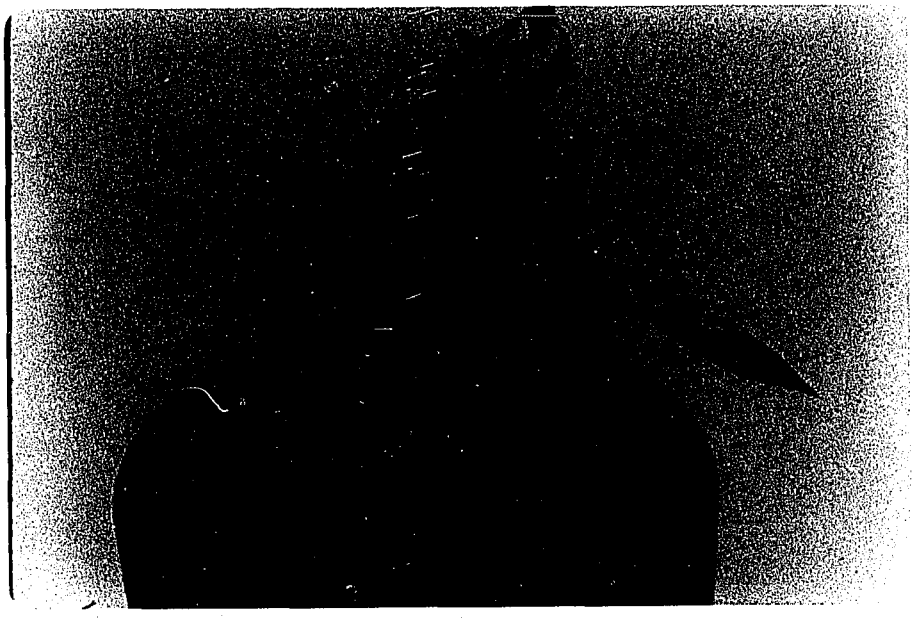

Fig. 2. Lethal top necrosis reaction of pepper plant (variety California Wonder) to sap inoculation of potato aucuba mosaic virus.

Plants which recovered from top and stem necrosis reaction were severely stunted and usually bore no fruits. Inoculation of older pepper plants approaching the flowering stage caused less severe reaction consisting of only light stem necrosis without complete death of the infected plants. The virus could not be easily recovered from surviving pepper plants.

An experiment was performed to evaluate the relative susceptibility of different pepper varieties to these virus isolates. Inocula prepared from potato plants infected with PAMV-M and PAMV-C, respectively, were inoculated onto 20 young pepper plants from each of 10 different varieties. The inoculated plants were kept under greenhouse conditions and observed at frequent intervals for symptom development for at least 20 days after inoculation. The results revealed that none of the 10 tested varieties, namely: King of the North, California Wonder, Calwonder, Cayenne Long Red, Yolo Wonder, Key Stone, Penn. Wonder, Hungarian Wax, Merrimack Wonder, and Tabasco, was found to be resistant to infection by sap inoculation with either PAMV-M or PAMV-C.

Symptoms in tomato (L. esculentum Mill. cv Rutgers) :

The reaction of tomato plants to these two virus isolates was erratic. No symptom could be observed on the plants inoculated with either PAMV-M or PAMV-C within the first three weeks. However, some infected tomato plants developed yellow spots or patches on the lower leaves when they became older. In some instances, if the main shoots of the infected plants were cut off, new side shoots showed a yellow

mosaic type symptom (Fig. 3). Inocula prepared from symptomless tomato plants which were previously inoculated with PAMV-M and PAMV-C, respectively, were reinoculated onto pepper plants and produced typical top necrosis on pepper. The response of tomato plants to both isolates was identical.

Symptoms in Chenopodium amaranticolor Coste & Reyn.: Plants of C. amaranticolor at about the 10-leaf stage were sap-inoculated with isolates PAMV-M and PAMV-C, respectively. Yellow local lesions (Fig. 4) occurred on the inoculated leaves 6-7 days after inoculation. Lesions induced by both isolates PAMV-M and PAMV-C were identical. The virus did not invade C. amaranticolor systemically since no systemic infection could be observed within a period of two months under either high or low temperature conditions. Further, leaves of these plants are thick and not easily injured in handling and local lesions produced are distinct and easily counted. All these characteristics revealed that C. amaranticolor is a satisfactory local lesion host for PAMV.

Responses of other species: N. tabacum L. var Havana, N. glutinosa L., Petunia hybrida Vilm., Datura Stramonium L., and four species of Physalis, proved to be symptomless hosts but virus carriers since virus could be recovered from these symptomless hosts a few days after infection by inoculating pepper plants. A number of other plant species showed neither symptoms nor the presence

Fig. 3. Yellow mosaic symptom on new side shoots of infected Rutgers tomato plant. The main shoot was cut off after infection with aucuba mosaic virus.

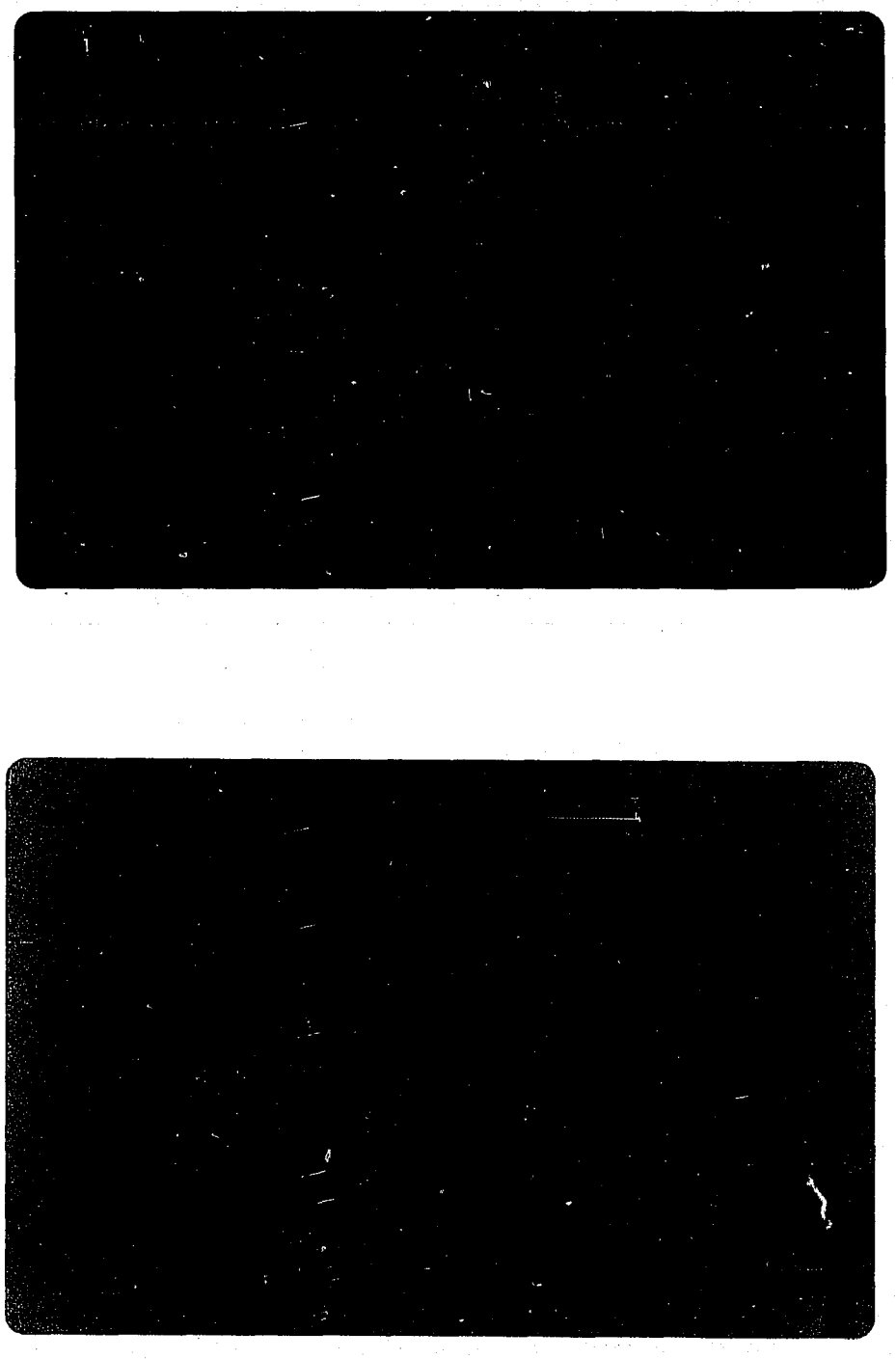

Fig. 4. Local lesions on C. amaranticolor infected with potato aucuba mosaic virus.

of virus when they were inoculated with either isolate PAMV-M or PAMV-C. See Table 1.

Single Lesion Isolation of the Virus

Knight (30), in 1961, reported that a discrete spot resulting from inoculation of diluted infectious sap onto leaves of a host which gives the local lesion response was derived from a single infectious unit. In view of the fact that C. amaranticolor developed yellow local lesions on the inoculated leaves 6-7 days after inoculation, the single lesion isolation method might be a valuable technique for pure culture isolation of PAMV-M and PAMV-C. Inocula prepared from tomato plants previously infected with PAMV-M and PAMV-C, respectively, were diluted in order to produce a small number of well-separated lesions, and inoculated onto leaves of C. amaranticolor. A single lesion was removed with a No. 2 cork borer just after the appearance of lesions and homogenized with a few drops of 0.05 M phosphate buffer at pH 7.0 in a suitable small container. Virus extract from a single lesion was reinoculated onto tomato or tobacco to build up virus concentration. Plants which were previously inoculated with single lesion virus extracts were tested for the presence of virus by inoculating pepper plants. The results are indicated in Table 2. Pure cultures of both PAMV-M and PAMV-C were derived from individual single lesions and maintained in tomato or tobacco through serial passages.

Table 1. The reaction of different plant species to PAMV-M and PAMV-C[a].

Plant species	Symptoms induced and recovery of virus			
	PAMV-M		PAMV-C	
	Symptom	Recovery of virus[b]	Symptom	Recovery of virus
Solanum tuberosum L.	AM	+	AM	+
Capsicum annuum L.	TN	-?	TN	-?
Lycoperisicon esculentum Mill.	YS?	+	YS?	+
Chenopodium amaranticolor L.	YLL	+	YLL	+
Nicotiana tabacum L.	-	+	-	+
N. glutinosa L.	-	+	-	+
Petunia hybrida Vilm.	-	+	-	+
Physalis floridana L.	-	+	-	+
P. ixocarpa Brotero	-	+	-	+
P. pruinosa L.	-	+	-	+
P. pubescens L.	-	+	-	+
Datura Stramonium L.	-	-	-	-
D. metel L.	-	-	-	-
Zinnia elegans Zacq.	-	-	-	-
Vigna sinensis (L.) Endl.	-	-	-	-
Phaseolus vulgaris L.	-	-	-	-
Vinca herbacea Waldst & Kit.	-	-	-	-

[a] The inoculated plants were kept under greenhouse conditions and observed at least 4 weeks for the development of symptoms, AM=aucuba mosaic, TN=top necrosis, YS=yellow spot, YLL=yellow local lesion, -=no symptom.

[b] Recovery of virus from inoculated plants was accomplished by inoculating pepper plants.

The pure cultures of both PAMV-M and PAMV-C were tested for their reaction on pepper and C. amaranticolor. No. variation in symptom expression in these hosts could be observed.

Table 2. Single lesion isolation of PAMV from C. amaranticolor[a].

Virus	No. of lesions removed	No. of plants inoculated	No. of plants infected[b]
PAMV-M	20	20	1
PAMV-C	20	20	2

[a] Virus extract from a single lesion was inoculated onto one plant only.

[b] The presence of virus in the inoculated plants was determined by inoculating pepper plants.

Physical Properties of PAMV-M and PAMV-C

Physical properties of the two virus isolates were determined using pepper as a test plant. Pepper was not a local lesion host, but is was used because of its super-sensitivity in response to sap-inoculation.

Thermal inactivation : The thermal inactivation determination was made by placing approximately 3 ml undiluted infectious sap which was obtained from infected tomato leaves in a 5 ml ampule. The ampules containing infectious sap were sealed in a flame and heated for 10 min in a constant temperature water bath. The temperatures used varied by intervals of approximately 2 C through the range of 40-70 C. After 10 min exposure, the ampules were immersed into tap water to ensure rapid cooling, and the extracts then were inoculated onto five young pepper plants to test for their infectivity. The thermal

inactivation was represented by the point at which none of the five inoculated plants in each experiment developed symptoms.

It was found that infective sap of both PAMV-M and PAMV-C had lost their infectivity when they were exposed to a temperature of 60 C, but both survived at 56-58 C.

Aging in vitro : Resistance to aging was determined by placing 1:5 dilutions of virus extracts from leaves of infected tomato plant in stoppered test tubes and storing them in the laboratory at a temperature of 24-25 C. Portions of the infectious sap were removed at 12-hr intervals and inoculated onto five young pepper plants. The results indicated that both PAMV-M and PAMV-C lost infectivity after standing in the laboratory for 60 hr.

