SHORT TITLE

CHARACTERIZATION OF AN UNKNOWN VIRUS
FROM PLANTAGO MAJOR

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THE CHARACTERIZATION OF AN UNKNOWN VIRUS FROM PLANTAGO MAJOR

by -

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ABSTRACT

M.Sc. ADIB ROWHANI Plant Pathology

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Plantago major (broad-leaved plantain) with symptoms typical of virus infection, was shown to be infected with a previously undescribed virus, designated as plantain virus (PV) throughout the investigation. PV was transmitted manually to a rather narrow host-range. Electron microscopy revealed that PV was a flexuous rod-shaped particle with a modal length of 607 nm in leaf-dip preparations. A satisfactory method for purification of the virus, involving the use of detergent Triton X-100 in borate buffer, pH 8.0, was developed. Determinations of the physical and chemical properties of the purified virus (sedimentation coefficient, UV absorbance, per cent RNA, molecular weight of viral components) showed that the virus was similar to those of the potato virus X (PVX) group. However, serological tests involving PV and members of the PVX and potato virus Y groups indicated that PV was serologically unrelated to any of the viruses tested.

RESUME

M.Sc. ADIB ROWHANI Phytopathologie

CARACTERISATION D'UN VIRUS INCONNU ISOLE DE PLANTAGO MAJOR

Il fut démontré que Plantago major (plantain à grandes feuilles), présentant des symptômes typiques d'infection virale, était infecté par un virus inconnu, nommé "virus du plantain" (VP) au cours de nos recherches. Le virus VP fut inoculé manuellement à un nombre restreint d'hôtes. L'étude au microscope électronique a révélé que le virus VP est une particule en forme de batonnet flexible, dont la longueur module, estimée à partir de préparations courantes, est de 607 nm. Une méthode satisfaisante de purification du virus fut développée en utilisant du détergeant Triton-X, dans un tampon borate, pH 8.0. Grâce à la détermination des propriétés physiques et cliniques du virus purifié (coefficient de sédimentation, absorbance à l'UV, pourcentage de l'ARN, poids moléculaire des composantes virales), il fut démontré que le virus est semblable aux virus du groupe X de la pomme de terre (PVX). Cependant, des tests sérologiques impliquant les virus PV et un certain nombre de virus du groupe PVX et du groupe PVY suggèrent que le virus PV ne présente aucun lien de parenté sérologique avec les virus étudiés.

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ABBREVIATIONS

Brome mosaic virus	BMV
Buoyant density	ρ
Cactus virus X	CVX
Cesium chloride	CsCl
Clover yellow mosaic virus	CYMV
Deoxyribonuclease	DNase
Deoxyribonucleic acid	DNA
Disodium ethylenediamine tetraacetate	Na_2EDTA
Miliampere	MA
Nanometer	nm
N,N,N',N', tetramethylenediamine	TEMED
Optical density	OD
Plantain virus	PV
Polyethylene glycol	PEG
Potato virus S	PVS
Potato virus X	PVX
Potato virus Y	PVY
Ribonuclease	RNase
Ribonucleic acid	RNA
Sodium diethyldithiocarbamate	NaDIECA
Sodium dodecyl sulfate	SDS
Sodium saline citrate buffer*	SSC
Southern bean mosaic virus	SBMV
Tobacco etch virus	TEV
Tobacco mosaic virus	TMV
Tris (hydroxymethyl) aminoethane	THAM
Tris, KCl, $MgCl_2$ buffer*	TKM
Ultraviolet	UV

*Molarity stated in materials and methods

INTRODUCTION

Although it is well known that weeds and non-economic plants may serve as hosts for some viruses, there has been little systematic study of weeds as virus hosts, or of the actual extent to which they serve as reservoir hosts, and usually knowledge of these factors has been obtained during epidemiological investigations of sources of infections affecting economic crop plants.

As reported by Hawthorn (1973) and Bassett (1973), Plantago major L. (broad-leaved plantain), and Plantago rugelii Decne. (Rugel's plantain) are common weeds in eastern Canada and the northern United States. During the past few summers locally growing plants of Plantago major L. (identified on the basis of seed size and other characters by Dr. D. E. Swales, McGill University Herbarium) often exhibited vein clearing, mottling, and leaf malformation suggestive of systemic virus infections. Preliminary observations indicated that these symptoms were caused by a flexuous rod-shaped virus. Relatively few viruses of this morphology have been reported to infect broad-leaved plantain.

The objectives of the research described were:

1. To determine the host range and to identify the virus, and find out whether it was a previously described virus whose host range has not been known to include Plantago major L., or an unknown virus.

2. To identify economic crop plants which might be susceptible to this virus.
3. To develop a suitable method of purification, to enable further characterization of the virus, and to enable production of an antiserum to the virus, for possible use in investigating its serological relationship to other viruses.

LITERATURE REVIEW

A. Distribution

Although they occur commonly as weeds, Plantago spp. have been reported as hosts of relatively few plant viruses. Holmes (1941) reported a strain of tobacco mosaic virus (ribgrass strain) occurring in Plantago lanceolata and in the broad-leaved plantain (Plantago major L.), growing as weeds in several widely separated locations near Princeton, New Jersey. Also, in the same locations, another strain of tobacco mosaic virus which caused tomato internal-browning disease was found on naturally infected broad-leaved plantain (Holmes 1950). Fulton (1948) has reported the occurrence of broad-leaved plantain naturally infected with tobacco streak virus, and Hollings (1958) has found hydrangea ringspot virus on Plantago lanceolata L. in Britain. As hydrangea ringspot virus is a flexuous rod-shaped virus having a length of 650 nm, causing local symptoms in plantain, Hollings believed that this virus should be placed in the potato virus X group. Beet yellows virus, another rod-shaped virus with a normal length of one to several microns, was reported by Bennett (1960) to infect Plantago erecta and Plantago insularis. Other reports are from Cropley (1961), indicating that cherry leaf-roll virus infects Plantago major L. producing systemic necrotic etched spots, and Hitchborn et al. (1966), who have found a virus which they refer to as Plantago mottle virus, infecting Plantago lanceolata and P. major in Britain. The virus

particles observed were bullet-shaped, and generally resembled those of vesicular stomatitis virus and lettuce necrotic yellows. Granett (1973) reported a new virus originally isolated from Plantago major L. which was designated as Plantago mottle virus and placed in the turnip yellow mosaic virus group on the basis of its properties. Broad-leaved plantain also can serve as the host of dodder latent mosaic virus (Smith 1972), and the leaf hopper transmitted agent causing aster yellows disease (Freitag et al. 1969).

B. Purification

Aggregation of rod-shaped plant viruses is one of the principal difficulties hindering their purification; it results in a physically heterogeneous population of virus particles and sometimes proceeds to the point at which most or all of the virus loses solubility. The aggregation is usually end-to-end and increases with purification and storage of the viruses (Brakke and Van Pelt 1969). The aggregation is not easily reversed once it has occurred. Addition of hydrogen ions, magnesium, calcium, and some polyamines will increase the rate of aggregation (Brakke 1967). The rate of aggregation is higher at higher temperatures, can be prevented by raising the pH, lowering the salt concentration, and adding chelating agents (Reichman 1959; Scott 1963; Black et al. 1963). Citrate at pH 8.0, or borate buffer at pH 8.0-9.0 prevents the aggregation of many rod-shaped plant viruses, but will not reverse it once it has occurred (Brakke 1967), and chelating agents such as citrate and EDTA do not bind polyamines and will not reverse their effect on aggregation or stability of virus.

Damirdagh and Shepherd (1970) used urea for purification of tobacco etch virus and several other members of the potato virus Y group. They reported that urea at concentration of 2 M or greater caused degradation of the virus, but at concentration of 0.5-1.0 M prevented, or reversed aggregation without causing degradation. They found that lateral aggregation of the virus particles could be prevented by using 0.5 M urea and 0.1 per cent 2-mercaptoethanol in the resuspension buffer during purification, and postulated that urea weakens the affinity of non-polar areas on the surface of the viral capsid, presumably weakening hydrophobic-type interactions between particles. Also, as a hydrogen bonding reagent, urea may break hydrogen bonds formed between particles (Schachman 1963). The role of 2-mercaptoethanol is related to its capacity to reduce disulfide linkages formed by oxidation of sulfhydryl groups on the viral protein.

Brakke (1959) reported the use of detergents for the dispersion of aggregated barley stripe mosaic virus. Igepon T-73 (active ingredient sodium N-methyl N-oleoyl taurate), was found to be the best of the detergents tested; 0.01 to 0.5 per cent of this detergent dispersed about 90 per cent of previously aggregated virus. The detergent also dispersed host colloidal particles of the size range of viruses to give particles of much lower density than the virus. These could be easily separated from the virus by density gradient centrifugation. However, Igepon T-73 appeared to cause a slight decrease in stability of the virus.

Bottomley (1970) reported the use of Triton X-100 (alkylphenoxy-polyethoxyethanol) for solubilization of plastids and disintegration of the organelles from pea etioplast preparations. Yuzo and Yamaura (1971) used Triton X-100 to purify TMV from plants other than tobacco. TMV from garden zinnia (Zinnia elegans) and spinach (Spinacea oleracea) isolated by this method showed the same characteristic ultraviolet absorption curve and infectious properties as that isolated from tobacco without using Triton X-100. It was also found, by electron microscopy, that the size and shape of TMV particles treated with Triton X-100 were the same as those of non-treated ones. When Triton X-100 was added to purified TMV particles, no appreciable effect was found at a final concentration of five per cent, but coagulation occurred at a concentration above ten per cent.

Van Oosten (1972) used Triton X-100 for the purification of the plum pox (Sharka) virus, and showed that infectivity was highest when this detergent was added, up to a concentration of about five per cent, to an extract clarified by low-speed centrifugation. Triton X-100 has also been used by DeWijs (1974) for the purification of a virus causing ringspot on Passiflora edulis (passionfruit ringspot virus), by adding it to a concentration of five per cent by volume.

Brakke and Rochow (1974) added Triton X-100 to the crude extract to a concentration of one per cent as a clarification procedure when purifying barley yellow dwarf virus. After one high speed centrifugation, the pellets were suspended in 0.1 M neutral potassium phosphate and clarified by low speed centrifugation. After the subsequent

addition of Triton X-100 the extracts were again concentrated by high speed centrifugation. Thouvenel et al. (1976) recently made use of Triton X-100 for the purification of Guinea grass mosaic virus a flexuous rod-shaped particle.

Polyphenol oxidase is a copper-containing enzyme involved in the oxidation of phenolic compounds in plant extracts, which have a highly inhibitory effect on plant viruses. Two chelating agents with more or less specificity for copper, diethyldithiocarbamate and potassium ethyl xanthate, have been used to obtain infectious preparations of several viruses (Matthews 1970). The viruses that cause prune dwarf, sour cherry recurrent necrotic ringspot, and necrotic ringspot diseases lose infectivity in tissue extracts within a few hours (Hampton and Fulton 1961), but addition of sodium diethyldithiocarbamate (NaDIECA) stabilizes the extract by making the copper unavailable and inhibiting the polyphenol oxidase. By combining stabilization with NaDIECA and a reducing agent with hydrated calcium phosphate (HCP) clarification, Fulton (1959) was able to purify prune dwarf and necrotic ringspot viruses. It seems probable that NaDIECA would have a favorable effect on the stability of other unstable viruses, and might facilitate their purification.

C. Sedimentation Coefficient

The rate at which a virus sediments through a gradient column in density-gradient centrifugation depends on its size, shape, density and the density and viscosity of the surrounding medium, which usually are

a function of temperature. Since these properties are constant for most viruses (Knight and Oster 1947), the sedimentation rate of a virus through a density-gradient column should also be a characteristic constant for each virus. Such sedimentation rates might be of considerable practical importance for characterization and identification of viruses because they can be measured on low concentrations of virus, because impure virus preparations can be used, and because the identification of the visible virus zones can be easily confirmed by infectivity assay.

Sedimentation coefficients are usually estimated from results of density gradient centrifugation by comparing the sedimentation of unknown and standard particles (Brakke 1958; Martin and Ames 1961). However, several procedures for estimating sedimentation coefficient by density gradient centrifugation without the use of markers have been published (McEwan 1967). Estimation of sedimentation coefficients with or without the use of markers, is simplified if the gradient is designed so that the sedimentation rate is independent of depth (so-called "isokinetic") (Harrison and Nixon 1959; Martin and Ames 1961; McCarty et al. 1968; Noll 1967). A gradient in which the logarithm of the depth is a linear function of the logarithm of the sedimentation coefficient or of the time of centrifugation is steeper than an "isokinetic" gradient and almost as convenient to use. For convenience, this type of gradient in which the logarithm of depth is a linear function of the logarithm of the sedimentation coefficient is called a "linear-log" gradient (Brakke and Van Pelt 1970a).

D. Ribonucleic Acid

Since isopycnic cesium chloride (CsCl) gradients were introduced in 1957 (Meselson et al.), this technique has become increasingly popular as a tool for studying the physical-chemical properties of macrospecies (nucleic acids, other macromolecules and particles such as ribosomes and viruses). Originally these buoyant density gradients were centrifuged in swinging bucket type rotors, but since 1966 (Flamm et al.) fixed angle rotors have also been employed. The use of fixed angle rotors increases the number of gradients that can be centrifuged simultaneously, and also increases the resolution of densities in the gradient (Flamm et al. 1966).

Brunk and Leick (1969), by using the equilibrium isopycnic CsCl gradients with an initial preformed CsCl gradient concentration, dramatically reduced the time required to achieve equilibrium conditions, from 36-50 hours to about 18 hours.

Estimation of protein and nucleic acid content of viruses can be made by chemical and colorimetric analysis (Schuster 1964) and ultraviolet (UV) light absorption spectrophotometry (Englander and Epstein 1957). Such methods are only reliable, however, when highly purified viral preparations are used. A distinct advantage of the equilibrium centrifugation procedure is that significant information on the physicochemical properties of macromolecules can be obtained while conserving the materials for other investigations (Sehgal et al. 1970). The relationship between buoyant density (ρ) in CsCl and ribonucleic acid (RNA) content of one bacterial and eight plant viruses was studied

by Sehgal et al. The viruses selected for this study varied in their RNA content from four to 42 per cent. A highly significant correlation ($R^2 = 0.99$) was found between ρ and the absolute amount of RNA in these viruses. The functional relationship indicated that the ρ of viruses increased at a greater than linear rate as the RNA content increased, but about 89 per cent of this relation was directly attributed to the amount of RNA in the virions. Based on these observations, they derived a mathematical formula to estimate the RNA content of a virus from its ρ value.

Methods for the isolation of pure, undegraded viral components are of interest for reasons apart from the study of the viral components themselves. Thus, viral nucleic acids have played a major part in our understanding of the properties and functions of nucleic acids. They have been used to study protein biosynthesis, enzyme action, nucleic acid interactions, radiation effects, and many other problems (Ralph and Bergquist 1967).

Numerous methods have been used to isolate viral nucleic acids from DNA- and RNA-containing viruses. Infectious nucleic acids have also been isolated from cells infected with viruses. In some cases infectious nucleic acid was obtained from infected cells when extraction of isolated virus failed to produce infectious material (Fraenkel-Conrat 1961).

Several factors must be considered during isolation of viral nucleic acid to avoid degradation or damage of the final product; shearing by rapid stirring, violent shaking, or pipeting of the viral

DNA or double-stranded RNA solutions should be avoided (Hersley and Burgi 1960). At extremes of pH, i.e., at values greater than 10, RNA phosphodiester bonds begin to hydrolyse, while at pH values greater than 12, ionization of the purine and pyrimidine bases occurs, and DNA is denatured as a result of strand separation. At pH values less than three, the purine bases of DNA and RNA are slowly released. The ionic strength and nature of the cations in solutions of nucleic acids can influence their stability. At very low ionic strength ($<10^{-4}$ M) double stranded DNA begins to denature and separate into single stranded DNA, while RNA has less base pairing and less secondary structure. Magnesium ions stabilize RNA base interactions, but cause RNA aggregation and precipitation at ionic strength around 0.01-0.1 M and greater. Strong aqueous salt solutions (>1 M) also precipitate high molecular weight RNA (Ralph and Burgquist 1967).

Viral nucleic acid is usually extremely susceptible to enzymatic degradation when released from its surrounding protective viral protein coat. During the preparations of viral nucleic acids, tissues, cell fragments, and subcellular particles present in virus preparations, frequently release nuclease following their disruption. It is necessary to either inactivate or inhibit the enzyme activity following the release of the viral nucleic acid. This can be accomplished by using nuclease inhibitors such as poly (vinyl sulfate), bentonite (Dunn and Hitchborn 1965) or diethylpyrocarbanate (Rosen and Fedorscsak 1966).

Bentonite has been reported to be an effective inhibitor of yeast ribonuclease (Brownhill et al 1959), and in sufficient

concentration completely inhibited the action of pancreatic ribonuclease and permitted the near-quantitative removal of the enzyme by centrifugation (Singer and Fraenkel-Conrat 1961). Many RNA preparations made in the presence of bentonite, or held in the presence of bentonite, lost little of their infectivity during 24 hours at 36°C in 0.1 M phosphate. Application of the RNA to the test plants in the presence of bentonite increased the local lesion response by a factor of 10-20. The mechanism of that inhibition appears to be a binding of enzymes by the clay, with an affinity which is very high for ribonuclease, and somewhat less for crude plant enzymes (Singer and Fraenkel-Conrat 1961).

In general, the more powerful the protein denaturant used to denature and remove the viral protein and release the viral nucleic acid, the more successful the inactivation of nucleases and the more intact the nucleic acid isolated (Ralph and Bergquist 1967). Of the reagents currently employed for isolating viral nucleic acids, the most successful are the protein denaturants phenol and sodium dodecyl sulfate (SDS). These have both been used successfully to prepare infectious viral nucleic acids from several viruses. Other methods have been used in special instances, but these are generally less successful in providing biologically active, pure viral nucleic acids. The mechanism of action of phenol as a protein denaturant has not been studied extensively, but it seems that it reacts with the various, more or less hydrophobic, groups in the inside of the proteins and effectively turns them inside out, causing their precipitation (Perutz 1963). SDS solutions do not usually precipitate viral proteins, although

they do denature viral coat proteins. The aliphatic group of the detergent presumably disrupts the internal forces stabilizing the viral protein shell in much the same way as does phenol. However, in this case the resulting protein subunits do not precipitate, since they are effectively dissolved in or surrounded by "shells" or "micelles" of the detergent (Ralph and Bergquist 1967).

Gierer and Schramm (1956) used phenol to extract the TMV-RNA by shaking a solution of TMV with an equal amount of water-saturated phenol. The RNA was released and recovered from the upper aqueous phase after centrifugation of the mixture. Following removal of the phenol from the water phase by ether extraction, the RNA was precipitated with two volumes of ethanol and recovered by low-speed centrifugation. Kirby (1964) reported that the addition of 0.1 per cent 8-hydroxyquinoline to water saturated phenol minimized the formation of metal catalyzed oxidation products, by chelating contaminating metal ions. The use of 8-hydroxyquinoline for minimizing enzyme action and also for facilitating the denaturation of protein contaminating bound metal ions has since become widespread. In some cases it has been found beneficial to make the phenol extraction after the virus structure has been opened up by a detergent such as SDS. Rushizky and Knight (1959) used such a technique to obtain infectious RNA from tomato bushy stunt virus, and Bachrach (1960) similarly obtained infectious RNA from foot-and-mouth disease virus.

Although the phenol extraction method is the most generally useful procedure for obtaining nucleic acid from a wide variety of

viruses, it is not satisfactory for some viruses, and trial and error may be needed to establish a satisfactory method for a new virus. Many other methods have been used for extraction of RNA and some of them are summarized here. Fraenkel-Conrat and Williams (1955), under carefully controlled conditions, used weak alkaline solution (pH 10-10.5) and degraded TMV without causing denaturation of the protein. Fraenkel-Conrat (1957) later observed that cold 67 per cent acetic acid split the virus and caused precipitation of the nucleic acid from the solution, and native protein free from nucleic acid or other gross contaminants could be isolated from the supernatant simply by dialysis.

Reichmann and Stace-Smith (1959) used guanidine hydrochloride to prepare infectious RNA from potato virus X, and Cox and Arnstein (1963) also used the same method to extract intact ribonucleic acid from rabbit-reticulocyte ribosomes. Guanidine-HCl, in the presence of LiCl, was used for extraction of turnip mosaic virus-RNA by Hill and Shepherd (1972).

Brakke and Van Pelt (1970b) used an ammonium carbonate-SDS buffer, pH 9 in the presence of EDTA, to extract infectious RNA from wheat streak mosaic virus. The same method was used to extract infectious RNA from maize dwarf mosaic virus, potato virus Y, and rye grass mosaic virus (Pring and Langenberg 1972; Makkouk and Gumpf 1974; Paliwal and Tremaine 1976).

Formaldehyde has been used extensively for chemically modifying proteins and for inactivating toxins and viruses, although the precise mode of its action is little understood (Haselkorn and Doty 1961). In

the case of tobacco mosaic virus, however, Fraenkel-Conrat (1954) and Staehelin (1958) have shown that formaldehyde is not only capable of reacting with the protein but also inactivation of the virus is due to its interaction with the amino groups of adenine, cytosine, and guanine in the ribonucleic acid component. They have reported that native DNA from several sources does not react with formaldehyde under the same conditions used for the reaction with RNA. The conclusion drawn was that amino groups involved in hydrogen bonds do not react with formaldehyde. Hoard (1960) has shown by means of a formol titration that the amino group of cytidilic acid combines with one molecule of formaldehyde in high formaldehyde concentration.

Formaldehyde is a very convenient denaturant, because RNA is completely denatured very rapidly at relatively low formaldehyde concentration (Boedtker 1968). Boedtker indicated that the sedimentation coefficient of a single stranded RNA depends on the molecular weight and the secondary structure. If the secondary structure of the RNA is destroyed by treatment with formaldehyde, the sedimentation coefficient should depend only on the molecular weight. She used formaldehyde to denature the helical regions of RNA and converted polynucleotides into random coils characterized by a single equation relating the logarithm of the sedimentation coefficient to that of the molecular weight. Brakke and Van Pelt (1970b), working with wheat streak mosaic virus, obtained similar results and devised a similar formula, $S = 0.083\ M^{0.38}$, for the relationship between the sedimentation coefficient (S) of formaldehyde-treated RNA and its molecular

weight before formaldehyde treatment (M). The same treatment was applied by other workers in characterizing RNA obtained from maize dwarf mosaic virus, and potato virus Y (Pring and Langenberg 1972; Makkouk and Gumpf 1974).

E. Protein

The application of electrophoresis in SDS-containing polyacrylamide gel to the determination of molecular weight of proteins has been reported by Shapiro et al. (1967). This method has utilized the linear relationship between the log of the molecular weight of the protein, and the distance which the protein migrates into the gel. Maizel (1966) has proven electophoresis in polyacrylamide gels in the presence of the anionic detergent SDS to be a useful tool for the separation and identification of polypeptide chains. Weber and Osborn (1969) followed the procedure of Shapiro et al. to study forty proteins with polypeptide chains of well characterized molecular weights by polyacrylamide gel electrophoresis in the presence of SDS. When the electrophoretic mobilities were plotted against the logarithm of the known polypeptide chain molecular weights, a smooth curve was obtained. The results showed that the method is reliable for determining the molecular weights of polypeptide chains for a wide variety of proteins. It appears that by this technique polypeptide molecular weights may be determined with an accuracy of at least ±10 per cent (Weber and Osborn 1969).

