Short Title

SEROLOGICAL STUDIES OF POTATO VIRUS Y

BORREL

SEROLOGICAL STUDIES OF POTATO VIRUS Y

by

Bernard Borrel

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Department of Plant Pathology,
Macdonald College of McGill University,
Montreal, Quebec.

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ABSTRACT

M. Sc. Bernard Borrel Plant Pathology

SEROLOGICAL STUDIES OF POTATO VIRUS Y

The efficacy of several compounds in depolymerizing potato virus Y (PVY) and the subsequent detection of the depolymerized products by immunodiffusion was investigated. PVY could be reliably diagnosed in crude expressed sap of infected tobacco leaves in immunodiffusion systems containing specific concentrations of the detergents sodium dodecyl sulphate (SDS) or sodium dibutylnaphthalene sulphonate (LSA), using undiluted antiserum prepared against intact PVY or SDS-degraded PVY. Antiserum prepared against PVY-protein did not react with degraded PVY antigens in immunodiffusion.

Evidence was obtained that the diagnosis of PVY in crude leaf extracts of potatoes is dependent on the concentration of the virus in the variety. Higher dilution endpoints of the virus, as determined by microprecipitin titrations, was correlated with an increased ability for detection of the antigen by immunodiffusion.

RESUME

M. Sc. Bernard Borrel Pathologie végétale

ETUDES SEROLOGIQUES DU VIRUS Y DE LA POMME DE TERRE

L'efficacité de plusieurs composés dans la dépolymérisation du virus Y (PVY) de la pomme de terre, et la détection subséquente par immuno-diffusion des produits dépolymérises, sont les sujets étudiés. Le PVY peut facilement se retrouver dans le jus du broyat de feuilles de plants de tabac infectés, en utilisant des antisérums non-dilués contre le PVY intact ou le SDS dégrade, par des systèmes d'immuno-diffusion contenant des concentrations spécifiques, de détergents, sulphate dodecyl de sodium (SDS) ou sulphonate dibutylnaphthalène de sodium (LSA). L'antisérum preparé contre le PVY protéinique ne réagit pas avec les antigènes dégradés de PVY en immuno-diffusion.

La mise en évidence du PVY dans un extrait brut de feuilles de plants de pomme de terre, dépend de la concentration du virus dans cette variété. La plus forte dilution finale du virus, déterminée par titrage en micro-precipitation, fut confirmée par une facilité accrue àdétecter l'antigène par immuno-diffusion.

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I. INTRODUCTION

Serological techniques have found wide application in the field of plant virology. They are commonly used in studies of relationships between viruses and the serological analyses of plant viruses and their antigenic components have contributed considerably to the concept of virus structure (Rappaport, 1965; Van Regenmortel, 1966).

In addition, various serological procedures have also proved extremely useful for virus identification and for the routine testing of plants on a commercial scale for freedom of virus infection.

Serodiagnostic methods for the detection of virus infection in plants are now increasingly used for a number of different crops, especially those for which vegetative propagation is essential for the maintenance of genetic characteristics, i.e., fruit trees, grape vines and potatoes. With these crops, selection of healthy propagating material requires that virus-infected plants be recognized and eliminated as early as possible to prevent further spread of the disease.

In many countries, including Canada, vegetatively reproduced plant material is often subject to rigid

certification standards which are enforced through periodic inspections. International movement of plants and plant products is governed in part by stringent phytosanitary regulations which usually require complete or substantial freedom from virus diseases. The necessity to meet certification standards in the production of seed potatoes makes the use of large scale screening and indexing programs imperative. The practicability of such programs is largely determined by the commercial value of the crop and the diagnostic techniques available.

For routine testing of large numbers of plants the use of indicator plants is often uneconomical because it requires extensive greenhouse facilities. Infectivity tests are extremely sensitive and reliable but results can only be obtained after several days or sometimes weeks. Serological procedures such as the microprecipitin test, which rely on prior clarification of the plant sap should also be avoided since they are too laborious for use in routine analysis of potato samples.

The least elaborate procedures are those whereby the presence of the virus can be detected in crude sap. In one such procedure, the gel diffusion method has become a valuable diagnostic tool in recent years. Initially, this test was restricted for use with the spherical viruses or the shorter rod-shaped viruses such as tobacco mosaic virus (TMV) and barley stripe mosaic virus (BSMV). The

elongate structure of most other rod-shaped viruses precluded their diffusion in agar gel.

Investigations by various workers have now demonstrated that diffusion of rod-shaped viruses into an agar matrix may be facilitated by the use of degrading compounds, which serve to depolymerize the virus into low molecular weight fragments or protein subunits, without the loss of serological activity. Specific examples will be dealt with in the literature review.

The objectives of the research reported in this thesis were (a) to investigate, qualitatively, the usefulness of several compounds for the degradation of potato virus (PVY) and the subsequent detection of the degraded products by immunodiffusion; (b) to prepare antisera to intact and depolymerized PVY for use in comparative serological tests; (c) to obtain an estimate of the capacity of the gel-diffusion method to detect PVY in crude extract of several plant species, with special emphasis on potatoes.

II. REVIEW OF THE LITERATURE

A. THE POTATO VIRUS Y

Potato virus Y (PVY) is the prototype of the POTY-virus group (Harrison et al., 1971). The group is characterized by particles of a diameter of about 15 nm and a length of 730-790 nm and by the presence of pin-wheel inclusion bodies in infected tissue. All members of the group are transmitted by aphids in a stylet-borne manner and induce diseases of the mosaic or mottle type in their host plants. They comprise the largest known category of plant viruses showing a common morphology, transmissibility and serological relatedness (Brandes and Bercks, 1965).

As reported by Delgado-Sanchez and Grogan (1966a) the normal length of PVY particles in purified preparations is about 684 nm, compared with about 730 nm for particles observed in tissue dip preparations. Varma et al. (1968) calculated the width of the particle to be 11 nm, helically constructed with a pitch of about 3.3 nm. Recently, Miki and Oshima (1972) found that PVY contains a single structural protein with a molecular weight of about 21,300, consisting of 187 amino-acid residues. The sedimentation coefficient (S_{20w}) of PVY is generally accepted to be 154 (Delgado-Sanchez and Grogan, 1966a), although lower values have been reported (Stace-Smith and Tremaine, 1970). The latter authors

calculated an extinction coefficient for the virus of 2.86 at 260 nm for 1 mg/ml of virus, on the assumption that the particle contained 5% RNA.

The thermal inactivation point (TIP) of the virus ranges with the strain; values of 52°C - 62°C have been reported. For most isolates the dilution end-point ranges from 10^{-2}-10^{-3}. PVY is usually inactivated *in vitro* within 48-72 hours (Delgado-Sanchez and Grogan, 1970) but it may remain infectious for 15 years in leaf-tissue dried and stored at 4°C (McKinney *et al.*, 1965).

Generally three groups of PVY strains, common, tobacco veinal necrosis and C, have been recognized (Beemster and Rozendaal, 1972; Delgado-Sanchez and Grogan, 1970) which can be distinguished according to the severity of systemic symptoms in potato, tobacco and other hosts. As far as is known, only the common strains occur in Canada. The tobacco veinal necrosis strain causes severe veinal necrosis in tobacco, but generally induces less severe symptoms in potato than does the common strain. The C-strain of PVY is characterized by causing severe stipple-streak symptoms in many potato varieties. Its reputed non-aphid transmissibility has recently been questioned by several investigators (Horváth, 1966a; Beemster and Rozendaal, 1972).

Symptoms of PVY in potato vary widely with strain and variety and range from light mottling to severe necrosis

and death of the infected plants. Any one variety may react differently with different strains. Sensitive varieties exhibit necrosis which ranges from veinal necrosis on the lower leaf surface to severe necrosis of leaves and stems. Ultimately, older leaves may collapse and drop from the plant. This condition is referred to as leaf-drop streak.

Necrosis is usually more severe after primary infection,i.e., infection which occurs during the growing season, than after secondary infection, i.e. infection arising from a previously infected tuber. Plants with secondary infection are less necrotic but dwarfed and brittle with leaves crinkled and bunched together. Yield losses of up to 80% have been reported (Beemster and Rozendaal, 1972). Combinations with other potato viruses like potato virus A (PVA), potato virus X (PVX) and potato virus S (PVS) cause severe diseases, sometimes destroying the crop. This synergistic effect, however, also depends very much on the combination of viruses and the potato variety.

In spite of the fact that PVY has long been studied in many parts of the world, it remains a major virus of potatoes and other crops because it is effectively spread by many species of aphids in a non-persistent manner. Wild solanaceous hosts usually serve as the source of inoculum. One report claims transmission of PVY by the mite Tetranychus telarius (Schulz, 1963) but this has not been confirmed.

B. PURIFICATION OF POTATO VIRUS Y

Like most other viruses of the POTY-virus group, PVY characteristically occurs in relatively low concentrations in its host plant (Bawden and Pirie, 1939) and it has a strong tendency to aggregate, both end-to-end and laterally, during extraction and purification procedures (Shepherd and Pound, 1960; Damirdagh and Shepherd, 1970a). Since aggregation may result in serious virus losses or physical heterogeneity of the virus preparation, conventional physical methods could not be routinely applied in the characterization of PVY preparations until recently with the development of improved purification procedures. Consequently, earlier purification procedures were developed mainly for the purpose of serological studies and little attention was paid to the state of aggregation or infectivity of the virus. Cremer (1951) and Bartels (1957) used low-speed centrifugation and ammonium sulphate precipitation to purify PVY. They produced a high-titered antiserum which gave satisfactory diagnostic results after having been absorbed with sap from healthy tobacco or potato plants. Wetter (1960) clarified a crude homogenate of PVY-infected tobacco leaves by using ether and carbon tetrachloride and further purified the virus by differential-and-zonal-centrifugation. The virus was used as an antigen in serological studies and no other properties were investigated. Van Regenmortel (1964) achieved good separation of host components and virus by using a zone-

electrophoresis method of purification. However, he did not comment on the state of aggregation of the final product nor did he indicate how much virus was lost during the initial extraction and clarification procedures. Venekamp and Mosch (1964) could not prevent serious aggregation of PVY after purification by chromatography on cellulose columns with solutions containing polyethylene glycol.

Since aggregation is usually most serious after the initial extraction and concentration of the virus, Delgado-Sanchez and Grogan (1966a) developed a procedure aimed at eliminating this problem. They used glass-distilled water containing 2-mercaptoethanol, ascorbic acid and sodium diethyldithiocarbamate (SDDC) as the extraction medium, followed by clarification of the extract with chloroform. Virus was purified and concentrated from the clarified extract by a combination of differential-and sucrose density gradient centrifugation. The preparation was apparently unaggregated as it sedimented in a single discreet band in the gradient and extracts were sufficiently pure to allow for analysis of some physical properties of the virus. Damirdagh and Shepherd (1970) developed a purification procedure for tobacco etch virus (TEV) and several other members of the POTY-virus group, including PVY. A high molarity phosphate buffer was used during extraction procedures followed by clarification of the crude sap with n-butanol and low speed centrifugation. The virus was precipitated

from the clarified supernatant with polyethylene-glycol (PEG). They found that lateral aggregation of the virus particles could be prevented by using 0.5M urea and 0,1% 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. Presumably, the role of 2-mercaptoethanol is related to its capacity to reduce disulfide linkages, formed by oxidation of sulfhydryl-groups on the viral protein.

Stace-Smith and Tremaine (1970) investigated several procedures for preliminary clarification of expressed sap of PVY-infected tobacco plants. Alcohol-or heat-precipitation prior to differential centrifugation were found to be most effective. Aggregation was reduced to a minimum by using a high molarity buffer (0.5M phosphate, pH 7.0) to resuspend the first high-speed pellet. Further purification was carried out by means of sucrose density gradient centrifugation, and the preparation was sufficiently pure to allow for the determination of its nucleic acid content and amino-acid composition of the protein.

In a recent investigation on the molecular size of the protein subunit of PVY, Miki and Oshima (1972) purified the virus from tobacco, by extraction with 0.02M phosphate buffer pH 7.0, containing 0.5M NaCl and 0.1% 2-mercaptoethanol. Clarification of the extract was with chloroform, followed by precipitation of the virus with PEG. Virus pellets

were subjected to 3-4 cycles of differential centrifugation and further separation of host components was achieved with sucrose density gradient centrifugation and ultracentrifugation in cesium chloride for 40 hours. Homogeneity of the purified preparation was assessed by electron microscopy and sucrose density gradient electrophoresis. No details regarding the ultraviolet spectrum of the virus were provided nor was any indication given as to the yield of purified virus per unit weight of plant tissue.

In a recent serological study on PVY, Purcifull and Gooding (1970) partially purified the virus from tobacco, by a combination of differential centrifugation and isoelectric precipitation at pH 5.6. The resuspended pellets were used as the inject-antigen and an antiserum was produced which reacted only weakly with antigens from crude sap of healthy tobacco plants, obtained by homogenizing tobacco leaves with ethanolamine-hydrochloride pH 10.5.

The preferred host for the culture of PVY is tobacco and several varieties have been reported to give satisfactory virus yield i.e., Havana 425 (Delgado-Sanchez and Grogan, 1970), Haranova (Stace-Smith and Tremaine, 1970) and Burley 21 (G.V. Gooding, Jr., personal communication). Usually, for purification purposes, young tobacco seedlings are inoculated in the 4-5 leaf stage and harvested 12-20 days later. Delgado-Sanchez and Grogan (1966 a and b) found that directly inoculated leaves of "Havana 425" were a better source of

PVY for purification than systemically infected leaves of the same host. They based their conclusions on results obtained from infectivity assays on Chenopodium quinoa with tissue extracts and purified preparations of PVY. In this respect, it should be observed that although several plant species have been reported to react with local lesions after inoculation with PVY (Horvath, 1967c), no reliable host for quantitative studies is believed to have been reported (A.F. Ross, personal communication).

It is generally accepted that PVY occurs in relatively low concentration in its host plant (Bawden, 1964) but few investigators have presented quantitative data to this effect. Stace-Smith and Tremaine (1970) reported a yield of 3-4 mg of purified virus/kg of leaves. The preparation exhibited an ultraviolet absorption spectrum typical of other tubular viruses, with absorption maximal at 260 nm and minimal at 247 nm. The A260:A280 ratio was 1.21 ± 0.04 and the A260:A247 ratio was 1.11 ± 0.02. The lack of similar data in reports presented by other investigators is presumably a reflection of the fact that PVY is characteristically difficult to manipulate in vitro and cannot easily be obtained in sufficient quantity and purity for physical and chemical studies.

C. DIAGNOSTIC PROCEDURES FOR PVY AND OTHER ROD-SHAPED POTATO VIRUSES

Because of the nature of the propagation of the potato plant and the nature of transmission of the infectious

agent, measures taken to control the incidence of virus diseases in potatoes are largely aimed at decreasing the rate of infection. Production of virus-free plant material is an essential part of any control-scheme designed to minimize virus infections and for this purpose reliable and sensitive indexing techniques are needed.

Horvath (1967c) lists 27 viruses as being pathogenic to potatoes (some of these "viruses" have since proven to be mycoplasmas) and listed among the major ones are potato virus A (PVA), potato aucuba mosaic virus (PAMV), potato virus S (PVS), potato virus M (PVM), potato virus X (PVX) and potato virus Y (PVY). All of these virus diseases are induced by rod-like particles with a length of 500 nm or greater.

The most commonly used techniques for the routine diagnosis of these elongated viruses in potato material are the serological precipitin or agglutination test and infectivity tests by means of indicator plants. Van Slogteren (1972) reported that in the Netherlands potato plants are routinely indexed for the presence of PVM, PVS and PVX by the micro-agglutination test. This method is extremely rapid, requires little antiserum and is reasonably reliable. Generally, the test-plant method is considered to be the most sensitive for plant viruses (Hollings, 1965) but the test requires extensive greenhouse facilities and it is often slow to give results. Sometimes, as is the case with

PVS and PVM, no reliable indicator plants are available.

Kahn et al. (1967) reported that the bentonite-flocculation test was more sensitive in the detection of PVM, PVS and PVX than the microprecipitin test, but Sampson and Taylor (1967) failed to obtain positive results. Bercks (1967) also reported that the bentonite-flocculation, barium-sulphate and latex-agglutination tests were more sensitive than the micro-precipitin test for the detection of many plant viruses including PVS, PVX and PVY, but Maat (1970) pointed out that the increased sensitivity of the bentonite-flocculation and the latex-agglutination tests for the detection of PVS in potato was only marginal and that, in contrast with the micro-agglutination test, clarification and/or dilution of the sap was often necessary.