Dilution end point : Freshly prepared infectious saps from tomato plants infected with PAMV-M and PAMV-C, respectively, were subsequently taken through a series of dilutions with 0.05 M phosphate buffer at pH 7.0. Each dilution was tested for its infectivity by inoculating five young pepper plants. It was found that dilution end points of both isolates differed from each other. PAMV-M retained its infectivity at a dilution of 1:90, while PAMV-C proved to be infectious at a dilution of 1:40.

The similarity of the physical properties of the two virus isolates as well as their identical symptom expression in pepper and C. amaranticolor proved them to be identical. For the purpose of the present investigation, only the pure culture derived from an infected tuber received from the U.S.D.A. Plant Industry Station, Beltsville, Maryland, was used

in all the subsequent experiments except in some special cases where indicated.

Quantitative Assay of PAMV in C. amaranticolor

In the past, no suitable local lesion host was available for quantitative work on this virus. Following the discovery of the local lesion host, C. amaranticolor, experiments were performed to evaluate the possibility of using this host for quantitative work. Plants of C. amaranticolor of the same age with 10 fully expanded leaves were selected and placed in the dark for 24 hours one day prior to inoculation. Inoculum used in this experiment was prepared from leaves of tomato plants infected with PAMV. All dilutions were made in a 0.05 M neutral phosphate buffer. Leaves which were dusted previously with carborundum were rubbed 3-4 times under uniform pressure and movement using the gauze-pad method of mechanical inoculation. After inoculation, the inoculated leaves were rinsed thoroughly with tap water to remove excess inoculum or foreign materials which might cause "burning" of the inoculated tissue. A total of 56 selected plants was randomly divided into 4 groups. Only 6 lower leaves from each plant were employed for inoculation since the lower leaves have a smooth upper surface suitable for inoculation, while the upper younger leaves not only lacked this characteristic but also produced fewer and poorer lesions after inoculation. Half of the leaves on each plant in each group were inoculated with a standard inoculum, usually at a dilution

of 1:8. The leaves on the opposite side of each plant were inoculated with a specific dilution of virus to compare the number of lesions produced in each case. A total of 84 leaves occurring on 14 plants was used for each comparison. Local lesion counts were made two weeks after inoculation. The average counts are recorded in Table 3. The progressively fewer local lesions formed on the inoculated leaves of C. amaranticolor which were inoculated with increasing dilution of PAMV revealed that C. amaranticolor is a satisfactory local lesion host for the determination of relative differences in virus concentration.

Table 3. Average number of lesions/leaf induced by different dilutions of PAMV on C. amaranticolor[a].

Dilution of test inoculum	Lesions/Leaf[b]	
	Standard dilution 1:8	Test inoculum
0	96	152
1/5	102	153
1/10	94	121
1/20	68	96
1/40	89	23
1/80	76	7
1/120	99	0

[a] Each inoculum was compared with a standard dilution in alternate leaves on 14 plants.

[b] Lesions/Leaf is the average count from a total of 42 leaves.

Virus Concentration in Various Hosts

It has been shown that different hosts differ greatly

in support of virus multiplication under identical conditions, although they are symptomless hosts (5). An attempt was made to determine the relative virus concentration in different hosts under the same conditions. All test plants employed in these experiments were grown in 4-inch pots filled with a 3:1:1 mixture of composted soil, peat and sand in a greenhouse at a temperature of 25-30 C until they reached a suitable size for inoculation. The daylight in the greenhouse was generally extended to 18 hours by a row of 150-watt incandescent bulbs or fluorescent lights suspended above the bench. Inoculum prepared from tomato plants infected with PAMV was rubbed onto leaves of test plants. The inoculated plants were kept in the greenhouse for approximately two weeks and then were checked for the presence of virus by inoculating pepper plants. When the presence of virus in the inoculated plants was demonstrated, inoculum prepared from those hosts at a dilution of 1:10 was inoculated onto 30 alternate leaves of 10 C. amaranticolor plants. At each assay, inoculum obtained from potato plants infected with virus at the same dilution was rubbed onto another 30 alternate leaves of the same group. The results as shown in Table 4 indicate that the relative virus concentration in tomato, tobacco, and P. floridana are considerably higher than in Petunia. In terms of virus content, they are a good source of virus for the preparation of virus extracts.

Table 4. Relative virus concentration in various hosts.

Test Plants	Average No. of lesions per leaf	
	Inoculum (1:10) from test plants	potato[a]
Tobacco	101	96
Tomato	186	102
Physalis floridana	129	94
Petunia hybrida	69	109

[a] Inoculum from infected potato plants was used as a standard.

Effect of Temperature on Virus Multiplication

Difficulties of recovering virus from tomato and tobacco plants which were previously infected with PAMV had been encountered when the infected plants were kept in the greenhouse where the temperature sometimes reached above 35 C during the summer time. When virus extracts prepared from infected tomato or tobacco plants which were kept in the greenhouse during midsummer were inoculated onto C. amaranticolor, only a few local lesions resulted, and in some cases no lesions developed at all. This evidence indicated that temperature plays an important role in virus multiplication. An experiment was designed to determine the exact effect of temperature on virus multiplication. Four temperature-controlled growth chambers which were maintained at 19, 25, 30, and 35+ C during the light period were used for this experiment. Each chamber had 16 hr of light daily.

Light was from fluorescent tubes supplemented with light from incandescent bulbs. The dark period temperature was about 5 C below the temperature of the light period in each chamber. The light intensity was 230 ft-c. The inoculated plants were maintained in the growth chambers and assayed for virus concentration every 3 days. Inoculum at a dilution of 1:5 with 0.05 M phosphate buffer was used throughout the course of this experiment. The multiplication of virus in tomato, tobacco, and other plants was determined by interval assay. Based on the results presented in Table 5, it was concluded that both low and high temperatures inhibit virus multiplication, but high temperature had the greater effect.

If the infected plants which had been previously kept in a high temperature (above 35 C) were returned to a growth chamber which was maintained at 25 C, the rapid virus multiplication could be detected within a short period of 5 days. It is obvious that high temperatures prevent virus multiplication but do not inactivate the virus.

Effect of Buffer Molarity and pH on Extraction of Virus

Experiments were conducted to determine buffer requirements necessary for retaining a high virus concentration in the extract.

Buffer concentration : Leaves from one tomato plant infected with PAMV were removed and used as the only source of virus in this experiment to avoid variability in virus content. Samples of infected tissue were homogenized

Table 5. Effect of temperature on concentration of PAMV in tomato and tobacco.

Host	Days after inoculation	Average No. of local lesions from plants grown at[a]			
		19 C	25 C	30 C	35+ C
Tomato	6	0	4	1	0
	9	8	49	18	0
	12	8	109	57	3
	15	34	128	73	0
	18	67	121	81	2
	21	72	190	70	4
	24	65	187	67	0
Tobacco	6	0	0	1	0
	9	2	21	5	1
	12	4	81	36	0
	15	22	98	67	6
	18	57	103	76	0
	21	48	96	64	3
	24	39	97	58	4

[a] Average number of lesions per leaf (18 leaves).

with 8 volumes (w/v) of distilled water and phosphate buffer of varying molarities at pH 7 in a mortar with pestle. The macerated samples were filtered through a double layer of cheesecloth and the infectivity of each virus extract was assayed on C. amaranticolor. The relative virus concentration in each preparation was determined by the number of local lesions produced on C. amaranticolor. The yield of virus, as shown in Fig. 5, was not significantly affected by the buffer molarities at the range tested.

Buffer pH : A similar experiment was performed with constant molarity (0.05 M) and varying pH to determine the optimum pH at which the most virus could be extracted from infected tissue, and the largest number of local lesions induced on C. amaranticolor. It was found that the highest infectivity, as indicated in Fig. 6, occurred between pH 6-8, and gradually decreased with increasing acidity or alkalinity.

Purification

Purification is a necessary step for the investigation of the chemical nature of a virus. Basically, purification of plant viruses is a problem of protein chemistry since plant viruses consist predominantly of protein. Therefore, protein fractionation techniques are commonly employed for plant virus purification. Furthermore, viruses are macromolecular substances which can be sedimented in a high gravitational field whereas noninfectious protein can not. A broad spectrum of techniques has been described in detail by various authors (7, 20, 23, 40, 48, 49, 51, 52, 54, 56). A number

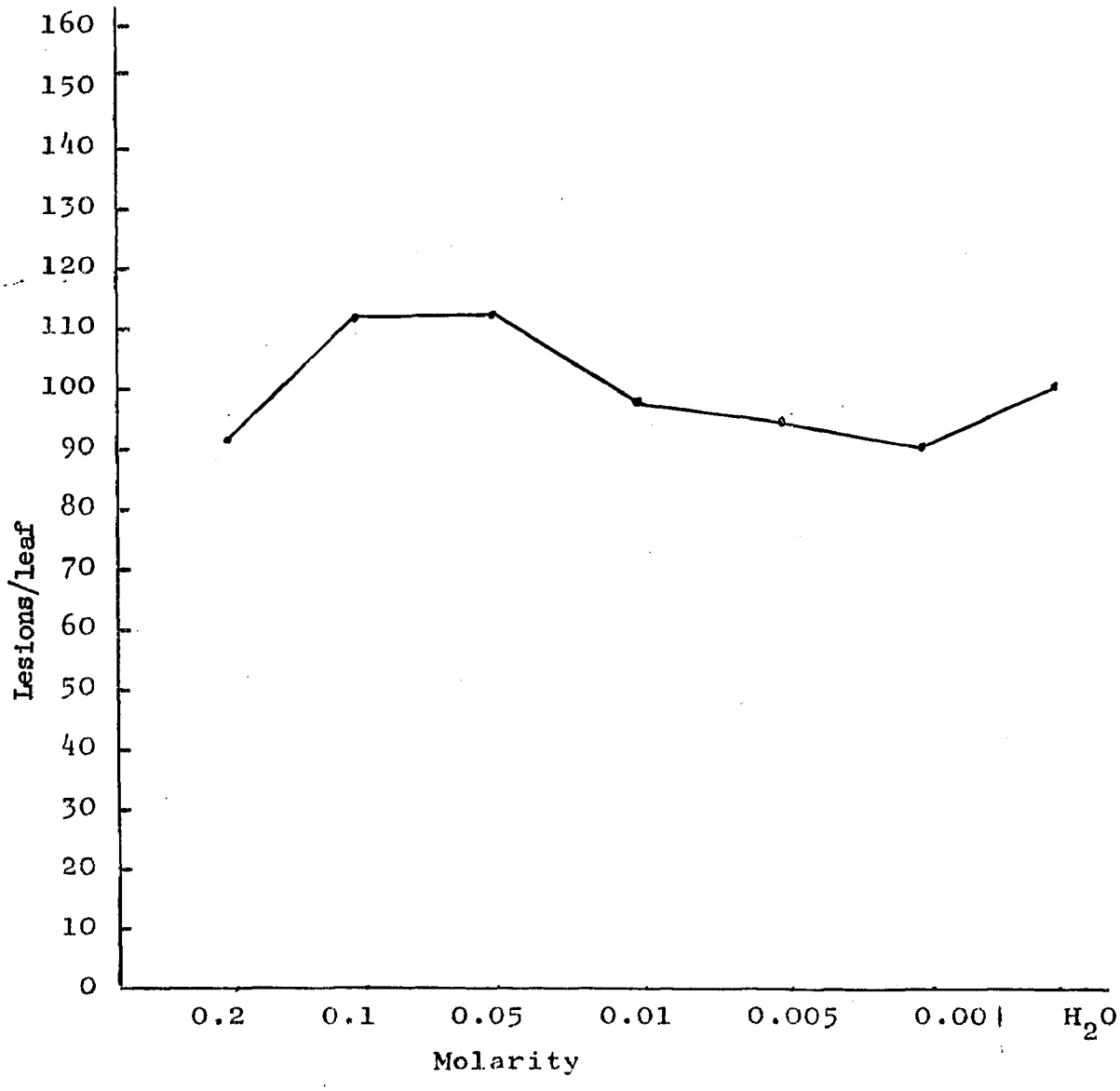

Fig. 5 Effect of buffer molarity at pH 7 on infectivity of PAMV on C. amaranticolor at a dilution of 1:8. Each point on the graph represents the mean number of local lesions per leaf based on a total of 18 leaves.