The tobacco necrosis virus coat protein has been shown by polyacrylamide gel electrophoresis to consist of one protein species (Lesnaw and Reichmann 1969). The minimum molecular weight of the protein subunit calculated from amino acid analysis is 33,300, and the molecular weight as determined by electrophoresis in SDS-containing polyacrylamide gel in the presence of markers of known molecular weight is 33,000, which is in precise agreement with the previous value. Hill and Shepherd (1972) estimated the molecular weights of the coat proteins of fifteen plant viruses by polyacrylamide gel electrophoresis in the presence of SDS. They have indicated that most of the well-characterized viral coat proteins gave values within about 10 per cent of that estimated by other means. Five viral proteins (cucumber mosaic virus, potato virus X, sowbane mosaic virus, tobacco etch virus, and white clover mosaic virus) gave values significantly different from those based on chemical and physical data. Two components were obtained with the coat proteins of tomato bushy stunt virus and white clover mosaic virus.

Hiebert and McDonald (1973), during their work with five different viruses of potato virus Y group, analyzed the viral coat protein by gel electrophoresis in polyacrylamide gels containing SDS. They observed that all five viral coat proteins separated into two molecular weight components (designated fast and slow according to the relative mobility). Preliminary evidence suggests the ratio of the two components appeared to depend on purification conditions and on the length of storage after purification, before dissociation with SDS.

F. Serology

Serological reactions are being used increasingly to identify plant viruses as well as to determine the degree of relationship among them (Bercks 1960). In both procedures, it is advisable to use antisera which react only with virus antigens and not also with those of the host plant. The outstanding characteristic of the serological reaction between a virus and its homologous antiserum is its specificity, which depends on the fact that antibodies react only with the antigen which was used for immunization or antigens chemically closely related to it. Because of this specificity, serological reactions are the most certain method of identifying a virus and the disease caused by it. An advantage of the serological diagnosis is that accurate results are obtainable in a short time (Wetter 1965). In most cases where a virus is to be identified, different isolates, mutants, or strains of the virus must be compared; therefore, the serological study of relationships between viruses is decisive for their identification and classification.

Of the different methods used in plant virology, the precipitin method, in its various modifications, is the most common. Tests which make the precipitate directly visible have a special advantage over those using indicator systems which necessitate extra controls (Wetter 1965). The microprecipitin reaction under paraffin oil is a modification of the precipitin test on slides (Dunin and Popova 1938), useful for potato-virus diagnosis (Stapp 1943) and also for titrating antigens during the course of their purification (Wetter 1960). The test offers

special advantages in that small amounts of antisera are required, and the reaction is quick, perhaps because of the special conditions prevailing on the surface of the drops (Wetter 1965).

Ouchterlony agar double diffusion tests (Ouchterlony 1958) have gained wide usage in recent years in the serological study of plant viruses. The technique has several advantages over more conventional tests. Lesser amounts of antigen and antiserum are required; the test has greater resolving power than other forms of the precipitin tests, particularly when impure antigen preparations are used. In addition, agar diffusion tests are convenient for the routine identification of virus in extracts of infected plants (Purcifull and Shepherd 1964), and the reaction of various antigens with a particular antibody, or the reverse, can be carried out simultaneously, under identical conditions.

Elongated plant viruses (longer than TMV) have a slow diffusion rate and are consequently unsuitable for the Ouchterlony test. This is attributable not only to their shape, but also to their tendency to aggregate end-to-end (Wetter 1965). With clover yellow mosaic virus (Bercks 1963), however, sharp bands close to the antigen depot could be obtained after prolonged incubation. The same was not true with the related potato virus X, probably because the virus was in an aggregated form (Ford 1964), but sharp precipitation bands were obtained if citrate-phosphate saline buffer at pH 8, which minimizes aggregation, was used (Ball et al. 1964). With barley stripe mosaic virus, the formation of precipitation lines could be facilitated by adding 0.1 per cent sodium dibutyl naphthalene sulfonate (Leonil S.A.) to the agar,

possibly because this substance breaks up the virus particles (Hamilton 1964).

The potential usefulness of immunodiffusion procedures for large-scale serodiagnostic programs has stimulated further work on the adaptation of this technique for use with rod-shaped plant viruses. Purcifull and Shepherd (1964) investigated the possibility of using fragments of viruses or protein sub-units, which would diffuse rapidly into the agar and react specifically with antiserum prepared against the intact virus. They demonstrated that some rod-shaped viruses could be degraded into serologically active components which readily diffused in the agar gel. Various chemical compounds were found effective but alkaline degradation using ethanolamine buffers yielded the greatest amount of serologically active protein from clover mosaic virus. It was pointed out, however, that conditions for degradation will vary with each particular virus. The use of chemical degradation products is also described by Shepard and Grogan (1967) for diagnosis of western-celery mosaic virus, by Milne and Grogan (1968) for watermelon mosaic virus, and by Purcifull and Gooding (1970), and Gooding and Bing (1970) for the detection of potato virus Y (PVY) and tobacco etch virus (TEV). The latter workers incorporated the detergent SDS in the agar gel and demonstrated the presence of PVY and TEV in crude sap of infected tobacco plants. Hiebert et al. (1971) used the same technique to disrupt purified TEV and PVY inclusion bodies and showed that they were serologically unrelated to the viruses that stimulated their formation.

Physical disruption of virus particles by ultrasonic treatment has been reported by Tomlinson and Walkey (1967) for potato virus X (PVX) and turnip mosaic virus (TuMV), and more recently by Koenig (1969) for viruses of the PVX group, and by deBokx and Waterreus (1971) for potato virus S (PVS). In all cases, ultrasonication of partially purified virus suspensions produced virus fragments which were serologically active and produced clear precipitin lines in agar gel with antiserum prepared against the intact antigen. However, deBokx and Waterreus stated that fragmentation of PVS particles in crude sap was not successful.

Shepard and Secor (1969) investigated the relative sensitivities of the Ouchterlony double diffusion and the single radial diffusion methods and demonstrated that both can be reliably used to detect pyridine-degraded PVX in crude expressed sap of infected potato leaves. With the radial-diffusion procedure the virus was detected in sap dilutions of 1:14,000, while the double-diffusion method did not detect the presence of the virus in sap dilutions exceeding 1:1400. In addition to having a significantly greater sensitivity than most other serological methods, the radial-diffusion test was found to be extremely rapid, as results could be obtained within 30 minutes. The technique required a minimum of sap manipulation and could be performed in the field with crude expressed sap. It was later reported useful for the detection of PVS (Shepard 1970) and potato virus M (Shepard et al. 1971), and was said to hold potential for the detection of other elongated plant viruses, depending on the selection of the virus-degrading agent and

the production of a suitable antiserum. The minimum amount of purified antigen that can be detected with the radial-diffusion technique compares favorably with other serological methods. Wright and Stace-Smith (1966) reported that PVX subunits could be detected by the tube precipitin and complement fixation tests at concentrations of 0.5 µg/ml at antiserum dilution end-points. Shepard and Secor (1969) detected purified PVX-protein at a concentration of 10 µg/ml with the double-diffusion method, while concentrations at 1 µg/ml were detected with the radial-diffusion method. Furthermore, large numbers of tests could be performed simultaneously in one agar plate and very little antiserum per test was required.

MATERIAL AND METHODS

A. The Virus

The virus used throughout this study was isolated from broad-leaved plantain (Plantago major) which occurred as a weed around the Macdonald Campus. A vegetative stock culture of the virus, designated hereafter as plantain virus (PV), was maintained in Plantago major. Permanent stock cultures were also maintained in leaves of Plantago major dried over anhydrous calcium sulfate at 3°C under vacuum.

B. Culture Conditions of Experimental Hosts

Plants used for host range determination, virus stock and assays were grown in pasteurized soil in clay pots. The potting medium consisted of two parts pasteurized soil, one part sand, and one part peat moss. Eight ounces of the commercial fertilizer "Osmocote" (14-14-14) were added to each bushel of the soil mixture.

All plants were grown in the greenhouse, maintained at about 22°C in the winter months and between 24°C and 32°C during the summer months. Supplemental light was supplied by incandescent and fluorescent lamps to give a minimal photoperiod of 16 hours. Excessive temperature fluctuations during the summer months were prevented with air coolers and shades. Pest control measures were carried out through weekly greenhouse fumigation with recommended fumigants.

C. Inoculum and Inoculation Procedures

Large quantities of inoculum were prepared by inoculating young tomato seedlings with a vegetative stock culture of the virus. Prior to inoculation, a suitable quantity of fresh infected leaf tissue was homogenized with a mortar and pestle in 0.1 M potassium phosphate buffer, pH 7.1. The leaves of the seedlings to be inoculated were dusted with carborundum and inoculations were performed by rubbing the leaves of plants to be inoculated with the thumb dipped in the inoculum. The same procedure was used to inoculate those plants which were chosen for host-range determination.

D. Host range

All test plants were raised from seed, and were inoculated at the cotyledon stage where practicable or at the four to eight leaf stage, according to species. At least five plants were used for each inoculation. Chenopodium amaranticolor Coste & Reyn., or Gomphrena globosa L. were chosen as assay hosts. Return inoculations were made in several cases, from inoculated and from non-inoculated leaves to the assay hosts, to test symptomless infections.

To determine the host range, various plant species or varieties were chosen among ten different families and inoculated with PV. Test plants were: from Amaranthaceae, Gomphrena globosa and Amaranthus retroflexus (Russian pigweed); Caryophyllaceae: Dianthus barbatus (carnation); Chenopodiaceae: Chenopodium quinoa, C. amaranticolor,

C. foetidum, C. album, C. capitatum, Spinacea oleracea (spinach, var. Epinard America, Epinard Northland, Hyb. 530), Beta vulgaris (sugarbeet, beet var. Betteraves ruby queen, Swisschard, Betteraves redpack, Improved dark red, I07A, I07 Detroit dark red); Cucurbitaceae: Cucumis sativus (cucumber), Cucurbita pepo (squash, var. Butternut), Citrulus vulgaris (watermelon, var. Charlston gray); Ficoidaceae: Tetragoina expansa (New Zealand spinach); Gramineae: Hordeum vulgare (barley, var. Moreval, Champlain, Montcalm, Betzes, Hypana), Triticum aestivum (wheat, var. winter wheat and Cornell wheat), Avena sativa (oats, var. Clintland and Roxton), Zea mays (sweet corn); Leguminosae: Phaseolus vulgaris (French bean, var. Black Valentine, Contender, Likalake, Bountiful, Kentucky Wonderwax, Pinto, Slender green, Highlander, Earlywax, and Sprite), Pisum sativum (pea, var Thomas Laxton, American Wonder pea, and Alaska), Phaseolus aureus (mung bean), Glycine max (soybean, var. Harosoy), Medicago sativa (alfalfa), Trifolium repens (white clover), Trifolium pratense (red clover), Vicia faba (broad bean), Vigna sinensis (cowpea); Plantaginaceae: Plantago major L. (broad-leaved plantain); Polygonaceae: Fagopyrum esculentum; Solanaceae: Nicotiana glutinosa, N. rustica, N. tabacum (tobacco, var. White burley, Xanthi, Turkish, Samsun, Haranova, Havana 425, Havana, Haranova T, Sylvestris, Havana 38), Lycopersicum esculentum (tomato, var. W 218 Armstrong, Rideau, Early Chateau, Asgrow Scarlet Dawn, Pink Gulf State, Jubilee, Michigan State Porune, Fire ball, Glamor, John Bear), Datura stramonium, Datura metel, Petunia hybrida, Solanum nigrum, S. dalcamara, S. tuberosum (potato), Capsicum annuum (sweet pepper) (see also results).

E. Stability in vitro

1. Thermal inactivation point

Frozen tomato leaves were used as the source of crude virus extract. The leaves were ground in a mortar and pestle, and the ground materials were squeezed through a double layer of cheesecloth and diluted with two volumes of 0.1 M borate buffer, pH 8.0. Tested temperatures were between 45 and 95°C, in 5°C increments. Tubes, made from Pasteur pipets, with their tips closed earlier by the gas flame, were used, and previously warmed to the desired temperature in a water-bath. After the tubes had been warmed, 1.5 ml of the crude sap was left in them for exactly 10 minutes at the temperature desired. After that the tubes (usually two) were removed from the water-bath and immediately cooled in running water. The treated juice and also an untreated control extract were then rubbed onto the leaves of Gomphrena globosa predusted with carborundum.

2. Dilution end point

Infected frozen tomato leaves were used as the source of inoculum. The leaves were ground in a mortar and pestle, and the resulting extract was filtered through cheesecloth as previously described. Dilutions were made on a logarithmic scale (from 10^{-1} to 10^{-10}), and each dilution was rubbed onto four Chenopodium amaranticolor leaves, predusted with carborundum.

F. Particle Length

Virus particle length measurements were made on photographic enlargements obtained from examination of "leaf-dip" preparations (Brandes 1957), which were negatively stained with neutralized 2% potassium phosphotungstate and examined in a Zeiss EM.9A electron microscope (Hitchborn and Hills 1965). Similarly stained purified virus preparations were also examined.

G. Purification

The virus was routinely purified from tomato (Lycopersicum esculentum var. Quebec 5). Young tomato plants were inoculated in the 3-5 leaf stage and for practical purposes, both directly inoculated and systemically infected leaves were collected 4-8 weeks later. Leaves were placed in polyethelene bags and stored at -6°C in the freezer before processing.

Various purification procedures were investigated and compared. They are presented here in stepwise fashion. Either Beckman Model L or a Beckman L2-65B ultracentrifuge was used for high-speed centrifugation and pelleting of the virus, while a Sorvall superspeed Rc-2-B refrigerated centrifuge with an SS-34 rotor was used for low-speed clarification.

1. Purification of PV by Triton X-100 clarification in borate buffer pH 8.0 and differential and density gradient centrifugation (this method was eventually adapted for routine use)

All steps were carried out at 3°C.

(a) Infected, frozen tomato leaves were homogenized in a Waring blender using two times their weight of 0.5 M sodium borate buffer pH 8.0, containing 1% 2-mercaptoethanol, 0.25 M urea, and 0.01 M sodium diethyl dithiocarbamate (NaDIECA). The borate buffer stock solution was made by adding solid NaOH to 1 M boric acid solution to give the desired pH.

(b) The homogenate was filtered through a double layer of cheesecloth.

(c) The sap was centrifuged at 10.000 rpm in the SS-34 rotor for 15 minutes and the pellet discarded.

(d) Triton X-100 was added dropwise to the supernatant, while stirring, to a final concentration of five per cent by volume.

(e) Stirring was continued for 60 minutes, after which the mixture was subjected to a low-speed centrifugation as above (c), and the pellet discarded.

(f) The supernatant was filtered through Miracloth (quick filtration material for gelatinous grindates; Chicopee Mills Inc.) to retain any pieces of pellet present, and centrifuged for 90 minutes at 27,000 rpm in a Beckman type 30 rotor, to pellet the virus.

(g) The pellet was overlaid with 0.1 M borate buffer pH 8.0 and shaken for a few hours.

(h) The resuspended pellets were pooled and centrifuged at 10,000 rpm in the SS-34 rotor for 15 minutes.

(i) The supernatant was layered on a 10 ml layer of sucrose in 0.1 M borate buffer, pH 8.0, in the appropriate tube and centrifuged at 27,000 rpm for 150 minutes in the type 30 rotor.

(j) The pellet was resuspended with 0.1 M borate buffer, pH 8.0.

(k) The resulting suspension was centrifuged at 10,000 rpm for 15 minutes and 1-2 ml of the supernatant was layered onto preformed gradients of 5.2, 11.0, 8.5, 5.8, 4.5, and 1.8 ml of 300, 260, 210, 160, 100, and 0 mg/ml of decolorized sucrose in 0.1 M borate buffer, pH 8.0, respectively (Jackson et al. 1973), and centrifuged for three hours in the Spinco Sw 27 rotor at 24,000 rpm at 3°C. (For comparison of different methods of purification, 0.2 ml of samples was layered onto preformed gradients of 1.7, 3.5, 2.8, 2.1, 1.6, and 1.1 ml of 300, 260, 210, 160, 80, and 0 mg/ml of decolorized sucrose in 0.1 M of the appropriate buffers, respectively, and centrifuged for 1.5-2 hours in the Spinco Sw 40 rotor at 36,000 rpm at 3°C.) All gradients were left for at least 12 hours at 3°C after layering, in order for a proper concentration gradient to form, before sample loading and centrifugation.

(l) After centrifugation, the gradient columns were spectrophotometrically analyzed with an ISCO model D density gradient fractionator coupled to an ISCO model UA5 absorbance monitor (Brakke and Van Pelt 1968). A pump speed of 2.5 ml/minute was routinely used, and the UV analyzer was set at 253.6 nm (the same apparatus and wave length was used for comparison of different methods of purification with a pump speed of 1 ml/minute when SW 40 rotor tubes were

fractionated). After scanning, the virus bands were collected, and after dilution with 0.1 M borate buffer, pH 8.0, they were centrifuged at 27,000 rpm in the type 30 rotor for three hours.

(m) The pellets of relatively pure virus were resuspended in 0.02 M borate buffer, pH 8.0, and kept at 3°C.

UV spectral analysis of virus suspensions was routinely carried out after purification procedures with a Unicam SP 800 A UV spectorphotometer with a 1 cm quartz cell. The concentration of purified virus preparations was determined by measuring the absorbance at 260 nm (A 260) and relating it to the extinction coefficient of 2.76 cm^2/mg, which was determined by the method used by Waterworth and Kaper (1972). The ratio of the absorbance at 280 nm (A 280) divided by A 260 (A 280/A 260) was calculated to determine the relative purity of the virus suspension (Brakke and Van Pelt 1968). No correction for light scattering was made.

The same procedure as method (1) was used with borate buffer having pH values of 8.5 and 8.8 to compare the effect of borate buffer's pH on purification, but the final pellets in each case were resuspended in 0.02 M borate buffer pH 8.0.

Purification of PV with sodium citrate buffer at three different pH values (8.0, 8.5, and 8.8), and potassium phosphate buffer at pH values 7.0, 7.5, and 8.0, were compared with each other, and with other methods. The procedures which were used were the same as method (1) except in each case the density gradient preparation and initial pellet suspension were done in citrate or phosphate buffer. However, the final pellets were resuspended in 0.02 M borate buffer pH 8.0.

2. Purification of PV by precipitation with polyethylene glycol (PEG) and differential and density gradient centrifugation

Initial homogenization and clarification were carried out as described in steps (a) to (d) under method (1).

(e) The virus was precipitated from the clear supernatant by dissolving five per cent (W/V) PEG (mol weight 6000), and the solution was incubated with stirring at 3°C for 2-3 hours.

(f) The precipitate was collected by low-speed centrifugation for 15 minutes at 10,000 rpm.

(g) The pellets were resuspended with 0.1 M borate buffer, pH 8.0, and steps (h) to (m) of method (1) were repeated.

3. Purification of PV by n-butanol clarification in borate buffer pH 8.0

Steps (a) to (c) were the same as mentioned in method (1).

(d) n-butanol was added dropwise to the supernatant while stirring to a final concentration of seven per cent (v/v) and stirring was continued for two hours.

(e) The mixture was subjected to a low-speed centrifugation for 15 minutes at 10,000 rpm, and the pellet discarded.

(f) Steps (f) to (j) of method (1) were repeated.

(g) The resulting suspensions were green and contaminated with host materials, so 0.2 ml of the samples were layered onto preformed gradients and centrifuged in the Spinco SW 40 rotor at 36,000 rpm for 1.5 hours.

(h) After centrifugation the gradient columns were scanned with the ISCO apparatus, as previously described.

4. Purification of PV by chloroform in borate buffer pH 8.0

All steps were the same as mentioned for method (3), except in step (d), instead of n-butanol, 7 per cent (v/v) chloroform was used.

5. Purification of PV by n-butanol-chloroform in borate buffer pH 8.0

Again the same procedure as mentioned in method (3) was used, except in step (d) instead of n-butanol, 7 per cent (v/v) 1:1 n-butanol: chloroform was used.

H. Extinction Coefficient

The extinction coefficient of PV was calculated using dry weight and spectrophotometry data from two experiments. This was done by centrifugation of about 10 mg of purified virus in 0.02 M borate buffer pH 8.0 at 10,000 rpm for 15 minutes immediately before the spectrophotometry, for removal of any aggregated virus. A small amount of the resulting supernatant was read at several dilutions at 260 nm without further correction for light scattering. The rest of the virus suspension in two ml borate buffer was evaporated to dryness in a Fisher Vacuum Oven at 90°C and then dried to constant weight and corrected for dry weight of the buffer (Waterworth and Kaper 1972).

I. Estimation of Sedimentation Coefficient (S20,W) of PV by means of Linear-log Sucrose Gradient (Brakke and Van Pelt 1970a)

The Spinco rotor model SW 40 was used for centrifugation; linear-log density gradients for the SW 40 rotor were prepared as previously

described (p. 29). Phosphate buffer, 0.02 M, pH 6.25, was used as the sucrose solvent, and the prepared tubes were kept at least twelve hours at 3°C to form a smooth gradient by diffusion.

Purification schedules were performed for tobacco mosaic virus (TMV) according to Brakke and Van Pelt (1969); Southern bean mosaic virus (SBMV) by Chenulu et al. (1968); Brome mosaic virus (BMV) by Brakke and Van Pelt (1970a), and PV according to the purification method (1) described previously.

After the gradient had stood overnight, 0.2 ml was removed from the top of the column just before 0.2 ml of sample solution was floated on the top of the column. The final concentration of each virus on the top of the column was about 0.1 mg. Then the tubes were placed in the cold rotor and centrifuged at 36,000 rpm in a Beckman model L2-65B ultra centrifuge for 90 minutes. Following centrifugation, the tubes were scanned with the previously described ISCO apparatus, and the depth to which each virus had sedimented was measured by ruler measurements of the distance between the meniscus and the center of the virus zones recorded on the fractionator chart. The log of sedimentation was plotted against the log of depth, and according to this line the sedimentation value of unknown virus was measured.

J. Analysis of Virus Components

1. Ribonucleic acid (RNA)

i. Cesium chloride (CsCl) density gradient

Equilibrium centrifugation was performed in a swinging bucket rotor, SW65K, in a Beckman L2-65B Ul centrifuge, using CsCl gradients prepared according to a procedure given by Sehgal et al. (1970). Two and a half ml of CsCl solution of buoyant density (ρ) = 1.5004 (about 6.753 g CsCl per 8.25 ml of buffer) was introduced into a cellulose nitrate tube and an equal volume of CsCl, (ρ) = 1.241 (about 2.973 g CsCl per 8.03 ml of buffer) was carefully layered on the denser solution. All CsCl solutions were prepared in 0.01 M potassium phosphate buffer pH 6.5. One-fifth ml of each of the virus preparations, purified as mentioned previously, at a concentration of about 0.5 mg/ml, were layered on the top of the CsCl gradients, and centrifuged for 21 hours at 40,000 rpm.

At the end of the centrifugation, the depth of the sedimentation of the viruses was observed and measured visually from the center of the sample at the meniscus to the center of the virus zone.

Before the bottom of the centrifuged tubes were pierced with a needle, the top of each tube was fixed with a stopper in which a needle was inserted to make it possible to control the number of drops per fraction. Three-drop fractions were collected, and the refractive index of each fraction was determined with a Carl Zeiss Abbe Refractometer at room temperature. The ρ of each fraction was

determined according to the table in "Handbook of Biochemistry," 2nd edition, p. j-292 - j-296. Then all fractions were diluted with 0.01 M phosphate buffer pH 6.5 for a spectrophotometric determination of their optical densities at 260 nm. Values were recorded and correlated with ρ values.

RNA content of viruses was estimated according to the following formula (Sehgal et al. 1970).

$$\text{RNA content} = 3.88462 + \frac{\sqrt{(0.00101)^2 - (0.00052)(1.31749 - \rho)}}{0.00026}$$

ii. RNA Extraction

RNA was extracted from BMV, SBMV, TMV, and PV, purified as described, according to the general methods of Ralph and Bergquist (1967), with minor changes, for gel electrophoresis. Five ml of virus suspensions were mixed with 5 ml 80 per cent phenol and 1 ml of 10 per cent sodium dodecyl sulfate (SDS), shaken for 15 minutes, and then the mixtures were put in ice for 20 minutes. One low-speed centrifugation at 10,000 rpm for 10 minutes separated the phenol phase, which contained denatured protein, from the aqueous phase. The aqueous upper phase was removed with a syringe, and the RNA was precipitated directly from it by addition of two volumes of 95 per cent ethanol and a few drops of 1 M sodium acetate at 2°C. The mixtures were stored in the freezer for 20 minutes, and the precipitate was recovered by a low-speed centrifugation for 10 minutes. After the supernatants were discarded, the tubes were put upside down on "Kimwipes" tissues in the freezer to evaporate

all alcohol from the pellet surface. The pellets were resuspended in 0.5 ml of 0.01 M Tris-HCl, 0.01 M potassium chloride, 0.001 M $MgCl_2$, pH 7.4 (TKM buffer), and dialyzed against TKM overnight.