DeBokx (1964) stated that PVA and PVY could not be reliably diagnosed in potatoes by means of serology and recommended the use of infectivity tests on detached leaves of Solanum demissum x Solanum tuberosum var. Aquila (clone "A6"). This test, although very sensitive, lacks specificity, as local lesions are produced not only by PVA and PVY but also by PAMV and severe strains of PVX.

In a comparative quantitiative study on the detection of elongated potato viruses by electron microscopy, infectivity and microprecipitin tests, deBokx (1969) found the sensitivity of electron microscopy about equal to that of an assay on the local lesion host. Only Gomphrena globosa

was more sensitive for the detection of PVX than electron-microscopy and local lesions were produced with sap dilutions of 1:30,000. The systemic host tobacco "White Burley" was found to be more sensitive than electron microscopy for the detection of PVY and PAMV. Sap from infected tobacco had an infectivity dilution end point of 1:8000 when assayed on White Burley; by electron microscopy the end point was 1:2000. The "A6" test was equally as sensitive as electron microscopy.

Similar studies were carried out by Sampson and Taylor (1968) with comparable results, except that for the diagnosis of PVY electron microscopy was found to be more sensitive than infectivity tests on tobacco. With the microprecipitin test PVY was only detected in sap dilutions of infected tobacco foliage not exceeding 1:50. Electron microscopy was found to be significantly more sensitive than indicator plants for the detection of PVX and PVY in potato tubers but less sensitive for the detection of PVX in potato foliage. The least sensitive technique was the microprecipitin test, for PVX was detectable in sap dilutions not exceeding 1:64.

It should be noted that results from comparative diagnostic studies based on antigen dilution end-points as a measure of sensitivity, have only limited value considering the variability of the plant material. Sampson and Taylor (1968) pointed out that serial dilution experiments

with PVS-infected potato sap indicated that the virus concentration in mature plants was higher than in younger plants. They also conceded that some of their PVX-infected plants might have contained more virus than plants used by other investigators. DeBokx (1964) could reliably detect the tobacco veinal necrosis strain of PVY by means of detached "A6" leaves only in leaves and parts of stems of secondarily infected potato plants. In foliage of potato plants with primary infection, detection of the virus was not possible shortly after infection. In an experiment with the variety "Bintje", inoculated 11 weeks after planting, virus could be detected in inoculated leaves of all plants tested 14 days after inoculated but in the top leaves of only 25% of the plants. In young tubers of plants with primary infection PVY could be detected shortly after harvesting if the period between inoculation and lifting was sufficiently long. This was taken as evidence that the diagnosis of the virus depends on the virus concentration in the tuber. Additional evidence was presented to demonstrate that the concentration of PVY in the potato plant is related to the virus resistance of the variety.

PVY could not be detected by means of the "A6" test in dormant tubers of plants with either primary or secondary infection. Virus activitywas restored immediately after artifically breaking the dormancy and the virus concentration was found to be higher in the sprouts than in the tuber itself.

Beemster (1967) provided evidence that the heel-end of a tuber is less frequently infected with PVY than the rose end.

Routine diagnostic procedures must be sensitive and applicable with high speed, two qualities which according to Sampson and Taylor (1968) are met by electron microscopy. They examined potato foliage known to be infected with PVS and processed 20 samples/hour, limiting the viewing time for negative samples to 5 minutes. However, de Bokx (1969) felt that, for the routine detection of viruses by electron microscopy, even a scanning time of 2 minutes per grid would be too long and that operating at this rate would not provide for a reliable diagnosis of PVM, PVS or PVY.

Recently, the Ouchterlony immunodiffusion procedure (Van Slogteren, 1954), has shown considerable potential for use in routine serodiagnosis of plant viruses with elongated particles, after Purcifull and Shephard (1964) demonstrated that these viruses could be degraded into shorter, diffusable fragments that would react with anti-serum prepared against intact virus (see Chapter II D, degradation and serology of rod-shaped plant viruses). 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. Furthermore, large numbers of tests could be performed simultaneously in one agar plate and very little antiserum per test was required. The technique required a minimum of sap manipulation and could be performed in the field with crude expressed sap. It was later reported to be useful for the detection of PVS (Shepard, 1970) and PVM (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. No precipitin lines were obtained in double-diffusion tests. Shepard and Secor (1969) detected purified PVX-protein at a concentration of 10 µg/ml with the double-diffusion method while concentrations of 1 µg/ml were detected with the radial-diffusion method.

D. DEGRADATION AND SEROLOGY OF ROD-SHAPED PLANT VIRUSES

Until recently, serological procedures which relied on diffusion of virus and antibody into a semi-solid medium such as agar, were thought to be suitable only for use with isometric viruses and the shorter rod-shaped viruses with lengths up to 300 nm. Diffusion of longer particles is precluded by their low diffusion coefficient and by their tendency to form aggregates (Van Slogteren,1969). A possible exception is PVX, where sharp precipitin lines were obtained using purified virus preparations (Ball, 1964). However, lines were obtained only when virus preparations were made in citrate-phosphate buffer pH 8.0, to minimize aggregation. Van Slogteren (1972) observed precipitin-lines with PVX in a double diffusion test by using crude untreated sap of infected tobacco plants. He did not elaborate on details of the immunodiffusion system.

The potential usefulness of immuno-diffusion procedures for large-scale serodiagnostic programs 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 anti-serum 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 to be effective but alkaline degradation using ethanolamine buffers yielded the greatest amount of serologically active protein from clover yellow mosaic virus. It was pointed out, however, that conditions for degradation will vary with each particular virus. Hamilton (1964) developed a method to detect the presence of barley stripe mosaic virus (BSMV) in sap from infected barley plants. He employed a gel double diffusion system in which Leonil S.A., (sodium dibutyl naphthalene sulphonate) an anionic detergent, had been incorporated. The detergent served to degrade the virus into more rapidly diffusing fragments. 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 PVY and tobacco etch virus (TEV). The latter workers incorporated the detergent sodium dodecyl sulphate (SDS) in the agar gel and demonstrated the presence of PVY and tobacco etch virus (TEV) in crude sap of infected tobacco plants. Hiebert *et al*. (1970) 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 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 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 (1971) stated that fragmentation of PVS particles in crude sap was not successful.

Purcifull and Shepherd (1964) questioned the degree of serological relatedness between intact and depolymerized virus particles since previous studies had suggested a difference in antigenic identity between TMV and its chemically degraded capsid protein (Kleczkowski, 1961; Takahashi and Gold, 1960; and Aach, 1959). In later studies several other viruses were shown to be antigenically different from their structural unit. Shepard and Shalla (1970) concluded from quantitative precipitation studies, in which they ascertained homologous and cross-reactive antibody levels in PVX and PVX-protein antisera, that PVX and the PVX depolymerized structural unit differ sufficiently in antigenic specificity to be considered as distinct but related antigens. In further studies (Shalla and Shepard, 1970) they demonstrated that after depolymerization, monomeric

and dimeric PVX structural units undergo a major change in conformation. According to a theory proposed by Rappaport (1965) and Rappaport et al. (1965) this change in conformation would result in the concomittant formation of new antigenic determinants.

Shepard and Shalla (1970) also pointed out that results obtained with BSMV (Atabekov et al., 1968) and TEV (Purcifull, 1966) suggest that the relationship between degraded virus and the intact nucleocapsid is contingent upon the presence of intermediate sized oligomers and not upon monomeric structure units. Expounding on this theory, they hypothesized that it would be the retention or reformation of stable intermediate aggregates upon degradation, which provides the majority of serological cross-reactivity between degraded protein preparations and virus antiserum. A drastic alteration in antigenic specificity would result with many plant viruses when depolymerized to the level of the monomeric or dimeric unit, resulting in a weak reaction or no reaction at all.

The practical significance of antigenic disparity between intact and degraded virus was recently demonstrated by Shepard and Secor (1969) who could not reliably diagnose the presence of pyridine-degraded PVX in crude potato sap by immunodiffusion, if antiserum prepared against the intact antigen was used. They found that the diagnosis was reliable only if antiserum prepared specifically against the degraded

virus was used. Little or no reactivity was observed between degraded virus and whole PVX antiserum and pyridine-degraded PVX antisera consistently possessed higher antibody titers to degraded virus than did whole PVX antisera. In accordance with results obtained for TMV by Loor (1967) it was shown that degraded PVX was less immunogenic than intact PVX and after injection into rabbits stimulated antibody production over a much shorter period.

In a study on the serological detection of PVS, Shepard (1970) demonstrated the importance of employing a suitable degrading agent. Pyridine, previously found suitable for degrading PVX, did not produce diffusable PVS fragments or protein although it reacted positively with degraded PVS antiserum in tube-precipitin tests. However, pyridine-degraded PVS was effective as an immunogen, producing an antiserum that, when used in immunodiffusion tests, would readily demonstrate the presence of pyrrolidine-degraded PVS in crude sap of potatoes. Pyrrolidine was also found to be an effective degrading compound for the serodiagnosis of PVM but in order to obtain antibody with a sufficiently high titer to degraded PVM it was found necessary to immunize a goat rather than rabbits.

Summarily, it may be stated that, in spite of the considerable potential which degrading agents have shown to facilitate the detection of elongated plant viruses, it has become apparent that no one method of degradation yields

viral products which behave in a predictable manner. Considering the differences in antigenic relationship that may exist between intact and degraded virus, especially when the latter is depolymerized to monomeric subunits, it seems advisable to prepare antisera to the degradation products in order to obtain maximum specificity.

Whether the immunogen has to be prepared with the same degrading compound which is used to prepare the antigen for the immuno-diffusion system remains to be investigated, but it likely varies with the virus. Low molecular weight viral fragments or subunits are not known to be good immunogens but the immune-response of different animal species to a particular immunogen should be investigated.

III. MATERIALS AND METHODS

A. THE VIRUS

The isolate used throughout this study was collected from field-grown tobacco at L'Assomption, Quebec, by Dr. W. E. Sackston, Department of Plant Pathology, Macdonald College, Ste Anne de Bellevue, Quebec.

Its identity as a normal strain (Horvath, 1966b) of potato virus Y (PVY) was verified with antisera received from Dr. G. V. Gooding, Jr., North Carolina State University, Raleigh, N.C. and from Dr. R. H. Bagnall, Canada Department of Agriculture Research Station, Fredericton, N. B. Further identification was carried out through a host range study using twenty-four differential host plants. Seed of most test plants was received from the Canada Department of Agriculture Research Station, Fredericton, N. B. After local-lesion passage through Physalis floridana a vegetative stock culture of the virus was maintained in Nicotiana glutinosa. Permanent stock cultures were also maintained in leaves of N. glutinosa dried over anhydrous calcium chloride at 3°C.

B. CULTURE CONDITIONS OF EXPERIMENTAL HOSTS

Plants used for virus stock and assays were grown in pasteurized soil in clay pots. The potting medium consisted

of 2 parts pasteurized or sterilized soil, one part sand and one part peatmoss. Eight ounces of the commercial fertilizer "Osmocote" (14-14-14) were added to each bushel of the soil mixture. Culture conditions were slightly different for potatoes. Prior to planting, potato seed-pieces, containing one or more eyes, were treated with the fungicide "Captan" dissolved in water at the rate of 5 lbs per 100 Imperial gallons. Seed-pieces were planted in 7-inch or 10-inch clay pots filled with a medium consisting of 3/4 loam and 1/4 black humus, without added fertilizer. After the plants had established a good root system they were fertilized at fortnightly intervals with the commercial fertilizer "Vigoro" (6-9-6).

All plants were grown in an insect-proof greenhouse, maintained at about 22°C in the winter months and between 24o C and 32°C during the summer months. Supplemental light was supplied by incandescent and fluorescent lamps to give a minimal photo-period 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 fumigations with recommended fumigants. Plants used for the preparation of inoculum were grown in growth chambers set at a temperature of 25± 2°C and a 16 hour illumination period.

C. INOCULUM AND INOCULATION PROCEDURES

Large quantities of inoculum were prepared by inoculating young tobacco seedlings (Nicotiana tabacum var. Burley 21) with a vegetative stock culture of the virus. At regular intervals, fully expanded green leaves showing clear symptoms were harvested, placed in plastic bags, and stored at -20°C (Wiersema, 1961). The virus retained its infectivity in frozen leaf tissue over a period of five to seven months, after which time it was necessary to regenerate it from permanent stocks maintained in leaves of N. glutinosa.

Prior to inoculations, a suitable quantity of frozen leaf-tissue was withdrawn from the freezer, thawed and homogenized with a mortar and pestle in 0.01M potassium phosphate buffer at pH 7.1. Celite* served as an abrasive and was added after grinding at a concentration of1-2%. Inoculations were performed by rubbing the leaves of plants to be inoculated with sterilized cheese-cloth dipped in the inoculum.

D. PURIFICATION PROCEDURES

The virus was routinely purified from N. tabacum var. Burley 21, or occasionally from N. tabacum var. Samsun.

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*Diatomaceous silica manufactured by Johns-Manville Co.

To eliminate the danger of contamination with tobacco mosaic virus (TMV), the variety "Burley 21", being hypersensitive to TMV, was the preferred host for virus culture despite the fact that "Samsun" was found to yield slightly higher virus quantities after purification. Seeds of "Burley 21" were kindly supplied by Dr. G. V. Gooding, Jr. of North Carolina State University.

Young tobacco plants were inoculated in the 4-5 leaf stage and for practical purposes, both directly inoculated and systemically infected leaves were collected 12-20 days later. Leaves were placed in polyethylene bags and cooled down to 3°C before being processed.

Two purification procedures were investigated and compared. They are presented here in stepwise fashion. Either a 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 or pelleting.

The method under (1) below was eventually adopted for routine use.

1. Purification of PVY by Differential Centrifugation and Iso-electric Precipitation (Purcifull and Gooding, 1970).

   All steps were carried out at 3°C.

a) Infected tobacco leaves, precooled to 3°C, were homogenized in 0.5M sodium citrate, pH 8.8 in a Waring blendor for 3-5 minutes. Prior to homogenization, 1% 2-mercaptoethanol was added. One hundred ml of sodium citrate was added to 100g of tissue.

b) The homogenate was strained through a double layer of Mira-cloth (Calbiochem, LaJolla, California,92037) and the sap collected.

c) N-butanol was slowly added to the constantly stirred filtrate, to a final concentration of 7%. The butanol had previously been washed with 40% sodium bisulfite (Damirdagh and Shepherd, 1970 (a)) a commercial mixture of sodium bisulfite, $NaHSO_3$, and sodium meta-bisulfite, $Na_2S_2O_5$.

d) Stirring was continued for 30 minutes, after which the mixture was subjected to a low speed centrifugation of 30 minutes at 10,000 rpm, to remove denatured host material and debris.

e) The supernatant was filtered through Mira-cloth and incubated for 10-12 hours at 3°C.

f) Additional denatured host material was removed by a centrifugation of 15 minutes at 10,000 rpm.

g) The supernatant was filtered through Mira-cloth, transferred to polycarbonate tubes and centrifuged at 30,000 rpm for 60 minutes using a Beckman Type 30 rotor, to pellet the virus.

h) Virus pellets were resuspended for 10-12 hrs in 0.02M borate buffer, pH 7.8.

i) The resuspended virus was pooled and centrifuged for 10 minutes at 10,000 rpm.

j) The supernatant was collected and centrifuged at 30,000 rpm for 60 minutes, to sediment the virus.

k) Steps h and i were repeated.

l) An estimation of the virus concentration in the suspension was obtained by spectrophotometric analysis. The concentration was adjusted to 1.0 mg/ml. (see Chapter III, E)

m) Sodium-chloride was added to the suspension to make a final concentration of 0.5%.

n) The pH of the suspension was lowered to 5.6 with 0.2% acetic acid in order to precipitate the virus.

o) The virus precipitate was pelleted by centrifugation for 15 minutes at 10,000 rpm.

p) The pellet was resuspended in0.02M borate pH 7.8 for 24 hours and then centrifuged for 10 minutes at 10,000 rpm.

q) The supernatant represented a suspension of partially purified virus.

2. Purification of PVY by Precipitation with Polythyleneglycol and Differential Centrifugation (Damirdagh and Shepherd, 1970 (a)).