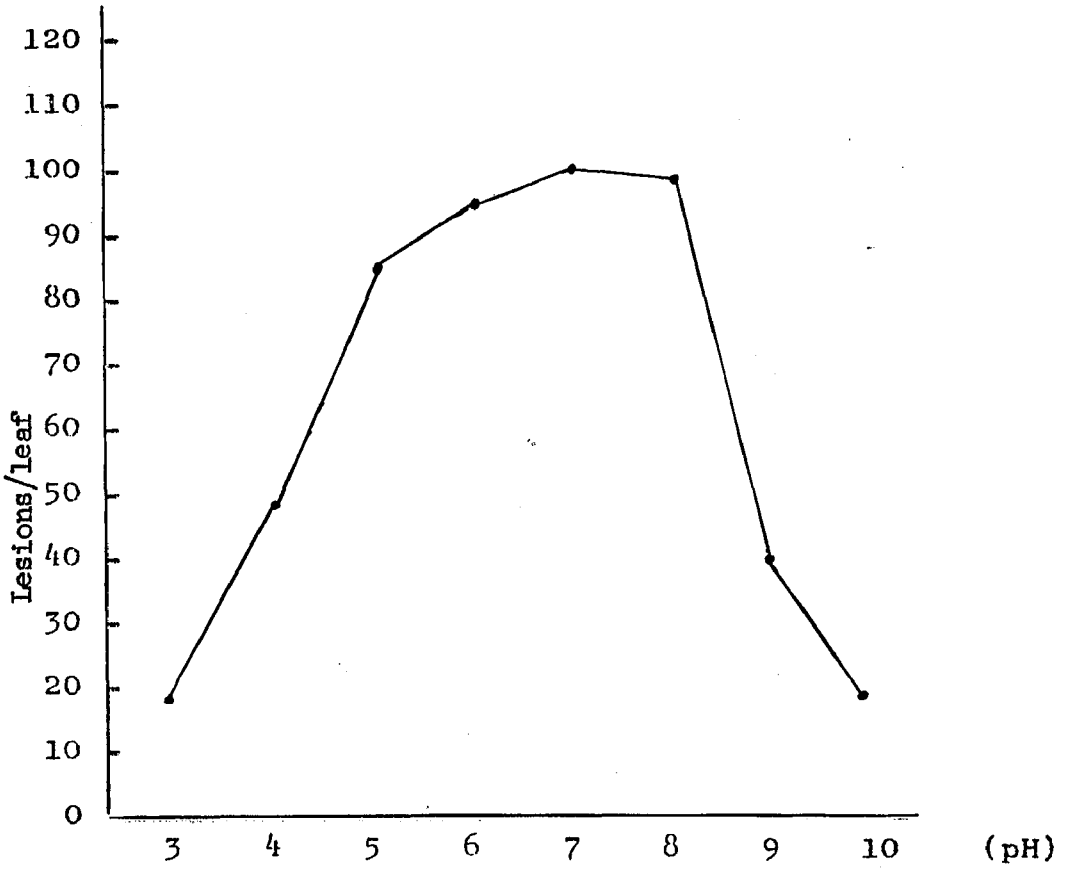

Fig. 6 Effect of pH on infectivity of PAMV on C. amaranticolor at a dilution of 1:8. Each point on the graph represents the mean number of local lesions per leaf based on a total of 18 leaves.

of these techniques were employed for the purification of PAMV in this investigation. Sap from tobacco plants infected with PAMV was used as a source of virus in all purification experiments since it was easily clarified in the preliminary extraction. All treatments of the virus extract were carried out in an ice-cooled water bath to avoid the possibility of high temperature inactivation. The purified virus obtained through different procedures was kept in screw-cap culture tubes or sealed in ampules, quick-frozen in a dry ice-alcohol bath, and stored in a dry ice chest. The Ouchterlony (56, 57, 60) double diffusion test was used to determine the antigenic purity of virus preparations prepared by the various methods.

Butanol and chloroform emulsions : Steere (48), in 1956, indicated that infectious sap treated with two volumes of 1:1 mixture of n-butanol and chloroform was free from noninfectious protein or other cell components. This technique was first used in an attempt to purify PAMV-M. Leaves from infected tobacco plants were homogenized with two volumes (w/v) of 0.05 M neutral phosphate buffer in a cooled chamber of an Omni-mixer and the sap was extracted manually by squeezing through a double layer of cheesecloth. Two volumes (v/v) of cooled 1:1 mixture of n-butanol and chloroform at a temperature of 5 C were added dropwise, and stirred continuously. The resulting emulsion was separated into three layers by low speed centrifugation for 30 min at 1,000 rpm in a Servall refrigerated centrifuge. The top aqueous layer was

removed and stored at 5 C. After an exposure of 6 hr at 5 C the suspension containing denatured protein was recentrifuged at 5,000 rpm for 30 min. The resulting supernatant was subjected to several cycles of differential centrifugation as indicated in Fig. 7. These procedures, which essentially followed those of Steere (48), led to complete loss of virus infectivity. None of the pepper or C. amaranticolor plants inoculated with purified virus which was obtained from the above procedure showed symptoms.

Tremaine and his coworkers (58), in 1964, reported that infectivity of prune dwarf virus was lost when it was extracted from petals with phosphate buffer containing 0.02 M sodium diethyldithiocarbamate (DIECA) and 0.02 M thioglycolic acid (TG) at pH 8, but serological activity was not lost. Bawden (4), in 1964, made a similar statement. In view of this fact, virus purified through these processes without showing infectivity was injected intramusuclarly into rabbits for the serological analysis.

DEAE-Sephadex purification : DEAE-Sephadex (Diethylaminoethyl-Sephadex) is an anion exchanger for ion exchange chromatography. At the outset, it was thought that the DEAE-Sephadex could be used to free virus from both color pigments and noninfectious proteins at a certain buffer molarity of a given pH. Experiments were performed to determine optimum buffer molarity at which the pigments and noninfectious proteins could be adsorbed onto DEAE-Sephadex, leaving the virus in the supernatant. Each 10-g sample from a tobacco

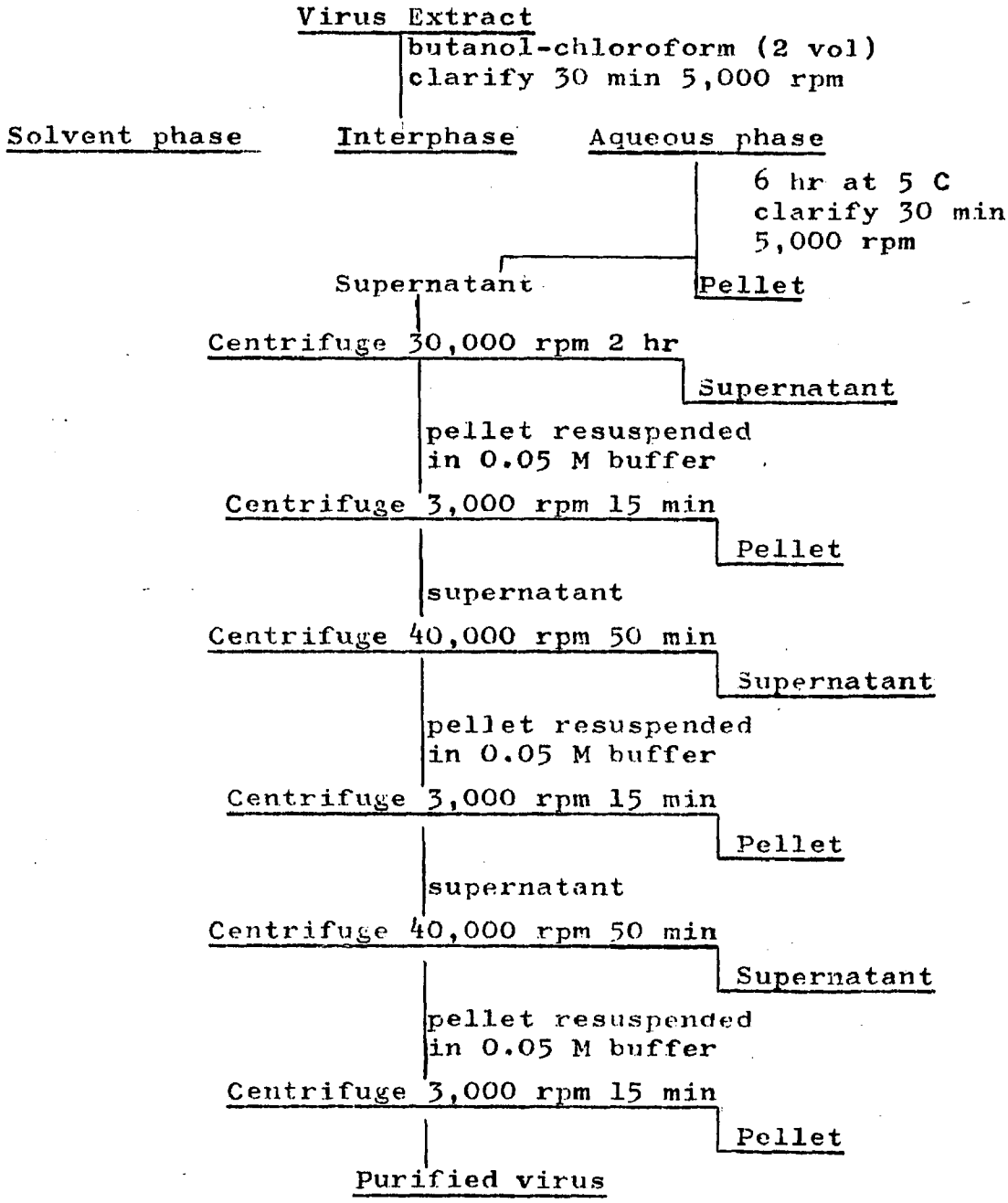

Fig. 7. Purification procedures for butanol-chloroform treatment.

plant infected with PAMV was macerated in 30 ml neutral phosphate buffer of varying molarities. The pulp was extracted through a double layer of cheesecloth and the extract was centrifuged for 30 min at 8,000 rpm to free it from all chloroplasts or chlorophyll-containing materials since DEAE-Sephadex did not adsorb chloroplasts at the molarities tested. It was found that chloroplasts or chlorophyll-containing materials could be easily eliminated from extracts prepared from young tobacco plants in this process. The supernatant was mixed with 0.5 g DEAE-Sephadex for 3-5 min and then the treated juice was filtered through a folded filter paper into a suitable container. The filtrate was divided into two portions, one of which was used to inoculate C. amaranticolor and pepper plants and the other part was subjected to high speed centrifugation for 1 hr at 40,000 rpm. Suspensions resulting from homogenizing the first high speed pellets were reinoculated onto pepper and C. amaranticolor. The results as shown in Table 6 reveal that buffer concentration is a critical factor for clarification of infective sap. When the buffer concentration was below about 0.005 M, both virus and noninfectious material were adsorbed onto DEAE-Sephadex, leaving an almost noninfectious filtrate. The appearance of high speed pellets after ultracentrifugation could be demonstrated only from filtrates which showed infectivity when they were assayed on indicator plants. This experiment clearly demonstrated that DEAE-Sephadex can be used in aiding purification of some chemically sensitive viruses.

Table 6. Effect of buffer molarity (pH 7) on adsorption of PAMV from DEAE-Sephadex.

Molarity	Infectivity of supernatant		Appearance of pellets after ultracentrifugation (45,000 rpm for 1 hr)[b].
	Pepper[a]	C. amaranticolor	
0.2	5/5	69	++
0.1	5/5	75	+++
0.05	5/5	59	+++
0.01	4/5	6	+
0.005	2/5	2	?

[a] Infectivity on pepper is indicated by number of plants showing top necrosis/number of plants inoculated. The number of local lesions produced on C. amaranticolor is recorded.

[b] +++ = large pellet; ++ = small pellet; + = nearly none.

For the purification purpose, a buffer concentration at 0.1 M was selected. Virus solution obtained through filtration of infectious sap which was treated with DEAE-Sephadex was subjected to differential centrifugation as indicated in Fig. 8. The pellets resulting from the first ultracentrifugation were homogenized with 1/10 of initial volume of 0.05 M phosphate buffer and allowed to stand at 5 C for at least 1 hr. The suspension was then centrifuged 15 min at 3,000 rpm, the pellets were discarded and the supernatant was termed as partially purified virus. The partially purified virus solution was divided into two equal parts, one of which received two additional differential centrifugations as indicated in Fig. 8, while the remaining part was subjected to acid preciptation treatment. For the

purpose of acid preciptation the partially purified virus solution was kept in an ice-cooled bath and its pH was lowered to 4-5 by dropwise addition of 10% acetic acid with constant stirring. The acidified solution was centrifuged immediately for 20 min at 10,000 rpm. The supernatant was removed and adjusted to pH 7 with 10% ammonium hydroxide and was assayed on pepper plants. The pellets resulting from acid preciptation were resuspended in 0.05 M phosphate buffer, made up to its original volume, and clarified by low speed centrifugation. The clear supernatant was subjected to another cycle of differential centrifugation (Fig. 8). The purified virus resulting from both differential centrifugation and acid preciptation was infectious (Table 7).

Table 7. Acid preciptation of PAMV in partially purified preparation from DEAE-Sephadex treatment at pH 5.

Number of pepper plants showing TN[a]/number of plants inoculated	
Supernatant	Pellet
2/10	10/10

[a] TN = Top necrosis in pepper plants.