An ammonium carbonate-phenol extraction (Brakke and Van Pelt 1970b) method was used to prepare RNA for linear-log density gradient, formaldehyde treatment, nuclease treatment, and infectivity assay. In this method one volume of purified virus (BMV, TMV, and PV) was added to an equal volume of RNA extraction buffer, pH 9.0, containing 0.2 M ammonium carbonate, 0.002 M Na2EDTA, 0.2 per cent NaDIECA, 2.0 per cent SDS, and about 200 µg/ml bentonite and kept at 3-4°C for one hour. Two volumes of water saturated phenol with 0.1 per cent 8-hydroxyquinoline were added to the virus-buffer mixture, which was stirred for ten minutes. The top aqueous layer was removed after a low-speed centrifugation, and bentonite was added to 100 µg/ml. The nucleic acid was then precipitated by adding two volumes of 95 per cent isopropanol and 0.1 volume of 1.0 M sodium acetate, pH 5.5. After storage at -20°C for three hours, the mixture was centrifuged at 10,000 rpm for 15 minutes and the pellets were suspended in 0.015 M sodium citrate buffer, pH 7.0, containing 0.15 M sodium chloride (SSC). After precipitation with one volume of isopropanol, pellets obtained by centrifugation were washed twice with isopropanol to remove traces of phenol. The final pellets were suspended in 0.035 M dibasic potassium phosphate, and 0.05 M glycine, pH 9.2.

As a necessary precaution to destroy or inactivate the ribonucleases (RNase), all glassware was autoclaved before use and all buffers made in sterilized water.

The bentonite used in this experiment was treated with Na_2EDTA according to Fraenkel-Conrat et al. (1961). Procedure: 2g of commercial bentonite (Fisher Scientific Company, Fair Lawn, New Jersey) were suspended in 40 ml of water and centrifuged at 2500 rpm for 15 minutes; the supernatant was recentrifuged at 8500 rpm for 20 minutes. The sediment from the centrifugation at 8500 rpm was resuspended and held in 0.1 M pH 7.0 Na_2EDTA for 48 hours at 25°C, and then again centrifuged differentially. The 8500 rpm sediment was suspended in 0.01 M pH 6 acetate, again centrifuged at 8500 rpm, and taken up in the acetate to a concentration of 1.5-6 per cent as determined by dry weight.

(a) Polyacrylamide-gel preparation

To make 3% acrylamide gel, 15 g and 0.75 g, and for 10 per cent gel, 22.2 g and 0.6 g of acrylamide and methylene-bis-acrylamide, respectively, were dissolved in water to give 100 ml of solution. Insoluble material was removed by filtration through Whatman no.2 filterpaper. The solutions were kept at 4°C in a dark bottle. The glass gel tubes used were approximately 10 cm long with an inner diameter of 6 mm. For a typical run of twelve 3 per cent gels for viral RNA's, 6.67 ml of tris buffer [1.45 g tris hydroxymethyl amino-ethane (THAM), 0.82 g sodium acetate, 3 H_2O, and 0.11 g Na_2EDTA per

100 ml of distilled water, adjusted to pH 7.2 with acetic acid], were mixed with four ml of acrylamide-bis mixture, 9.11 ml distilled water, 0.2 ml of 10 per cent freshly made ammonium persulfate solution, and 0.045 ml of N,N,N',N'-tetramethylethylene diamine (TEMED) (Adesnik 1971). After mixing, each tube was filled with about two ml of the solution. Before the gel hardened, a few drops of water were layered on top of the gel solution. After 10-20 minutes an interface could be seen, indicating that the gel had solidified.

To make 10 per cent gel for protein electrophoresis, the procedure was the same as used for three per cent gel, except in this procedure the gel buffer contained 7.8 g $NaH_2PO_4-H_2O$, 38.6 g of $Na_2HPO_4-7H_2O$, and 2 g of SDS per liter (Weber and Osborn 1969). Thirteen and a half ml of acrylamide solution (made for 10 per cent gel), 15 ml of gel buffer, 1.5 ml of freshly made ammonium persulfate solution (15 mg/ml) and 0.045 ml of TEMED were mixed and used for a typical run of 12 gels.

For RNA electrophoresis, the chamber buffer was tris buffer (the same buffer used for gel preparation) diluted three times with distilled water. The power supply (ISCO model 490) was adjusted so that a constant current of approximately 6-8 milliamperes per gel (which usually involved running at about 60 volts) was applied for 30 minutes before the application of the RNA samples. The samples were prepared by adding ribonuclease-free sucrose to a final concentration of five per cent. Samples of 0.03-0.05 ml, having a concentration of about 1.0 OD, were layered on the top of the gels. As a control, one tube was run with bromophenol blue dye.

Electrophoresis was continued until the control dye released from the anode and then the power was cut off. The gels were removed from the tubes by "rimming" with a 22-gauge syringe needle attached to a water supply so that a fine water stream lubricated the gel tube interface.

For staining, the gels were immersed in the tubes containing 0.3-0.5 per cent toluidine blue (Adesnik 1971) for about three hours until the visible RNA bands appeared in the gel. Distances of the bands from the top of the gels were measured and recorded.

(b) Linear-log density gradient
(Brakke and Van Pelt 1970b)

"Linear-log" gradients were made for RNA according to the pipetting formula of Jackson et al. (1973): 2.1, 3.7, 2.6, 1.7, 1.3, and 1.4 ml of 325, 270, 210, 160, 100, and 0 mg/ml of decolorized sucrose, respectively, dissolved in SSC, were layered in SW 40 cellulose nitrate tubes, and the procedure described previously for intact viruses was carried out. After the tubes were centrifuged at 6°C for five hours at 36,000 rpm, the gradients were fractionated with an ISCO apparatus. BMV- and TMV-RNA standards for S-value estimation, were prepared according to the same method described for PV-RNA extraction. During fractionation, the PV-RNA band was collected for infectivity assay on Gomphrena globosa.

(c) Formaldehyde treatment
(Boedtker 1968)

The formaldehyde treatment used by Boedtker (1968) was used to enable a more accurate estimation of the RNA molecular weight by centrifugation in linear-log density gradients. Thirty-seven per cent reagent grade formaldehyde was diluted to 30 per cent by adding one-fifth volume of 0.45 M Na_2HPO_4 + 0.05 M NaH_2PO_4; one volume of the latter was then added to nine volumes of RNA solution. The resultant solution, which was 1.1 M formaldehyde and 0.09 M Na_2HPO_4 + 0.01 M NaH_2PO_4, was heated to 63°C for 15 minutes, cooled rapidly in ice water and used for density gradient centrifugation immediately.

The preparation and centrifugation of linear-log density gradients were the same as described in the previous experiment (b)

(d) Nuclease treatment
(Makkouk and Gumpf 1974)

PV-RNA preparations were incubated for 30 minutes at 37°C with 3 µg/ml of RNase, and the same treatment was done with DNase at the concentration of 50 µg/ml. As a control, instead of nuclease, the same amount of distilled water was added to the RNA preparation.

After incubation for 30 minutes, one part of the preparation was used for linear-log density gradient as described previously in experimental procedure (b). The remainder of the sample, after the addition of about one per cent celite, was inoculated to Gomphrena globosa for infectivity assay, which was carried out according to the procedures described by Brakke and Van Pelt (1970b). The finger was

dipped in a bentonite suspension, then in the nucleic acid solution, and leaves of the assay plant (Gomphrena globosa) were rubbed. The nucleic acid solution contained a low concentration of bentonite (about 100 µg/ml) and one per cent suspended celite.

2. Protein

i. Sample preparation and electrophoresis (Hill and Shepherd 1972)

The molecular weight of the PV-protein subunit was estimated by its comparison with five proteins of known molecular weight as the standards. These proteins included: TMV-protein subunit, alcohol dehydrogenase, myoglobine, ovalbumin, and carbonic anhydrase.

All proteins were prepared for electrophoresis by dissolving weighed amounts of non-viral proteins and virus concentration by certain OD (viral proteins) in 0.01 M phosphate, pH 7.2, containing one per cent SDS, and one per cent 2-mercaptoethanol. The protein-SDS solutions were then heated for one minute at 100°C. Approximately 50-100 µg of each sample was mixed with one drop of glycerol and one drop of aqueous 0.05 per cent bromophenol blue and applied to the top of the gels, prepared as previously described in (a) with a micropipet. The chamber buffer, which was the same as the gel buffer, was diluted 1:1 with distilled water, and one per cent 2-mercaptoethanol was added.

Electrophoresis was carried out at eight mA/gel until the bromophenol tracking dye approached the opposite end of the gel. The migration distance of the tracking dye and length of the gels were measured.

ii. Staining and destaining
(Hill and Shepherd 1972)

For staining the gels were immersed in 0.05 per cent aniline blue black in 15 per cent acetic acid and 50 per cent ethanol overnight. The gels were then washed successively with seven per cent acetic acid for one hour, seven per cent acetic acid in 50 per cent ethanol until the protein bands were evident, and seven per cent acetic acid in 25 per cent ethanol until the remaining dye was removed. Migration distance of the stained protein components and gel length were then measured and the relative electrophoretic mobility was calculated in the manner of Weber and Osborn (1969), and the relative mobility of proteins in the gel was plotted against the logarithm of the protein molecular weight. Gels were stored in seven per cent acetic acid in 25 per cent ethanol.

K. Serology

1. Antiserum preparation

Antiserum to PV was prepared in two rabbits through a series of intramuscular injections with a known quantity of purified PV in 0.02 M borate buffer, pH 8.0. The virus suspension was emulsified with an equal volume of Freund's incomplete adjuvant (Ball 1964). Rabbits were injected at weekly intervals for four weeks (3, 4, 4, and 4 mg of virus respectively). Prior to the first injection, normal serum was prepared from blood obtained by bleeding each rabbit from the marginal ear vein. The blood was collected in a plastic petri dish, incubated at 4°C for

12 hours, and the serum was collected and clarified by centrifugation for 15 minutes in an International Clinical Centrifuge. The serum was stored in 1-2 ml aliquots at -20°C with 0.02 per cent sodium azide added as preservative.

Bleedings for immune serum were made about two weeks after the final injection. It was collected as described for normal serum and titrated by the microprecipitin technique of Van Slogteren (1954), reacting one drop aliquots of a two-fold dilution series of antiserum against one drop aliquots of a two-fold dilution series of the virus (1.0 mg/ml) (Ball 1961).

2. The microprecipitin test

The microprecipitin test of Van Slogteren (1954) was used as a diagnostic method to establish the presence or absence of PV in clarified expressed sap of infected leaf tissue and to measure the titer of the antisera. Usually dilution titrations were performed in a grid pattern (Ball 1961). Small droplets of two-fold dilutions of the antiserum were dispensed with a micropipet and placed on the bottom of a plastic petri dish. Equally small droplets of two-fold dilutions of the antigen were added to the antiserum droplets and mixed. The bottom of the dish and the droplets were then covered with white light paraffin oil (Saybolt viscosity 125/135) to prevent evaporation. The plates were incubated for one hour at room temperature and examined by a dark field binocular microscope for the presence of a precipitate, indicating a positive antigen-antibody reaction. A confirmatory reading was made after the plates had been stored overnight at room temperature.

Testing of each antigen-antibody system was done by reacting the antigen with normal serum and clarified sap of healthy plants with immune serum as controls. Dilutions of antigens and antisera were made in 0.05 M Tris-HCl pH 7.1, containing 0.85 per cent NaCl. Clarification of the plant sap was accomplished by homogenizing the leaf tissue in 0.05 M Tris-HCl, pH 7.1 (1 g tissue/1 ml buffer). The homogenate was placed in a water bath at 40°C for one hour, then centrifuged for 20 minutes at 10,000 rpm to remove denatured host proteins and debris. The supernatant was pipetted off and used immediately.

3. The Ouchterlony double diffusion test

PV antiserum was prepared according to the method discussed previously, and the same method was used to prepare potato virus Y (PVY) antiserum. PVX (PV.As #47), PVS (PV.As #43), cactus virus X (CVX) (PV.As #79), clover yellow mosaic virus (CYMV) (PV.As #56), and tobacco etch virus (TEV) (PV.As #87) antisera, and TEV (PV.69), and mild strain of PVX (PV.179), were obtained from the American Type Culture Collection (ATCC). Antisera to carnation vein mottle virus (CVMV) was kindly supplied by Dr. M. Hollings of the Glasshouse Crops Research Institute, Sussex, England.

PV was grown on tomato plants; TMV on Nicotiana tabacum var. Turkish; TEV on N. glutinosa; PVX on N. tabacum, N. rustica; and PVY on N. tabacum var. White burley. All mentioned viruses were purified partially by grinding the frozen tissues with an equal weight of 0.8 per cent NaCl in potassium phosphate buffer, pH 7.0 (V/W), filtration

through a double layer of cheese cloth, and incubation for one hour at 40°C in the water bath. After a differential centrifugation, the high-speed pellets were resuspended in 0.02 M borate buffer, pH 8.0. The resuspended pellets were given a low-speed centrifugation for ten minutes at 10,000 rpm and the supernatants were collected and used for this experiment. The same method was applied for healthy tomato leaves used as a control.

Immunodiffusion plates were prepared by pouring 2.5 ml of agar solution into each quadrant of a quadrant-type plastic petri dish. The agar mixture consisted of 0.8 per cent Difco special agar-Noble, 0.2 per cent SDS, and 0.1 per cent sodium azide in distilled water (Gooding and Bing 1970) which was heated to about 90°C for 30 minutes in the water bath. The degrading agents (SDS and sodium azide) were added to the warm agar (60°C) just before it was pipetted into the diffusion chamber. These were left level on a table for a few minutes for gelation to occur, and then wells were cut with a no. 2 cork borer (5.5 mm) in a hexagonal arrangement such that the center of each well was 8-10 mm from the center of a central well and the distance between the centers of the neighbouring peripheral wells was 8-10 mm.

Usually the central well was charged with antiserum and the peripheral wells were charged with the partially purified viruses, and the plates were incubated for 10-12 hours in a moist chamber at room temperature. The plates were then examined for the presence of a visible precipitation line, in indirect light against a dark background, in a laboratory-constructed cardboard device.

EXPERIMENTS AND RESULTS

A. Host Range and Symptomatology

Plantago major L., the apparent natural host, infected with PV under natural conditions, showed general mottling and leaf malformation. The same symptoms were observed under greenhouse conditions (Figure 1-A). Eighty-five other species and varieties of plants from ten different families were inoculated with PV to determine its host range. Among these plants, 30 species and varieties from seven families showed symptoms (Table 1), and 56 species and varieties out of six families did not show any symptoms (Table 2). Extracts from most of the symptomless plants were back-inoculated to assay plants (Gomphrena globosa) or the natural host (Plantago major) to detect possible symptomless infections, but none of them showed any reaction.

Most of the plants in Chenopodiaceae showed local lesion symptoms, mainly necrotic local lesions (Figure 1-E and 1-F). Some beet varieties showed ringspot symptoms on inoculated leaves and some were not a host of the virus. One species (Chenopodium capitatum) showed systemic vein clearing, mottling, followed by vein necrosis and death of the plant, while Chenopodium foetidum was not a host of the virus. In Amaranthaceae, Gomphrena globosa reacted with necrotic local lesions (Figure 1-C and 1-D) and Amaranthus retroflexus showed primary necrotic local lesions and vein necrosis, which a few days later,

TABLE 1. Symptom development on various host plants after inoculation with PV

Scientific name	Common name	Family	Incubation period* (days)	Description of symptoms
1. *Amaranthus retroflexus***	Russian pigweed	Amaranthaceae	8	Necrotic local lesion, and vein necrosis. After a few days the inoculated leaves dropped down while they were still fresh and green. A few days later, systemic vein necrosis was developed.
2. *Gomphrena globosa*	Globe-amaranth	Amaranthaceae	3	Necrotic local lesions; at first they were small, but after 2 days they enlarged, with a red margin.
3. *Dianthus barbatus***	Carnation	Caryophyllaceae	20-23	Mottling and proliferation.
4. *Beta vulgaris*	Beet	Chenopodiaceae	6	
var. Betteraves red pack	"	"	"	Ringspot symptoms, with reddish margins, on inoculated leaves.
5. var. Betteraves ruby queen**	"	"	"	Ringspot symptoms, with reddish margins, on inoculated leaves.
6. var. Improved dark red**	"	"	"	Reddish chlorotic spots, and ringspots with reddish margins on inoculated leaves.
7. var. I07 Detroit red	"	"	"	Reddish chlorotic spots on inoculated leaves.

TABLE 1 (continued)

Scientific name	Common name	Family	Incubation period* (days)	Description of symptoms
8. Beta vulgaris**	Sugarbeet	Chenopodiaceae	6	Reddish chlorotic spots, some of which then changed to necrotic lesions on inoculated leaves.
9. Chenopodium album	-	"	5	Papery necrotic local lesions; after a few days the inoculated leaves dropped down while they were green and fresh.
10. C. amaranticolor	-	"	4-5	Small necrotic local lesions which enlarged after a few days.
11. C. capitatum**	-	"	6	First symptom was chlorotic local spotting, and after a while the inoculated leaves dried out and dropped. Then the symptoms changed to systemic vein clearing, mosaic and after a few days vein necrosis and the whole plant dried out.
12. C. quinoa	-	"	4	Chlorotic local lesions which changed to necrosis few days later.
13. Spinacea oleracea	Spinach	"	7-8	
var. Epinard America**	"	"	"	Chlorotic local lesions which changed to necrotic lesions few days later.

TABLE 1 (continued)

Scientific name	Common name	Family	Incubation period* (days)	Description of symptoms
14. Spinacea oleracea	Spinach	Chenopodiaceae	7-8	
var. Epinard North Land**	"	"	"	Chlorotic local lesions which changed to necrotic lesions few days later.
15. var. Hyb.530**	"	"	"	Chlorotic local lesions which changed to necrotic lesions few days later.
16. Tetragonia expansa	New Zealand spinach	Ficoidaceae	5	Chlorotic local lesions.
17. Medicago sativa**	Alfalfa	Leguminosae	13-15	Very mild vein clearing which changed to mild mosaic.
18. Plantago major L.	Broad-leaved plantain	Plantaginaceae	10-12	Vein clearing and mottling, developing into leaf malformation.
19. Datura metel**	-	Solanaceae	11-13	Systemic chlorotic lesions.
20. D. stramonium**	-	"	11	Chlorotic local lesions.
21. Lycopercicum esculentum	Tomato	"	8-10	
var. Asgrow scarlet dawn**	"	"	"	Mottling and leaf malformation.
22. var. Armstrong W218**	"	"	"	" " " "

TABLE 1 (continued)

Scientific name	Common name	Family	Incubation period* (days)	Description of symptoms
23. Lycopercicum esculentum	Tomato	Solanaceae	8-10	
var. Early chateau**	"	"	"	Mottling and leaf malformation.
24. var. Fire ball**	"	"	"	" " " "
25. var. Glamor**	"	"	"	" " " "
26. var. John Bear	"	"	"	" " " "
27. var. Jubilee**	"	"	"	" " " "
28. var. Michigan State porume	"	"	"	" " " "
29. var. Pink Gulf State	"	"	"	" " " "
30. var. Rideau**	"	"	"	" " " "

*Denotes the time of first symptom appearance.

**Reinoculated to Gomphrena globosa to detect the presence of PV.

TABLE 2. List of non-host plants inoculated with PV

	Scientific name	Common name	Variety	Family
1.	Beta vulgaris	Beet	I07 A	Chenopodiaceae
2.	" "	"	Swisschard	"
3.	Chenopodium foetidum*	-	-	"
4.	Citrulus vulgaris	Watermelon	Charlston gray	Cucurbitaceae
5.	Cucurbita pepo*	Squash	Butternut	"
6.	Cucumis sativus*	Cucumber	-	"
7.	Avena sativa	Oats	Clintland	Gramineae
8.	" "	"	Roxton	"
9.	Hordeum vulgare	Barley	Betzes	"
10.	" "	"	Champlain	"
11.	" "	"	Hypana	"
12.	" "	"	Montcalm	"
13.	" "	"	Moreval	"
14.	Triticum aestivum	Wheat	Cornell	"
15.	" "	"	Winter wheat	"
16.	Zea mays	Sweet corn	-	"
17.	Glycine max*	Soybean	Harosoy	Leguminosae
18.	Phaseolus aureus	Mungbean	-	"
19.	Phaseolus vulgaris	French bean	Black valentine*	"
20.	" "	" "	Bountiful*	"
21.	" "	" "	Contender	"
22.	" "	" "	Early waxbean	"
23.	" "	" "	Highlander	"
24.	" "	" "	Kentucky wonderwax	"
25.	" "	" "	Likalake*	"
26.	" "	" "	Pinto*	"
27.	" "	" "	Slender green*	"
28.	" "	" "	Sprite	"

TABLE 2. (continued)

	Scientific name	Common name	Variety	Family
29.	Pisum sativum	Pea	Alaska*	Leguminosae
30.	" "	"	American wonderpea*	"
31.	" "	"	Thomas Laxton	"
32.	" "	"	Laxton's progress	"
33.	" "	"	Little wonder	"
34.	Sesbania exaltata	-	-	"
35.	Trifolium pratense	Red clover*	-	"
36.	Trifolium repens	White clover*	-	"
37.	Vicia faba	Broad bean*	-	"
38.	Vigna sinensis	Cow pea*	-	"
39.	Fagopyrum esculentum	-	-	Polygonaceae
40.	Capsicum annum	Pepper	Sweet pepper	Solanaceae
41.	Nicotiana glutinosa	Tobacco*	-	"
42.	Nicotiana rustica	" *	-	"
43.	Nicotiana tabacum	"	Haranova	"
44.	" "	"	Haranova T	"
45.	" "	"	Havana*	"
46.	" "	"	Havana 38*	"
47.	" "	"	Havana 425*	"
48.	" "	"	Samsun*	"
49.	" "	"	Sylvestris	"
50.	" "	"	Turkish*	"
51.	" "	"	White burley*	"
52.	" "	"	Xanthi*	"
53.	Petunia hybrida*	-	-	"
54.	Solanum dalcamara*	-	-	"
55.	Solanum nigrum*	-	-	"
56.	Solanum tuberosum*	Potato	-	"

*Reinoculated to Gomphrena globosa to detect the presence of PV.

Figure 1-A. Systemic symptoms of PV on detached leaves of Plantago major.
Right: infected leaf; left: healthy leaf.