All steps were carried out at 3°C.

a) Infected tobacco leaves precooled to 3°C, were homogenized in a Waring blendor for 3-5 minutes, using 120 ml of 0.5M phosphate buffer pH 7.1 containing 1% 2-mercaptoethanol for each 100g of leaf tissue.

b) The homogenate was strained through a double layer of Mira-cloth.

c) N-butanol, previously washed with 40% sodium-bisulfate, was slowly added to the constantly stirred filtrate to a final concentration of 7-8%.

d) Stirring was continued for 30 minutes after which the mixture was subjected to a low-speed centrifugation of 30 minutes at 10,000 rpm to remove denatured host material and debris.

e) The supernatant was filtered through Mira-cloth and incubated at 3°C for 10-12 hrs.

f) Additional denatured host material was removed by a centrifugation of 15 minutes at 10,000 rpm.

N.B. Omission of steps e and f, although not part of the published procedure, resulted in the precipitation of large

amounts of host-material upon the addition of PEG (see step g).

g) The virus was precipitated from the clear supernatant by dissolving 4.0g of polyethylene glycol (PEG), MW 6,000, per 100 ml of extract.

h) The solution was incubated at 3°C for 90 minutes while stirring.

i) The virus precipitate was collected by centrifugation for 10 minutes at 10,000 rpm.

j) Virus pellets were resuspended overnight in 0.025M phosphate buffer pH 7.4, containing 0.5M urea and 0.1% 2-mercaptoethanol.

k) Resuspended virus pellets were pooled and centrifuged for 10 minutes at 10,000 rpm.

l) The supernatant was collected and centrifuged for 90 minutes at 30,000 rpm using a Beckman Type-30 rotor.

m) Steps k and l were repeated twice.

n) Final pellets were resuspended in 0.025M phosphate buffer, pH 7.4, containing 0.5M urea and 0.1% 2-mercaptoethanol.

o) The resuspended virus pellets were pooled and centrifuged at 10,000 rpm for 10 minutes.

p) The supernatant represented a partially purified suspension of PVY.

All purified virus suspensions were kept at 3°C and utilized as soon as possible, since the tendency of virus to aggregate increased with time. Evidence was also obtained that upon prolonged storage in dilute borate buffer the antigenic specificity of the virus is altered (Shepard, 1970).

E. SPECTROPHOTOMETRIC ANALYSIS

Ultraviolet spectral analysis of virus suspensions was routinely carried out during and after purification

procedures, during degradation studies and during the preparation of viral protein.

Analysis was performed with a Unicam SP 800A ultraviolet spectrophotometer with a 1 centimeter quartz-cell. The concentration of virus in purified preparations was determined by measuring the absorbance at 260 nm and relating it to the published extinction-coefficient of 2.86 cm^2 mg^{-1} (Stace-Smith and Tremaine, 1970). The ratio of the absorbance at 260 nm divided by the absorbance at 280 nm (A260/A280 ratio) was calculated and compared with the published value of 1.21 ± 0.04 (Stace-Smith and Tremaine, 1970). The value was used as one of several criteria to determine the relative purity of the virus suspensions. In all optical density measurements, appropriate corrections were made for the absorbance of the suspending medium. No corrections were made for light scattering.

F. SUCROSE DENSITY GRADIENT CENTRIFUGATION ANALYSIS

The sucrose density gradient centrifugation technique was used to analyse sedimentation profiles of purified or degraded PVY preparations, as a means of obtaining information on the homogeneity of the preparation or the relative degree of degradation of the virus. Beckman SW 40 (9/16 x 3 3/4 inch) or SW 27 (1.0 x 3 1/2 inch) cellulose nitrate tubes were used to hold a preformed sucrose density gradient

column. Virus samples to be analysed were carefully floated on top of the columns which were then centrifuged in a Beckman SW 40 or SW 27 rotor at 40,000 rpm or 27,000 rpm. All centrifugations were carried out in a Beckman L2-65-B ultracentrifuge set at a constant operating temperature of 3°C. Duration of the run was varied to suit the aim of the experiment.

Sucrose density gradient columns to be used for centrifugation with an SW 40 rotor (hereafter referred to as SW 40 gradients) were prepared using an ISCO model 570 gradient former. Solutions of 10% and 40% sucrose (w/v) were made by dissolving ribonuclease-free sucrose (Schwartz-Mann, Orangeburg, N.Y.) in 0.02M borate buffer, pH 7.8. Two flasks containing these sucrose solutions were used as reservoirs for two 10 ml syringes, which were controlled by independent pumps. Pump speed was adjusted so that the syringes would deliver the two sucrose solutions in the tube at a linear concentration of 40% at the base and 10% at the top in a total volume of 12 ml. When desired, columns with a 5-20 percent (w/v) sucrose gradient were prepared in a similar manner, by preparing stock solutions of 5% and 20% sucrose.

Occasionally, SW 40 gradients were made by hand by layering 3 ml each of 400, 300, 200 and 100 mg of sucrose per ml in the cellulose nitrate tubes, using a pipette with a wide orifice. Sample volumes for SW 40 gradients were 0.2 ml of known virus concentration.

Sucrose density gradient columns to be centrifuged with a SW 27 rotor (hereafter referred to as SW 27 gradients) were made by hand by layering 9 mls each of 100, 200, 300 and 400 mg of sucrose per ml for 10-40% gradients. For 5-20% gradients sucrose concentrations were 50, 100, 150 and 200 mg/ml. Sample volumes for SW 27 gradients were 2.0 mls of virus or viral protein at known concentration.

SW 40 gradients made with the ISCO model 570 gradient-former, were used within 24 hours. Handmade SW40 or SW 27 gradients were stored at 3°C for at least 12 hours in order for a proper concentration gradient to form.

After centrifugation, gradient columns were analyzed with the ISCO model D density gradient fractionater coupled to an ISCO model 610 "Lab graph" bench top recorder.

The fractionater consisted of a pump which pushed the sucrose at a constant rate past an ultraviolet light source. The absorbance was recorded on a chart which graphically resolved the absorbing material at any point within the gradient column. A pump speed of 1.0 ml/minute was routinely used and the ultraviolet analyzer was set at 254 nm. If desired, specific fractions of the gradient column were collected for further experimentation.

G. VIRUS DEGRADATION

1. Preliminary Studies

Preliminary degradation studies of the virus were made using modifications of the procedures of Purcifull (1964).

Initially, ten degrading compounds were selected arbitrarily on the basis of their demonstrated or potential usefulness for the depolymerization of viruses into serologically active diffusable fragments, i.e.:

Sodium dodecyl sulphate (Gooding and Bing, 1970)
Pyrrolidine (Shepard, 1970)
Guanidine-hydrochloride (Reichmann, 1960)
Sodium dibutylnaphthalene sulphonate (Leonil SA or LSA) (Hamilton, 1964)
Urea (Spitnik-Elson, 1965)
Ethanolamine-hydrochloride (ETA) pH 10.5 (Purcifull and Gooding, 1970)
Pyridine (Shepard and Secor, 1969)
2-Dimethyl-amino-ethanol pH 10.2 (Purcifull and Shepherd, 1964)
Igepon T-73 (Brakke, 1959)
Glycine-NaOH pH 12.0 (Purcifull, 1966)

Stock solutions of the degrading compounds were prepared with distilled water at twice the desired concentration. A small aliquot of each compound (1.0-2.0 ml) was mixed in a glass tube with an equal volume of a purified virus preparation of known concentration, suspended in 0.02M borate pH 7.8.

Degradation of the viral material was evaluated visually by observing the relative loss of opalescence of the suspension as compared with control tubes containing untreated virus at the same concentration or buffer only. Optical properties of opalescence could be judged in a dark room by shining a strong beam of light through the tubes.

Tubes were incubated at 25°C for 4 hrs and then transferred to the coldroom (3°C) for an additional 44 hrs. Exposure of the virus to the degrading compound was terminated by dialysis against several changes of 0.02M borate

buffer, pH 7.8. At certain stages of the experiment, aliquots were withdrawn from each tube for analysis by sucrose density gradient centrifugation and immunodiffusion, in order to observe qualitative changes in the sedimentation properties of the virus and to evaluate the effectiveness of the degrading agents in producing diffusable virus fragments or subunits that would react with antiserum prepared against intact virus. The immunodiffusion system consisted of 0.8% agar, 0.85% sodium chloride and 0.04% sodium azide. In analyzing sucrose density gradient profiles of treated PVY suspensions degradation of the virus was indicated by low molecular weight, ultraviolet light-absorbing material, which sedimented a shorter distance in the gradient than did untreated PVY in a corresponding control tube.

The degrading compounds were also evaluated for their ability to degrade virus in expressed crude sap of infected plant tissue. To this effect, infected tobacco leaves, 12-15 days after inoculation, were homogenized with a mortar and pestle. An aliquot of the crude sap was mixed with an equal volume of the degrading compound under study. The mixture was incubated for 4 hrs at 25°C and an additional 44 hrs at 3°C. Aliquots were withdrawn at each stage for use in immunodiffusion tests.

Variations of the above procedures were achieved by homogenizing infected leaves directly with the degrading compound for subsequent use in immunodiffusion or by charging

the wells of the immunodiffusion system simultaneously with equal volumes of infected crude sap and degrading compound.

The efficacy of the degrading compounds was also tested by incorporating them in the immunodiffusion medium (Hamilton, 1964; Gooding and Bing, 1970) which consisted of 0.8% Difco agar-Noble, dissolved in distilled water. Sodium chloride (0.85%) or sodium azide (0.1%) was added to serve as the electrolyte (Crowle, 1961). Stock solutions of the degrading compounds were added to the agar at 60°C, in appropriate quantity to give the desired final concentration in the agar, and 2.5 mls of the mixture was pipetted into each quadrant of a quadrant-type petri dish.

After gelation, immunodiffusion tests were made with antigen wells deployed in a ring around a central antiserum well. Size of the wells and distance between wells were varied as part of the experimental procedure. Antigen wells were charged with purified PVY or expressed crude sap of infected and healthy tobacco plants. Undiluted antiserum was used. Plates were normally incubated at room temperature in a moist chamber. The formation of a precipitin line midway between the antiserum and antigen well indicated that the degradation treatment had resulted in producing serologically active and diffusable viral fragments.

2. Studies with Sodium Dodecyl Sulphate (SDS), Ethanolamine-Hydrochloride pH 10.5 (ETA) and Sodiumdibutylnaphthalene sulphonate (Leonil SA or LSA)

Based on recent reports in the literature (Purcifull and Gooding, 1970; Gooding and Bing, 1970) and after analysis of results obtained from preliminary studies, three degrading compounds, i.e., SDS, ETA and LSA were selected for further experimentation. Methods and techniques employed were essentially the same as those used during previous preliminary tests, with the objective of determining the minimum time and concentration of the degrading compound required to degrade purified PVY into serologically active diffusable fragments or subunits to be used in the preparation of an antiserum. Degraded PVY was analyzed by sucrose gradient centrifugation and immunodiffusion.

The anionic detergents SDS and LSA, when incorporated into the agar at specific concentrations, appeared to be the only degrading compounds analyzed that allowed for rapid diagnosis of PVY in infected crude sap of tobacco. Subsequently, all detailed and quantitative serodiagnostic tests of PVY-infected tobacco and potato plants were carried out employing SDS or LSA as the degrading agent and using antisera prepared against intact virus, SDS-degraded virus and viral protein.

H. PREPARATION OF VIRAL PROTEIN

Since the chemical properties of potato virus Y approximate those of tobacco etch virus (TEV) (Damirdagh and Shepherd, 1970b; Stace-Smith and Tremaine, 1970) and the alkali-derived antigens of the two viruses were shown to

be serologically related (Purcifull and Gooding, 1970) the method of Purcifull (1966) for the preparation of TEV protein was adopted to prepare a protein suspension of PVY. The procedure is presented here in stepwise fashion:

1. Approximately 15-20 mg of PVY, suspended in 0.02M borate pH 7.8 was pelleted by ultra-centrifugation at 40,000 rpm for 60 minutes, using a Beckman Type 65 rotor.

2. The pellet was covered with 3 ml of 0.1M glycine-NaOH buffer at pH 12.0.

3. The solution was incubated at room temperature for 6-8 hours with frequent agitation in order to facilitate resuspension of the protein.

4. To remove undegraded virus, the solution was centrifuged at 40,000 rpm for 90 minutes in a Beckman Type 65 rotor.

5. Two volumes of distilled water were added to the supernatant.

6. The solution was made 50% of saturation with the addition of 2.8g of granular ammonium sulphate (Green and Hughes, 1955) and incubated for 60 minutes at room temperature.

7. The flocculent protein precipitate was removed by low speed centrifugation (3000g) for 15 minutes.

8. The pellet was washed in distilled water, centrifuged and resuspended in 9 ml of 0.1M glycine-NaOH pH 12.0.

9. Steps 6, 7 and 8 were repeated and the final pellet was resuspended in 2 ml of the glycine buffer.

10. The protein solution was dialysed against 0.02M borate pH 7.8 for 48 hrs at $3^{o}C$.

11. Insoluble material formed during dialysis was removed by centrifugation for 20 minutes at 15,000 rpm.

12. The clear supernatant containing the protein was collected for use as the immunogen.

13. On the assumption that PVY has a nucleic acid content of 5% (Stace-Smith and Tremaine, 1970), it was calculated spectrophotometrically that approximately 60% of the original protein present could be recovered by this method. An extinction coefficient of 1.0 for PVY-protein was assumed.

I. PREPARATION OF ANTISERA

1. Preparation of Antiserum against Intact PVY

Antiserum to PVY was prepared in two rabbits through a series of intramuscular and intravenous injections with a known quantity of purified PVY in 0.02M borate pH 7.8 containing 0.85% NaCl. For intramuscular injections, 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. Prior to the first injection, normal serum was prepared from blood obtained by bleeding each rabbit from the marginal earvein. The blood

was collected in a plastic petri dish and incubated at 37°C for 1 hour. As the blood clotted it was ringed several times to prevent adhesion to the walls of the petri dish. Following an additional incubation at 4°Cfor 12 hours, 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 -20C with 0.02% NaN_3 added as a 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 micro-precipitin 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 PVY (0.5 mg/ml) (Ball, 1961). Both reactants were diluted in 0.05M Tris-HCl pH 7.1, containing 0.85% NaCl. It was found that the use of phosphate buffer as the diluent caused aggregation of the PVY particles which sometimes resulted in precipitation of the virus. Booster injections were administered to each rabbit after a significant decrease of the antibody level of the immune serum was observed.

2. Preparation of Antiserum against SDS-degraded PVY

The immunogen was prepared by adding to 1.5 ml of purified PVY at a concentration of 3-4 mg/ml, an equal volume of 0.5% SDS and incubating this mixture for 5 hrs at 3°C. It had been established by previous experimentation that

this exposure time and temperature would result in total degradation of the virus. After incubation, the preparation was dialysed against 0.02M borate pH 7.8 for 24 hrs at 3°C.

To ensure complete removal of any undegraded virus and to effect analysis of the degradation products, 2 ml of the preparation were layered on a 10-40% SW 27 sucrose density gradient and centrifuged for 3 hrs at 27,000 rpm. After fractionation of the gradient, the top 8 ml of the sucrose column, showing a high optical density, was collected and concentrated to 2 mls by transferring the fraction to a dialysis sac which was placed in granular PEG (MW 20,000) for approximately 20 minutes. A control tube, on which 2 ml of untreated PVY was layered, showed that no undegraded virus remained in the top 8 mls of the sucrose column.

The virus concentration of the final preparation was determined spectrophotometrically before emulsification with an equal volume of Freund's incomplete adjuvant. The emulsion was injected intramuscularly. Four injections were given at weekly intervals to an animal previously bled for normal serum. The animal was bled 2 weeks after the last injection. Antiserum and normal serum was collected as described under 1.

In determining the concentration of viral material in the SDS-degraded virus preparations, the extinction coefficient for PVY was used, since the absorbance spectrum

indicated only a slight loss of nucleic acid after density gradient centrifugation.

No attempt was made to remove the sucrose from the final antigen preparation as it apparently does not affect the immugenicity or alter the serological specificity of the antigen (C.E. Tanner, personal communication).

3. Preparation of Antiserum against PVY-protein

The preparation of the antiserum against PVY-protein was carried out in a manner described under 1, except that only three injections of the protein solution were given at weekly intervals. The rabbit was bled 3 days after the last injection at 3-day intervals as it had been observed for other flexuous rod-shaped viruses that titers to viral protein dropped rapidly shortly after the final injection (Shepard and Secor, 1969).

An extinction coefficient of 1.00 cm^2mg^{-1} at 280 nm was used to estimate the concentration of protein in PVY protein preparations. This value was assumed because there are no reports of the extinction coefficient for PVY protein; a value of 1.07 was reported for turnip mosaic virus, a virus with chemical and physical properties similar to PVY (Hill and Shepherd, 1972).