Butanol method: Kellmer and Larson (32) reported that the "Butanol Method" used by Tomlinson, Shepherd and Walker (56) for the purification of Y-strain of CMV was successful in partially purifying isolate 1 of PAMV, and the high speed pellets obtained from this method were highly infectious. An attempt was made to purify this virus by this

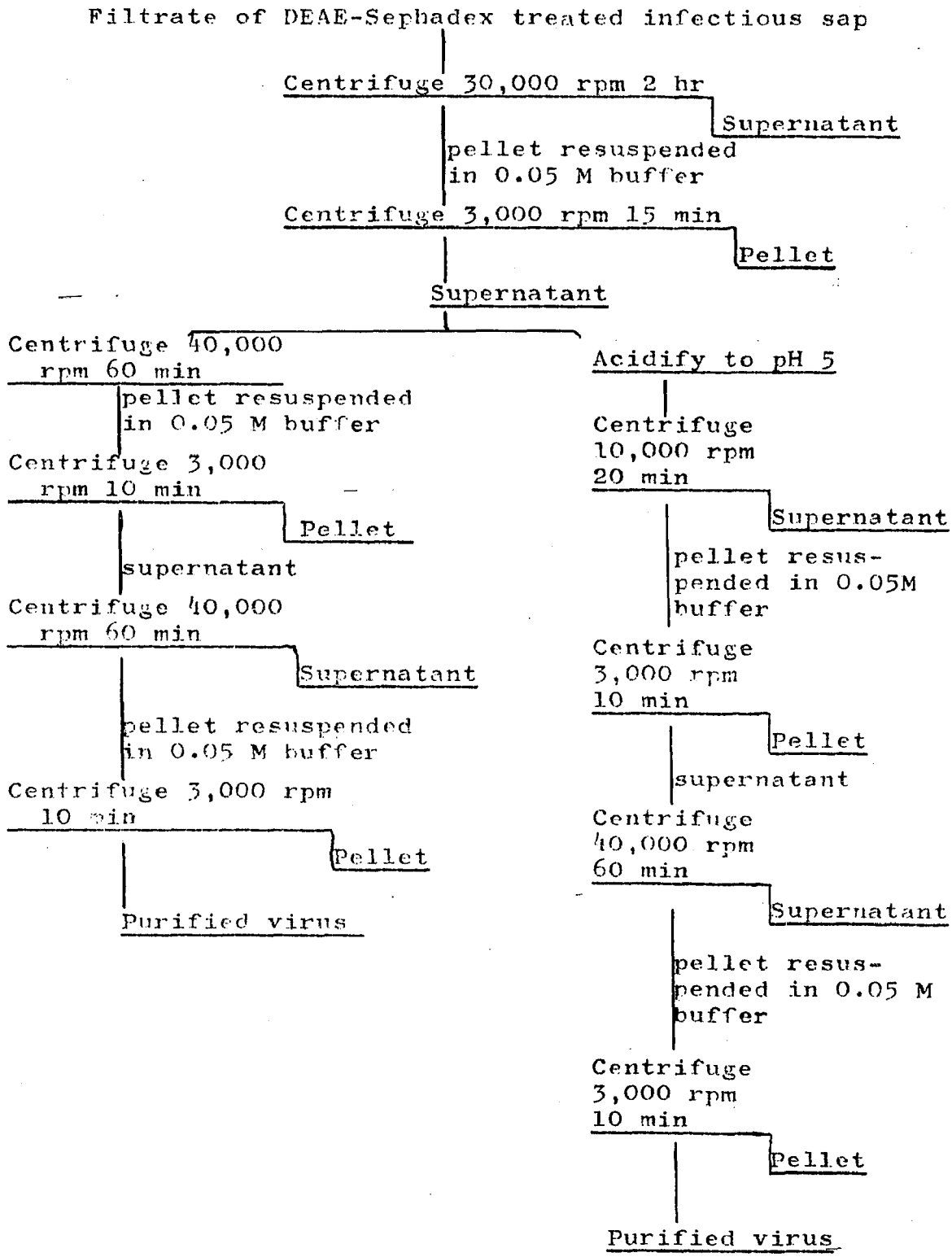

Fig. 8. Procedures for DEAE-Sephadex purification

method. Fifty grams of infected leaves from tobacco plants infected with PAMV were homogenized, and the pulp was filtered through glass wool. Butanol was added dropwise to the filtrate to give a final concentration of 8.5%, and the filtrate was stirred for an additional 30 min in an ice-cooled water bath. The solution was then centrifuged for 15 min at 5,000 rpm, the residue discarded and the supernatants were dialyzed against 0.05 M phosphate buffer for 8 hr at 5 C. After dialysis, the solution was centrifuged at 5,000 rpm for 10 min. The resulting supernatant was subjected to one cycle differential centrifugation to obtain partially purified virus. The solution of partially purified virus was acidified by lowering the pH to 4-5. Pellets obtained from centrifugation of acidified virus solution at 10,000 rpm for 20 min were resuspended in 0.05 M phosphate buffer and followed by another cycle of differential centrifugation. Detail of these procedures is presented in Fig 9. Purified virus resulting from this method was infectious, but not as infectious as that from DEAE-Sephadex method.

Bentonite purification : Dunn and Hitchborn (20), in 1965, used purified magnesium bentonite for purification of a number of plant viruses. The preparation of magnesium bentonite in the present study followed essentially the method described by Dunn and Hitchborn. One hundred grams of crude bentonite obtained commercially was suspended in 2 liters of solution containing 10^{-2} M phosphate buffer at pH 7.4. The mixture was shaken for 2 hr in a gyratory shaker,

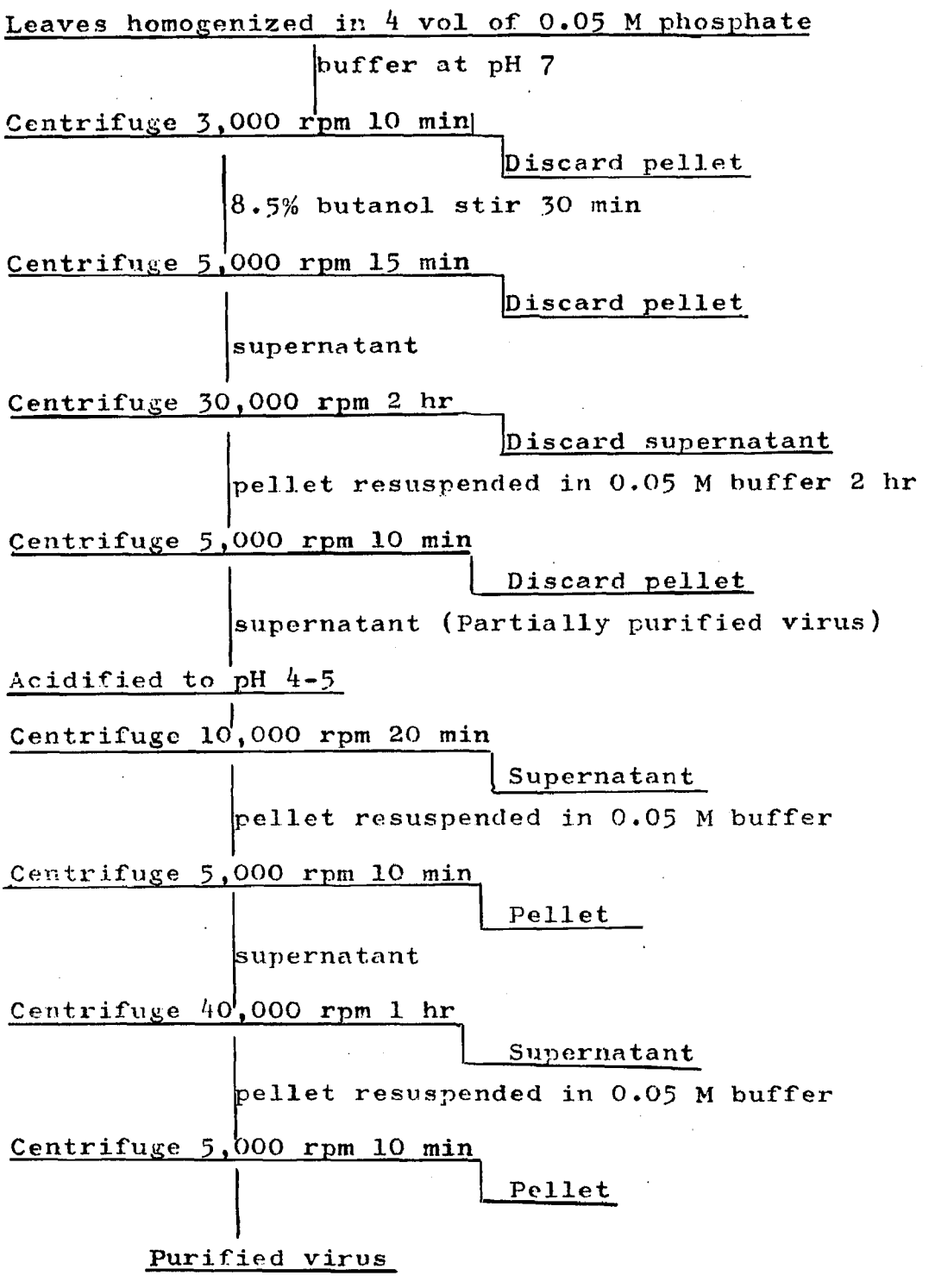

Fig. 9 Procedures for butanol method purification.

and it was then centrifuged for 1 min at 1,500 rpm. The resulting supernatant was recentrifuged for 15 min at 2,000 rpm and the pellets were added to 1 liter of solution containing 10^{-3} M $MgSO_4$ and 10^{-3} M phosphate buffer and shaken again for 2 hr. The low and high speed centrifugations were repeated once and finally the pellets were suspended in 500 ml of the above 10^{-3} M salt mixture. The bentonite salts suspension was mixed with an equal volume of 90% ethanol and centrifuged at 2,500 rpm for 10 min. The pellets were washed once with 90% ethanol. The bentonite suspension was adjusted so as to contain 50 mg per ml in a solution of 5×10^{-3} M $MgSO_4$ and 5×10^{-2} M phosphate buffer, and stored until required.

Leaves of tobacco plants infected with virus were ground with 2 volumes of phosphate buffer with varying molarities at pH 7. The pulp was centrifuged at 5,000 rpm for 30 min to sediment all cell debris. The virus extract was then mixed with 1/5 vol of purified magnesium bentonite obtained previously. The mixture of virus extract and magnesium bentonite was centrifuged at 5,000 rpm for 30 min. The resulting supernatant was assayed on C. amaranticolor and pepper plants. The virus in the low speed pellets could be eluted by resuspending it in 0.1 M phosphate buffer. As indicated in Table 8, when the buffer concentration was below 0.005 M, most of the virus and host materials were adsorbed onto bentonite, leaving a nearly noninfectious supernatant. This experiment demonstrated again that buffer concentration was a critical factor for the bentonite adsorption test.

Accordingly, phosphate buffer at a concentration of 0.05 M was selected for purification of virus, using bentonite. At this concentration, green host materials were adsorbed onto bentonite and sedimented. The supernatant resulting from low speed centrifugation of a mixture of virus extract and bentonite was subjected to three cycles of differential centrifugation. The detailed procedures are illustrated in Fig. 10. The virus purified through these processes was clear and infectious.

Table 8. Effect of molarity of phosphate buffer (pH 7) on adsorption and elution of potato aucuba mosaic virus from purified magnesium bentonite.

Buffer conc. used in preparation of virus extract	Supernatant		Pellet Elution	
	Lesions/ leaf on C. amaranticolor[a]	Top necrosis on pepper[b]	Lesions/ leaf on C. amaranticolor[a]	Top necrosis on pepper[b]
0.2 M	86	6/6	5	6/6
0.1 M	104	6/6	4	6/6
0.05 M	139	6/6	5	6/6
0.01 M	20	6/6	50	6/6
0.005 M	11	6/6	59	6/6
0.001 M	9	6/6	56	6/6

[a] Average lesion counts based on 18 leaves.

[b] Ratio of number of inoculated plants showing top necrosis/number of plants inoculated.