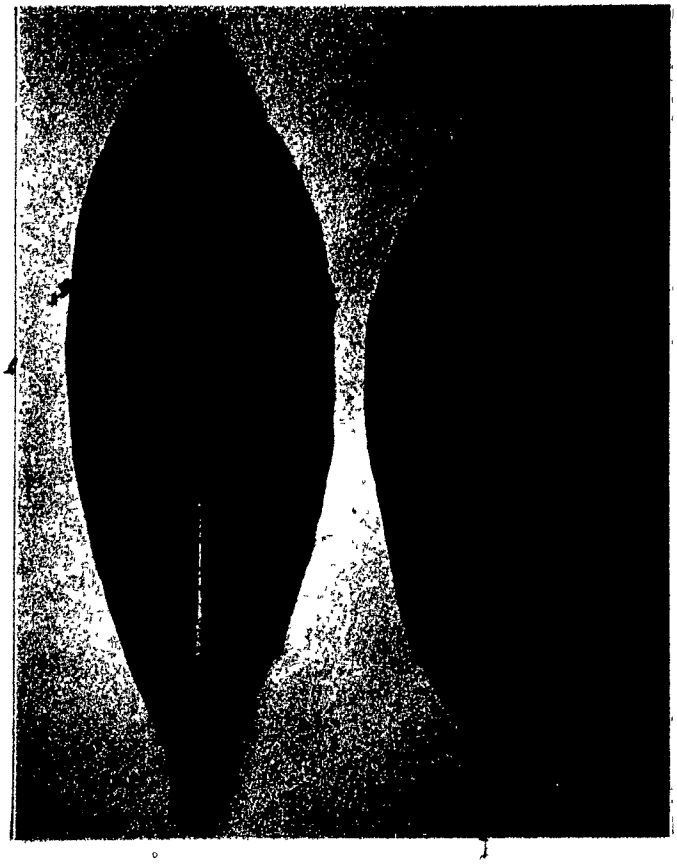
Figure 1-B. Systemic symptoms of PV on detached leaves of tomato.
Right: infected leaf; left: healthy leaf.

1-A

1-B

Figure 1-C. Local lesion symptoms of PV on detached leaves of Gomphrena globosa.
Right: healthy leaf; left: infected leaf.

Figure 1-D. Detached leaves of Gomphrena globosa showing local lesion symptoms caused by PV.

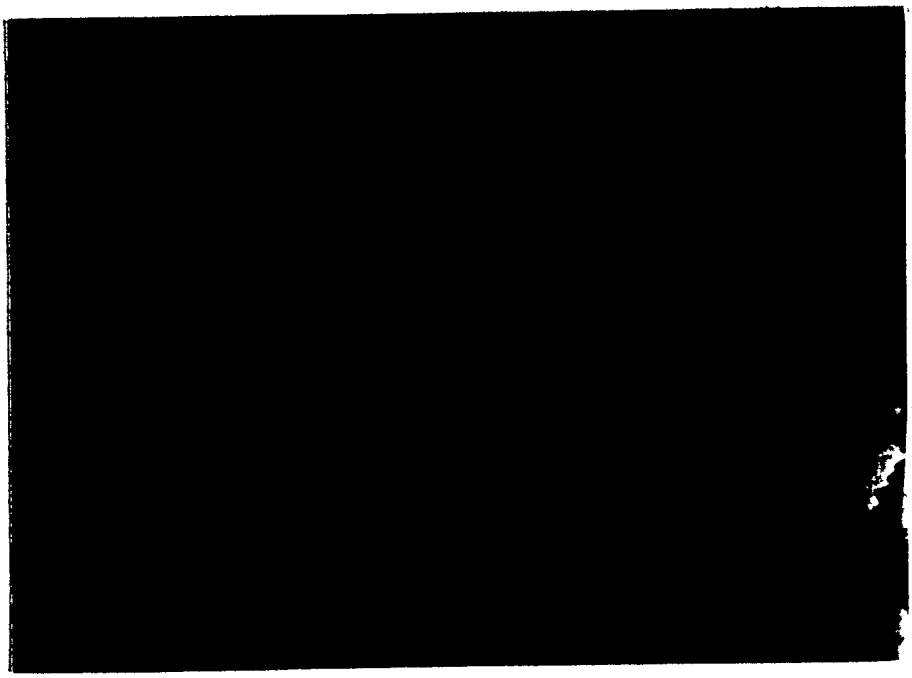


1_C

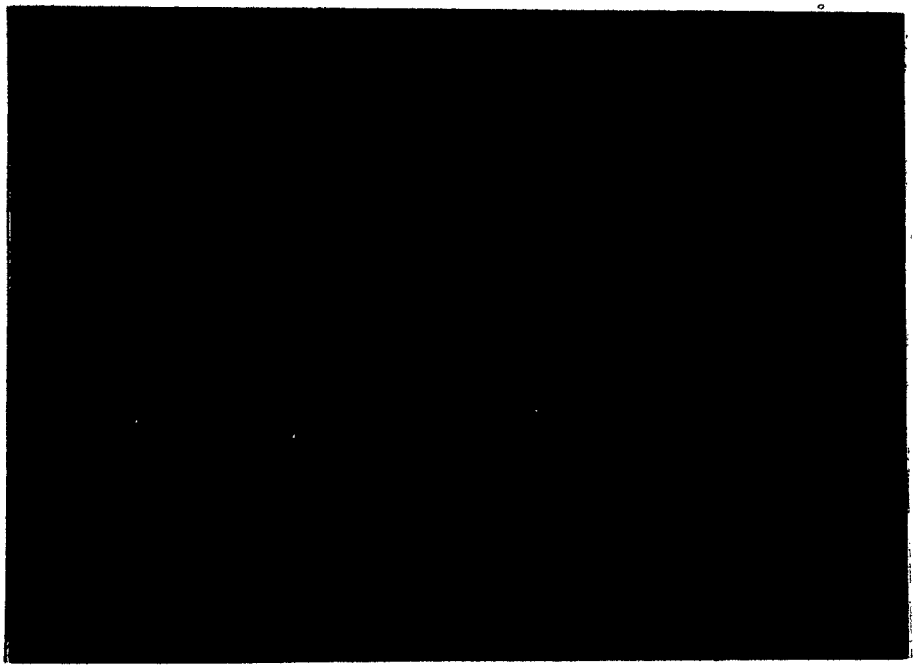
1_D

Figure 1-E. Local symptoms caused by PV on detached leaves of Chenopodium amaranticolor.
Right: healthy leaf; left: infected leaf.

Figure 1-F. Local lesion symptoms caused by PV on detached leaves of beet (Improved dark red).

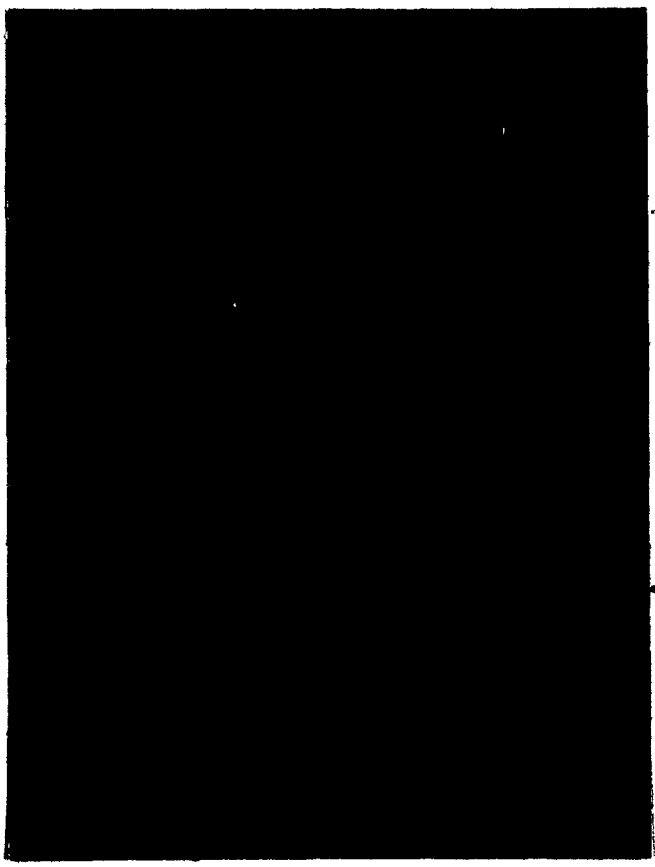


1-E



1-F

Figure 1-G. Detached leaves of Dianthus barbatus showing systemic mottling symptoms of PV.



1_G

developed into the systemic vein necrosis symptom. From Caryophyllaceae, Dianthus barbatus (carnation) showed systemic mottling and proliferation symptoms (Figure 1-G), and in Ficoidaceae, Tetragonia expansa showed chlorotic local lesions. Only Medicago sativa from the Leguminosae family showed very mild vein clearing and mild mosaic symptoms; the rest of the species and varieties which were tested did not show any symptoms and were not hosts of PV. From Solanaceae, Datura stramonium reacted with chlorotic local lesions, Datura metel with systemic chlorotic lesions and all varieties of tomato which were tested reacted with systemic mottling and malformation (Figure 1-B), but none of the tobacco varieties, potato, or pepper, showed any symptoms. The plants from Cucurbitaceae, Gramineae, and Polygonaceae which were tested were not hosts for PV.

B. Physical Properties of PV in Plant Sap

1. Thermal inactivation point

Results obtained for the thermal inactivation point studies are presented in Table 3. At 70°C several local lesions were observed on test plants but not at 75°C.

2. Dilution end point

Young Chenopodium amaranticolor plants with about 10-12 leaves were used as the local lesion hosts. In all treatments the same number of leaves per plant (four leaves) were inoculated, and lesions were counted eight days after inoculation.

TABLE 4. Infectivity dilution end point of the PV in crude extracts of infected tomato

Dilution of crude sap	Number of local lesions per plant
10^{-1}	34
10^{-2}	21
10^{-3}	6
10^{-4}	5
10^{-5}	2
10^{-6}	0
10^{-7}	0
10^{-8}	0

TABLE 3. Thermal inactivation point of PV

Temperature °C	Number of local lesions			
	Test plants		Control	
	1	2	1	2
45	79	232	32	322
50	24	343	36	308
55	95	315	38	206
60	24	217	37	304
65	28	31	28	184
70	1	9	47	406
75	0	0	47	304
80	0	0	30	183
85	0	0	42	316

Table 4 shows the results obtained in the infectivity dilution end point studies of PV. Sap was still infectious at a dilution of 10^{-5} but no lesions were formed at 10^{-6}.

3. Particle length

Particle length was measured from electron microscope photography of 255 particles. Particles were prepared for electron microscopy by the leaf-dip method, followed by conventional negative staining procedures. Electron micrographs of preparations from leaf-dip technique (Figure 2) showed flexuous rod-shaped particles measuring about 202-648 nm in length. The length of 90 per cent of 254 measured particles ranged between 567 and 627 nm. The histogram (Figure 3) of 234 particles in the 546-648 nm range, which represented 93.5 per cent of the population sampled, showed the modal length to be 607 nm.

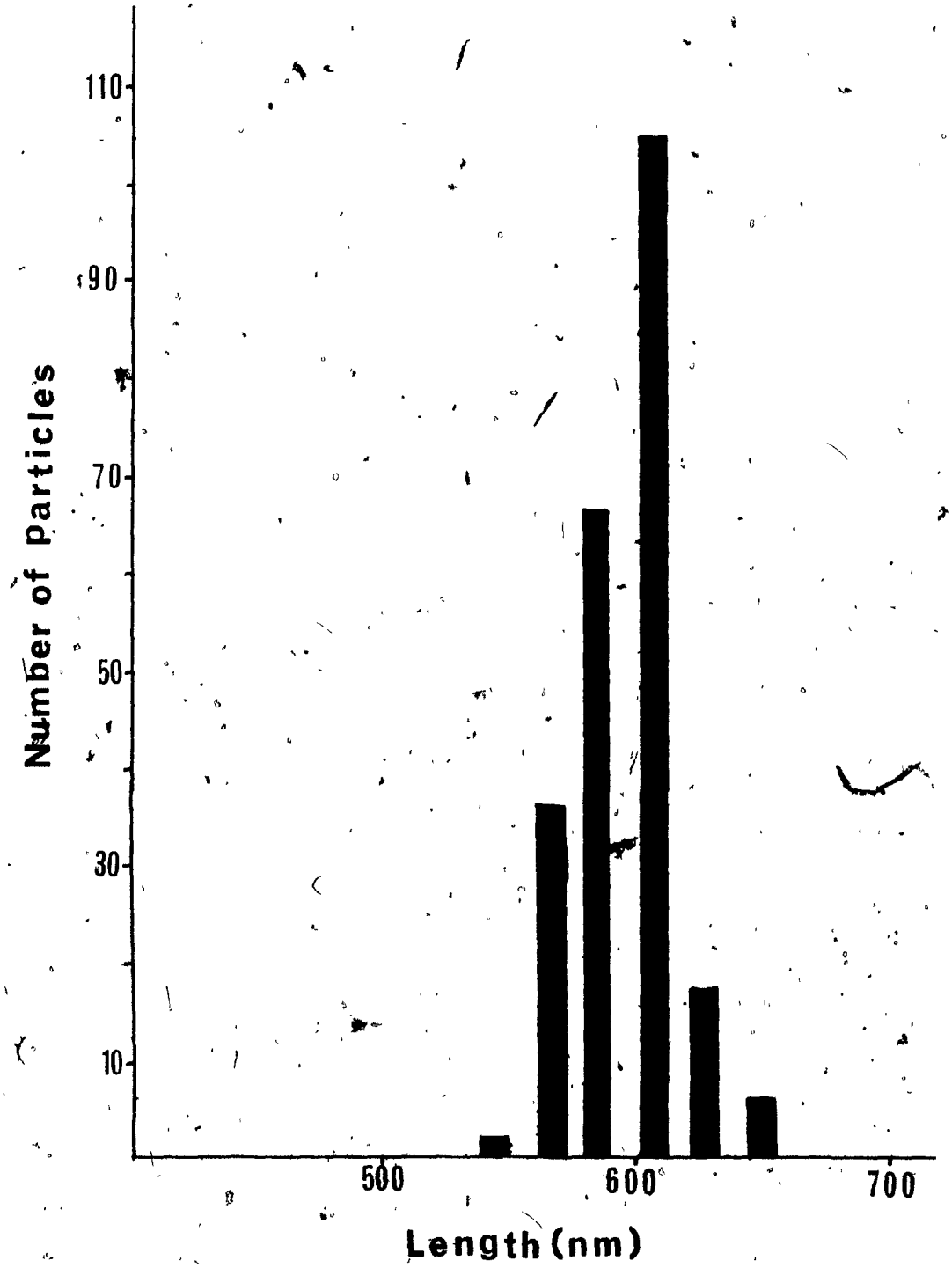
C. Assessment of Purification Procedure

1. Purification of PV by Triton X-100 clarification in borate buffer pH 8.0 and differential and density gradient centrifugation

For the purpose of obtaining a sufficient quantity of partially purified virus, to be used as the injected antigen for antiserum production, and for determination of some other physicochemical properties of PV, this procedure was found the most satisfactory of several methods investigated. Virus yield from infected tomato plants was relatively high in concentration, averaging 200-260 mg/kg of leaves. After stirring for 1-2 hours following the addition of

Figure 2. Electron micrograph of PV particles, prepared by leaf-dip method and negatively stained with two per cent phosphotungstic acid.
Magnification approximately 21700 X.

Figure 3. Histogram showing the length distribution of 234 PV particles in the 567-627 nm range.


110
90
70
50
30
10
Number of Particles
500
600
700
Length (nm)

Triton X-100 (steps d and e in the materials and methods) the first high-speed pellets were clear and transparent. The aggregation problem was very much decreased, compared with other methods, and the low-speed pellet after resuspending the first high-speed pellet contained relatively little infectivity, probably due to virus aggregates that were not broken up by detergent.

Different molarities and pH values of borate buffer were used for homogenizing tissue and tested for their effect on the loss of virus during low-speed centrifugation. Half molar borate buffer pH 8.0 gave the best result for clarification.

The sucrose density gradient centrifugation was used as a tool for assessing the relative state of aggregation of the virus, and the homogeneity of the preparation. After centrifugation of PV for 2.5 hours in the SW 27 rotor at 24,000 rpm, a visible band was located approximately 1.9-4.5 cm from the meniscus. Infectivity was mostly associated with this band.

Experiments with borate buffer pH 8.5 and 8.8 gave different results from pH 8.0, as shown in Figure 4-A, 4-B, and 4-C. When the pH was increased, the virus was concentrated in two closely occurring zones, presumably a monomer, followed by a dimer aggregate.

Purification of PV in citrate buffer caused extensive aggregation. The yield of virus, as shown in Table 5, was much lower than that obtained by other methods. The absorbance peaks on scanning pattern were much broader and smaller in comparison with other methods (Figure 4-D, 4-E, and 4-F), reflecting a more diffuse distribution of virus throughout the gradient column.

Density gradient centrifugation showed more sedimented material in the bottom of the density gradient tubes containing citrate buffer extract, and less sedimenting material with borate extracts.

Aggregation was also extensive in phosphate buffer; the yield of virus as shown in Table 5 was about half of the amount gained from the method with borate buffer. The scanning patterns also did not show a sharp smooth peak (Figure 4-G, 4-H, 4-I). These patterns showed a more rapidly sedimenting second peak, indicating the presence of virus aggregates near or at the bottom of the tubes.

2. Purification of PV by precipitation with PEG and differential and density gradient centrifugation

This method also gave satisfactory results. For PV five per cent PEG and three hours stirring with a magnetic stirrer provided relatively clean virus in a high concentration as judged by the infectivity test of the supernatant (a low number of local lesions on assay plants) and the concentration of final pellet. Partially purified virus was reasonably clean, and the scanning pattern of centrifuged density gradients showed a sharp, smooth peak (Figure 4-J). However, an extra peak was shown close to the virus peak, which was probably due to some virus aggregation.

Figure 4. Sucrose density gradient centrifugation profiles of partially purified PV in 0-30 per cent sucrose gradients after centrifugation in an SW 40 rotor at 36,000 rpm for 90 minutes.

A. Profile of PV immediately after purification with Triton X-100 clarification, in borate buffer, pH 8.0.

B. Profile of PV immediately after purification with Triton X-100 clarification, in borate buffer, pH 8.5.

C. Profile of PV immediately after purification with Triton X-100 clarification, in borate buffer, pH 8.8.

D. Profile of PV immediately after purification with Triton X-100 clarification, in citrate buffer, pH 8.8.

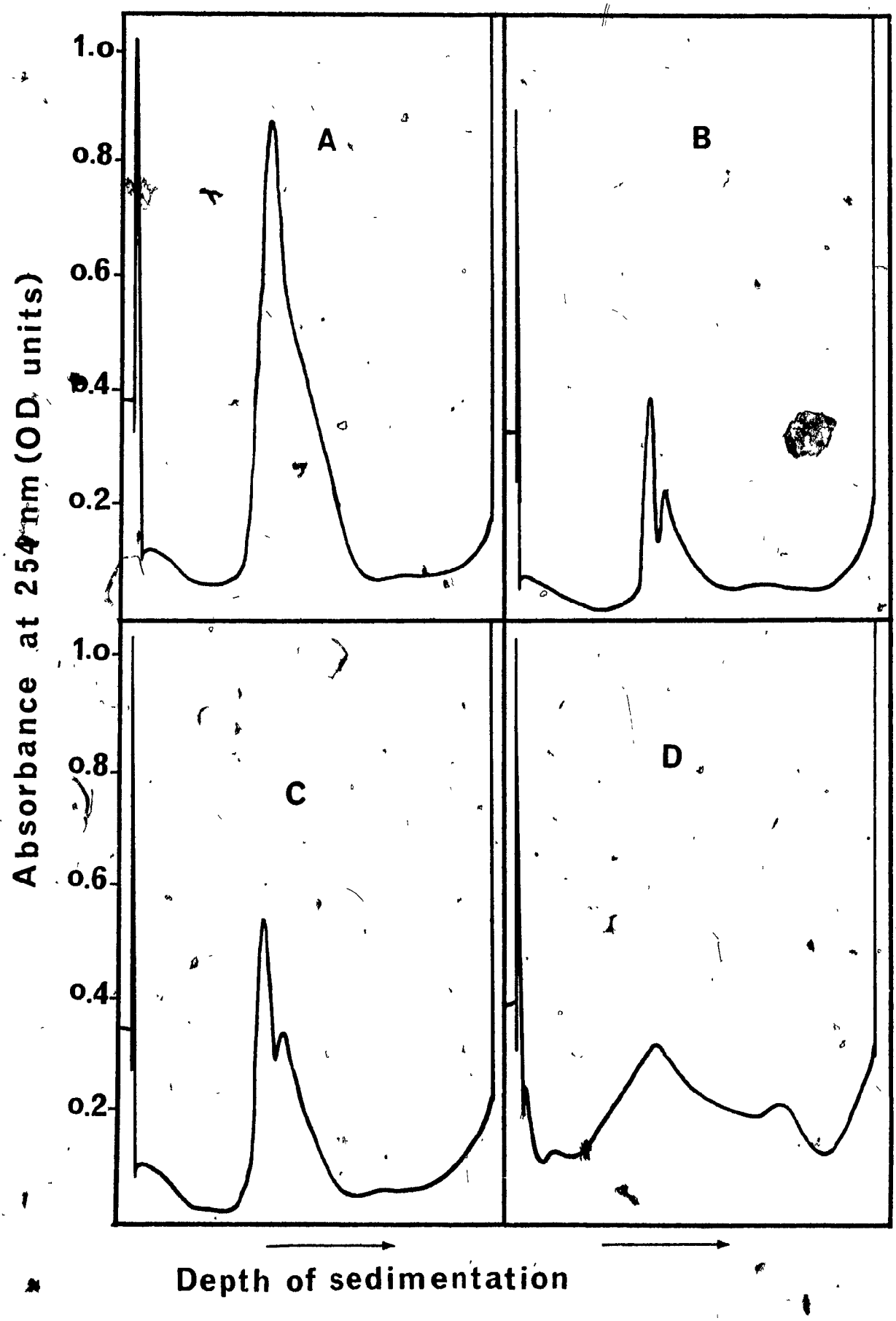

A
B
C
D
1.0
0.8
0.6
0.4
0.2
Absorbance at 254 nm (OD units)
Depth of sedimentation

Figure 4. (continued)

E. Profile of PV immediately after purification with Triton X-100 clarification, in citrate buffer, pH 8.5.

F. Profile of PV immediately after purification with Triton X-100 clarification, in citrate buffer, pH 8.0.

G. Profile of PV immediately after purification with Triton X-100 clarification, in phosphate buffer, pH 8.0.

H. Profile of PV immediately after purification with Triton X-100 clarification, in phosphate buffer, pH 7.5.

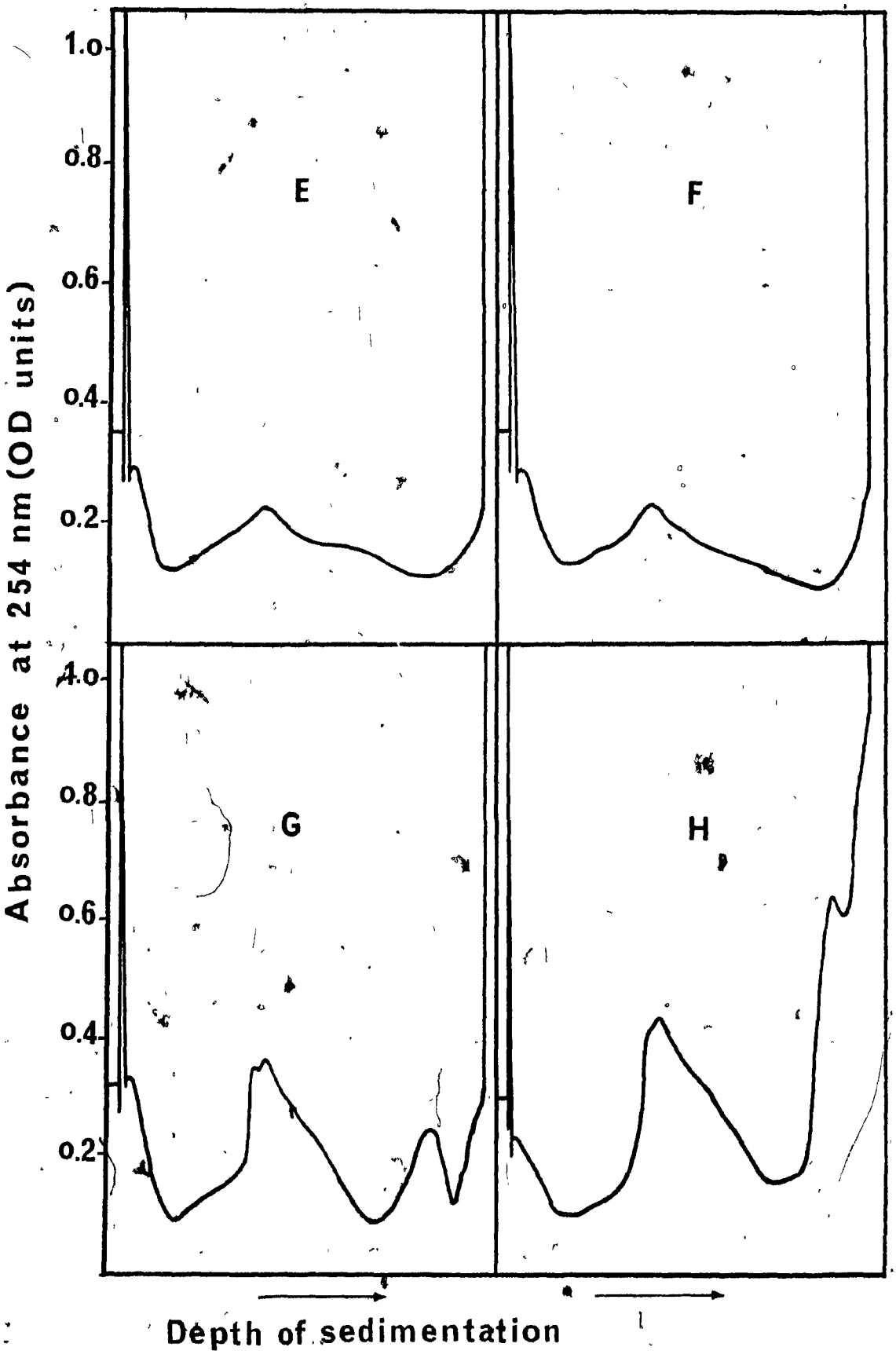

E
F
G
H
1.0
0.8
0.6
0.4
0.2
Absorbance at 254 nm (OD units)
Depth of sedimentation