4. Fractionation of the Immunoglobulins from PVY Antiserum

Occasionally, non-specific precipitates occurred in

the immunodiffusion systems containing SDS or LSA. These precipitates were thought to be due to serum components other than the immunoglobulin. Attempts were made to isolate the immunoglobulin fraction by a method described by Bercks (1960) who successfully used it to increase the titer of different antisera.

Ten ml of PVY antiserum was made 40% of saturation by addition of 2.43g of granular ammonium sulfate (Green and Hughes, 1955). The solution was gently shaken at room temperature for 60 min and then incubated at 4°C for 12 hrs. It was centrifuged for 30 min at 3000 rpm and the supernatant was discarded. The white precipitate representing the crude globulin fraction was washed three times with a 40% saturated ammonium sulphate solution. After the third washing, the supernatant was discarded and the globulin fraction was re-suspended in 10 ml of 0.01M phosphate-buffered saline (PBS), pH 7.4. It was dialysed against PBS until the dialysate was sulphate-negative with a 1% solution of barium chloride. A precipitate that had formed during dialysis was removed by centrifugation at 3000 rpm for 10 mins.

J. PREPARATION OF ANTIGENS FROM HEALTHY TOBACCO

Antigens from healthy tobacco plants were prepared according to a method described by Shepard and Grogan (1967). The preparation served as a control in some immunodiffusion experiments and was occasionally used to absorb antibodies

against protein from healthy plants present in the immune serum.

Leaves from healthy tobacco plants var. Burley 21 were homogenized in a Waring blendor with 0.5M borate buffer pH 8.7. One ml of buffer was added for each gram of tissue. The homogenate was pressed through several layers of Mira-cloth and the expressed sap was emulsified with an equal volume of chloroform. The mixture was incubated at $4^{o}C$ for 60 minutes, then centrifuged for 10 minutes at 10,000 rpm to break the emulsion. The supernatant was pipetted off and centrifuged for 2 hrs at 30,000 rpm in a Beckman Type 30 rotor. The pellets were resuspended in 0.005M Tris-HCl pH 7.5, in 0.033 of the original volume and centrifuged at 10,000 rpm for 5 minutes.

The ultraviolet absorption spectrum of the preparation exhibited a maximum at 260 nm and a minimum at 243 nm. The A260/A280 value was 1.65, indicating the probable ribosomal nature of the preparation.

K. SEROLOGICAL TESTS

1. The Microprecipitin Method

The microprecipitin test of van Slogteren (1954) was used as a diagnostic method to establish the presence or absence of PVY in clarified expressed sap of infected leaf tissue and to estimate the relative concentration of the virus in plant extracts by determining its dilution

end-point (DEP). Usually, dilution titrations were performed in a grid pattern (Ball, 1961). Small droplets of two-fold dilutions of antiserum were dispensed with a micropipette 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 37°C and examined by a dark field microscope for the presence of a precipitate, indicating a positive antigen-antibody reaction. A confirmatory reading was made after the plates had been stores overnight in an incubation chamber maintained at 10°C.

Each antigen-antibody system was controlled by reacting the antigen with normal serum and clarified sap of healthy plants with immune serum. Dilutions of antigens and antisera were made in 0.05M Tris-HCl pH 7.1, containing 0.85% NaCl. Clarification of the plant sap was accomplished by homogenizing the leaf tissue in 0.05M Tris-HCl pH 7.1 (1g tissue/ to 1 ml buffer). The homogenate was placed in a water bath at 40 C for 1 hr, then centrifuged for 20 minutes at 5,000 rpm to remove denatured host proteins and debris. The supernatant was pipetted off and used immediately.

2. The Ouchterlony Double Diffusion Test

Originally developed in 1943 by Ouchterlony (1958), this test was adapted for use with plant viruses by van Slogteren (1954) and has since become a valuable tool for the serodiagnosis of many plant viruses.

In order to demonstrate the presence of PVY in crude sap of infected plants, the principles of this method were used in these investigations but all variables of the test were subject to experimentation, i.e., concentration of agar, concentration of the degrading compound, distance between wells, buffer, electrolyte, time and temperature of incubation. Eventually, preparative procedures and optimum conditions were determined and the following three immunodiffusion systems were routinely used.

1. 0.8% Difco special agar-Noble
   0.2% sodium dodecyl sulphate
   0.1% sodium azide

2. 0.8% Difco special agar-Noble
   0.5% sodium dodecyl sulphate
   1.0% sodium azide

3. 0.8% Difco special agar-Noble
   0.85% sodium chloride
   1.0% Leonil SA
   0.04% sodium azide

The immunodiffusion plates were prepared by pouring 2.5 ml of the agar solution into each quadrant of a quadrant type plastic petri dish. The solvent for the agar was distilled water, containing sodium chloride or sodium azide as the electrolyte. The degrading agents were added to

the warm agar (60°C) just before it was pipetted into the diffusion chambers. After gelation, wells were cut with a no. 2 cork borer (5.5mm) in a hexagonal arrangement on the circumference of a circle 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 neighboring peripheral wells was 8-10 mm.

Usually, the central well was charged with antiserum and the peripheral wells were charged with sap from infected plants or with the virus preparation. Sap from healthy plants or antigens prepared from healthy tobacco and normal serum were included in every experiment to serve as controls. Plates were incubated for 10-12 hrs in a moist chamber at room temperature.

Immunodiffusion systems of similar composition were also formed on 76 mm x 26 mm glass slides. Ten ml of a 0.8% agar solution, containing the detergent and the electrolyte at desired concentrations, were layered on six glass slides held in an LKB 6801A slide frame. A thin layer of hot adhesive agar solution (0.1% agar, 0.05% glycerol in distilled water) was brushed onto the slide and allowed to dry prior to the layering of the agar solution. Wells were cut in the gel with an LKB 6807A gel punch equipped with die 6866A, with cutters set in the six-way test pattern. The distance between the peripheral wells and the center well, center to center, was 8 mm.

The wells were 3 mm in diameter and held approximately 20 µl of fluid. After charging the wells, the slide frames were inserted in a holder, transferred to a humid chamber and incubated for 10-12 hrs at room temperature.

3. The Single Radial Diffusion Test

This test is a modification of the Oudin agar single diffusion test (Ball, 1961) and was successfully used by Shepard and Secor (1969) for the detection of low concentrations of pyridine-degraded PVX. The method is based on the diffusion of antigen from wells cut in the solidified mixture of antibody and agar. Precipitates formed by the antigen-antibody complex appear as rings surrounding the well and the distance of the ring from the well is directly proportional to the antigen and antibody concentration.

In these experiments, undiluted antiserum was mixed with an equal volume of a 2% agar-Noble solution, in distilled water, containing the degrading compound and the electrolyte at twice the desired final concentration. The liquid agar was mixed with the antiserum at 50°C and 10 ml was immediatley poured into a 100 mm petri dish. After solidification, the antigen wells were cut with a no. 2 corkborer, and were spaced at regular intervals over the agar surface. Antigens were prepared by homogenizing infected leaves of tobacco or potato with a mortar and pestle. Normal serum and sap from healthy plants were used as controls in all tests.

Variations of the procedure were carried out by homogenizing the leaf samples directly with various concentrations of the degrading compounds, while omitting the compounds from the immunodiffusion system.

L. ELECTRON MICROSCOPY

Samples of purified virus at a concentration of 0.1 mg/ml were negatively stained by combining equal volumes of the virus suspension and 2% phosphotungstic acid dissolved in distilled water and adjusted to pH 6.5 with 2N NaOH (Brenner and Horne, 1959). Formvar-coated, carbon reinforced grids were dipped into the mixture for 60 seconds. They were then dried with filter paper and observed under an AEI EM6B electron microscope.

IV. EXPERIMENTS AND RESULTS

A. IDENTIFICATION OF THE ISOLATE BY INOCULATION TO DIFFERENTIAL HOSTS

Following the initial identification of the virus with PVY antisera obtained from different sources, the identity of the isolate as a normal strain of PVY was further established by mechanical inoculation of twenty-four differential host plants with undiluted sap extract of infected tobacco which showed clear symptoms of PVY. The host plants were selected on the basis of previous host range studies carried out by deBokx (1964) and Horvath (1966a, b; 1967a, b, c). The observed host reactions, incubation periods and attempts to recover the virus are tabulated in Table 1. Datura stramonium L., which reacted negatively in this study, is known to be immune to infection by all tested strains of PVY but is susceptible to tobacco etch virus, which has a host range similar to PVY. Species of Capsicum showed faintly discernible symptoms or no symptoms at all, but after about 25 days virus was recovered from all inoculated plants. Symptoms on Solanum demissum L. "Y" were obscured by the poor state of growth of the host plant,

Figure 1A. Systemic symptoms of PVY on detached leaves of Nicotiana tabacum L. cv. Burley 21.

From right to left: healthy leaf and infected leaves, 9, 16 and 23 days after inoculation, respectively.

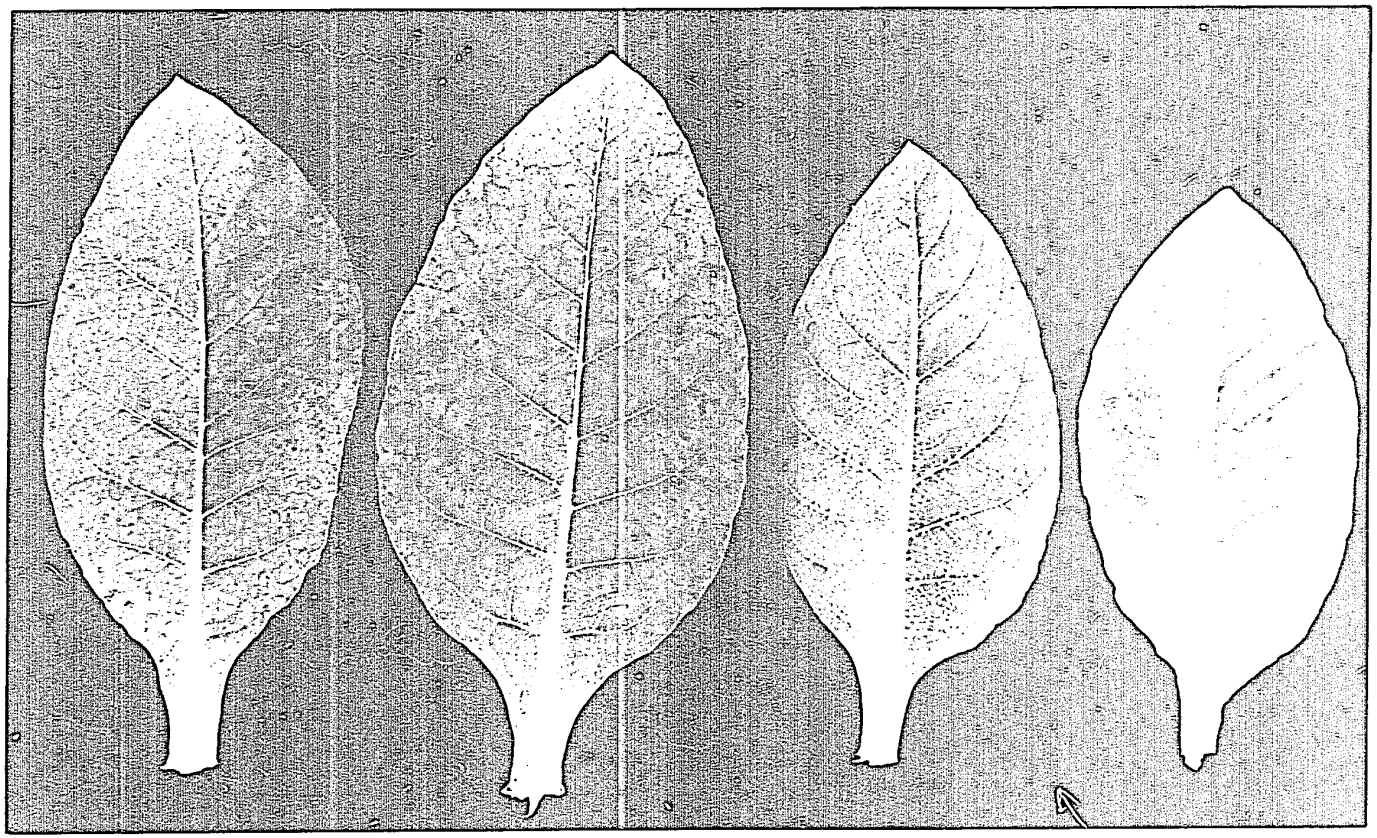


Figure 1B. Young leaf of Nicotiana tabacum cv. Burley 21, showing systemic vein clearing, 9 days after inoculation with PVY.

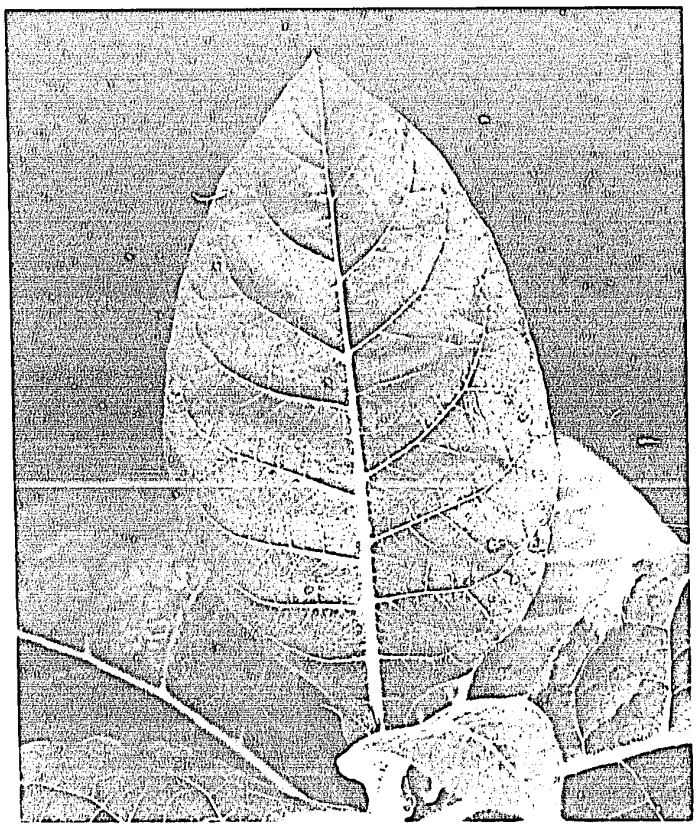


Figure 1C. Leaf of Nicotiana tabacum L. cv. Burley 21 showing severe systemic mottling 23 days after inoculation with PVY.

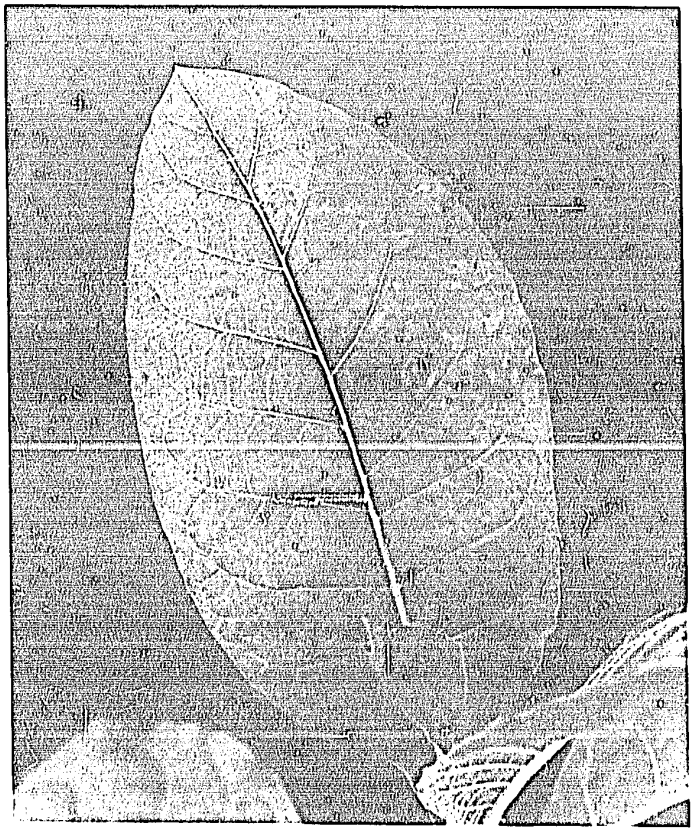


Table 1. Symptom development on various host plants after inoculation with PVY.