Hydrated calcium phosphate purification: Fulton (23), in 1959, indicated that cherry necrotic ringspot and prune dwarf viruses were adsorbed onto hydrated calcium phosphate (HCP) in 0.002 M phosphate buffer at pH 7.5 but

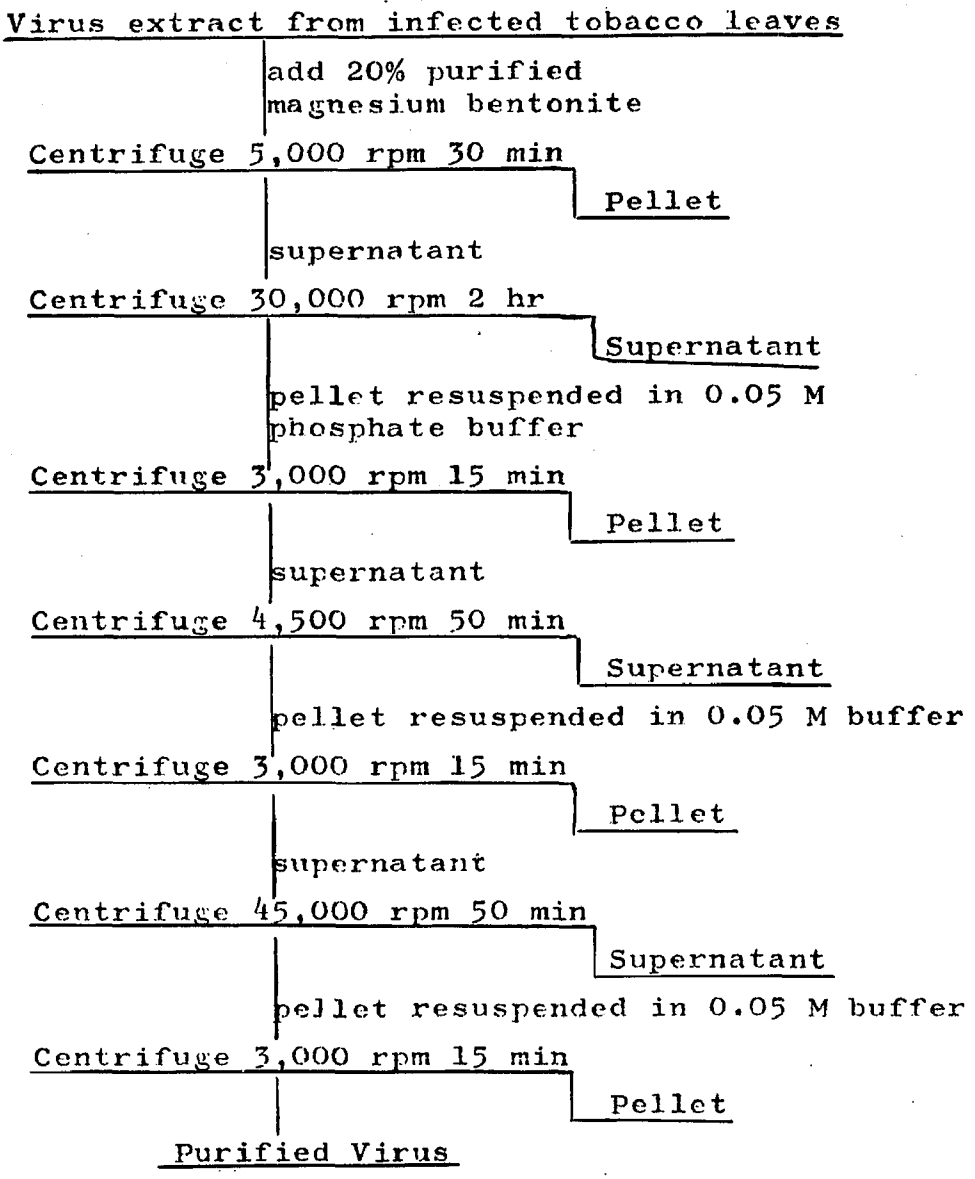

Fig. 10 Procedures for bentonite purification of virus.

failed to be adsorbed in 0.003 M buffer. An attempt was made to employ this technique for the purification of PAMV. Hydrated calcium phosphate was prepared by adding an equal volume of 0.1 M calcium phosphate to 0.1 M solution of Na_2HPO_4, and allowed to stand at room temperature for 4 hr. The supernatant was removed, and the white flocculent precipitate was washed 15-20 times to remove soluble salts. Finally, the white precipitate was packed by centrifuging at 2,000 rpm for 10 min. The resulting gel-like HCP was stored until required.

Leaves from plants infected with PAMV were homogenized in 20 vol (w/v) of phosphate buffer (pH 7) containing 10% HCP with varying molarity. The pulp was centrifuged at 5,000 rpm for 30 min. The resulting supernatant was assayed on pepper and C. amaranticolor to test for infectivity, and centrifuged at 45,000 rpm for 1 hr determining the appearance of pellet. It was found that larage high-speed pellets could result only from supernatants which were highly infectious. However, a high-speed pellet did occur when the buffer concentration was below 0.001 M but it appeared green in color and contained chloroplast fragments and other chlorophyll-containing substances (Table 9).

It appeared that buffer concentrations of 0.1 M and 0.05 M were satisfactory for HCP purification. Therefore, virus extract resulting from homogenizing infected tissue with 20 vol (w/v) of 0.05 M phosphate buffer containing 10% HCP was centrifuged at 5,000 rpm for 30 min, and the resulting

supernatant was subjected to 2-3 cycles of differential centrifugation as indicated in Fig. 11. The purified virus resulting from this method was clear and highly infectious.

Table 9. Effect of concentration of phosphate buffer (pH 7) containing 10% HCP (v/v) on adsorption of PAMV.

Buffer concentrations of virus 1:20 (w/v) extract	Infectivity of supernatant		High speed pellet resulting from ultracentrifugation of supernatant	
	Pepper	C. amaranticolor	Pellet	Infectivity
0.2 M	6/6	59	+	+
0.1 M	6/6	49	+	+
0.05 M	6/6	51	+	+
0.01 M	1/6	3	-	-
0.005 M	1/6	1	-	-
0.001 M	4/6	6	+(green)	+

Sephadex gel filtration : Sephadex gel filtration is a relatively recent innovation in separation and fractionation of macromolecules and particles. The use of Sephadex in gel filtration was first introduced by Porath and Flodin (40). The principle of separation (54) is based on difference in molecular weight and size of substances to be fractionated. Sephadex in the gel acts as a molecular sieve. Sephadex is made by cross linking the polysaccharide dextran. Thus, it consists of a three-dimensional network of polysaccharide chains. A high degree of cross linkage gives a compact structure with low porosity, and a low cross-linkage gives a highly porous structure. The porosity

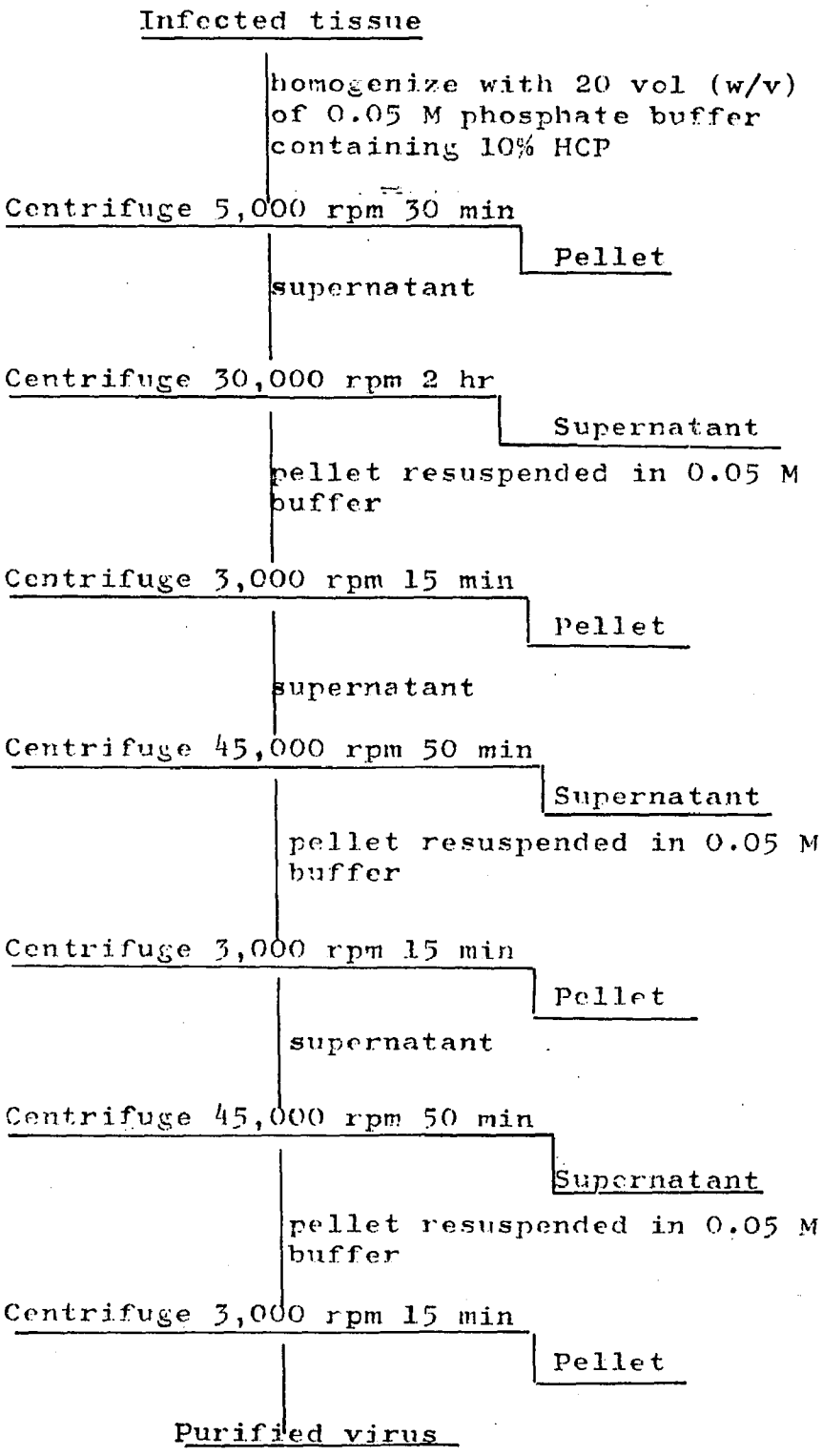

Fig. 11 Procedures for HCP purification.

of cross linkage determines the nature of separation. Molecules of larger dimension than the matrices of the gel particles do not penetrate the swollen gel and are completely excluded from the gel. These excluded molecules migrate without retention in the interstitial fluid. On the other hand, smaller molecules can penetrate the gel to an extent which is determined by their molecular dimension and degree of porosity of gel particles. In a packed column, the non-penetrating particles remain in the interstitial volume and appear in the effluent following an elution volume equal to the void volume of the column. Smaller molecules appear in a larger volume of eluent, depending on their degree of penetration. When a volume equal to the total solvent volume of the column has been reached, all solutes have been eluted from the column, except when adsorption has occurred.

The exclusion limit of dextran homologues varies with types of Sephadex which differ in porosity. Sephadex G-200 was employed in this experiment and its exclusion limit is 200,000. This means that molecules with a molecular weight of more than 200,000 will be completely excluded from penetrating the gel. Particles with molecular weight less than 200,000 penetrate the gel and migrate through it at different speeds and finally appear in the eluate in the order of decreasing molecular weight (Fig. 12).

The reason for using this technique in purification of PAMV was that viruses are macromolecules with molecular weights much higher than 200,000, and they should be com-

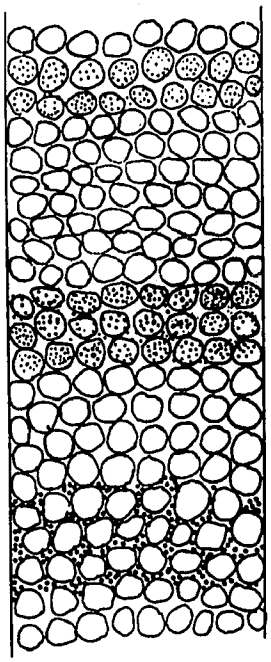

Fig. 12 Diagram illustrating the principle of gel filtration in a bed of gel particles. Three kinds of solute molecules move faster with increasing size and consequently lower permeability. The direction is downward.

pletely excluded from penetrating the gel and should appear in the effluent first, and thus result in separation of virus from substances which have molecular weights below 200,000.

(1) Theory of gel filtration : A Sephadex-packed column is made of gel matrix (Vg) and water (or buffer). Water in the column consists of two parts : the internal water (Vi) which is inside the gel granules and the external water (Vo) which is outside the gel granules. Thus the total volume of the column = Vo + Vi + Vg.

The appearance of molecules which are completely excluded from penetrating the gel in the eluate is determined by void volume (Vo). This simply means that excluded molecules will appear in the effluent after a volume Vo while the non-excluded molecules appear after an additional volume of Vi. When a volume equal to the total solvent volume is reached, all solute molecules have been eluted from the column, except when adsorption has occurred.

(2) Packing of column : In order to get good results, a carefully packed column is absolutely necessary. The column employed in our laboratory was prepared by adding dry Sephadex G-200 to an excess amount of 0.005 M phosphate buffer pH 7.5 and the Sephadex allowed to swell until equilibrium was attained. Swelling of gel particles took a minimum of 24 hr. After complete swelling, the supernatant should be decanted to remove the finest gel particles, and the homogenous suspension transferred to a 2.5 x 55 cm chromatographic tube through a

funnel. The best packing was obtained by mounting the tube in a vertical position and filling it to about 1/3 of its height with buffer. A funnel was then placed on top of the tube and the suspension was slowly added to the funnel. Gel granules gradually sedimented to the bottom of the tube. When a column of 2-5 cm was formed, the orifice at the bottom of the tube was gradually opened to allow a slow flow of buffer. After the formation of a uniform column, a circle of filter paper, made to fit the inside diameter of the tube was placed on the top of the column to protect the upper surface from disturbance during sample introduction. The column should be washed thoroughly with buffer, and the buffer never allowed to drain below the top surface.