Figure 4. (continued)

I. Profile of PV immediately after purification with Triton X-100 clarification, in phosphate buffer, pH 7.0.

J. Purification of PV with Triton-PEG method.

K. Purification of PV with n-butanol clarification method.

L. Purification of PV with chloroform clarification method.

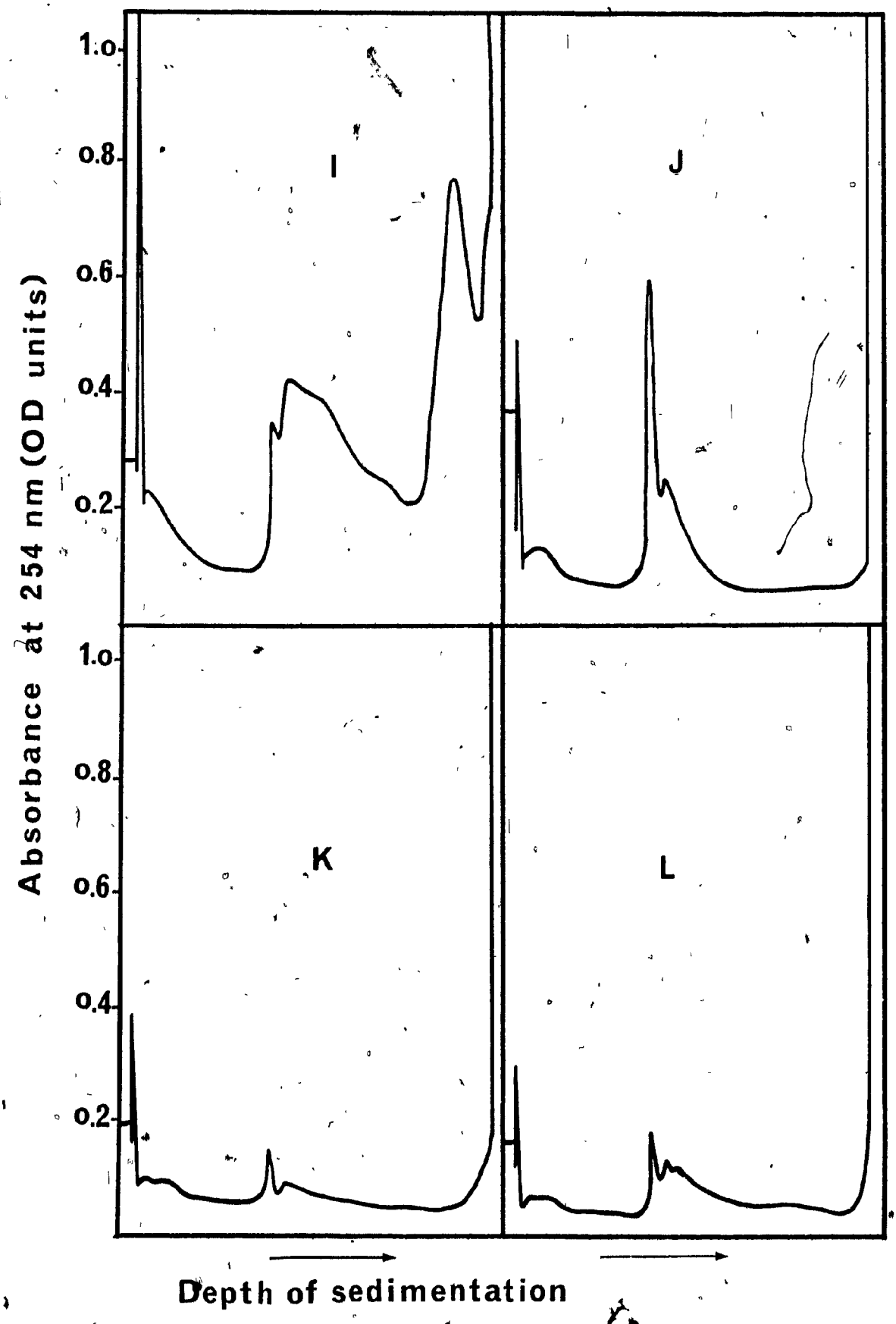

I
J
K
L
1.0
0.8
0.6
0.4
0.2
Absorbance at 254 nm (OD units)
Depth of sedimentation

Figure 4. (continued)

M. Purification of PV with n-butanol/chloroform clarification method.

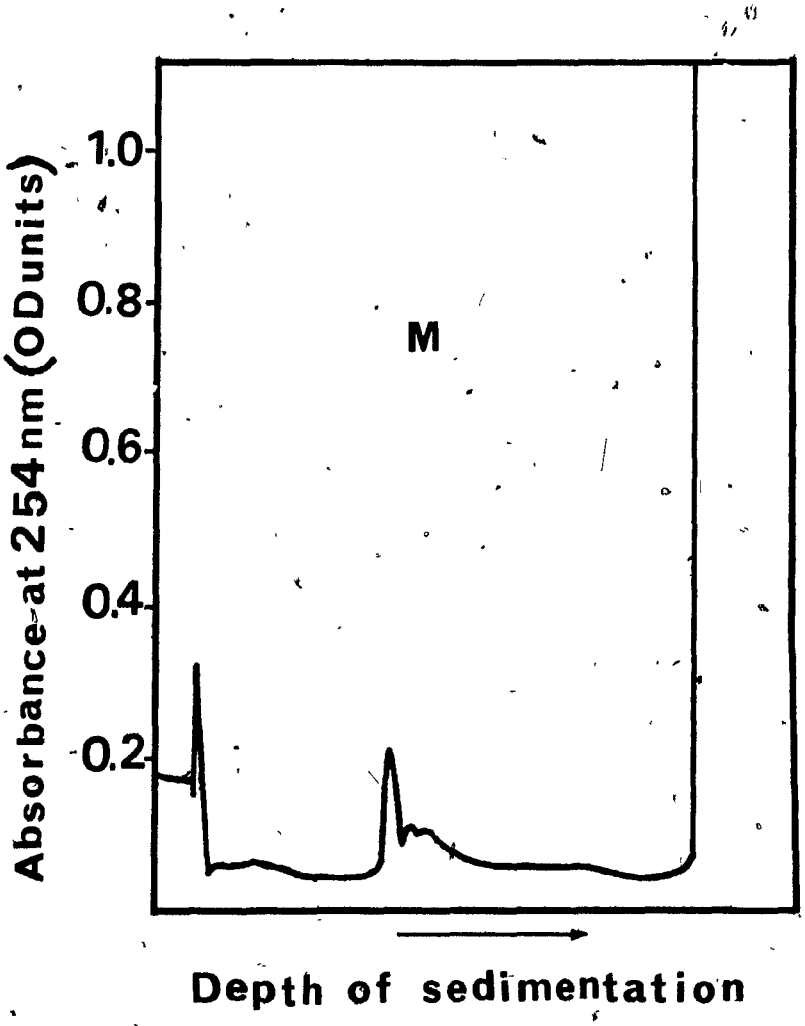

Absorbance at 254 nm (OD units)
1.0
0.8
0.6
0.4
0.2
M
Depth of sedimentation

TABLE 5. Yield data and spectrophotometric analysis of separate preparations of PV

Preparation number	Purification method	Yield mg/kg	Ratio A260/A280	Absorption Min.	Absorption Max.
1	Borate buffer pH 8.0 + Triton	216	1.28	244	259
2	Borate buffer pH 8.5 + Triton	184	1.32	244	259
3	Borate buffer pH 8.8 + Triton	201	1.32	244	259
4	Sodium citrate buffer pH 8.0 + Triton	50	1.29	246	260
5	Phosphate buffer pH 7.5 + Triton	121	1.29	245	260
6	PEG + Triton	178	1.35	245	259
7	N-Butanol*	-	-	-	-
8	Chloroform*	-	-	-	-
9	N-Butanol/chloroform*	-	-	-	-

*The high-speed pellets were too green and impure to be used for further purification and spectrophotometric analysis.

3. Purification of PV by N-Butanol, Chloroform, or N-Butanol/Chloroform clarification

Organic solvents such as n-butanol and chloroform were not effective for PV purification. The virus was apparently severely aggregated, as judged by the great number of local lesions of Gomphrena globosa caused by the first low-speed centrifugation pellets. The results obtained from n-butanol, chloroform, and n-butanol/chloroform clarification were similar. The first high-speed pellets were green and contaminated with host materials. The second high-speed pellets (after layering the virus suspension on 20 per cent sucrose in rotor No. 30 tubes and centrifuging them as described in materials and methods) were still green. The scanning pattern of density gradient centrifugation (Figure 4-K, 4-L, 4-M) showed small peaks, which resembled those of the non-aggregated PV, and an additional amount of UV absorption, below the first peak, presumably due to virus aggregation.

D. Properties of "Purified" PV

The virus suspension at concentrations of 5-10 mg/ml were colorless and showed strong stream birefringence. Maximum yields obtained were about 260 mg/kg of tissue. The ultraviolet absorption spectra of several separate preparations of purified PV suspended in 0.02 M borate buffer, pH 8.0, showed a maximum absorption at 259-260 nm and a minimum at 244-246 nm (Table 5). The ratio A260/A280 in all cases was above 1.25, which is not in agreement with that of most flexuous rod-shaped viruses (usually less than 1.25). In general, this

Figure 5. Typical UV absorption curve of a purified suspension of PV.

A. Purified suspension of PV in 0.02 M borate buffer, pH 8.0.

B. Absorbance of the 0.02 M borate buffer pH 8.0.

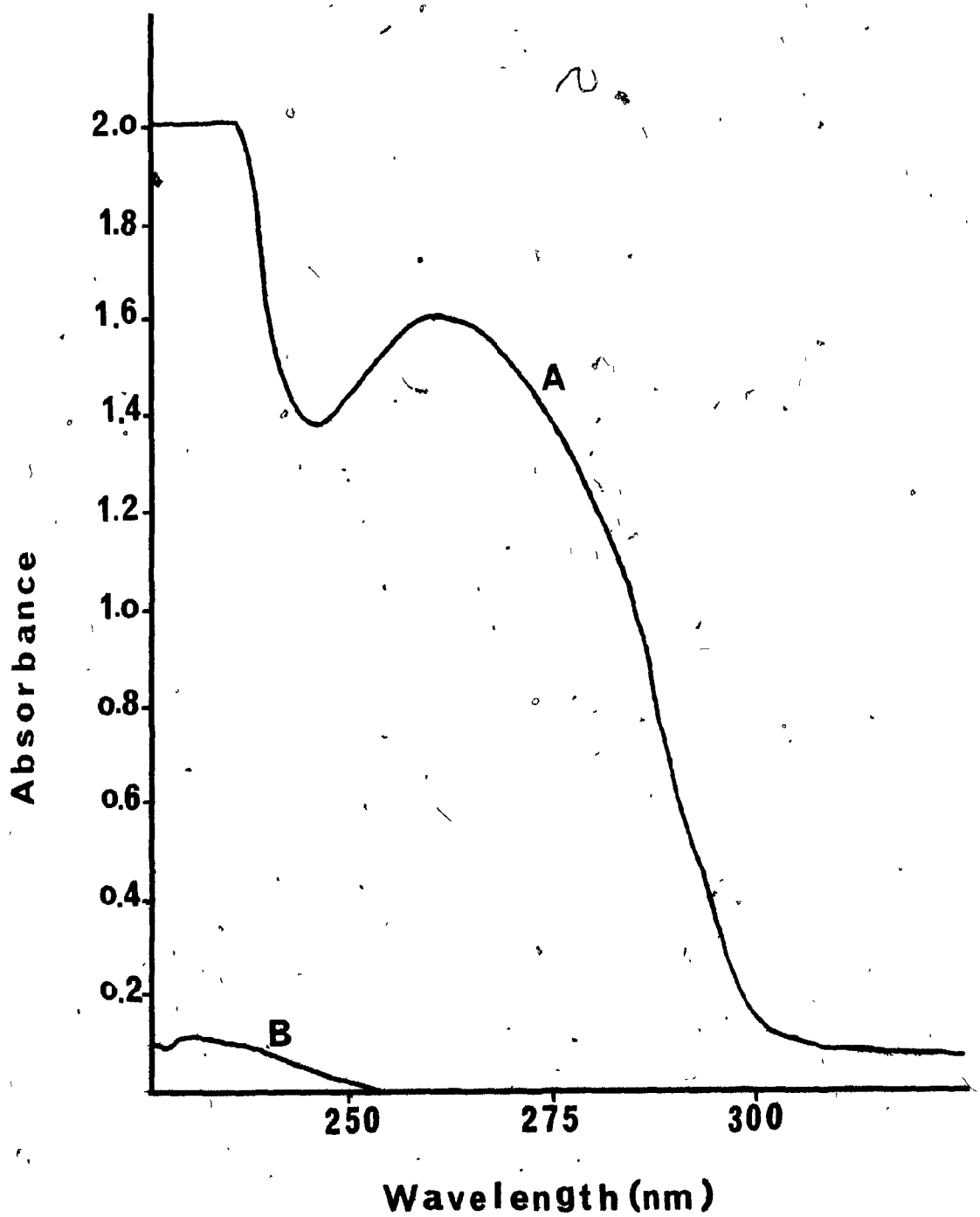
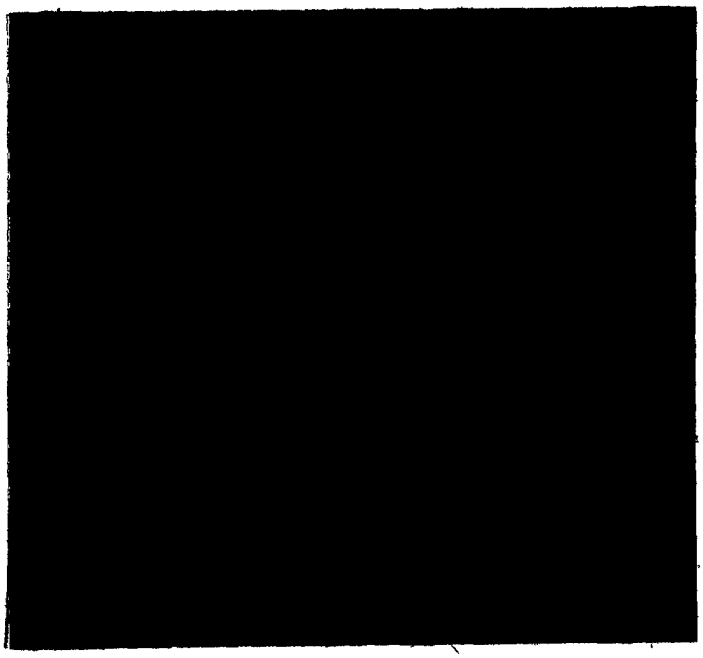

2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Absorbance
A
B
250
275
300
Wavelength (nm)

Figure 6. Electron micrograph of a purified preparation of PV, negatively stained with two per cent phosphotungstic acid (PTA).
Magnification approximately 21,700 X.



ratio was between 1.28 and 1.32, suggesting some host impurities, especially in the latter case.

A typical UV absorption curve of a purified PV suspension is shown in Figure 5. An extinction coefficient of 2.7-2.8 at 260 nm was calculated on the basis of dry weight determination.

Examination of electron micrographs of purified preparations revealed some heterogeneity of particle length in the preparations. Pieces of rods and lateral aggregates as well as end-to-end aggregates were detected (Figure 6).

E. Sedimentation Coefficient

This experiment was repeated three times; in all cases similar results were obtained. The depths of sedimentation for BMV, SBMV, TMV, and PV were measured as described in materials and methods from the scanner charts (Figure 7-A to 7-I) and the results were recorded (see Table 6). As the results of these three experiments show, the depth of sedimentation of PV is the same as SBMV. To make sure that no differences existed between the sedimentation rate of PV and SBMV, the experiment was repeated and centrifugation continued for 150 minutes instead of 90 minutes, and again the same estimate of sedimentation coefficient (115 s) was obtained (Table 7).

The logarithm of sedimentation was plotted against the logarithm of depth (Figure 8) by use of the least squares method. The plotted line has a slope of 0.75, in comparison with 0.8 obtained by Brakke and Van Pelt (1970a).

Figure 7. Linear-log density gradient profile of purified preparations of BMV, SBMV, TMV, and PV in an SW 40 rotor at 36,000 rpm for determination of S-value of PV.

A. Profile of BMV centrifuged for 90 minutes.
B. Profile of SBMV centrifuged for 90 minutes.
C. Profile of TMV centrifuged for 90 minutes.
D. Profile of PV centrifuged for 90 minutes.

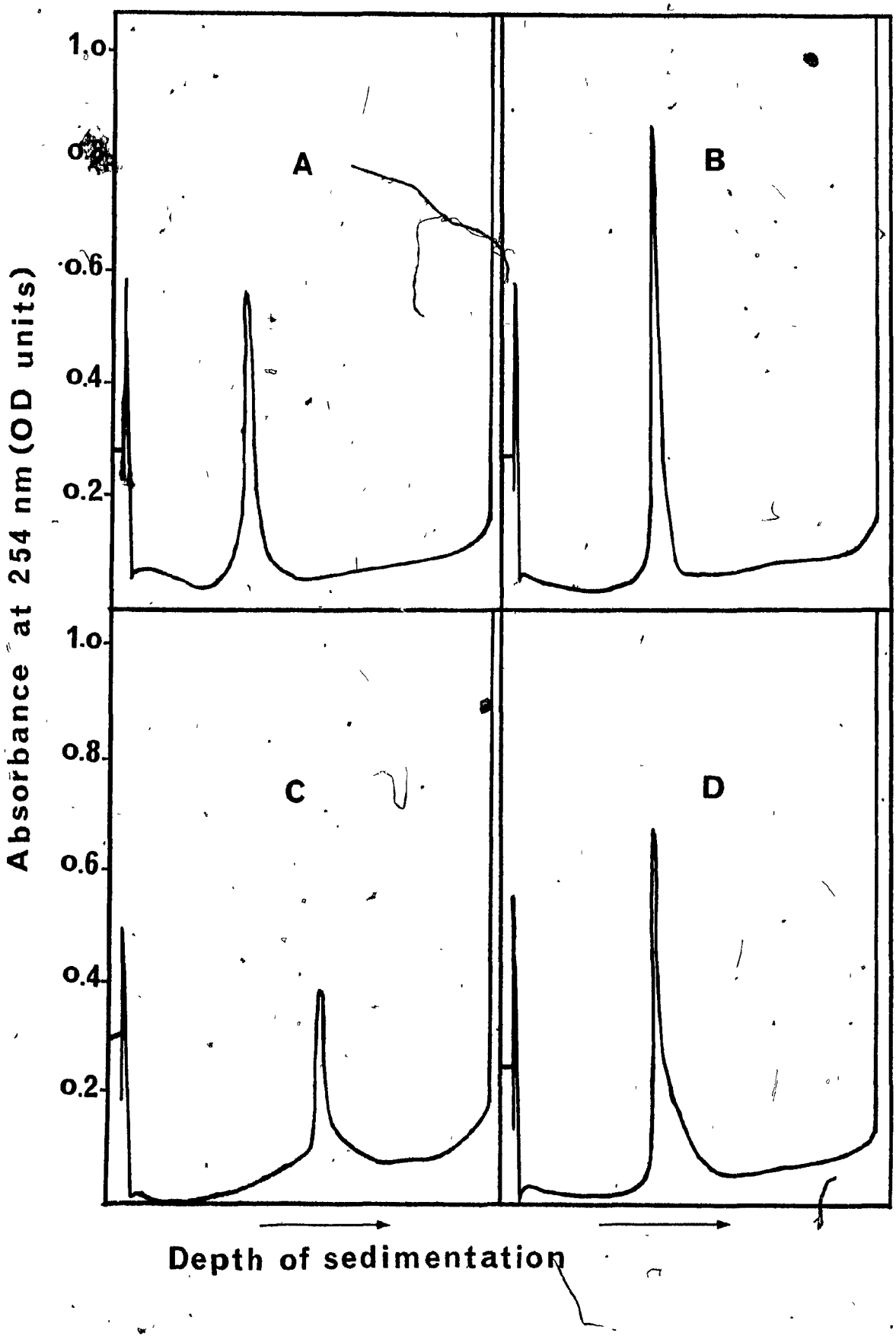

1.0
0.8
0.6
0.4
0.2
A
B
C
D
Absorbance at 254 nm (OD units)
Depth of sedimentation

Figure 7. (continued)