Host	Description of Symptoms	Incubation* Period (days)	Recovered**
Capsicum annuum L.	very mild mosaic, vein clearing	23	+
Capsicum frutescens L.	no symptoms	--	+
Chenopodium album L.	chlorotic local lesions	7-8	+
C. amaranticolor Coste & Reyn.	chlorotic local lesions	7-8	+
C. quinoa Willd.	chlorotic local lesions	7	+
Datura ferox L.	systemic mosaic, vein clearing	14	+
D. stramonium L.	no symptoms	--	-
Gomphrena globosa L.	no symptoms	--	-
Lycium hamilifolium Mill.	necrotic local lesions, leaf distortion, leaf drop	9-10	+
L. barbarum L.	necrotic local lesions, leaf drop	7-8	+
Lycopersicon esculentum Mill.	mild systemic mosaic	14	+
Nicotiana debneyi Domin.	severe vein clearing, systemic mosaic	9-10	+

Table 1 (continued)

Host	Description of Symptoms	Incubation* Period (days)	Recovered**
Nicotiana glutinosa L.	diffuse mottling, leaf distortion	10	+
N. glauca Grah.	no symptoms	--	-
N. tabacum L.			
cv. Havana 425	severe vein clearing, systemic mottle	8-10	+
cv. Samsun	-ditto-	7-9	+
cv. White Burley	-ditto-	8-10	+
Petunia hybrida Vilm.	light vein clearing, mild mosaic	14	+
Physalis floridana Rydb.	necrotic lesions, systemic mosaic, systemic necrosis, leaf drop, leaf distortion	12-14	+
Solanum demissum L. "Y"	necrotic lesions	4	+
S. melongena L.	no symptoms	--	-
S. nigrum L.	vein clearing, mild mosaic,leaf drop	22-25	+
S. tuberosum L. cv. USDA 41956	local necrotic lesions, systemic necrosis leaf drop	12-14	+

* Denotes the time of first symptom appearance.

** Recovered by mechanical inoculation to *N. tabacum* cv. White Burley.

but it is well documented that this seedling cannot be adequately grown under short day conditions even with supplementary lighting (deBokx, 1964). The isolate caused severe systemic necrosis and leafdrop on Physalis floridana Rydb. and Solanum tuberosum L. cv. USDA 41956. Species of Lycium reacted with circular necrotic lesions on the inoculated leaves, which dropped off the plant after 2-3 weeks. No systemic symptoms were observed. Fig. 1A, B, C shows symptoms caused by PVY on the tobacco cultivar "Burley 21", the host from which PVY was routinely purified.

B. ASSESSMENT OF PURIFICATION PROCEDURES

1. Purification by Differential Centrifugation and Isoelectric Precipitation (pH 5.6).

For the purpose of obtaining a sufficient quantity of partially purified virus to be used as the inject antigen for antiserum production, this procedure was found the most satisfactory of several methods investigated. After initial experimentation, virus yield from infected tobacco plants was found to be relatively predictable and uniform, averaging 5-8 mg/kg of leaves during the winter months (November-February) and 4-5 mg/kg during the summer months (May-August). In general, the yield of purified virus remained fairly constant over a two-week period commencing 10-14 days after inoculation but declined rapidly in older plants, especially without the application of supplementary nutrients.

The ultraviolet absorption spectra of twelve separate preparations of purified PVY suspended in0.02M

borate-NaOH buffer, pH 7.8, showed a maximum absorption at 258-260 nm and a minimum at 243-244 nm (Table 2). The ratio A260/A280 varied from 1.34 to 1.46 suggesting the presence of host impurities.

However, further purification of the suspension at this stage resulted in too great a virus loss and did not appreciably lower the A260/A280 ratio. A typical UV absorption curve of a partially purified PVY suspension is shown in Fig. 2. No corrections for absorbance due to light scattering were made. The data presented in Table 2 are representative of a great number of purifications carried out during these investigations. Tobacco cv. Burley 21 was used as the increase host for the virus; leaves were harvested 14-20 days after inoculation, kept at 3°C and processed within 3 days after harvesting.

No attempts were made to assess virus losses which occurred during the various stages of the purification procedure, mainly because the local lesion hosts Chenopodium quinoa and C. amaranticolor were found to be unsuitable for a quantitative analysis of PVY-containing sap extracts. The appearance of chlorotic lesions on inoculated leaves seemed to be erratic and the infectivity of the virus preparations appeared to be influenced by the suspending medium, the age of the preparation, temperature and other factors. However, no detailed investigation of these aspects was carried out.

Table 2. Yield data and spectrophotometric analyses of separate preparations of PVY.

Preparation	Season	Yield mg/kg	Ratio A260/A280	Absorption Minimum (nm)	Absorption Maximum (nm)
1	S[a]	4.8	1.40	244	259
2	S	5.2	1.43	244	260
3	S	3.8	1.36	243	259
4	S	4.0	1.34	244	259
5	S	4.5	1.46	244	259
6	S	5.0	1.34	244	259
Average	S	4.55	1.39	244	259
7	W[b]	5.1	1.40	243	259
8	W	5.2	1.36	244	260
9	W	8.6	1.46	243	258
10	W	8.3	1.39	244	258
11	W	7.3	1.37	244	259
12	W	6.0	1.40	244	259
Average	W	6.78	1.40	244	259

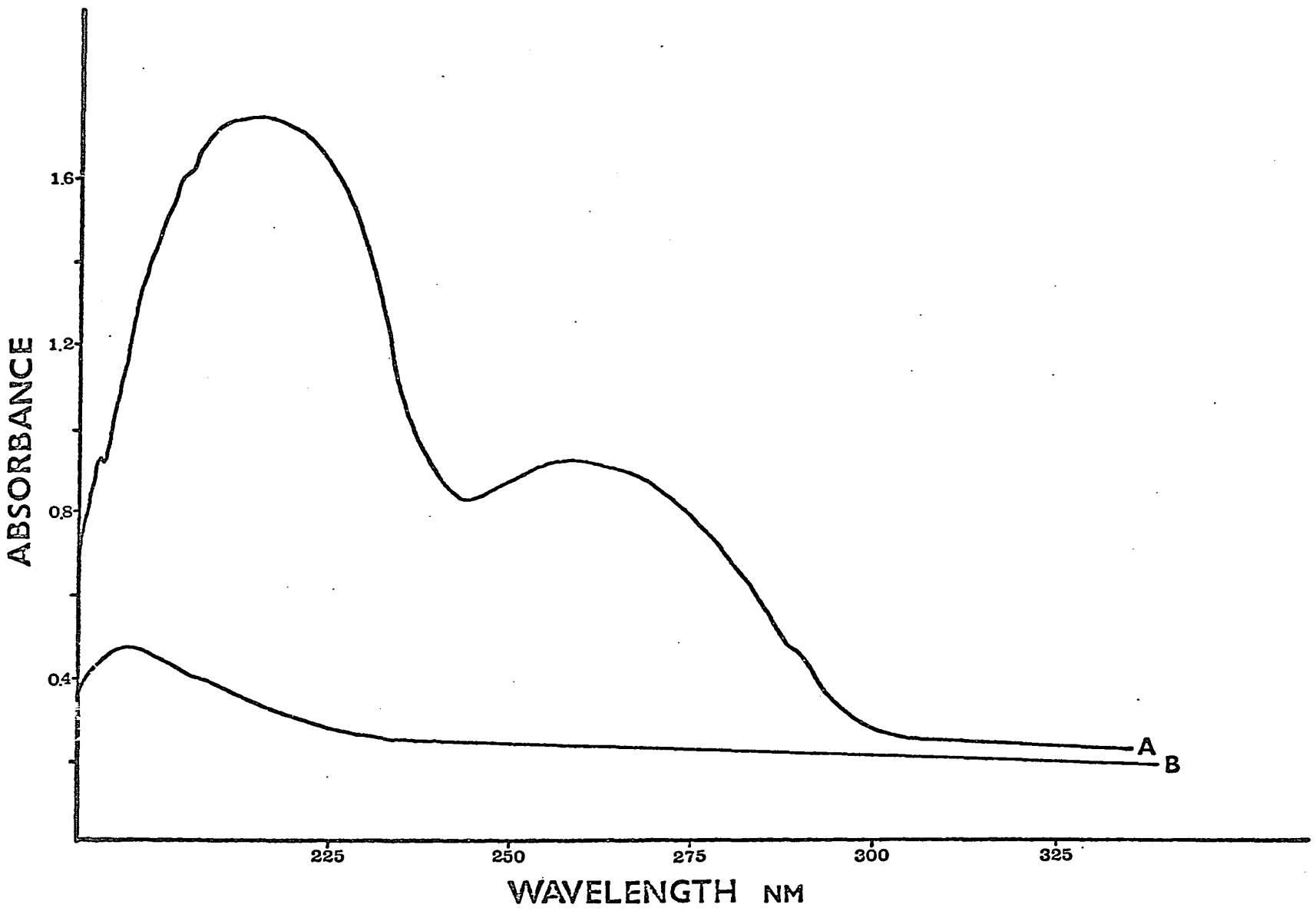
[a]S denotes summer (May-August).

[b]W denotes winter (November - February).

Figure 2. Typical ultraviolet absorption curve of a partially purified suspension of PVY.

A. Partially purified preparation of PVY suspended in 0.02M borate-NaOH buffer, pH 7.8.

B. Absorbance of the borate buffer.


ABSORBANCE
1.6
1.2
0.8
0.4
225
250
275
300
325
WAVELENGTH NM
A
B

The homogeneity of the preparation and the relative state of aggregation of the virus were occasionally assessed by sucrose density gradient analysis. Ultraviolet scanning patterns of a purified PVY suspension after rate-zonal centrifugation showed that the virus sedimented in a single, if somewhat diffuse, peak in the gradient column. Aggregation was minimal immediately after purification but increased considerably upon storage at 3°C (Fig. 3), as indicated by the decreased area under the absorption peak corresponding to the monomer form of the virus. After fractionation of the gradient column, PVY infectivity was found associated with the fraction which corresponded with a peak on the recording chart, located approximately 3.3-3.5 cm from the meniscus. Measurements were taken from the recording chart as visual bands could hardly be discerned in the gradient or were too diffuse for precise measurement. No infectivity could be recovered from any other gradient fractions, as determined by rub-inoculation to N. tabacum.

It should be noted that infectivity test results, even with the fractions representing major absorbance peaks, were extremely variable. Age of the preparation or frequent manipulation of the preparation at room temperature were factors contributing to reduced infectivity. Since infectivity tests generally proved to be unreliable, some confirmatory tests were carried out by serological microprecipitin tests. Successive one ml fractions were collected from a centrifuged gradient which had previously been layered with

Figure 3. Sucrose density gradient centrifugation profiles of partially purified PVY in a 10-40% sucrose gradient in 0.02M borate-NaOH buffer pH 7.8 after centrifugation in an SW40 rotor at 40,000 rpm for 90 minutes. Direction of sedimentation is to the left.

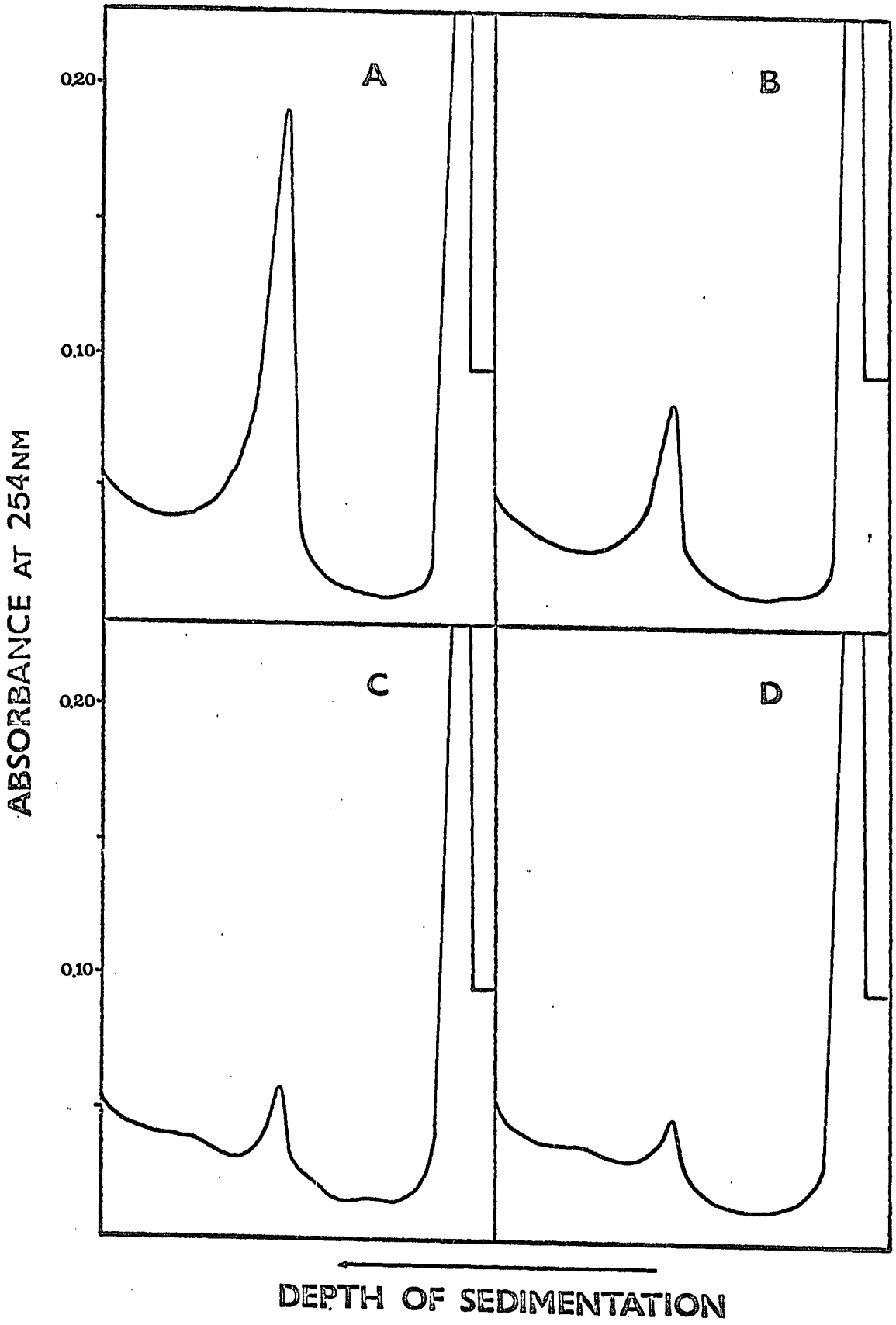
Virus was purified by differential centrifugation and acid precipitation(pH 5.8).

A. Profile of PVY (1.0 mg/ml) immediately after purification.

B. Profile of PVY (1.0 mg/ml), after storage at 3°C for 6 days.

C. Profile of PVY (1.0 mg/ml), after storage at 3°C for 2 weeks.

D. Profile of PVY (1.0 mg/ml) after storage at 3°C for 3 weeks.


A
B
C
D
0.20
0.10
0.20
0.10
ABSORBANCE AT 254NM
DEPTH OF SEDIMENTATION

0.2 ml of a recently purified PVY suspension (1 mg/ml). The fractions were serially diluted in 0.02M borate pH 7.8 and were reacted with PVY antiserum in grid-fashion to determine the dilution end-point (DEP). Results are tabulated in Table 3 and it is evident that fraction 7, which corresponded to the centrally located peak on the recorder chart (Fig. 3A), contained the highest amount of viral antigen. Serological activity associated with fraction 1 (top-fraction) indicated that the antiserum contained some antibodies to normal host material, since fractions 1, 2 and 3 from a control gradient on which 0.2 ml of clarified sap from healthy tobacco had been layered showed a similar low reactivity with the antiserum. Scanning patterns from the control gradient revealed a much larger area of absorbance at the top of the gradient than did those from gradients layered with a purified preparation of PVY and in subsequent purification procedures the width of the UV-absorbing zone at the meniscus was taken as a relative indication of the purity of the preparation.

Positive serological reactions with fractions 8 and 12 (bottom fraction) almost certainly indicate the presence of PVY aggregates, although no infectivity could be associated with these fractions.

Although virus losses resulting from aggregation are adequately illustrated by the sedimentation profiles in Fig. 3, it should be observed that the degree to which aggregation occurred was often variable and unpredictable, depending on the initial virus concentration of the suspension, the temperature and probably other factors. At

Table 3. Serological dilution end-point (DEP) determination and infectivity of fractions collected from sucrose density gradients after rate-zonal centrifugation.