(3) Preparation of sample: Young leaves from tobacco plants previously infected with PAMV were macerated with an equal volumne of 0.1 M phosphate buffer (pH 7) containing 0.1 M NaCl. Juice was extracted from pulp by squeezing through a double layer of cheesecloth and clarified by centrifuging for 30 min at 5,000-8,000 rpm. Virus extract prepared in this manner should be completely free from chloroplasts. If infected leaves from old tobacco plants were employed, higher molarities of both phosphate buffer and sodium chloride were necessary to eliminate the chloroplasts or chlorophyll-containing substance.

(4) Introduction of sample: Buffer solution above the surface of the column was removed by pipette just to the level of the upper surface of the column. The

5-7 ml sample to be fractionated was then slowly pipetted onto the filter paper disc resting on the surface of the column, by placing the outlet of the pipette immediately above the filter paper. When all the sample had entered the column, a small volume of buffer was added in the same manner and allowed to enter the gel surface. A large amount of buffer was then added to the tube to start elution.

(5) Collection of sample : Before introduction of the sample, the column was attached to a Gilson Medical Electronics fraction collector, adapted with a UV photometer (265 mu) and a recorder, since viruses absorb this wave length (Fig. 13). The virus sample which was eluted from the column entered the collecting chamber in the ultraviolet photometer (265 mu) which gave an output to drive a recorder. Continuous flow of virus through the UV photometer resulted in peaks on the recorder. The details of these processes are illustrated in Fig. 14. Samples under the peak areas were automatically collected through the collector and assayed for infectivity on indicator plants. Two peaks (Fig. 15) which showed 265 mu UV absorption spectra were recorded on the recorder. The first one was a clear solution and appeared about 50 min after introduction of the sample, while the second one was a yellowish-brown solution which came out slowly and continued for more than 2 hr. However, if the crude virus extract was centrifuged for 50 min at 30,000 rpm and the pellet resuspended before it was introduced onto the column, the second peak was eliminated. Refractionation

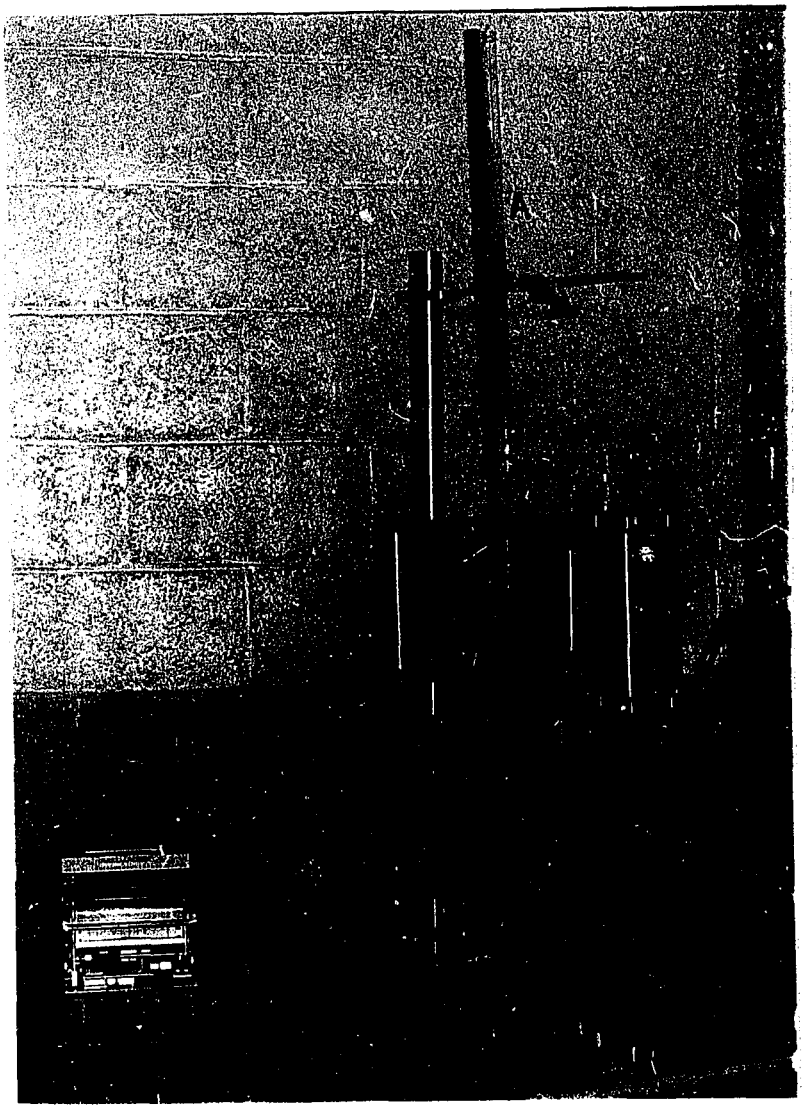

Fig. 13 Apparatus used for gel filtration and fractionation. A: Sephadex column; B: UV photometer; C: fraction collector; D: recorder.

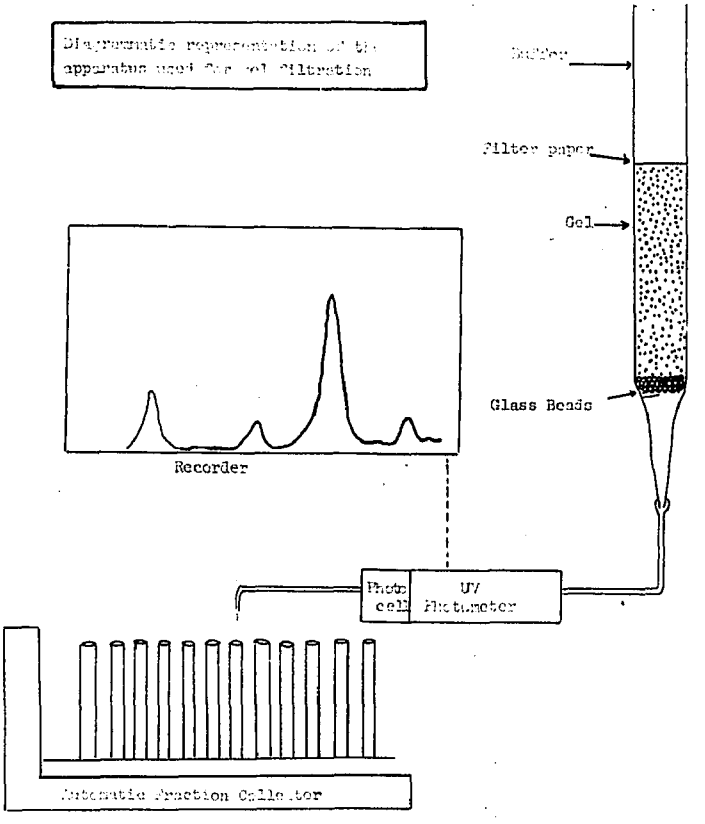

Fig. 14 Diagrammatic representation of the apparatus used for gel filtration.

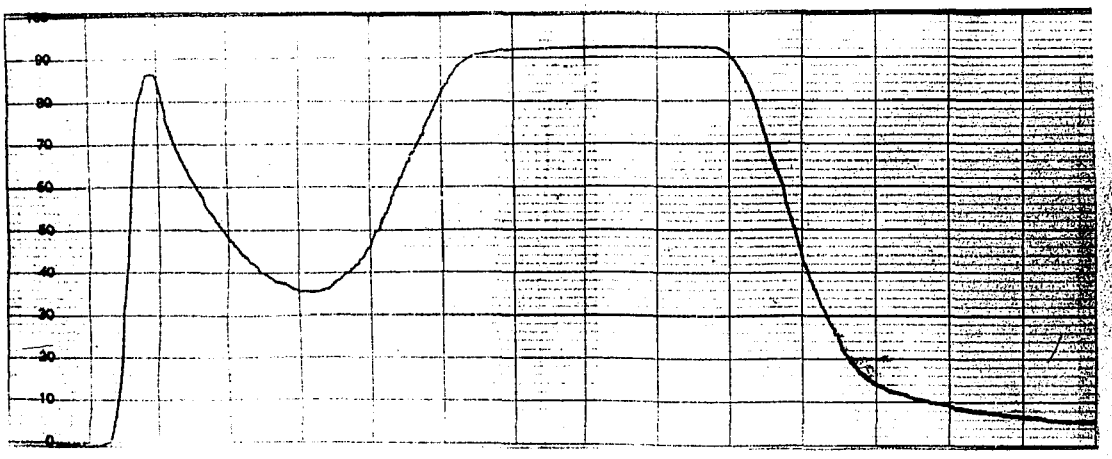

Fig. 15 Separation of PAMV from chloroplast-free infectious sap in a Sephadex column. The fraction is recorded through UV photometer at 265 mu. The first peak is virus, and the second peak (yellowish-brown solution) contains substances with a molecular weight of less than 200,000.

of samples which were previously collected under the first peak area resulted in a symmetrical peak (Fig. 16). The infectivity tests (Table 10) indicated that samples from the first peak contained virus, and samples from the second one occasionally caused top necrosis on pepper. It is probable that infection from the sample under the second peak was due to either contamination or small fragments of virus particles which were still infective.

Table 10. Infectivity of samples collected from Sephadex gel filtration under 265 mu absorption spectrum.

Fraction	Infectivity	
	Pepper	C. amaranticolor[a]
Fraction I (clear solution)	20/20	91
Fraction II (Yellowish-brown solution)	4/20	13

[a] Average counts of a total of 50 leaves.

Samples collected under the first peak area were centrifuged at 30,000 rpm for 50 min. The resulting pellets were homogenized in 0.05 M phosphate buffer and recentrifuged 15 min at 5,000 rpm. The supernatant (partially purified virus) was subjected to acid precipitation treatment to precititate the virus from solution. This was done because the same kind of preparation from healthy tobacco plants also showed the same UV absorption spectrum at 265 mu. The purified virus solution resulting from acid precipitation was clear and infectious.

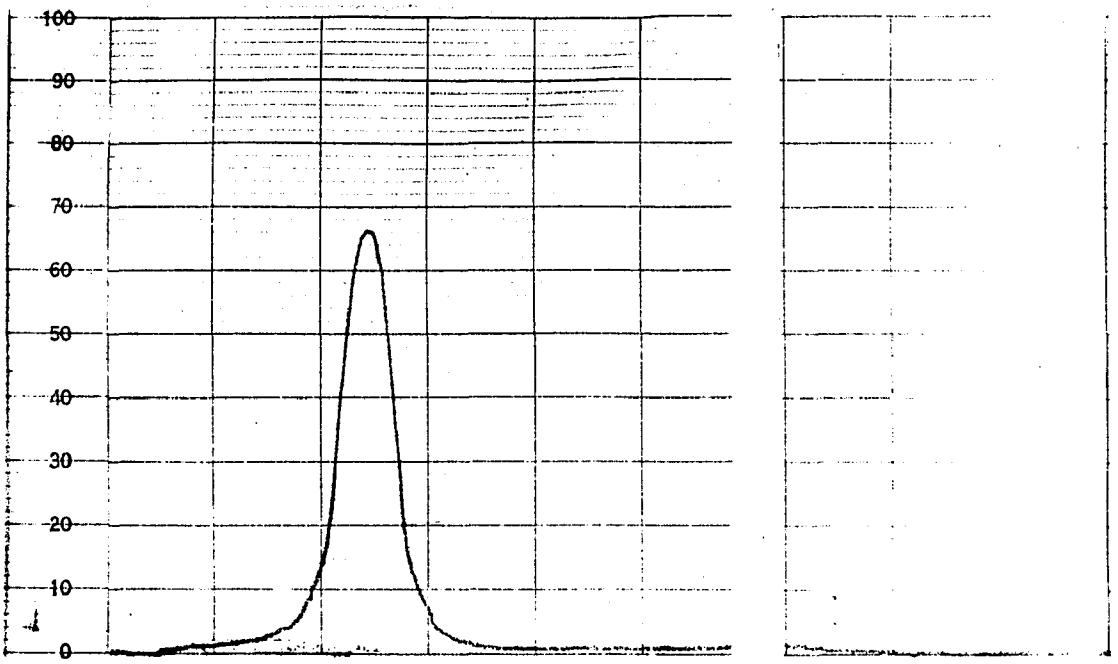

Fig. 16 Symmetrical pattern of refractionation of sample which was previously collected from first peak.

Within a period of 4 hr, a total of 3 columns could be finished since only the samples under the first peak were collected. When samples from the first peak were completely collected, the column was replaced with another and a new sample was introduced.