E. Profile of SBMV + PV centrifuged for 90 minutes.

F. Profile of BMV + SBMV + PV centrifuged for 90 minutes.

G. Profile of SBMV centrifuged for 150 minutes.

H. Profile of PV centrifuged for 150 minutes.

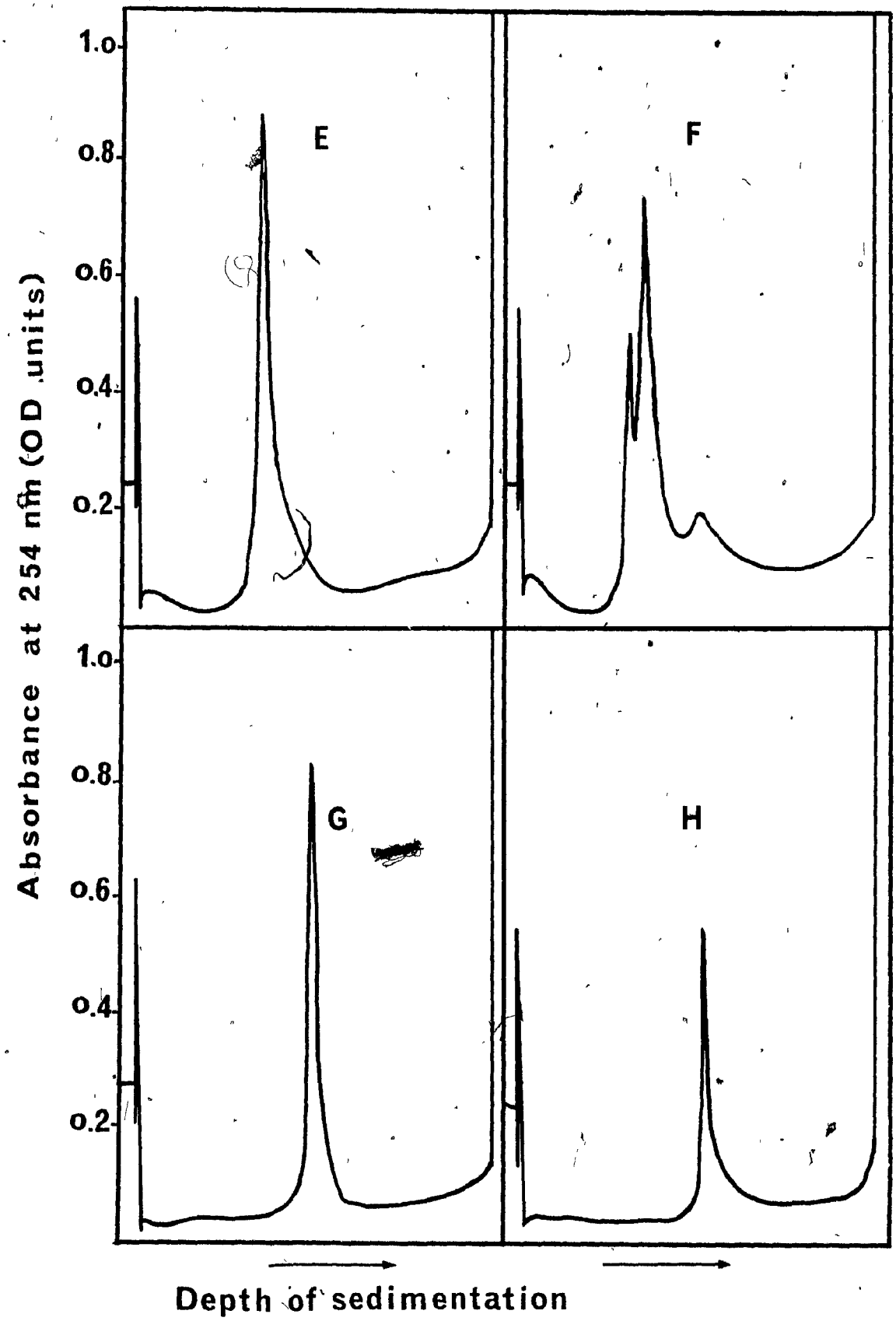

1.0
0.8
0.6
0.4
0.2
E
F
G
H
Absorbance at 254 nm (OD units)
Depth of sedimentation

Figure 7. (continued)

I. Profile of BMV + SBMV + PV centrifuged for 150 minutes.

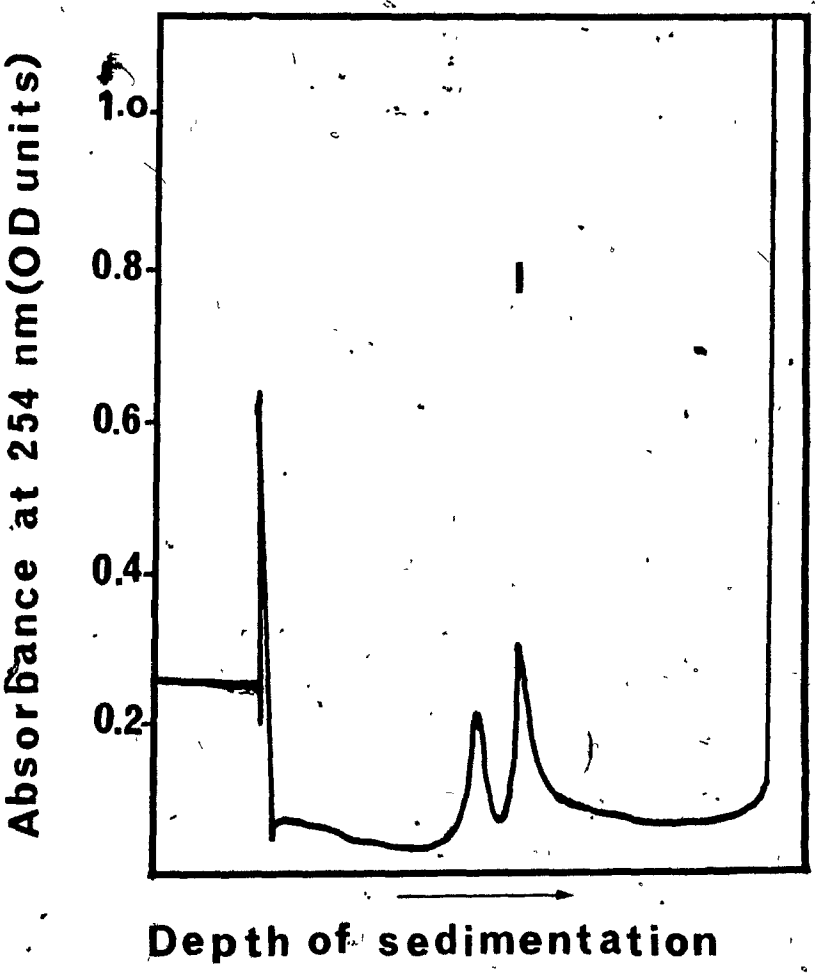

Absorbance at 254 nm (OD units)
1.0
0.8
0.6
0.4
0.2
I
Depth of sedimentation

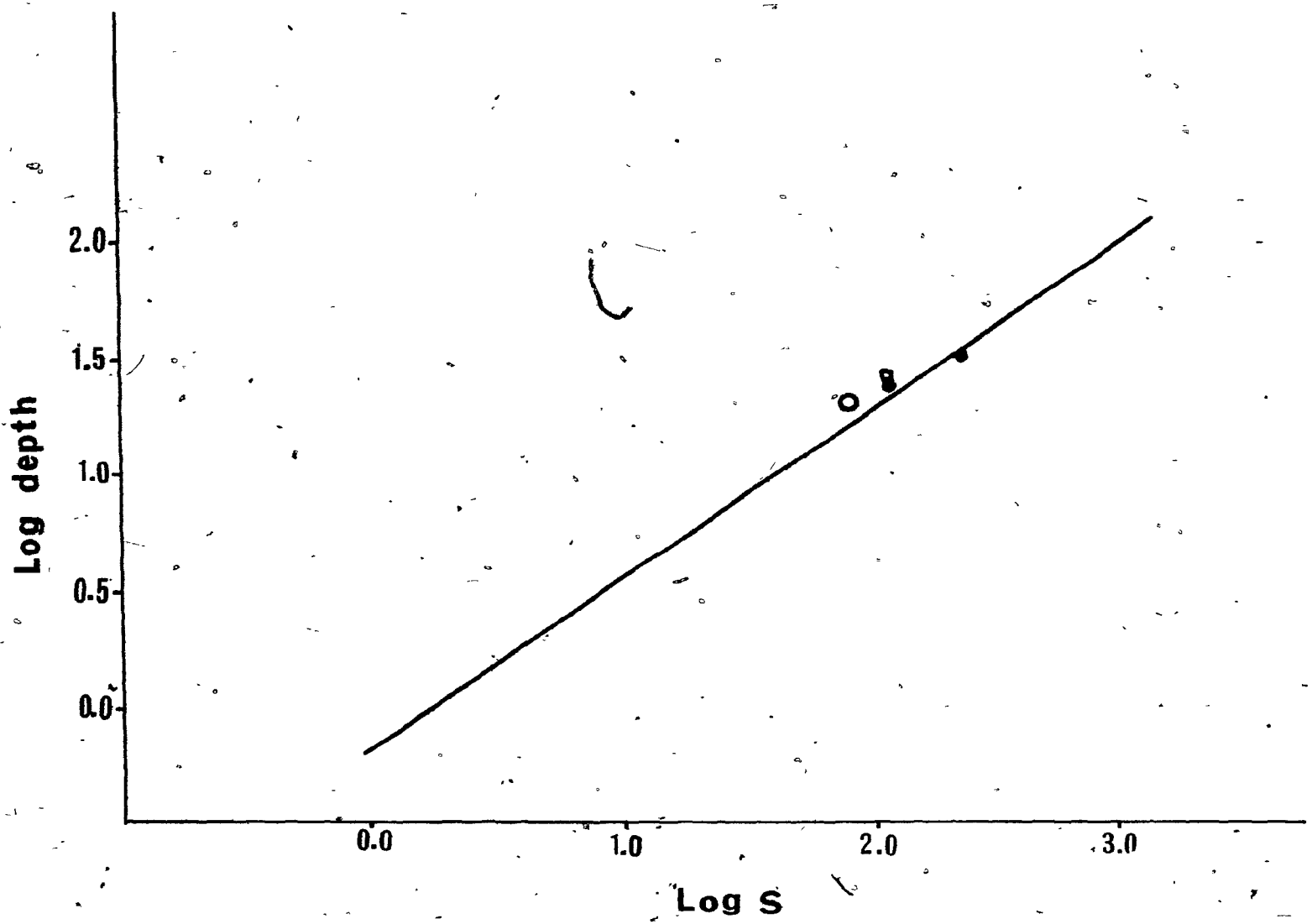
TABLE 6. Depth of sedimentation of BMV, SBMV, TMV, and PV after 90 minutes centrifugation in SW 40 rotor

Virus	Depth (mm)			Known s-values
	Rep.1	Rep.2	Rep.3	
BMV	21.5	21.0	21.0	79 (Brakke and Van Pelt 1970a)
SBMV	23.5	23.0	23.5	115 (Brakke and Van Pelt 1970a)
TMV	34.5	33.5	34.0	187 (Lauffer 1944)
PV	23.5	23.0	23.5	115 (experimentally determined)

TABLE 7. Depth of sedimentation of BMV, SBMV, and PV after 150 minutes centrifugation

Virus	Depth (mm)
BMV	28.5
SBMV	34.0
PV	34.0

Figure 8. Logarithm of depths of BMV (○), SBMV (●), TMV (■), and PV (□) plotted against logarithm of their respective sedimentation coefficients.


Log depth
2.0
1.5
1.0
0.5
0.0
0.0
1.0
2.0
3.0
Log S

F. Analysis of Viral Components

1. Ribonucleic acid

a. CsCl density gradient

To calculate the per cent of RNA, it is necessary to have a suitable buoyant density (ρ), i.e., the density of CsCl at which the particular virus sediments. The estimation of ρ values of each fraction was described previously (materials and methods). The suitable ρ value for use in the formula given in the materials and methods belongs to the fraction with highest absorbance at 260 nm (Figure 9-A, 9-B, 9-C). However, sometimes this value does not fit the formula, because negative values may be obtained under the square root sign; therefore, the values of lower fractions were used. Table 8 shows the ρ values which were used for the calculation in this experiment.

The buoyant density gradient centrifugation experiment was repeated three times; the results are summarized in Table 8. As this table shows, the depth of sedimentation of PV and TMV in all three replications was more or less the same, which indicates that the sedimentation rates of these two viruses through CsCl gradient are the same. In the first and second replications, the table shows exactly the same amount of RNA calculated for both TMV and PV (5.27 per cent), but the third replication did not follow the mentioned amount. This variation might be due to some error during the experimental procedure. Knight and Woody (1958) reported a value of 5.0 per cent RNA for TMV, which is close to 5.27 per cent obtained in this experiment. The

Figure 9. Optical density profile of viruses following equilibrium CsCl column centrifugation.

A. OD profile of PV and its related buoyant density.

B. OD profile of TMV and its related buoyant density.

C. OD profile of BMV and its related buoyant density.

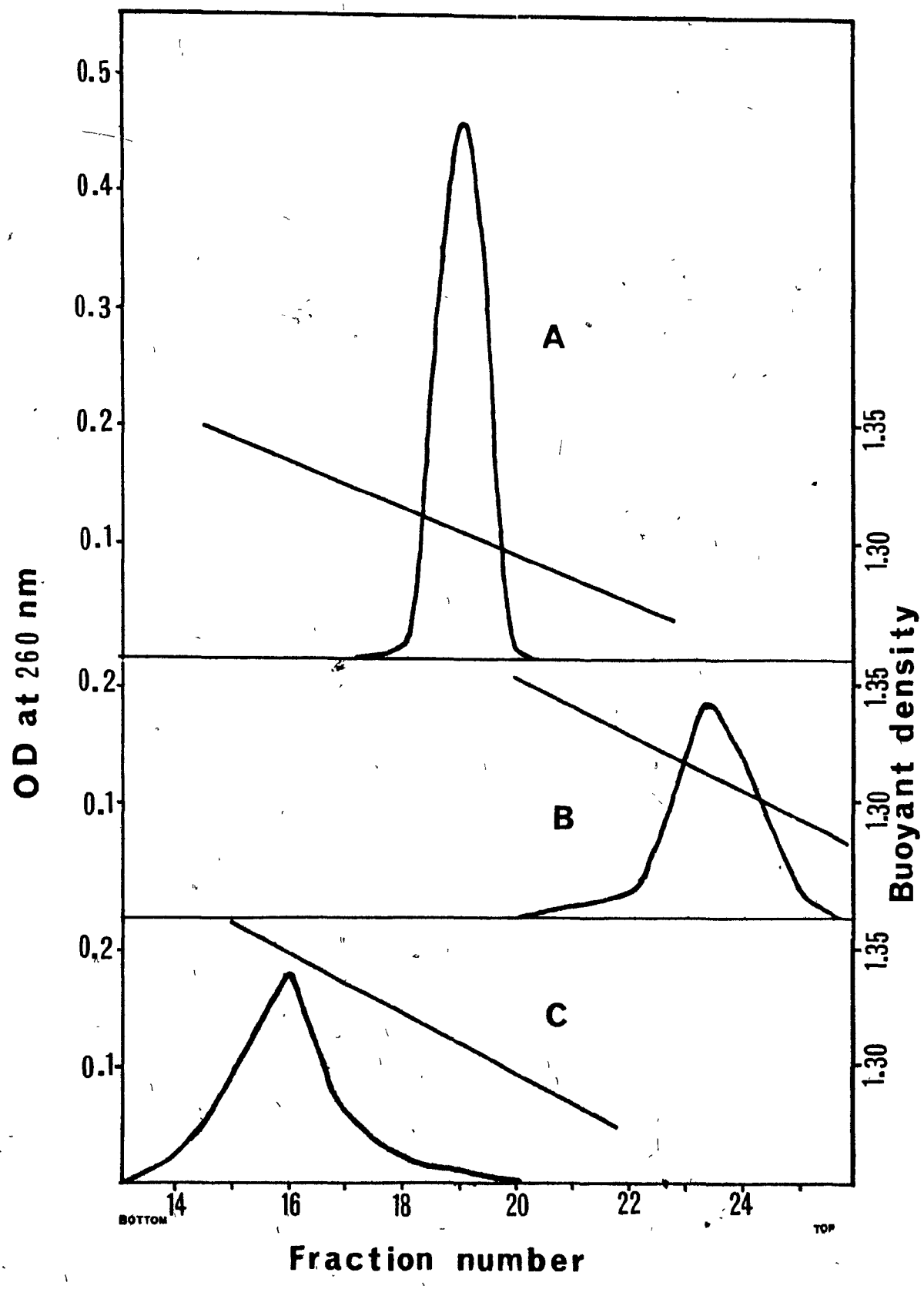

0.5
0.4
0.3
0.2
0.1
A
0.2
0.1
B
0.2
0.1
C
OD at 260 nm
1.35
1.30
1.35
1.30
1.35
1.30
Buoyant density
BOTTOM
14
16
18
20
22
24
TOP
Fraction number

TABLE 8. The depth of sedimentation of BMV, TMV, and PV through buoyant density gradient, the desired ρ-values which were used in the formula, and the calculated amounts of RNA

Virus	Rep.1			Rep.2			Rep.3			Per cent of RNA (known values)
	Depth of sed. (mm)	RNA-content %	Desired ρ-value	Depth of sed. (mm)	RNA-content %	Desired ρ-value	Depth of sed. (mm)	RNA-content %	Desired ρ-value	
BMV	22.0	21.04	1.35379	22.5	21.28	1.35487	22.0	-*	-*	21 (Bockstahler and Kaesberg 1961)
TMV	18.5	5.27	1.31578	17.5	5.27	1.31578	17.5	6.08	1.31795	5 (Woody 1958)
PV	18.0	5.27	1.31578	17.0	5.27	1.31578	16.5	8.38	1.32012	-

*The gradient was mixed before fractionation.

higher value obtained in these experiments may have been caused by aggregation of TMV particles. The calculated amounts of RNA for BMV in two different replications were 21.04 per cent and 21.28 per cent, which are very close to 21 per cent reported by Bockstahler and Kaesberg (1961). When the value for ρ was plotted against the fraction number (fractions around the maximum absorbancy), in all cases a straight line was obtained, indicating the relationship between the fraction numbers and ρ-values.

b. Properties of PV-RNA

The molecular weight of PV-RNA was determined by coelectrophoresis with well-characterized marker RNA values (BMV, SBMV, and TMV-RNA). This experiment was repeated three times and the results are recorded in Table 9.

The ultraviolet absorption spectrum of PV-RNA (Figure 10) prepared by SDS-phenol method and taken in TKM, had a maximum at 258 nm and a minimum at 231 nm. The A258/A280 value was 2.43.

All of the marker RNAs gave well defined, homogeneous components during gel electrophoresis (Figure 11). Four RNA components were obtained with BMV-RNA (Lane and Kaesberg 1971), a double pattern with TMV-RNA (which is not well illustrated in Figure 11), as reported by Fowlks and Young (1970), and a broad single band with PV-RNA.

The distance of reference RNAs' migration through the gel on a linear graph was plotted against the log of their molecular weights (Figure 12). By extrapolation of the mobility of PV-RNA to that of

molecular weight, values of 2.17-2.21 x 10^6 daltons were obtained for PV-RNA molecular weight.

The sedimentation coefficient of PV-RNA was determined by comparing its sedimentation with TMV and BMV-RNA values. The ammonium carbonate-phenol method for RNA extraction, as described in materials and methods, was used hereafter for the rest of this experiment.

The nucleic acids were centrifuged for five hours through linear-log gradients in the spinco SW 40 rotor at 36,000 rpm, so that the logarithm of the depth of sedimentation was a linear function of the logarithm of the sedimentation coefficient.

The depths of the peaks were measured on the scanning patterns (Figure 13-A to 13-C), from the middle of the peaks to the meniscus, and the log of depth was plotted against the log of sedimentation coefficient by using the least squares method (Figure 14). The S-value of PV-RNA was estimated as 37.2 s by comparing its sedimentation with those RNA values used as reference.

The PV-RNA band was collected during scanning and used for infectivity assay on Gomphrena globosa which showed a low number of necrotic local lesions, indicating the presence of intact, functional PV-RNA.

A value of 20.7 S was obtained for the sedimentation coefficient of PV-RNA after treatment with formaldehyde. As the results showed (Table 10 and Figure 13-D to 13-F), this value is the same as sedimentation value for TMV-RNA as reported by Brakke and Van Pelt (1970b).

TABLE 9. The distance of RNA migration in 3 per cent polyacrylamide gel from 3 different replications, and the molecular weights of the known RNA's

Viruses	Distances of RNA migration in 3 per cent gel (cm)			Known RNA mol.wt.	Reference
	Rep.1	Rep.2	Rep.3		
TMV	0.40	0.70	0.75	$2.05\ 10^6$	Knight (1952)
SBMV	0.80	1.26	1.70	$1.40\ 10^6$	Tremaine (1966)
PV	0.25	0.47	0.60	-	-
BMV_1	1.90	1.85	2.10	$1.09\ 10^6$	Lane and Kaesberg (1971)
BMV_2	2.00	1.90	2.15	$0.99\ 10^6$	
BMV_3	2.40	2.52	2.80	$0.75\ 10^6$	
BMV_4	4.00	4.60	4.85	$0.28\ 10^6$	

Figure 10. Typical UV absorption curve of a PV-RNA in TKM, prepared by SDS-phenol extraction method.

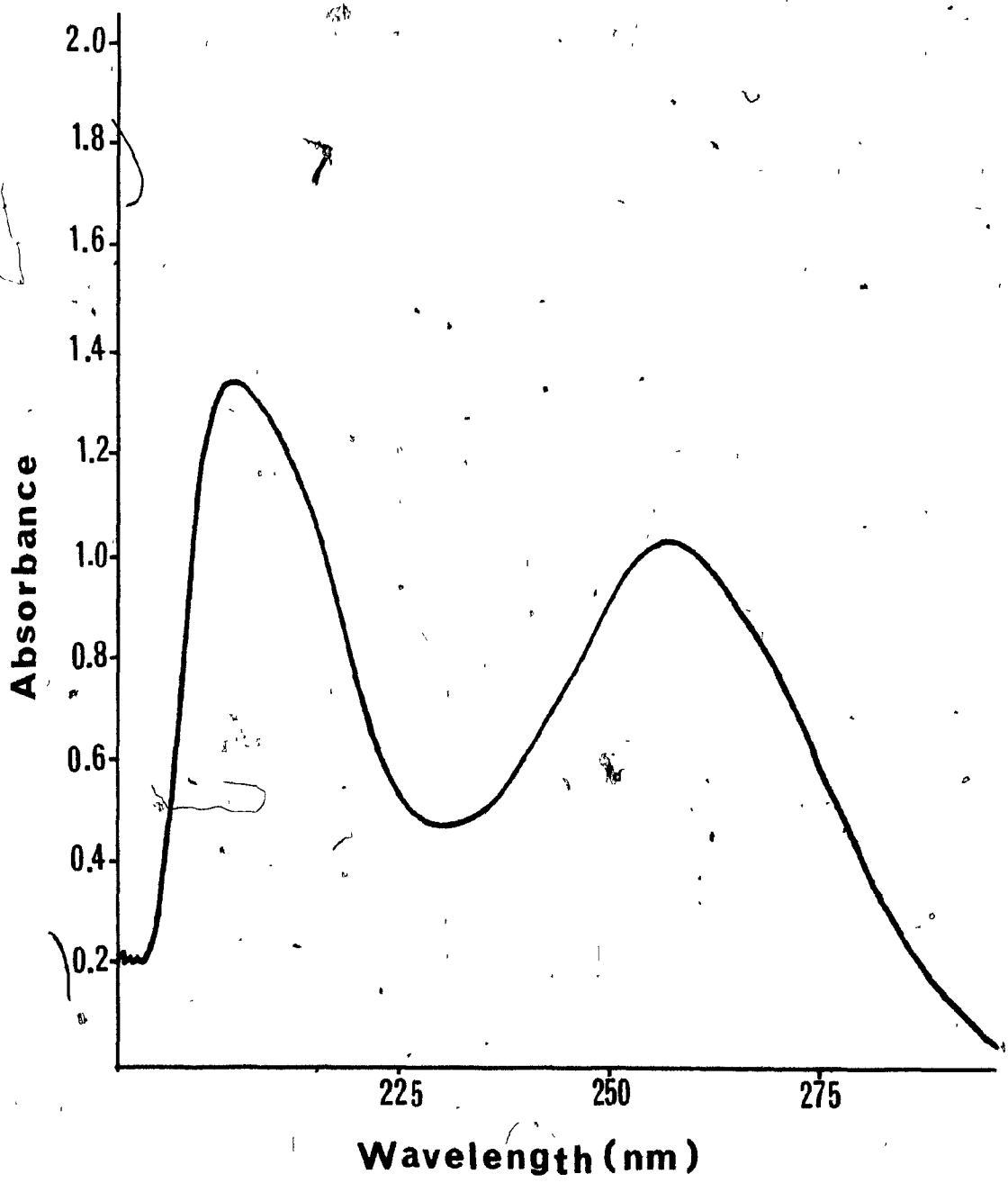

2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Absorbance
225
250
275
Wavelength (nm)

Figure 11. PV, TMV and BMV-RNA bands in 3 per cent polyacrylamide gel.