Fraction No.	Gradient Sample: 0.2 ml clarified sap from healthy tobacco		0.2 ml partially purified PVY	
	DEP	Infectivity[a]	DEP[b]	Infectivity[a]
1	2	-	2	-
2	2	-	-	-
3	2	-	-	-
4	-	-	-	-
5	-	-	-	-
6	-	-	2	-
7	-	-	16	+
8	-	-	4	-
9	-	-	-	-
10	-	-	-	-
11	-	-	-	-
12	-	-	2	-

[a]Determined by mechanical inoculation to N. tabacum cv. White Burley.

[b] Reciprocal of highest dilution showing precipitate.

higher concentrations (4-5 mg/ml) and upon prolonged storage the virus often sedimented spontaneously as a white flocculent precipitate. On occasion, even recently purified preparations at a concentration of 1 mg/ml showed a great amount of spreading, indicative of aggregation, upon rate zonal centrifugation in a sucrose density gradient column. Upon further storage and subsequent sucrose density gradient analysis spreading increased, often with sedimentation of the virus to the bottom of the gradient tube.

In general, since recently purified preparations of PVY showed the least evidence of aggregation and could be manipulated in vitro with little virus loss, care was taken to utilize as much as possible such preparations for experimental purposes.

Additional indication of the purity of purified PVY suspensions was provided by electron micrographs. No obvious contaminants were observed in photographs of negatively stained preparations of the virus, which showed the presence of numerous flexuous rod-shaped particles (Fig. 4A). No similar rods were seen in preparations from control plants or in leaf dips of control plants. Fig. 4B shows the presence of virus particles in a leaf dip preparation of PVY-infected tobacco.

2. Purification by Precipitation with Polyethylene Glycol and Differential Centrifugation.

Because the sedimentation of PVY from clarified extracts of infected tobacco involved the centrifugation of

Figure 4A. Electron micrograph of a partially purified preparation of PVY negatively stained with 2% phosphotungstic acid pH 6.5 x 104,000.

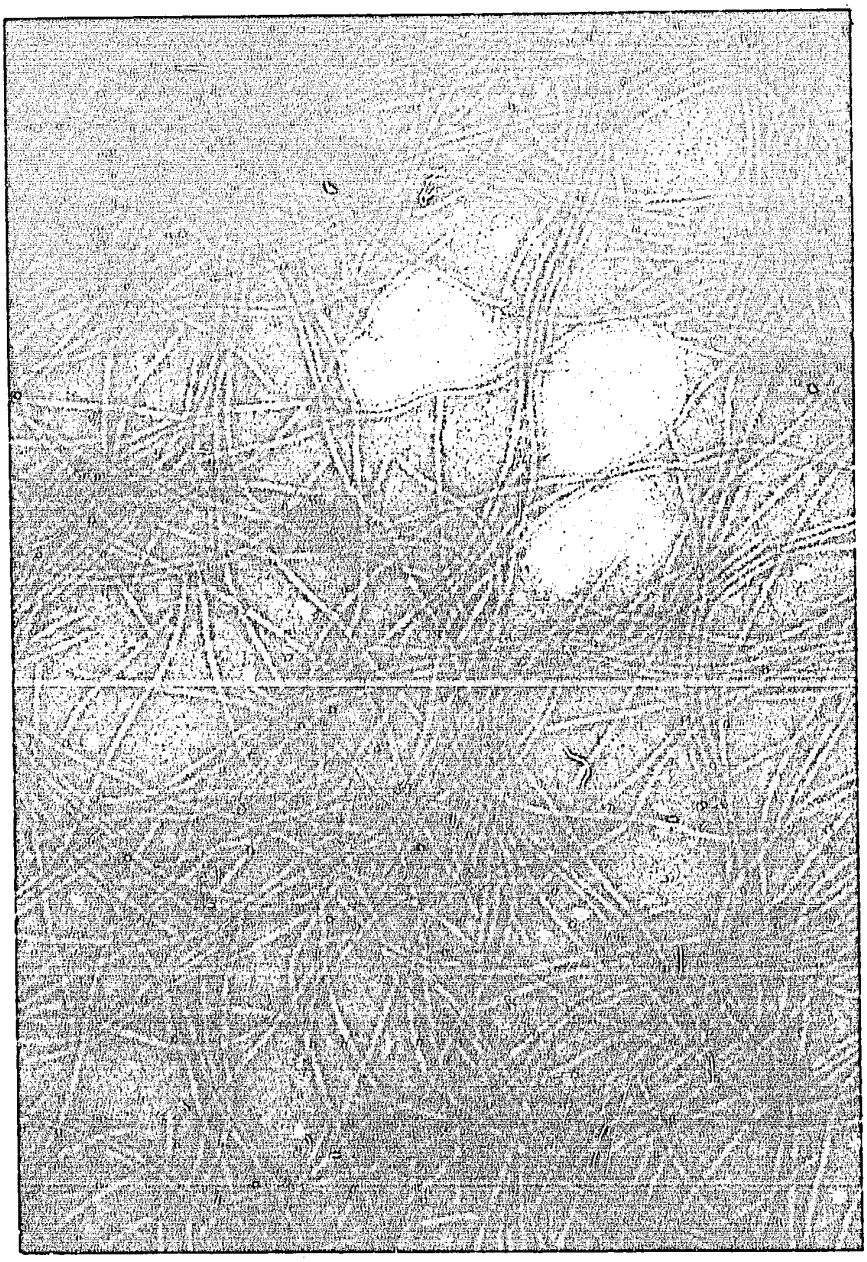
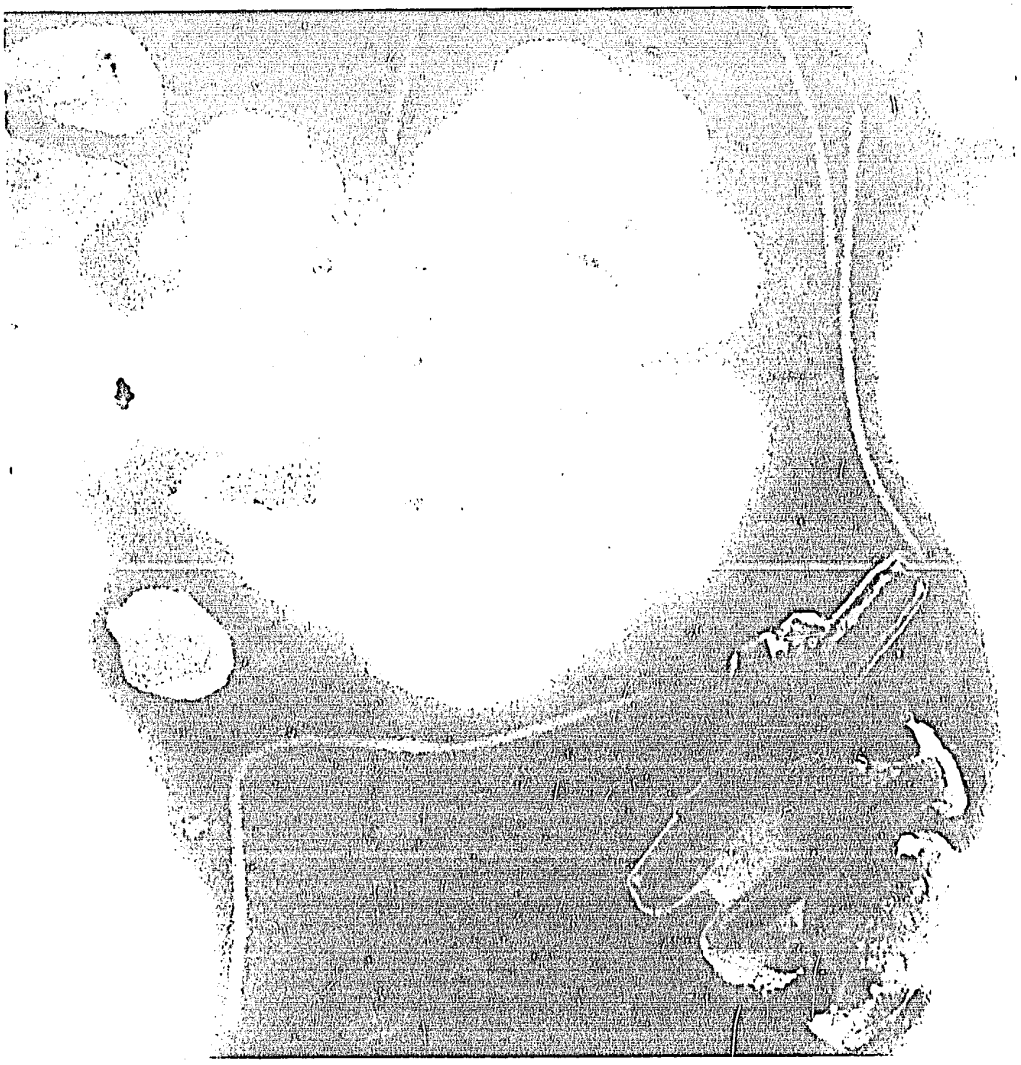


Figure 4B. Electron micrograph of a leaf-dip preparation of PVY-infected tobacco, negatively stained with phosphotungstic acid pH 6.5 x 122,200.



large volumes of sap, the usefulness of polyethylene glycol (PEG) to precipitate the virus was briefly investigated. Initially, a considerable amount of host material was co-precipitated with PVY when 4-5% PEG was added to a clarified extract. To solve this problem, the extract was incubated for an additional period of 12 hours at 3°C followed by the addition of PEG. The precipitated virus was resuspended in phosphate buffer pH 7.4. After 2 or 3 cycles of differential centrifugation the virus pellet was finally resuspended in 0.025M phosphate buffer containing 0.5M urea and 0.1% 2-mercaptoethanol to prevent aggregation (Damirdagh and Shepherd, 1970a).

Although the yield of purified virus appeared to be approximately 10-14 mg/kg of leaves, as determined by measuring the absorbance at 260 nm, sucrose density gradient analysis of the preparation after rate zonal centrifugation revealed a considerable aggregation of ultraviolet light-absorbing material, just below the zone corresponding to virus (Fig. 5B). Increasing the molarity of the urea in the resuspension buffer to 1.0M apparently resulted in degradation of the viral material, for the sedimentation profile indicated a small additional peak just above the one corresponding to virus (Fig. 5C). Attempts to use 0.5M citrate as the extraction medium and 0.02 borate-NaOH buffer, pH 7.8, containing 0.5M urea and 0.1% 2-mercaptoethanol as the resuspension buffer, resulted in even more severe degradation of the virus (Fig. 5D).

Infectivity tests with ultraviolet light-absorbing fractions from the gradient were again very erratic although, on occasion, tobacco plants showed typical PVY symptoms two weeks after they were rub-inoculated with the virus-containing fraction of the gradient. Once, inoculation of C. quinoa with such a fraction produced a small number of chlorotic lesions on the inoculated leaf.

In order to demonstrate conclusively that the ultraviolet light-absorbing material in the gradient after centrifugation represented PVY, or aggregates and degradation products thereof, the following experiment was carried out. To one ml of PVY (1.0 mg/ml), purified with PEG and resuspended in 0.025M phosphate buffer containing 1.0M urea and 0.1% 2-mercaptoethanol, 0.05 ml undiluted PVY antiserum (obtained from G. V. Gooding Jr.) was added. The mixture was incubated overnight to allow a precipitate to form; then 0.2 ml of the clear supernatant was carefully withdrawn with a pipette and layered on a SW 40 gradient column for centrifugation and subsequent analysis. Normal serum from a different rabbit was used as a control in a similar manner. Results in Fig. 6 show that all UV-absorbing material is removed with PVY antiserum whereas normal serum does not remove it. The difference between the gradient profile of the untreated virus (Fig. 6A) and the gradient profile of the virus treated with normal serum (Fig. 6B) is not necessarily the result of the treatment, because such differences often occurred between identical untreated preparations as well, presumably due to the formation of

Figure 5. Sucrose density gradient centrifugation profiles of partially purified PVY in a 10-40% sucrose gradient in 0.02M borate buffer after centrifugation in an SW 40 rotor at 40,000 rpm for 90 minutes. Direction of sedimentation is to the left.

A. Purified by differential centrifugation and acid precipitation. Final pellet resuspended in 0.02M borate buffer pH 7.8.

B. Purified by precipitation with PEG followed by 2 cycles of differential centrifugation. Final pellet resuspended in 0.025M phosphate buffer, pH 7.4 containing 0.5M urea and 0.1% 2-mercaptoethanol.

C. As in B, with concentration of urea at 1.0M.

D. Purified by clarification with 0.5M sodium citrate followed by precipitation with PEG and 2 cycles of differential centrifugation. Final pellet resuspended in 0.02M borate buffer pH 7.8, containing 0.5M urea and 0.1% 2-mercaptoethanol.

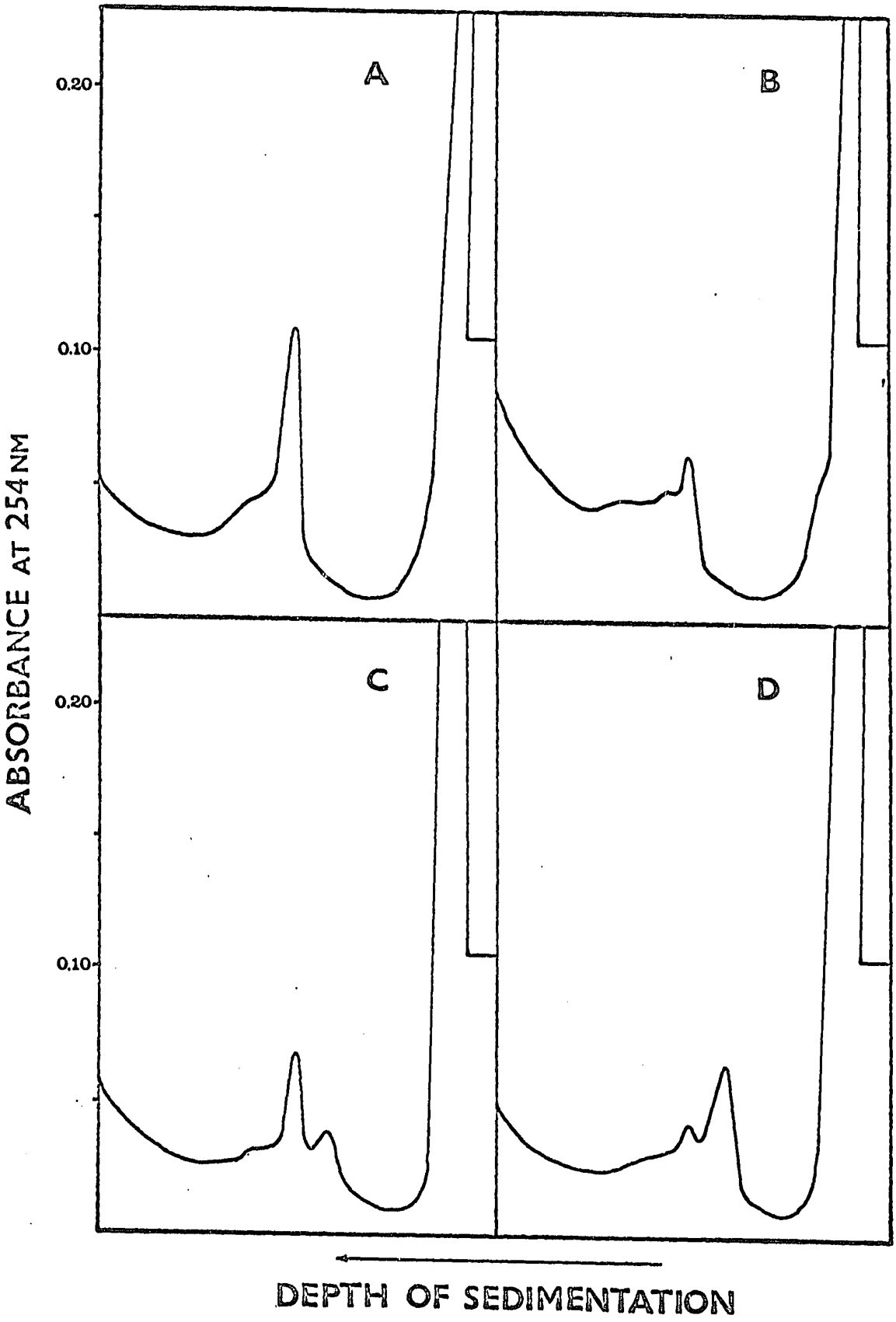

A
B
C
D
0.20
0.10
0.20
0.10
ABSORBANCE AT 254 NM
DEPTH OF SEDIMENTATION

Figure 6. Density gradient centrifugation of PVY after incubation with normal serum or PVY antiserum.