Steere (52), in 1962, reported that tobacco mosaic and southern bean mosaic viruses can be separated, using a 4% agar granule column. In view of this fact, PAMV purified through Sephadex gel filtration was introduced onto an agar column. A symmetrical curve resulted which appeared in the eluent at about the same time interval as when the Sephadex column was used. The agar column used in the present study was prepared by autoclaving a 4% agar suspension at 15 lb for 30 min, after which it was allowed to gel, then chopped into small pieces and sorted by washing through sieves to obtain the granules which passed through a 40 mesh but not through a 60 mesh screen. The agar granule suspension was packed and washed in a chromatographic tube in the same manner as that for the Sephadex column.

Serology Experiment

The Ouchterlony agar double diffusion test (57, 60) was employed to determine the purity of the antigenic virus preparation and the identity of two virus isolates which were used in the present investigation.

Antigen preparation: Purified virus obtained from different purification methods was used for immunization of

experimental animals.

Immunization of experimental animals: Gelatinous aluminum hydroxide was used as an adjuvant throughout all immunizing processes. The adjuvant was prepared by addition of 13.9 g of aluminum hydroxide to one liter of sterile distilled water and adjusted to pH 7. One part of antigen preparation was mixed with 10 parts of aluminum hydroxide solution, and the mixture was administered to the rabbit subcutaneously. A total of four injections was given at weekly intervals in all cases except with the Sephadex gel preparation which was injected every three days. Rabbits were bled from the heart one week after the last injection. Antisera against different virus preparations were secured and stored in ampules or screw-cap culture tubes in a dry ice chest.

Preparation of Ouchterlony agar plates: Ouchterlony agar plates were prepared with an autoclave medium consisting of 1% agar in 0.005 M citrate neutral phosphate buffer to which was added either methiolate to give a final concentration of 1:10,000 or sodium azide at a concentration of 1:4,000. Ten ml of this medium was added to a small petri plate (65 x 16 mm) and allowed to solidify. Reservoir holes in which antiserum and antigen were to be placed were then cut with No. 2 and No. 3 cork borers, respectively, and the plugs were removed from the agar. A small amount of melted agar was then added with a pipette to the bottoms of the holes to prevent the liquid reactants from spreading between the glass

and the agar. The pattern of the holes, as generally arranged, is illustrated in Fig. 17. The distance between the edge of the center antiserum hole and the edge of each peripheral antigen hole was 10 mm.

Ouchterlony agar double diffusion test: The 0.005 M citrate neutral phosphate buffer was used as a diluent for all the reagents in this test. The peripheral antigen holes were filled with crude sap of varying dilutions prepared from tomato or tobacco plants. Three drops of antiserum at different dilutions were placed in the center holes by means of a capillary pipette 24 hr after the antigen was introduced into the peripheral holes. This was delayed because long-rod virus particles migrate very slowly, and the band formed between antigen and antiserum would otherwise be too close to the outer peripheral hole. A control test was set up by replacing antigenic virus with healthy plant sap. The plates were kept in a moist chamber at room temperature to allow diffusion of the two reactants. They were examined for four weeks by observing for the formation of a precipitation band between antigen and antiserum (Fig. 17). The results of this experiment are presented in Table 11. No precipitation bands were formed in the plates containing antiserum obtained from the rabbit which was injected with butanol-chloroform preparation. More than one band occurred between antigens and antisera which were prepared from injection of bentonite and HCP preparations, respectively. Virus preparation for DEAE-Sephadex treatment appeared to be

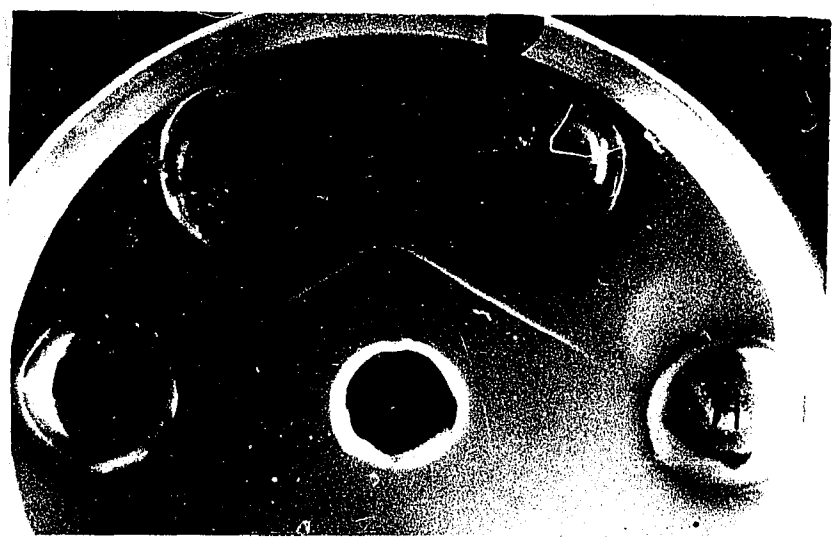

Fig. 17 Ouchterlony agar diffusion test demonstrating reaction between antigen and antiserum. V_1 = undiluted extract from tomato plants infected with PAMV; V_2 = 1:2 dilution; V_3 = 1:4 dilution; H = extract from healthy tomato plant (undiluted).

the best method for purification since only one band was observed consistently between antigen and antiserum. The Sephadex gel filtration and butanol methods gave good results also, but an additional band was observed once.

Table 11. Infectivity and serological activity of various purification preparations.

Manner of preparation	Infectivity	Antigen-antibody reaction	Pattern of precipitin band
Butanol-chloroform	-	-	No band
DEAE-Sephadex	+	+	1 band
DEAE-Sephadex and Acid ppt.	+	+	1 band
Purified magnesium bentonite	+	+	2-3 bands
Hydrated calcium phosphate	+	+	2 bands
Sephadex gel filtration	+	+	1 band ?
8.5% Butanol	+	+	1 band ?

Serological Identity of PAMV-M and PAMV-C

An experiment was performed to determine the serological relationship of the two virus isolates used in the present studies. This was determined by using antiserum against DEAE-Sephadex purified aucuba mosaic virus. A band formed in the appropriate zone between reservoirs containing PAMV-M antiserum and antigen prepared from tobacco or tomato plants infected with PAMV-C, indicating a positive relationship between these two virus isolates.

Ultraviolet Absorption Spectra of Purified Virus Preparation

The ultraviolet absorption of virus which was prepared from DEAE-Sephadex purified method was determined with a Beckman DU spectrophotometer in 0.05 M phosphate buffer at pH 7. The virus appeared to give a typical nucleoprotein absorption curve with maximum near 260 mu and minimum near 240 mu (Fig. 18).

Electron Microscopy of PAMV

An electron microscopic study of PAMV, indicating flexible rod shape, was made by Paul and Bode (38) using Johnson's (28) crude exudate method. No such observation had been made using purified virus preparation in the past. The purpose of the present investigation was designed to reevaluate the morphology of PAMV by using a purified virus preparation.

Preparation of supporting film: Specimen support films can be prepared from many different materials. The films used in this study were prepared from Formvar (Polyvinyl formal). The film was formed on a standard 1 x 3 inch microscope slide by dipping the slide into a 0.125% solution of Formvar in dichloride ethylene and held there for a few seconds. The slide was then withdrawn and drained as quickly as possible. The flat plane of the glass should be held vertically, the long axis of the slide should be held obliquely so that excess solution drains quickly across the short axis of the glass. After a film was dried on the glass surface,

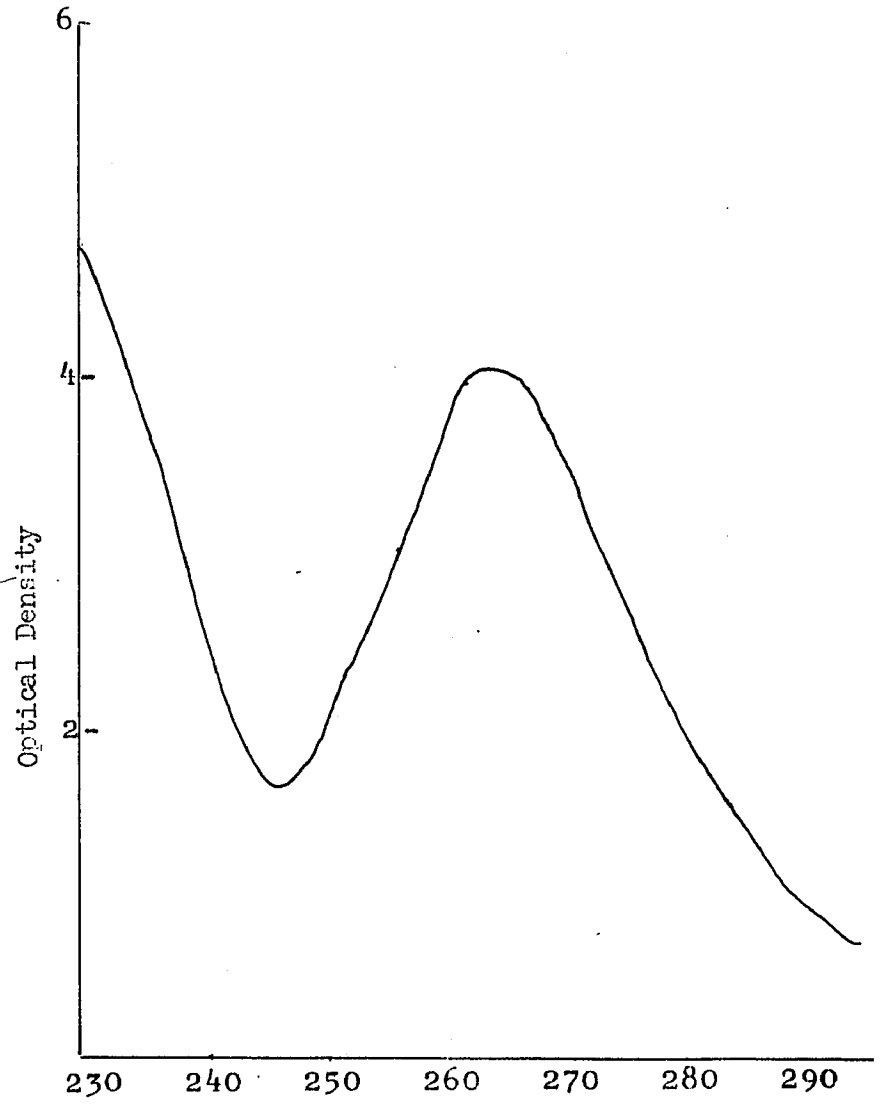

Fig. 18 Ultraviolet absorption spectra of purified preparations of PAMV in 0.005 M phosphate buffer at pH 7.

a rectangular score marked close to the edges of the glass was made with a sharp scalpel around the area that was to be removed. The film was floated off the slide on a distilled water surface. For the convenience of film observation, a container with black background was preferred. Grids with 200 wire mesh were placed in rows on top of the floating film. The floating film with grids on it was picked up by dropping a strip of filter paper on top of the film, after which it was removed and dried (39, 50).

Specimen preparation: The purified virus solution was sprayed on the prepared grids with a de Vilbiss nebulizer and allowed to air dry. The droplets thus produced were small and dried quickly.

Shadow casting and morphological examination: The specimen prepared previously was shadowed with uranium in a vacuum chamber. A tungsten filament mounted in the vacuum chamber containing uranium metal was heated to a temperature sufficient to evaporate the introduced metal. The specimen was placed so that its surface formed an acute angle with the filament in all directions and the evaporated metal was deposited on the surface of the substance with which it came in contact. This shadow casting process caused a significant improvement in contrast in the specimen when viewed with the electron microscope.