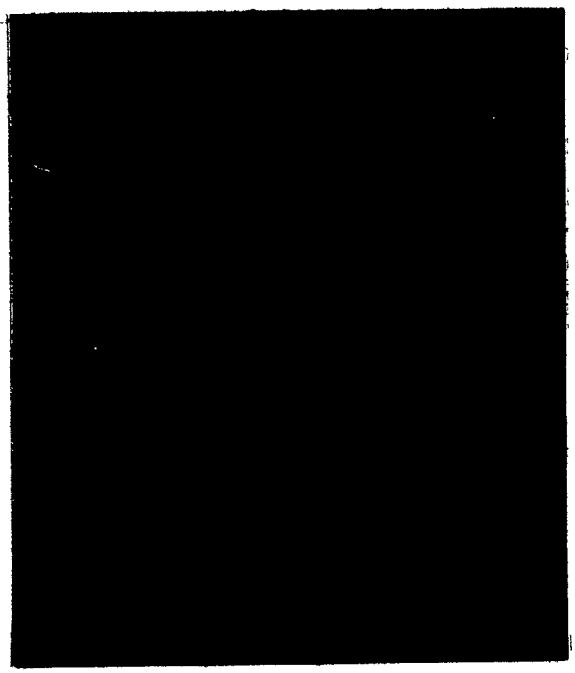


Figure 12. Logarithm of molecular weight of BMV (●), SBMV (⊙), TMV (■), and PV-RNA (◘), plotted against their respective migration through the 3 per cent polyacrylamide gel in three different experiments (1, 2, and 3).

B_1 = BMV_1

B_2 = BMV_2

B_3 = BMV_3

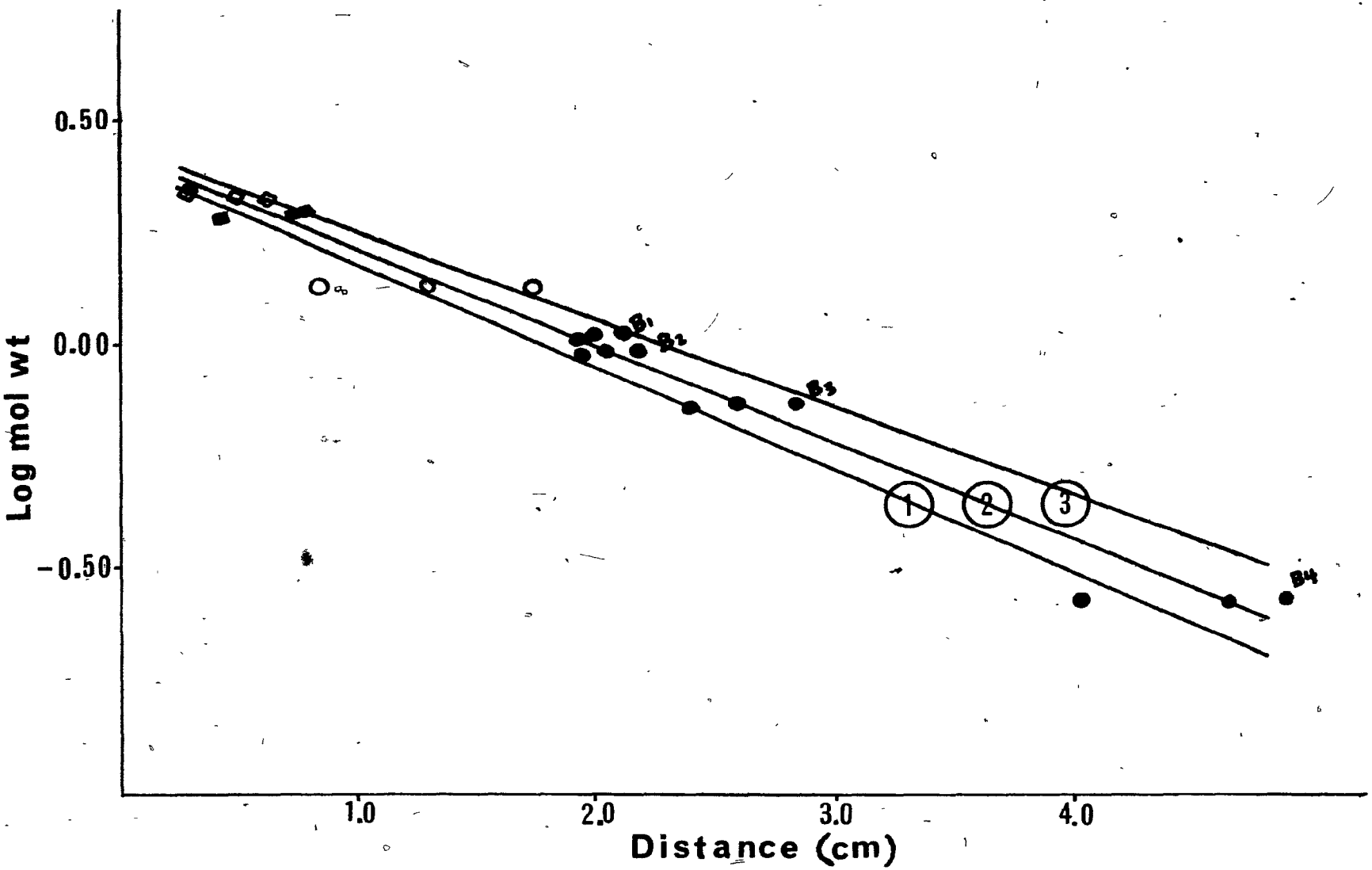

0.50
0.00
−0.50
Log mol wt
1.0
2.0
3.0
4.0
Distance (cm)
1
2
3

Figure 13. Linear log sucrose density gradient centrifugation profile of BMV, TMV, and PV-RNA, extracted by ammonium carbonate-phenol method, centrifuged in SW 40 rotor for five hours at 36,000 rpm.

A. Profile of TMV-RNA
B. Profile of BMV-RNA
C. Profile of PV-RNA
D. Profile of TMV-RNA after formaldehyde treatment.

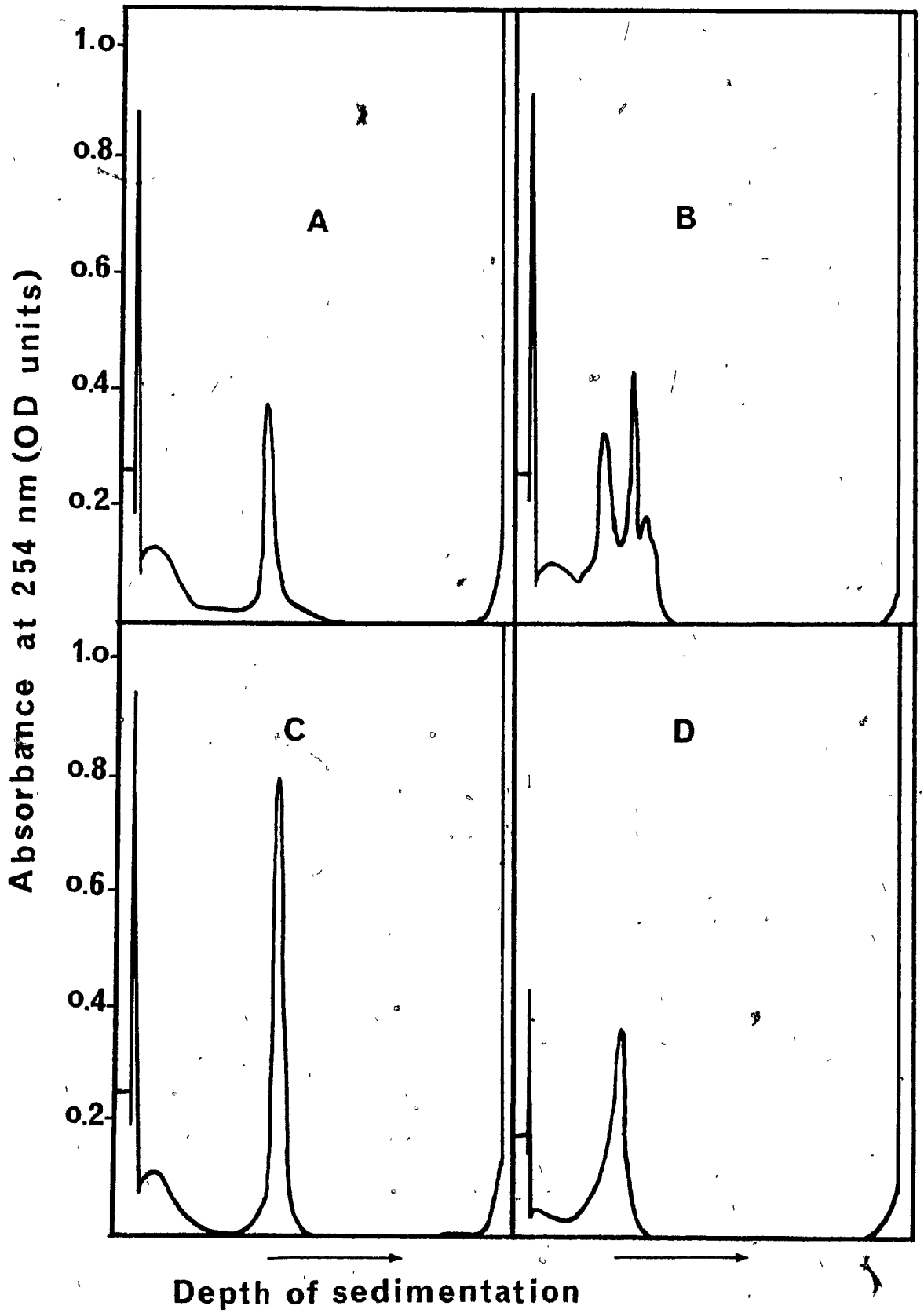

1.0
0.8
0.6
0.4
0.2
A
B
C
D
Absorbance at 254 nm (OD units)
Depth of sedimentation

Figure 13. (continued)

E. Profile of BMV-RNA after formaldehyde treatment

F. Profile of PV-RNA after formaldehyde treatment

G. Profile of PV-RNA after treatment with distilled water

H. Profile of PV-RNA after DNase treatment

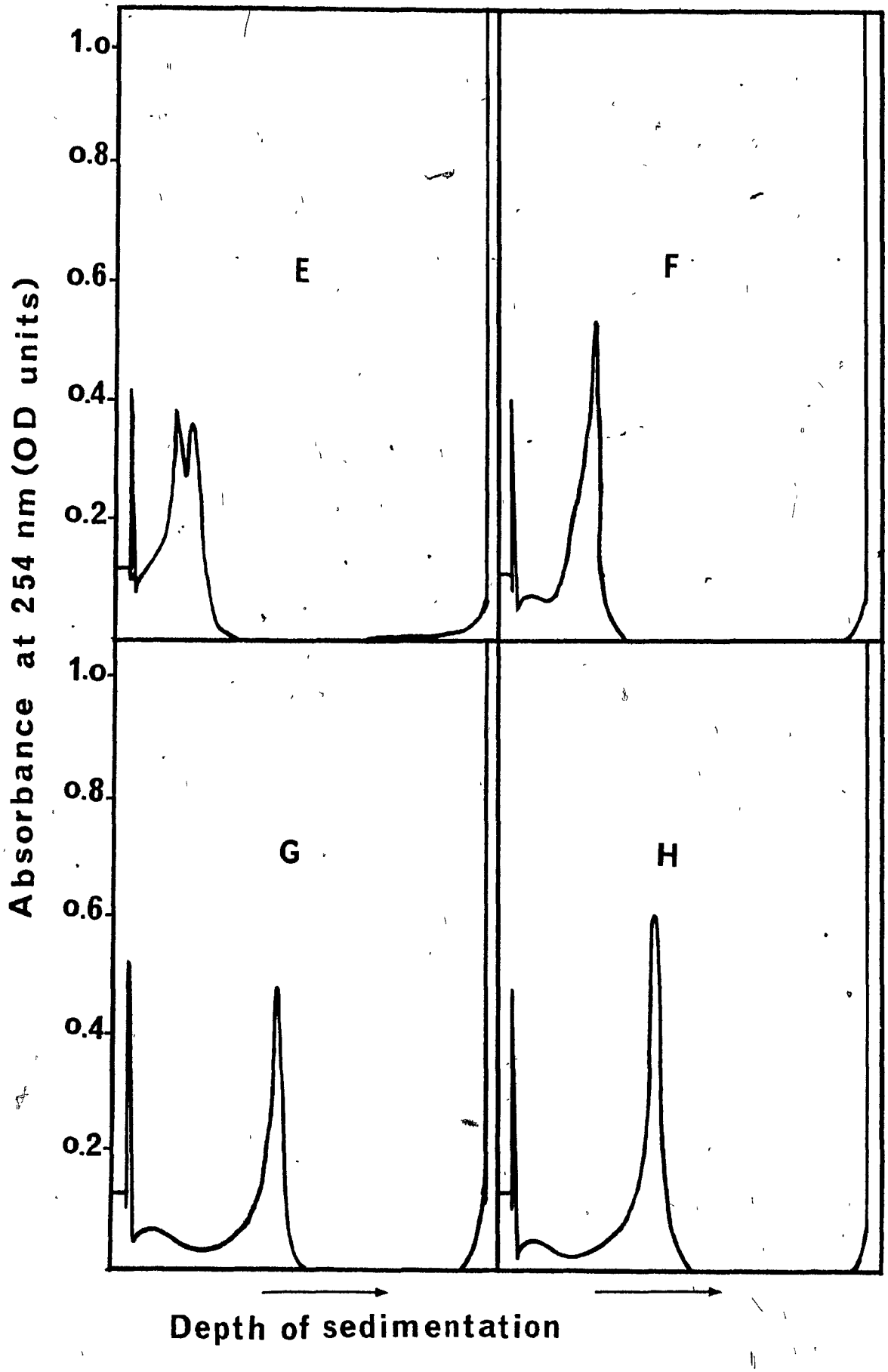

1.0
0.8
0.6
0.4
0.2
E
F
G
H
Absorbance at 254 nm (OD units)
Depth of sedimentation

Figure 13. (continued)

I. Profile of PV-RNA after RNase treatment.

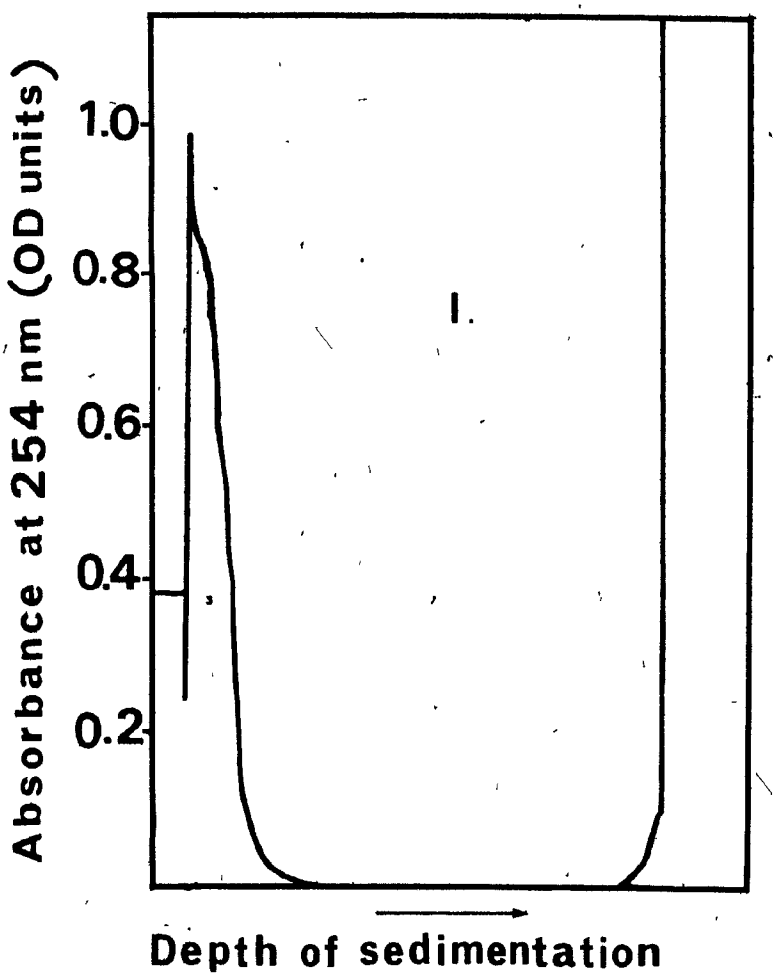

Absorbance at 254 nm (OD units)
1.0
0.8
0.6
0.4
0.2
I.
Depth of sedimentation

Figure 14. Logarithm of sedimentation coefficients of BMV (●), TMV (■), and PV-RNA (▫), plotted against their respective logarithm of depth, before (1), and after (2) formaldehyde treatment.

B_1 = BMV_1

B_2 = BMV_2

B_3 = BMV_3

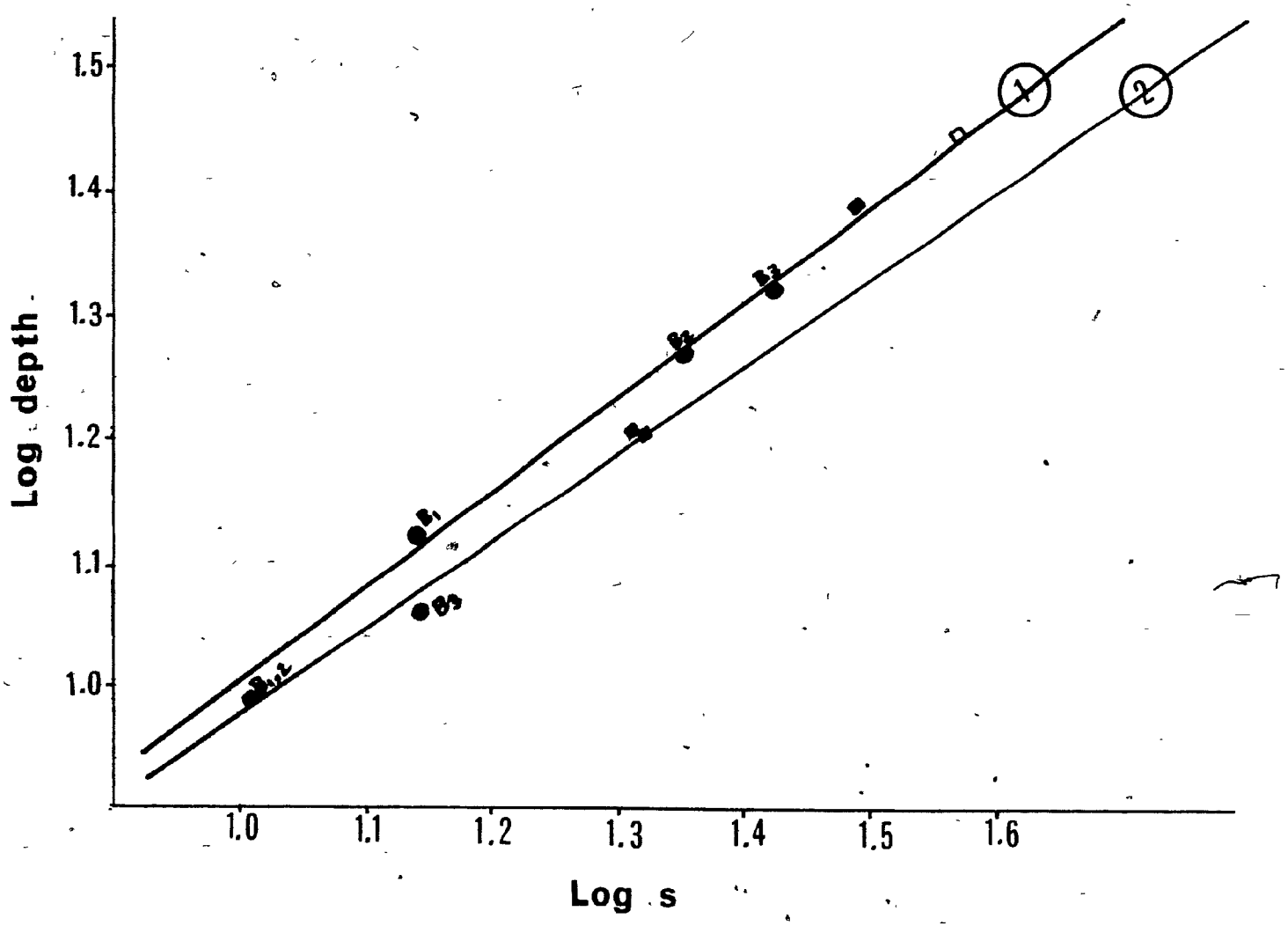

1.5
1.4
1.3
1.2
1.1
1.0
Log depth
1.0
1.1
1.2
1.3
1.4
1.5
1.6
Log s
1
2

TABLE 10. Depth of sedimentation of BMV, TMV and PV-RNAs before and after formaldehyde treatment, and the BMV and TMV-RNA's known S-values

Virus	Depth of sedimentation (mm)		Known S-value	
	Before formaldehyde treatment	After formaldehyde treatment	Before formaldehyde treatment	After formaldehyde treatment
TMV	24.50	16.00	31.1[a]	20.7[a]
PV	27.00	16.00	-	-
BMV_1	21.00	11.50	26.8[b]	15.6[a]
BMV_2	18.50	11.50	22.3[b]	13.9[a]
BMV_3	13.25	8.50	14.0[b]	10.1[a]

[a]Brakke and Van Pelt (1970b)

[b]Bockstahler and Kaesberg (1965)

Table 10 also shows the comparison of S-values of RNAs of PV, TMV, and BMV before and after treatment with formaldehyde.

By using the formula $S = 0.083\ M^{0.39}$ for the relationship of sedimentation coefficient (S) of formaldehyde-treated RNA and its molecular weight before formaldehyde treatment (M) (Brakke and Van Pelt 1970b), a molecular weight of 2.03×10^6 daltons was obtained for PV-RNA.

No nucleic acid zone was obtained after density gradient centrifugation of a preparation that had been treated with 3 µg/ml RNase. Treatment with 50 µg/ml DNase had no effect (Figure 13-G to 13-I). It was therefore concluded that nucleic acid of PV was RNA.

2. Protein

For an independent determination of the molecular weight of the PV-protein subunit, polyacrylamide gel electrophoresis in the presence of SDS was used (Shapiro et al. 1967). Typical gels with PV and standard proteins are shown in Figure 15. PV-protein subunit showed a single, well-stained band which moved slower than TMV-protein subunit in the gel. The mobility of the polypeptide chains through polyacrylamide gel was calculated according to the formula given by Weber and Osborn (1969):

$$\text{Mobility} = \frac{\text{Distance of protein migration}}{\text{length after destaining}} \times \frac{\text{length before staining}}{\text{distance of dye migration}}$$

The calculation of the mobility must include the length of the gel before and after staining as well as the mobility of the protein and of the marker dye, because the gels swell some five per cent in the acidic solution used for staining and destaining (Weber and Osborn 1969).

Figure 15. Representative gels after electrophoresis with PV-protein subunit (P); TMV-protein subunit (T); myoglobine (M); alcohol dehydrogenase (A); ovalbumine (O); and carbonic anhydrase (C).