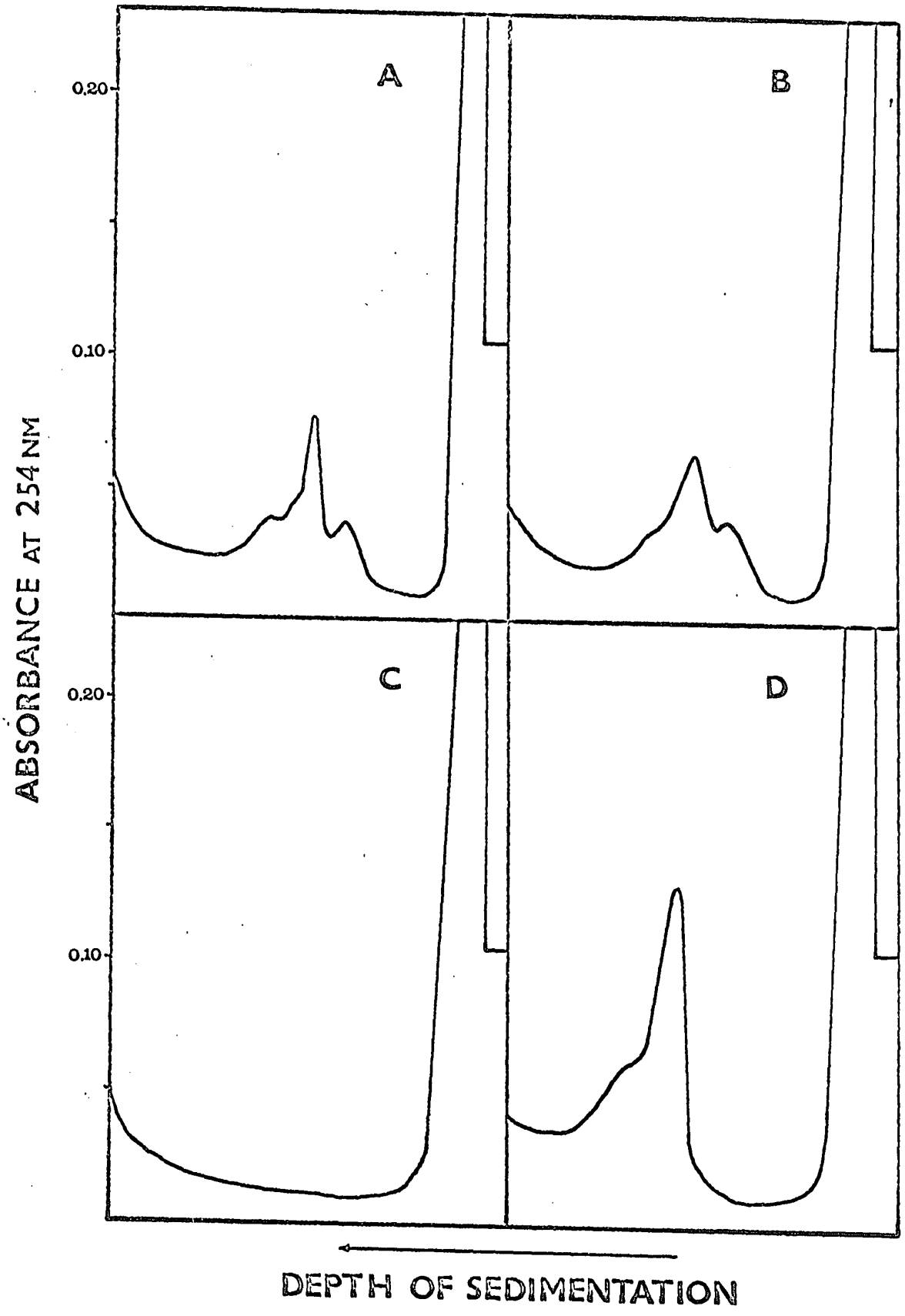
Sucrose density gradient centrifugation profiles in a 10-40% sucrose density gradient in 0.02M borate buffer pH 7.8 after centrifugation in a SW 40 rotor at 40,000 rpm for 90 minutes. Direction of sedimentation is to the left.

A. PVY, purified by PEG precipitation followed by 2 cycles of differential centrifugation. Final pellet resuspended in 0.025M phosphate pH 7.4, containing 0.5M urea and 0.1% 2-mercaptoethanol.

B. As in A, incubated with normal rabbit serum for 14 hours.

C. As in A, incubated with heterologous PVY antiserum for 14 hours.

D. PVY, purified from the same batch of infected tobacco plants, by differential centrifugation and acid precipitation.


A
B
C
D
0.20
0.10
0.20
0.10
ABSORBANCE AT 254 NM
DEPTH OF SEDIMENTATION

differently sized aggregates of the virus.

In spite of the fact that purification of PVY by PEG showed considerable potential in terms of virus yield, the procedure was rejected as a means of obtaining purified virus for use as the inject antigen. Aggregation could not be prevented effectively with a urea concentration of 0.5M whileurea at 1.0M caused considerable degradation of the virus. Since the objective of this research was partly to study and compare the effectiveness of antisera prepared against intact and degraded virus for the detection of PVY by immunodiffusion, it was not thought advisable to work routinely with PVY exposed to urea of such high molarity. Figure 5D shows that the degrading action of urea may be enhanced by borate or a higher pH or perhaps a combination of these factors.

The use of PEG, alone or in combination with certain buffer salts, appeared to result in some degradation of the virus. Evidence was obtained that degradation of the virus resulted from precipitation with PEG if 0.5M citrate was used as the extraction buffer and 0.02M borate pH 7.8, without urea, as the resuspension buffer. In this experiment the virus was purified according to the procedure outlined in Chapter III D, 1, except that the virus was precipitated from the clarified sap with 4% PEG instead of by high speed centrifugation. Sucrose density gradient analysis of this preparation showed a sedimentation profile similar to the one presented in Fig. 5C.

It seemed clear therefore that the purification of this particular PVY isolate with the method described by Damirdagh and Shepherd (1970a), required some detailed preliminary experimentation and that in the absence of such experiments the use of PEG for routine purification could not be justified.

3. Yield of PVY from Inoculated and Systemically Infected Tobacco Leaves.

Experiments by Delgado-Sanchez and Grogan (1966a) had indicated that directly inoculated leaves of the tobacco cultivar "Havana 425" were a better source of virus for purification 10-20 days after inoculation than systemically infected leaves of the same plant. These conclusions were based on infectivity tests and virus titers were ascertained by the number of local lesions produced on eight carborundum-dusted leaves of Chenopodium quinoa, after rub-inoculation with homogenates of composite leaf samples of infected tobacco in 0.05M borate pH 8.2.

Such findings warranted investigation, but, in view of the fact that previous infectivity tests with the PVY isolate were found to be unreliable, the virus content of several tobacco varieties was determined by direct purification of inoculated leaves and systemically infected leaves two weeks after inoculation. To this effect, 15 plants each of the tobacco cultivars White Burley, Havana 425, Burley 21 and Samsun were inoculated in the 5-6 leaf stage with a leaf

extract of PVY-infected N. glutinosa. Four leaves from each plant were inoculated. After harvesting, leaves from each variety were kept separate and 200 g samples were purified over a period of 48 hours. Virus was purified according to the procedure of Purcifull and Gooding (1970) and total virus yields were determined spectrophotometrically by measuring the absorbance at 260 nm.

Table 4. Yield of purified PVY from inoculated and systemically infected leaves of various tobacco cultivars, two weeks after inoculation.

Tobacco Cultivar	Inoculated Leaves mg/200g	Systemically Infected Leaves mg/200g
Burley 21	0.54	1.78
Havana 425	0.55	0.95
Samsun	0.65	1.91
White Burley	0.47	1.53

Results in Table 4 show that directly inoculated leaves of all cultivars yielded a significantly smaller amount of virus than did systemically infected leaves of the same plant.

This does not, however, invalidate the results obtained by Delgado-Sanchez and Grogan (1966b), for these investigators carried out their infectivity tests under strictly controlled

environmental conditions, measuring infectious virus only. Although results from a single experiment cannot serve to demonstrate conclusively the merits of one system over another, for practical reasons the cultivar "Burley 21" was selected for routine purifications of PVY. In spite of the fact that the cultivar "Samsun" yielded slightly more virus than "Burley 21", the latter produces a greater quantity of leaves and is hypersensitive to tobacco mosaic virus, i.e., the plant responds to infection with TMV by producing necrotic local lesions followed by mosaic disease. Proximity of TMV infected plants, used for other research purposes, made the use of the extremely TMV-susceptible cultivar "Samsun" prohibitive.

C. DEGRADATION STUDIES

1. Preliminary Investigation of Compounds for their Efficacy in Degrading PVY.

a) Changes in opalescence of PVY preparations after exposure to degrading compounds.

Changes which occurred in the opalescence of the virus suspension after varying periods of exposure to the degrading compound and also after subsequent dialysis against borate buffer, are tabulated in Table 5. The virus concentration was 2 mg/ml before addition of an equal volume of the degrading compound. The mixtures were incubated for 4 hours at 25°C and for an additional 44 hours at 3°C followed by

Table 5. Relative degree of opalescence of partially purified PVY suspensions after exposure to various degrading compounds.

Degrading Compound (final concentration)	Opalescence[a] after			
	1 minute	4 hrs	48 hrs	dialysis
PVY (1 mg/ml) control	++++	++++	++++	++++
0.25% Igepon T-73[b]	++++	++++	++++	+++
0.5M Glycine-NaOH pH 12.0	+++	++	++	+++
o.1% DMAE[c]	++	++	++	++
1% Leonil S.A. (LSA)[d]	++	++	+	+
4M Urea	++	++	+	+
30% Pyridine	++	+	+	+
0.25M ETA pH 10.5[e]	++	+	-	+
2M Guanidine-HCl	+	+	+	++
0.2% SDS[f]	+	-	-	±
2.5% Pyrrolidine	-	-	-	-
0.02M Borate-NaOH pH 7.8	-	-	-	-

[a]Relative degree of opalescence is indicated by (-), (±), (+), (++), (+++), (++++), with (-) indicating no opalescence and (++++) indicating high opalescence.

[b]Contains 20% N-methyl-N-oleoyl taurate as the active ingredient.

[c]2-dimethyl amino ethanol

[d]sodium dibutylnaphthalenesulfonate.

[e]ethanolamine-hydrochloride buffer.

[f]sodium dodecyl sulphate.

dialysis against 0.02M borate buffer pH 7.8 for 24 hours. Concentrations of the degrading compounds, as shown in the table, are final concentrations.

It was observed that after dialysis against borate buffer, the opalescence of most suspensions increased, indicating a certain degree of polymerization or re-aggregation of previously depolymerized or unaggregated virus.

Following the 48 hr degradation treatment, aliquots were withdrawn from each tube and again after dialysis. The samples were analyzed by immunodiffusion in gels and by density gradient centrifugation. Gradients were made up with 10-40% sucrose in 0.02M borate buffer pH 7.8.

b) Sucrose density gradient analysis of degraded PVY suspensions.

The sedimentation profiles of the degraded virus samples after rate zonal centrifugation of SW 40 gradients were analyzed and compared with the sedimentation profile of the untreated virus control. The relative degree of degradation of the virus into lower molecular weight fragments was judged by the amount of UV-absorbing material which had remained at the meniscus or which had sedimented to a depth above the position to which PVY should have sedimented.

The sedimentation profile of the untreated PVY control showed that the virus, which represented several pooled preparations, was heavily aggregated due to prolonged storage and manipulation at room temperature (Fig. 7A). It was further observed that treatment with 0.1% 2-dimethyl amino ethanol did not have any noticeable effect

on the degradation of the virus (Fig. 7B) while treatment with 2M guanidine-HCl (analyzed after dialysis) and 0.25% Igepon-T73 apparently caused complete removal of the virus since no increase in the width of the absorbance zone at the meniscus was observed (Fig. 7C). Glycine-NaOH, pH 12.0, caused depolymerization of PVY into variable sized fragments or aggregates of fragments, which sedimented only slightly higher up in the gradient column than intact PVY (Fig. 7D). Prominent zones of UV-absorbing material of low molecular weight were observed at the meniscus of gradients containing virus which had been treated with SDS, ETA, Urea, pyrrolidine, pyridine or LSA. Fig. 8 shows that significant quantitative differences in the amount of low molecular weight material resulted from the various treatments, as judged by the width of the absorbing zone at the meniscus in gradients containing pyrrolidine-degraded virus (Fig. 8A) and SDS-degraded virus (Fig. 8C). The increased absorbance at the meniscus could not have been due to absorbance of the degrading compound because it was determined by spectrophotometric analysis that, at low concentrations, only pyridine, guanidine-HCl and LSA absorbed UV light at wavelengths where PVY showed absorbance. Consequently virus treated with these compounds was only analyzed after removal of the degrading compound by dialysis.

In order to observe changes in the sedimentation profiles of degraded virus due to re-polymerization of the

Figure 7. Effect of degrading compounds on the sedimentation of PVY.

Sucrose density gradient centrifugation profiles in a 10-40% sucrose gradient in 0.02M borate buffer pH 7.8 after centrifugation in an SW 40 rotor at 40,000 rpm for 90 minutes.

Direction of sedimentation is to the left.

A. Untreated PVY control (1.0 mg/ml). The preparation was heavily aggregated due to prolonged storage and manipulation at room temperature.

B. PVY (1.0 mg/ml), treated with 0.1% 2-dimethyl amino ethanol for 4 hours at 25°C and 48 hours at 3°C.

C. As in B, treated with 0.25% Igepon-T73.

D. As in B, treated with 0.5M glycine-NaOH pH 12.0.

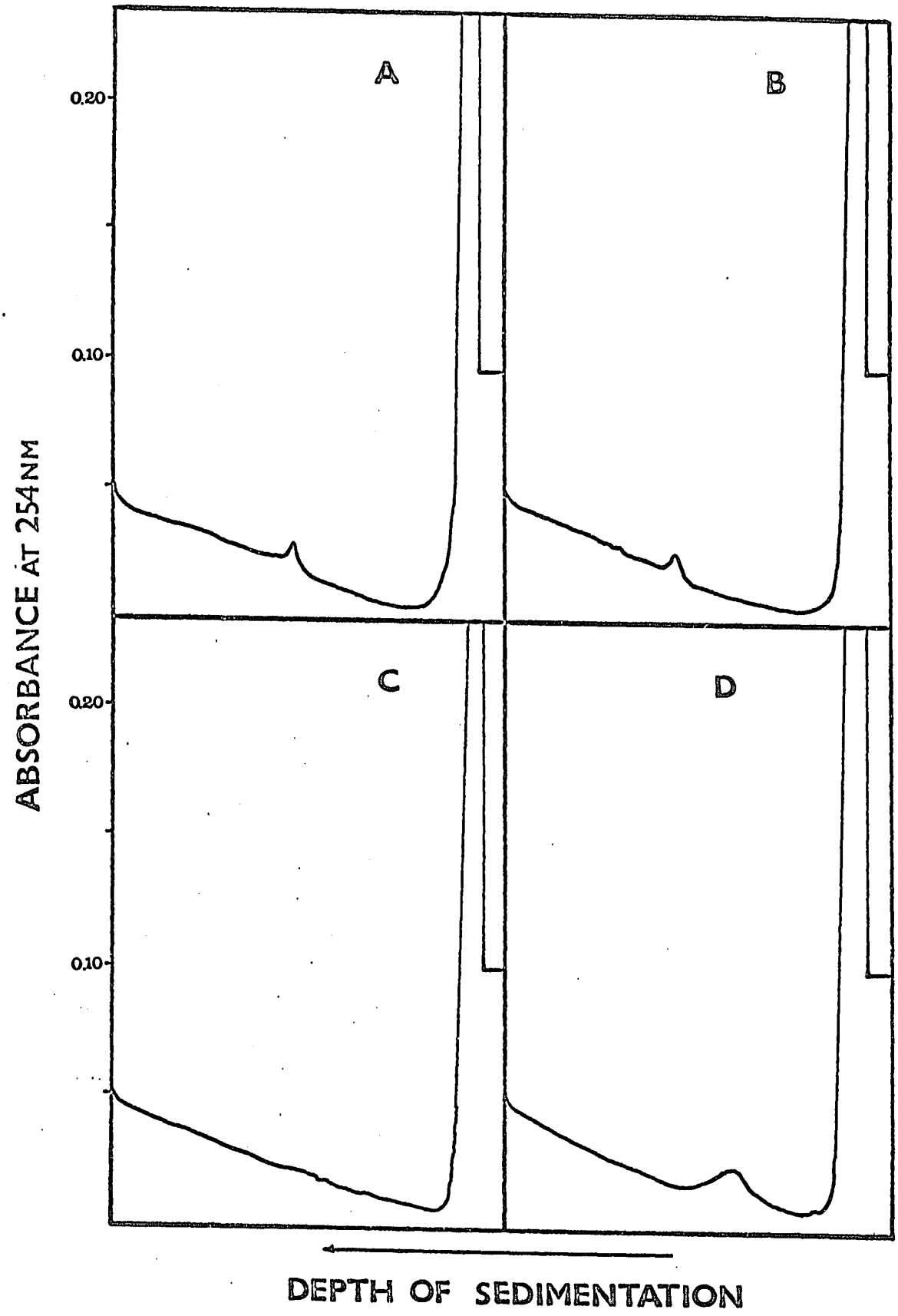

A
B
C
D
0.20
0.10
0.20
0.10
ABSORBANCE AT 254 NM
DEPTH OF SEDIMENTATION

Figure 8. Effect of degrading compounds on the sedimentation of PVY.