A preliminary morphological study was also attempted, using Johnson's crude exudate method. Water line pressure (Fig 19) was applied to the bottom of a cut shoot of a tomato

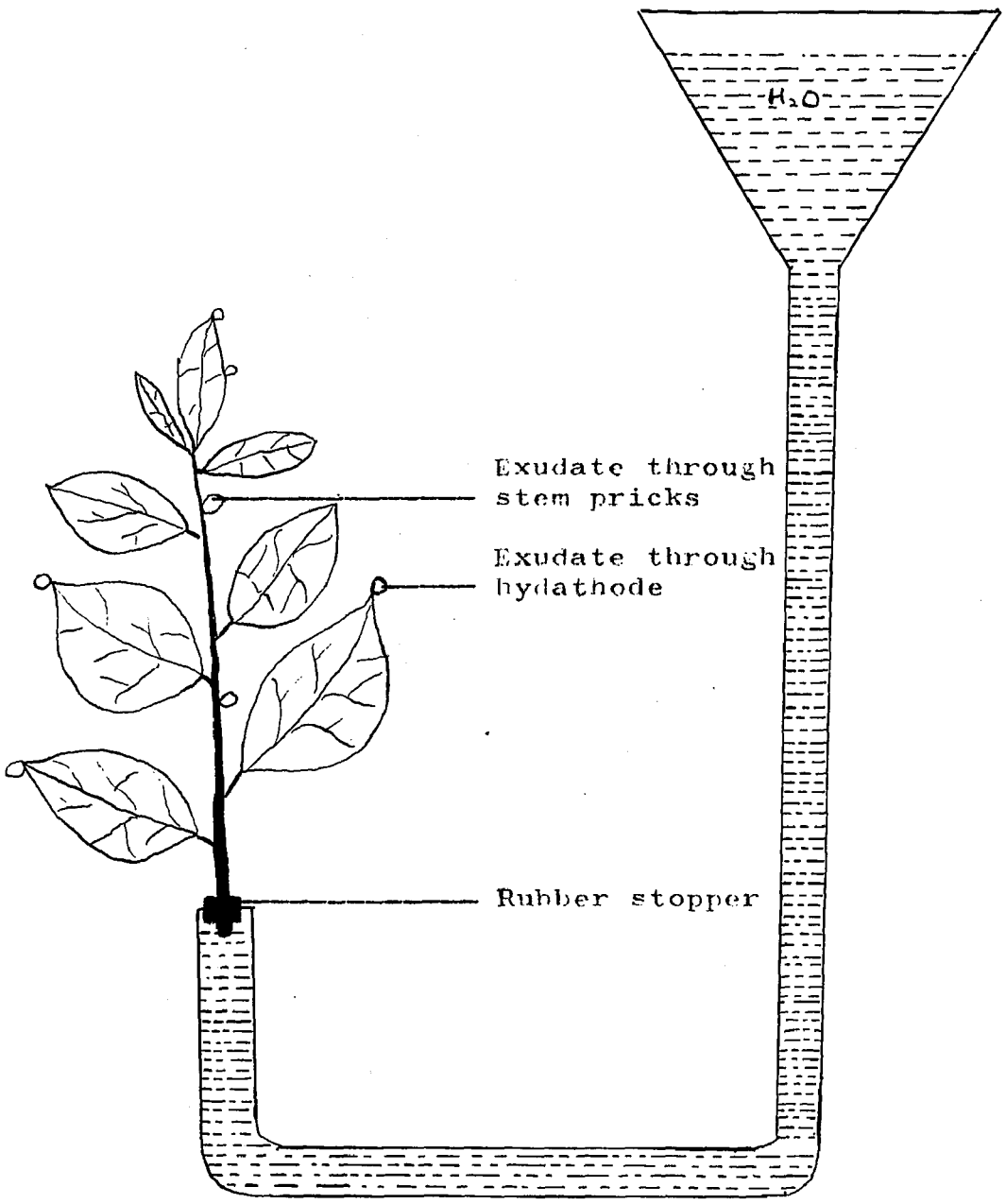

Fig. 19. Tomato shoot under water pressure, showing droplets, used for preparation of virus sample in electron microscope studies.

plant infected with PAMV. The exudates obtained from either stem pricks or hydathodes were dropped onto prepared grids and shadowed with uranium. No virus particles could be observed under the electron microscope in this preparation. This is probably due to the extremely low virus concentration. The electron micrograph of PAMV in Paul and Bode's original publication showed only two or three particles in one electron microscope observation field.

However, a great number of virus particles were observed from specimens which were prepared using purified virus preparation. The virus particles in a microscopic droplet are shown in Fig. 20. They are flexible rods but vary in length. The average length, based on 25 measurements, is 516 mu. The average diameter of the particles is 15 mu; this value is probably larger than the true one because of shadow casting. The virus particles were almost invisible under the electron microscope before shadow casting.

Fig. 20 Electron micrograph (approximately 80,000 x) of potato aucuba mosaic virus purified through DEAE-Sephadex method. The particles were shadowed with uranium at an angle of 26°.

DISCUSSION

The virus isolates (PAMV-M and PAMV-C) used in this investigation were obtained from two different locations. The identity of these two isolates was unknown when they were obtained. First, it was essential to conduct a series of experiments to determine the relationship between PAMV-M and PAMV-C. Identification of plant viruses or strains of virus has paralleled the growth of knowledge in all phases of plant virology. Some viruses or strains of virus can be identified on the basis of precise information concerning only a few properties while with others a judgement can be made only after the acquisition of information on all of the properties that can be obtained. Historically, symptomatology has been the most widely used criterion for identification. This by itself, of course, is not reliable. The inadequacy of symptomatology as a basis for identification has long been recognized, but it still can provide valuable information. The results from inoculation of different plant species indicated that the two virus isolates are readily sap-transmissible from potato to pepper, C. amaranticolor, tomato, Petunia, tobacco and Physalis spp. Identical symptom expressions in both pepper and C. amaranticolor caused by PAMV-M and PAMV-C reveal an obvious relationship between them. Similarity in physical properties provides supplementary evidence on their identity. Furthermore, a positive serological reaction between antigen PAMV-C and

antiserum against DEAE-Sephadex purified virus confirms their identity.

Quantitative assays of virus on a local lesion host make it possible to determine infectivity of virus suspensions or purified virus preparations relative to other preparations, and to correlate the infectivity with particles which are predominant in purified virus preparations. C. amaranticolor is the only local lesion host available at present for this purpose. This plant grows readily under greenhouse conditions, and its leaves are thick and possess a smooth upper surface, thus not easily injured during the process of inoculation. A comparison of six to eight leaves can be made on a single plant. Local lesions appearing in the inoculated leaves are discrete and easily counted. No systemic infection and spots of noninfectious origin have been observed throughout the course of these experiments. The number of lesions produced on the inoculated leaves decreased with increasing dilutions. All of this evidence indicates that C. amaranticolor is a satisfactory host for quantitative studies.

Comparison of sensitivity of pepper and C. amaranticolor plants to inoculation of PAMV-M and PAMV-C, respectively, reveals that pepper is far superior to C. amaranticolor for very dilute inocula, but C. amaranticolor is reliable in detecting large differences in concentrations of active virus in different preparations. For indexing and virus detection purposes, pepper is the best and most reliable indicator plant.

The main purpose of the present study is to perform a series of experiments using different techniques which will determine the most satisfactory method for purification of this virus. The problems of purification of plant viruses are numerous and far from completely solved. The various techniques used in the purification have been reported and reviewed by various authors, but no one method has proven to be applicable for the purification of all plant viruses. Procedures that work well with some viruses may be destructive or unsatisfactory for others. However, certain requirements are common for the purification of nearly all plant viruses. The basic demands for purposes of purification are to remove nonvirus constituents and to concentrate the virus. Before purification can be started, a suitable host is required for the mass production of virus. In addition, a good local lesion host is also desired to assay for the infectifity of a purified preparation. The quantitative assays of relative virus concentration in different plant species indicate that tomato, tobacco, and Physalis spp. are satisfactory hosts for supporting virus multiplication and production. For purification purposes, only tobacco plants infected with PAMV were selected because the sap is easily clarified in preliminary extraction. Virus extracts prepared from young tobacco plants with 0.1 M phosphate buffer containing 0.1 M NaCl showed no chloroplast fragments nor chlorophyll-containing substances. Extract from older tobacco plants infected with PAMV, prepared in the same way,

contained variable amounts of chloroplast fragments, thus making the extracts undesirable for fractionation or purification.

A purification process involving the use of chemicals such as n-butanol and chloroform led to the inactivation of virus. As shown in Table 11, purified virus preparation through the use of two volumes of 1:1 mixture of n-butanol and chloroform resulted in complete destruction of both infectivity and serological activity of the virus. It is obvious that this procedure could not be employed in the purification of PAMV. Substantial evidence has been presented to show that n-butanol-chloroform is indeed effective in removing noninfectious constituents or antigenic materials from infected plants since no antigenic component could be detected in this preparation, using the Ouchterlony agar double diffusion test.

Treatment of virus extract with 8.5% n-butanol was successful in retaining virus infectivity, but failed to remove all the noninfectious proteins or other antigenic materials which were present in both infected and healthy plants. This was demonstrated by the reaction between antibody against butanol-purified virus and antigen from a healthy plant in the Ouchterlony agar diffusion plate. Tomlinson et al (56) who initiated this method in purification of the Y-strain of cucumber mosaic employed infected cucumber corollas as a source of virus. It is probable that antigenic constituents, presumebly normal plant proteins, behave

differently in different plant species.

The results of adsorption experiments using DEAE-Sephadex, purified magnesium bentonite and HCP, respectively, revealed that buffer molarity is a critical factor in applying these techniques for purification. Among them DEAE-Sephadex appears to be the best material for purification of this virus at buffer molarity of 0.1-0.05 M. The presence of brown pigment, presumably polymerized polyphenols, in the virus extract could be easily eliminated to a large extent, if not entirely, by DEAE-Sephadex treatment. Virus purified through this process is antigenically pure as no band was formed between the wells containing antibody and virus-free plant extracts. On the other hand, purified preparation through the use of magnesium bentonite or HCP contains one or more antigenic components of normal plant origin which can be detected, using an agar gel double diffusion test.

Gel filtration employed in the present investigation promises to be a useful technique at least for partial purification. Samples collected under the first peak with the 265 mu absorption spectrum are clear and highly infectious. The second fraction which also showed 265 mu absorption moved slowly and far behind the virus fraction. When the high speed pellet suspensions resulting from ultracentrifugation of samples collected under the first peak were refractionated on a Sephadex column, a symmetrical curve resulted. Steere and Ackers (52), in 1962, reported that separation of tobacco mosaic and southern bean mosaic viruses can be accomplished

by means of a 4% agar granule column. The agar in the column has a sufficiently large pore size that the southern bean mosaic virus can move through, whereas the rod-shaped tobacco mosaic virus is excluded. The excluded rod-shaped particles of tobacco mosaic virus migrate without retention in the interstitial fluid and appear in the effluent earlier than spherical particles of southern bean mosaic virus. A symmetrical curve was obtained when virus which was collected from the Sephadex column was subjected to agar gel filtration analysis. The mechanism of separation of PAMV from a Sephadex column is exactly the same as that for separation of tobacco mosaic virus from an agar gel column. A Sephadex column can not be used for separation of different viruses since the pore size of Sephadex is not big enough to allow virus particles to penetrate the gel. The problem we encountered in Sephadex gel filtration is that some normal plant nucleoproteins with a molecular weight above 200,000 (similar to virus particles) were eluted simultaneously with virus. This could be partially eliminated by acid precipitation. Sephadex gel filtration can be extended to a large preparative scale by using a large column. The separation is gentle to the virus and easily accomplished. It is an excellent method for partial purification of plant viruses.

An electron micrograph of PAMV obtained previously by Paul and Bode (38) with unpurified extracts showed the virus as a flexible rod-shaped particle, 580 X 11 mu. An electron micrograph of purified preparation of the virus in

the present investigation has confirmed the earlier observation on shape of particles but demonstrated that they are variable in length. The diameter of the virus particles found in the present investigation was larger than those reported by Paul and Bode in 1956.

SUMMARY

Identical symptom expressions in both pepper and C. amaranticolor plants caused by potato aucuba mosaic virus (PAMV) isolates M and C, respectively, demonstrate an obvious relationship between them. Similarity in physical properties and positive serological reaction between antiserum against PAMV-M and antigen prepared from tomato plant infected with PAMV-C confirm their identity. Pepper plants inoculated with PAMV-M or PAMV-C developed severe top necrosis, usually followed by death. This host is extremely sensitive to inoculation with sap containing virus and promises to be an excellent indicator host for indexing purposes.

C. amaranticolor proved to be a local lesion host. Yellow local lesions occurred in leaves of this plant five to six days after inoculation with either PAMV-M or PAMV-C. The results of quantitative studies indicated that this host is useful for assaying virus concentration. The number of local lesions produced in the inoculated leaves decreased with increasing dilutions. This proves that C. amaranticolor is a reliable host for detecting large differences of active virus concentration in different preparations.

Tomato is unsatisfactory as an indicator host due to erratic symptom expressions. However, side shoots which developed following the removal of main shoots of infected tomato plants showed a yellow mosaic pattern symptom.

Temperature has a significant effect on virus multiplication. Both high and low temperatures inhibit virus multiplication but high temperature has the greater effect. It has been demonstrated that these unfavorable temperatures only prevent virus multiplication but do not inactivate the virus.

The antigenic purity of virus preparation prepared through the use of various methods is determined by the Ouchterlony double diffusion test. Virus extracts treated with two volumes of 1:1 mixture of n-butanol and chloroform led to complete destruction of both infectivity and serological activity of the virus. Treatment of virus extract with 8.5% n-butanol alone was successful in retaining virus infectivity but failed to remove all the non-infectious antigenic materials which were presumably normal plant proteins or nucleoproteins. Neither magnesium bentonite nor hydrated calcium phosphate was satisfactory for purification of the virus as evidenced by the presence of one or more antigenic components in addition to the virus in agar diffusion plates.

DEAE-Sephadex appears to be an excellent material for purification of this virus. Virus prepared by this method proved to be antigenically pure. Buffer molarity is a critical factor in application of this purification technique since both virus and normal plant materials were adsorbed onto DEAE-Sephadex when the buffer molarity used in the preparation was below 0.005 M.

Gel filtration is a useful technique for partial

purification of this virus. The problem in employing this method for purification is that some normal plant nucleoproteins or proteins with molecular weights similar to those of virus particles were eluted simultaneously from the column with the virus.

An electron micrograph of PAMV, using a purified preparation of the virus, indicated that the average length of particles is 516 mu and the average diameter is 15 mu.

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