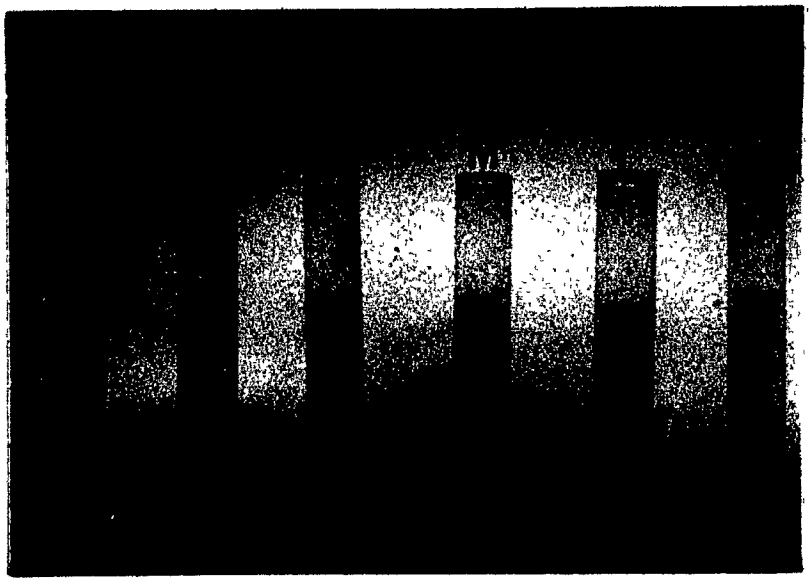


TABLE 11. The mobility of proteins in 10 per cent polyacrylamide gel, and molecular weights of their polypeptide chains, taken from the literature

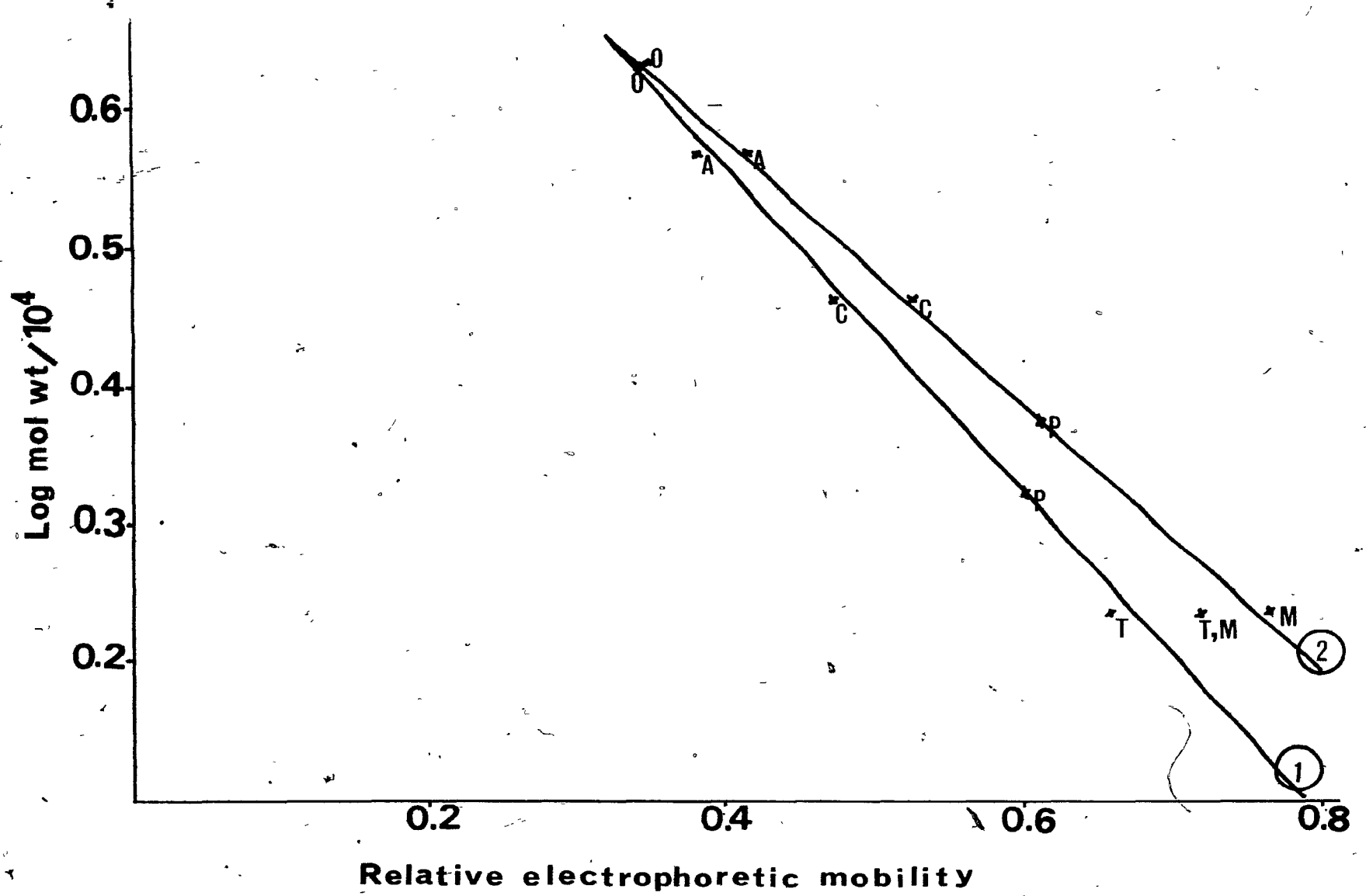
Proteins	Mobility of protein in 10 per cent gel*		Mol. wt. of polypeptide chains	Reference
	Rep.1	Rep.2		
PV-protein subunit	0.61	0.60	-	
TMV-protein subunit	0.72	0.66	17500 dal.	Harris and Knight (1955)
Myoglobine	0.76	0.72	17500 dal.	Tanford et al. (1967)
Alcohol dehydrogenase	0.41	0.38	37000 dal.	Weber and Osborn (1969)
Ovalbumine	0.35	0.34	43000 dal.	Castellino and Barker (1968)
Carbonic anhydrase	0.52	0.47	29000 dal.	Armstrong et al. (1966)

*Calculated from the experimental data.

Figure 16. Relationship between mobility and molecular weight of PV-protein subunit and reference proteins in 10 per cent polyacrylamide gel. Abbreviations are:

A, alcohol dehydrogenase
C, carbonic anhydrase
M, myoglobine
O, ovalbumine
P, PV-protein subunit
T, TMV-protein subunit

1. Plotted from the first experiment's data
2. Plotted from the second experiment's data


0.6
0.5
0.4
0.3
0.2
Log mol wt/10^4
0.2
0.4
0.6
0.8
Relative electrophoretic mobility
O
O
A
A
C
C
P
P
T
T,M
M
1
2

This experiment was repeated twice; the calculated mobilities according to the experimental data are recorded in Table 11. The mobility of the marker proteins was plotted against the logarithm of their molecular weight (Figure 16) by using the least squares method. By extrapolation of the PV-protein subunit mobility to that of molecular weight, values of 21,400 and 23,700 were obtained for protein subunit molecular weight.

G. Serology

The rabbits were bled once per week for five successive weeks; each time about 10 ml of blood was taken, as described in materials and methods. The microprecipitin test showed that the titer of antisera (for both rabbits) was low, about 1/128. The antisera were also tested against clarified crude healthy sap, but no reaction was observed. The normal sera did not show any reaction with PV.

The immunodiffusion test was done in quadrant-type petri dishes by the method described. The PV-antisera showed a strong precipitin line only with PV (Figure 17), and not with other antigens (TMV, TEV, PVY, and PVX) or healthy sap. The other available antisera (PVX, CVX, PVS, PVY, TEV, CYMV, and CVMV) also did not show any reaction with PV.

PV and other viruses (mentioned above), degraded by five per cent pyrolidine (in distilled water) were used similarly in immunodiffusion tests, as described previously. The gel diffusion plates were made of 0.9 per cent agarose in 0.05 M Tris saline buffer, pH 7.2

Figure 17. Immunodiffusion test with PV antisera (central well) and antigens of:

1, 2. PV

3, 4. TMV

5. PVX

6. Healthy sap

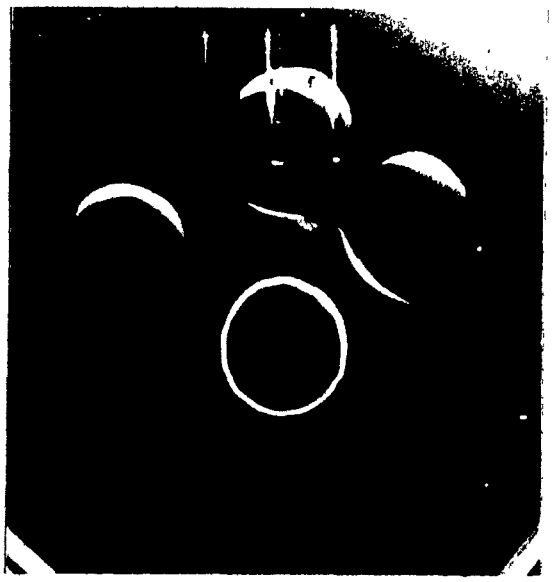
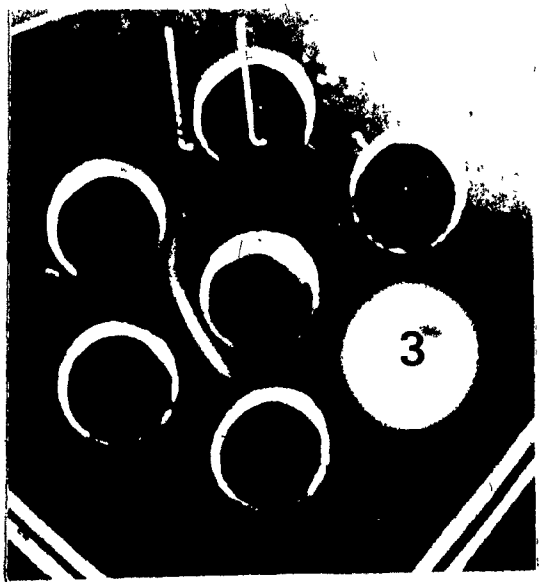


Figure 18. Immunodiffusion test in agarose-tris saline buffer.

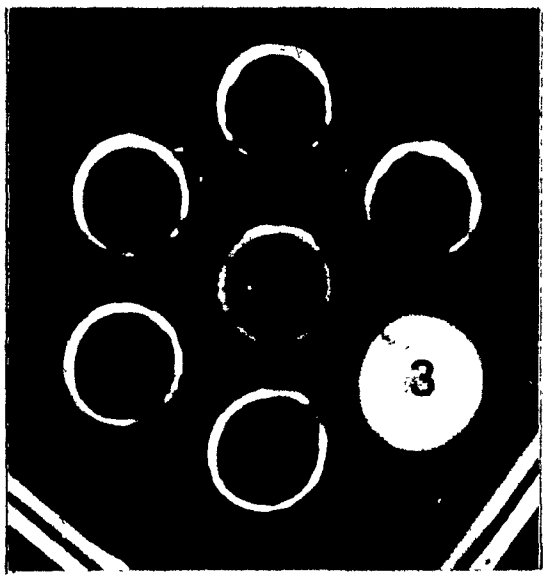
A. Central well charged with PVY-antiserum,

B. Central well charged with TEV-antiserum,

and peripheral wells charged with: (1) PV; (2) healthy sap; (3) TMV; (4) TEV; (5) PVY; (6) PVX.



18-*A*



18-*B*

(0.85 per cent NaCl in 0.05 M Tris-HCl buffer, pH 7.2). In this experiment, also, each virus only reacted with its homologous antiserum (Figure 18-A and 18-B), and there were no indications of a serological relationship between PV and any of the other viruses.

DISCUSSION

PV is thought to be a representative of the PVX group of filamentous viruses because of its gross morphology and of its properties in vitro. Two other flexuous rod-shaped viruses have been reported to infect plantain, i.e., Hydrangea ringspot virus (Hollings 1958) and beet yellows virus (Bennett 1960), but neither of them has all the characters of PV.

PV was mechanically transmitted to 30 species and varieties in seven families of plants, and, like other groups of viruses with similar morphology, had a rather narrow host range. Most of the host plants reacted with local symptoms. This virus did not infect any of the legume plants (Tables 1 and 2) which were tested for host-range determination, except alfalfa; and, also unlike most potato viruses, PV did not infect tobacco cultivars. Tomato plants appeared to be suitable for culturing quantities of PV for purification and characterization because of their high susceptibility to the virus, and because of their fast growth in greenhouse conditions.

The purification method reported here for PV, employing borate buffer, pH 8.0, and Triton X-100 clarification, gave much better results than other methods, at least as far as purity of the final preparation was concerned, and ease of performance.

Some of the virus in purified preparations apparently was aggregated, as indicated by the presence of infectious material below the discrete bands and in the pellets at the bottom of tubes after density-gradient centrifugation. While some of this aggregation probably occurred during processing, it may have been due in part to the aggregation of the virus in the infected host tissue before extraction (Delgado-Sanchez and Grogan 1966). Electron micrographs of thin sections of systemically infected tissues suggest that this is so. Thus it would be necessary to disaggregate this virus to obtain it in a monodispersed condition.

The loss in infectivity (infectivity assay on Gomphrena globosa) was mainly attributed to aggregation of virus and host material in the pellets after high-speed centrifugation and subsequent removal of large aggregates by low-speed centrifugation. Adding Triton X-100 resulted in dissolution of cell membranes and chloroplasts before high-speed centrifugation (Van Oosten 1972). This apparently led to a release of more virus and a reduction of the losses caused by aggregation of virus and host material in the high-speed centrifugation pellet.

It should be emphasized that the use of urea and mercaptoethanol in fractionation does not prevent end-to-end aggregation, but is largely effective in preventing the lateral type of aggregation, and concomitant insolubility (Damirdagh and Shepherd 1970). It appears that interactions of a hydrophobic type may be responsible for the lateral type of polymerization of some filamentous plant viruses.

If so, it seems likely that the role of urea is to weaken the affinity of nonpolar areas on the surface of the virus coat and thus to weaken hydrophobic-type interactions. This action of urea may be due to increased solubility of nonpolar groups on the protein rather than solely as a hydrogen bond-breaking reagent (Bruning and Holtzer 1961).

The aggregation effect of certain salts on the PV has been observed with other viruses of similar morphology. Phosphate has been observed to induce aggregation and to inactivate PVX, particularly at elevated temperatures (Bawden and Crook 1947). Proteins and the materials in plant sap protected against this inactivation. Several other salts were also found to have similar inactivating effects, but not borate or veronal buffers (Shepherd and Pound 1960). The latter buffer, but not borate, was found to cause aggregation of turnip mosaic virus.

The characteristics of PV obtained experimentally as described in this thesis compared with several elongated plant virus groups (TMV, PVX, carnation latent virus, and PVY groups) as shown in Table 12. The physical properties of PV are more or less similar to those of viruses of the PVX-group. Values of 70°C and 10^{-5} were obtained for thermal inactivation point and dilution end point respectively, which are very close to the amounts reported for viruses in PVX-group.

Particle length was measured according to the leaf-dip preparation method, and the normal length of 607 nm was obtained from the electron micrographs. This measurement is also in agreement with the length of viruses in PVX-group. Many factors may affect particle

length during the preparation of virus for electron microscopy. Francki (1966) proved that the pH of the buffers used in purifying TMV plays an important role in determining the length distribution of virus particles. He reported that acid conditions cause end-to-end aggregation of the rods, whereas under neutral or slightly alkaline conditions the particles tend to fragment. Hampton et al. (1974) believed that the particle lengths of pea seedborne mosaic virus (a virus of PVY group) in leaf-dip and in partially purified preparations fixed with formalin, were significantly shorter than those derived from either type of preparation when fixed with glutaraldehyde, and the length of the virus in purified preparation was longer than in leaf-dip and partially purified preparations.

The concentration of PV in infected leaves was high, often more than 0.3 mg/ml, compared with 0.5 mg/ml reported for viruses in PVX group, and the calculated extinction coefficient was 2.7-2.8, which is very close to 2.97 reported for PVX group (Table 12).

The linear-log density gradient method for estimating sedimentation rate is of potential values as an aid in the characterization of plant viruses. For this purpose, it has several advantages over the analytical ultracentrifuge method. Impure virus preparations can be used, since the identity of the virus zone is easily established by infectivity assays. With many viruses, crude extracts can be used after partial denaturation of the normal proteins by freezing or heating. Low concentrations of virus can be used. The equipment necessary for density gradient centrifugation is not as expensive as that required for analytical ultracentrifugation (Brakke 1958).

Linear log gradients have given good reproducibility and resoltuion. The estimates of sedimentation coefficients from separate experiments have usually differed by less than five per cent (Brakke and Van Pelt 1970a). The most frequent deviation from expected results has been in the slope of the plot of log depth vs. logs, not in the linearity. Therefore, use of more than one marker particle is desirable (Brakke and Van Pelt).

Values of 79, 115, and 187 s were used for the sedimentation coefficients of BMV, SBMV, and TMV (markers) respectively. The plots of logarithms of depths of the three markers against logarithms of the sedimentation coefficients have always been linear with the slope of about 0.8 (Brakke and Van Pelt 1970a), whereas a value of 0.75 was obtained by the experiments in this thesis. Results of three different experiments showed that the depth of sedimentation of PV was exactly the same as SBMV, and a value of 115 s was obtained for its sedimentation coefficient, which is in good agreement with the value of 118 s reported for PVX (Berks 1970).

The amounts of RNA calculated for TMV and BMV according to buoyant density gradient centrifugation were similar to those reported by other investigators (Woody 1958; Bockstahler and Kaesberg 1961), indicating the reliability of the procedure used. The calculated amount of RNA for PV in two experiments was the same and equal to the amount calculated experimentally for TMV (5.27 per cent). The commonly quoted RNA content of PVX is five per cent (Markham 1959), but Reichmann (1959) reported 0.59 per cent phosphorus, which suggests

six per cent RNA. In any case, neither of these values is far from the amount calculated here.

Some intrinsic viral features can markedly affect the ρ of viruses in CsCl, e.g., presence of lipids and carbohydrates as structural viral components can markedly reduce the ρ of viruses in CsCl (Murant et al. 1969). Physical alterations in the macromolecular configuration of virions which do not affect the protein to nucleic acid ratio can also affect that ρ of viruses in CsCl (Sehgal et al. 1970). Ideally, for the relationship between ρ and RNA content to be fully valid, there should be no interaction between Cs^+ and the virus particles.

The RNA of PV was extracted by a phenol-SDS extraction method (Ralph and Bergquist 1967) for the purpose of gel electrophoresis. BMV, SBMV, and TMV-RNA were used as markers, and a three per cent acrylamide gel was used for measurement of PV-RNA molecular weight. Staining with toluidine blue showed sharply stained blue bands in the gels readily visible after staining. Each band represented a discrete species of RNA. With BMV four bands were observed in the gel which represented the four different species of RNA, and the two heavier RNAs were precipitated close to each other in the gel (Figure 11). A double pattern band was obtained with TMV-RNA and with SBMV a hazy gel was obtained. The hazy gel may be due to too much sample, incomplete deproteinization, or RNA degradation (Morris and Wright, personal communication). With PV-RNA a broad single band was obtained (Figure 11).

The calculated molecular weight for PV-RNA is 2.17-2.21 x 10^6, and the amount reported for PVX (Brakke and Van Pelt 1970b) is 2.1 x 10^6 (Table 12). The value obtained for sedimentation coefficient of PV-RNA in linear-log density gradients was 37.25 when determined by using the reference RNAs.

The sedimentation coefficient of single stranded RNA depends on the temperature at which it is measured (Mitra and Kaesberg 1965) and on the buffer. The values reported in the literature for the sedimentation coefficient of BMV-RNA and TMV-RNA were obtained under the same conditions described in this thesis, and have thus been used as standard markers in estimating the sedimentation coefficient of PV-RNA.

According to Boedtker (1968), when the secondary structure of RNA is destroyed by formaldehyde treatment, the sedimentation coefficient should depend only on molecular weight. Brakke and Van Pelt (1970b) obtained a relationship between the sedimentation coefficient of formaldehyde treated RNA (s), and its molecular weight before formaldehyde treatment (M) as described previously in the results. Duplicating the experimental conditions of Brakke and Van Pelt, a molecular weight of 2.03 x 10^6 daltons was obtained for PV-RNA. As shown in Figure 13-D, 13-E, and 13-F, the rate of sedimentation for BMV, TMV, and PV-RNAs after formaldehyde treatment are much less than before formaldehyde treatment (Figure 13-A, 13-B, 13-C). Two species of BMV-RNA (BMV_1 and BMV_2) were overlapped during the centrifugation through linear-log density gradient (Figure 13-B), but after

formaldehyde treatment, 3 RNA species were overlapped (BMV_1, BMV_2, and BMV_3) and only two peaks were obtained after scanning the density gradient tube (Figure 13-E).

The binding of formaldehyde by RNA is affected by several factors. The reaction rate is very temperature-dependent. Variations of pH over a range of five to eight have no marked effect on the reaction which is consistent with the theoretical consideration that the amino groups of the bases are not dissociated within this range, but the amount of formaldehyde bound is, however, greatly affected by the salt concentration of the medium (Staehelin 1958).

The basis for the conclusion that nucleic acid from PV is an infectious single-stranded RNA is its susceptibility to RNase, resistance to DNase, infectivity tests, and its reaction with formaldehyde.

Polyacrylamide electrophoresis of SDS dissociated PV suggests that the capsid of this virus is made of a single kind of protein subunit having a molecular weight around 21,400-23,700, which is more or less similar to the molecular weights reported for protein subunits of the members of PVX group (Table 12). The gels containing PV-protein subunit after staining and destaining showed a single clear band, indicating a single type of protein subunit (Figure 15).

The binding of dodecyl sulfate ions to proteins has been shown for several protein molecules, and was assumed to be the basis of the separation of the denatured proteins upon SDS electrophoresis on polyacrylamide (Shapiro et al. 1967). The electrophoretic mobilities

TABLE 12. Comparison of different characters of PV obtained according to the experiments discussed in this thesis with the members of TMV, PVX, carnation latent virus, and PVY groups

Characters	PV	TMV	PVX	Carnation latent virus group	PVY
Thermal inactivation point °C	70	90[h]	65-75[h]	55-70[h]	50-60[h]
Dilution end point	10^{-5}	10^{-6}[j]	10^{-5}-10^{-6}[a]	10^{-2}-10^{-3}[m]	10^{-2}-10^{-3}[e]
Particle length nm	607	300[h]	480-580[h]	620-690[h]	720-780[h]
Concentration in sap (mg/ml)	>0.3	>1[h]	0.5[h]	0.02-0.1[h]	0.005-0.025[h]
Extinction coefficient	2.7-2.8	3.24[b]	2.97[b]	-	2.86[k]
$S_{20,W}$ (particle)	115	190[h]	118[a]	160[l]	154[d]
Per cent of RNA	5.27	5[h]	6[h]	6[h]	5[k]
RNA mol.wt/10^6	2.17-2.21	2.05[c]	2.1[c]	-	3.1[g]
$S_{20,W}$ (RNA)	37.25	31.1[c]	-	-	39[g]
Mol.wt.of protein subunit	21,400-23,700	17,500[h]	22,000-24,000[i]	-	28,000 and 34,000[f]

a) Berks 1970; b) Brakke 1967; c) Brakke and Van Pelt 1970b; d) Delgado-Sanchez and Grogan 1966; e) Delgado-Sanchez and Grogan 1970; f) Hiebert and McDonald 1973; g) Makkouk and Gumpf 1974; h) Matthews 1970; i) Miki and Knight 1968; j) Smith 1972; k) Stace-Smith and Tremaine 1970; l) Varma et al. 1970; m) Wetter 1971.

of different proteins through polyacrylamide gel are independent of the isoelectric point and the amino acid composition and seem to be governed solely by the molecular weights of their polypeptide chains (Weber and Osborn 1969). Also, the good resolution, and the fact that an estimate of the molecular weight can be obtained within a day, together with the small amount of protein needed, makes SDS gel electrophoresis strongly competitive with other methods commonly employed.

The antiserum prepared against PV had a low titer (about 1/128), but the titer was high enough for some serological tests. The immunodiffusion tests in nobel agar-SDS, or with pyrolidine-degraded viruses in agarose, showed that each antiserum only reacted with its dependent antigen (Figures 17 and 18), and PV only reacted with PV-antiserum. The serological tests did not show any relationship between PV and the other members of the flexuous rod-shaped groups which were tested. Therefore, unless further investigation shows that the virus described in this study is related to some previously described virus, its designation as "Plantain virus" seems appropriate.

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