Sucrose density gradient centrifugation profiles in a 10-40% sucrose gradient in 0.02M borate buffer pH 7.8 after centrifugation in a SW 40 rotor at 40,000 rpm for 90 minutes.

Direction of sedimentation is to the left.

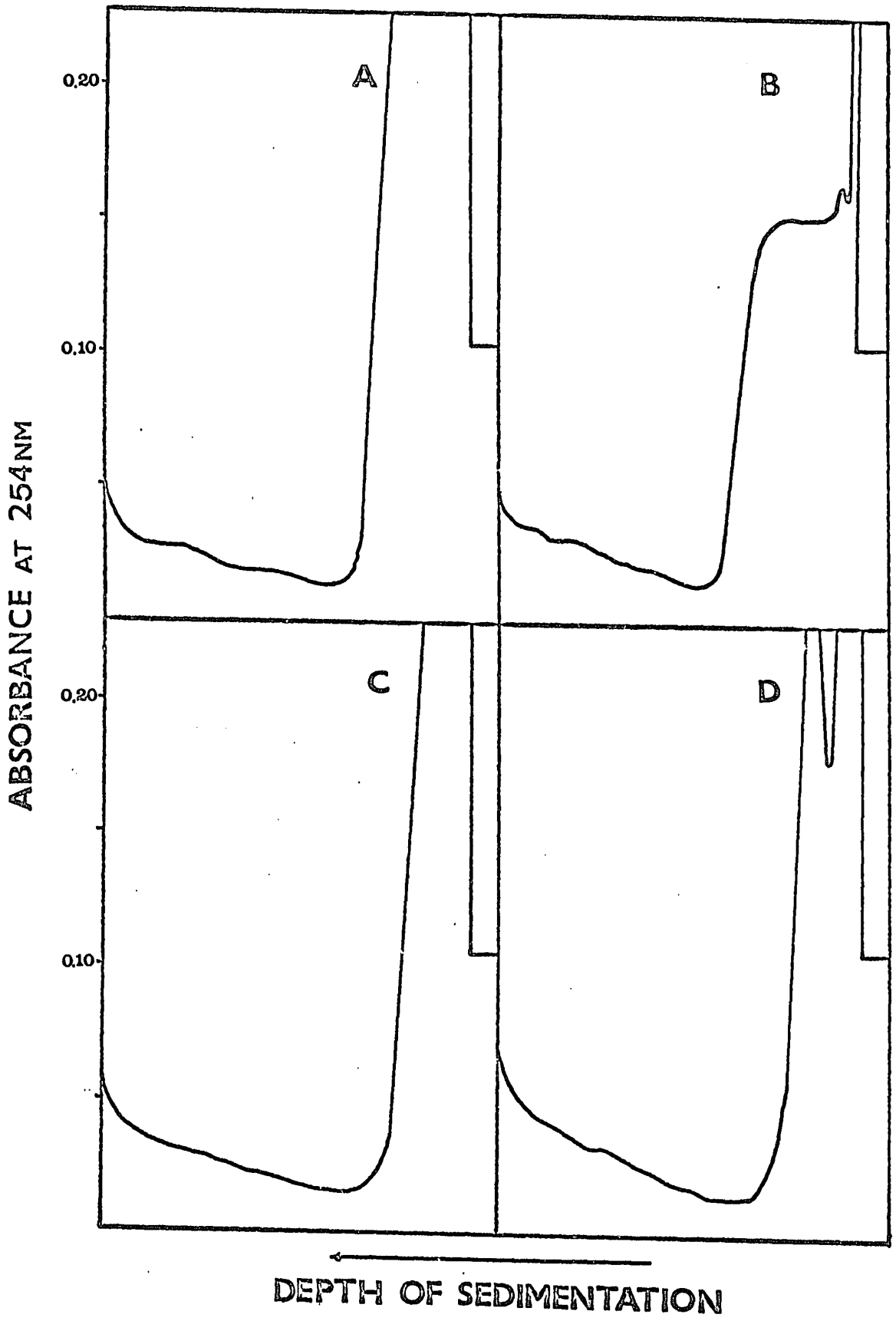
A. PVY (1.0 mg/ml), treated with 2.5% pyrrolidine for 4 hours at 25°C and 44 hours at 3°C.

Note increased area of absorbance at the meniscus.

B. As in A, after subsequent removal of the degrading compound by dialysis.

C. PVY (1.0 mg/ml), treated with 2.5% pyrrolidine for 4 hours at 25°C and 44 hours at 3°C.

D. As in C, after subsequent removal of the degrading compound by dialysis.


A
B
C
D
0.20
0.10
0.20
0.10
ABSORBANCE AT 254NM
DEPTH OF SEDIMENTATION

protein oligomers after removal of the degrading compound, samples were also analyzed after dialysis.

Results indicated that no marked differences in sedimentation resulted after removal of the degrading compound, even in preparations which had shown a noticeable increase in opalescence after dialysis. Only virus treated with SDS or pyrrolidine showed some evidence for the presence of higher molecular weight material after dialysis, indicating that probably a certain degree of repolymerization or aggregation had taken place. However, at the position to which intact PVY should have sedimented, no increased absorption was observed (Fig. 8).

The serological activity of all UV-light absorbing fractions from the gradients was briefly investigated. All such fractions reacted positively with PVY antiserum in microprecipitin tests but most did not produce precipitin lines in double diffusion tests, presumably because the concentration of diffusable and serologically active virus in the gradient fractions was too low. Fractions representing SDS-, LSA-, and pyrrolidine-treated virus produced weak precipitin lines with PVY antiserum.

c) Immunodiffusion tests with degradation products of partially purified PVY.

In order to observe whether the various degradation treatments to which the purified PVY suspensions had been subjected would produce serologically active fragments that would diffuse into an agar matrix and react specifically

Table 6. Serological reactions between degraded PVY and PVY antisera in immunodiffusion and microprecipitin tests.

Treatment of antigen[a]	Immunodiffusion				Microprecipitin			
	Antisera				Antisera[b]			
	PVY-MC	PVY-NC	PVY-NB	NS	PVY-MC	PVY-NC	PVY-NB	NS
0.2% SDS	+[c]	+	-[d]	-	+	+	+	-
2.5% Pyrrolidine	+	+	-	-	+	+	+	-
2M Guanidine-HCl	-	-	-	-	+	+	+	-
1% LSA	+	+	-	-	+	+	+	-
4M Urea	+	+	-	-	+	+	+	-
0.25M ETA pH 10.5	+	+	-	-	+	+	+	-
30% Pyridine	-	-	-	-	+	+	+	-
0.1% 2-DMAE pH 10.2	-	-	-	-	+	+	+	-
0.25% Igepon T-73	-	-	-	-	+	+	+	-
0.5M Glycine-NaOH pH 12.0	+	+	-	-	+	+	+	-
Untreated PVY (control)	-	-	-	-	+	+	+	-

[a]PVY at 1 mg/ml was subjected to the degrading compound for 48 hours followed by dialysis against borate buffer.
[b]Serum was diluted 1/8 to avoid reactions with normal host protein.
[c](+) indicates formation of precipitate. [d](-) indicates absence of precipitate.

Figure 9. Double diffusion test with PVY, treated with degrading compounds for 4 hours at 25°C and 44 hours at 3°C.

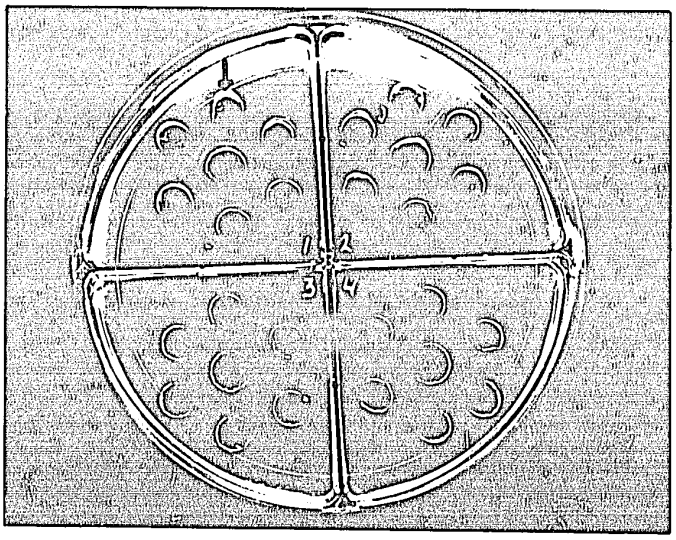
A. Peripheral wells (clockwise from the arrow in each quadrant) contained the following antigens:

1. treated with 0.2% SDS
2. treated with 2.5% Pyrrolidine
3. treated with 4M Urea
4. treated with 0.25M ETA pH 10.5
5. treated with 0.5M Glycine-NaOH pH 12.0
6. treated with 1% LSA

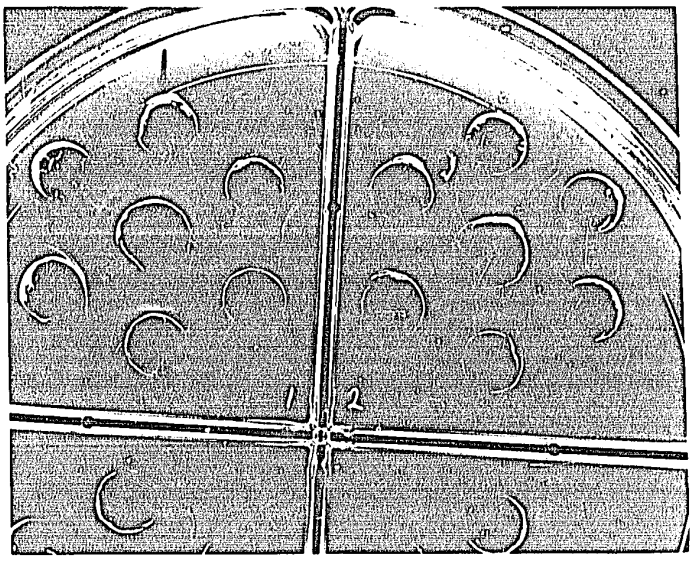
Central wells contained the following antisera:

Quadrant 1: PVY-MC
2: PVY-NC
3: PVY-NB
4: Normal serum

B. 1.8 x enlargement of quadrants 1 and 2 of A.



A



B

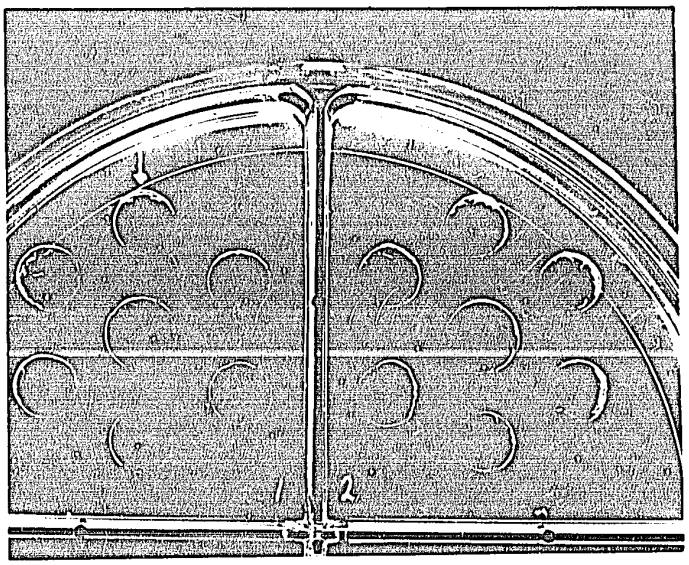
Figure 10. Double diffusion test with PVY, treated with degrading compounds for 4 hours at 25°C and 44 hours at 3°C, then dialysed against 0.02M borate buffer pH 7.8.

Left quadrant: PVY-MC antiserum

Right quadrant: PVY-NC antiserum.

Peripheral wells (clockwise from arrow in both quadrants) contained the following antigens:

1. treated with 0.2% SDS
2. treated with 2.5% Pyrrolidine
3. treated with 4M Urea
4. treated with 0.25M ETA pH 10.5
5. treated with 0.5M Glycine-NaOH pH 12.0
6. treated with 1% LSA



with PVY antiserum, aliquots from each treatment were withdrawn after 48 hours and also after dialysis, for use in immunodiffusion tests. A recently prepared antiserum to PVY (PVY-MC) was used in one quadrant of a quadrant-type petri dish and its effectiveness in detecting degraded viral antigens was compared with antisera received from Dr. G. V. Gooding, Jr. (PVY-NC) and Dr. R. H. Bagnall (PVY-NB). Normal serum was used as a control in the fourth quadrant. The microprecipitin titers of the PVY antisera were previously found to be 1/512, 1/512 and 1/128 for PVY-MC, PVY-NC and PVY-NB respectively. The immunodiffusion titer of PVY-NC antiserum was known to be 1/4 against SDS-degraded PVY (G. V. Gooding, Jr., personal communication).

Results indicated that six degrading compounds, i.e., SDS, pyrrolidine, urea, ETA, glycine-NaOH and LSA, were effective in degrading purified PVY into diffusible fragments (Table 6). Figs. 9 and 10 show that treatment of the virus with these compounds, resulted in the formation of precipitin lines in agar gel double diffusion, particularly in the quadrants containing antiserum to PVY-MC and PVY-NC. The antibody content of the PVY-NB antiserum was apparently too low to give observable reactions with the degraded virus antigen, as was already indicated by its lower microprecipitin titer. No reactions were observed between normal serum and degraded PVY antigens. When purified undegraded PVY or clarified

sap from healthy tobacco plants was used as the antigen, a very weak, straight line midway between the antigen and antiserum well sometimes developed. This occurred with all three antisera and clearly demonstrated that they all contained antibodies to normal host proteins. However, such precipitin lines could be recognized and distinguished from precipitin lines resulting from the presence of viral antigens. It was not thought advisable to absorb the antisera with extracts from healthy tobacco, for this would have resulted in a considerable dilution. Antisera PVY-MC and PVY-NC were equally effective in detecting viral antigens but only if used undiluted. A two-fold dilution resulted in a weak precipitin line and no lines resulted with four-fold diluted antiserum.

Fig. 10 shows that removal of the degrading agent by dialysis results in a better precipitin line formation. Presumably, this is not the result of antigenic differences due to repolymerization of the viral fragments, but rather because the addition of highly alkaline chemical compounds to the wells adversely affects the antigen-antibody reaction or perhaps alters the agar structure which would interfere with the diffusion of the antigens.

Degraded virus preparations were also reacted with the various antisera in microprecipitin tests and positive reactions were obtained between all reactions (Table 6).

It was concluded from these results that, although exposure of the virus to the degrading compound does not

affect the serological activity of the antigen, it does not always result in the production of diffusable viral fragments. For example, sucrose density gradient analysis of pyridine-degraded PVY conclusively demonstrated that the degradation treatment produced low molecular weight material, which reacted with PVY antiserum in a microprecipitin test. Yet, the antigen did not react in the immunodiffusion system. Presumably then, pyridine treatment affects the diffusibility of the viral fragments as has been suggested by Shephard (1970) who encountered a similar phenomenon when working with pyridine-degraded PVS. Negative immunodiffusion results obtained with PVY which had been exposed to treatment with guanidine-HCl, Igepon T-73 or 2-dimethyl amino ethanol were predictable because sucrose density gradient analyses of these preparations indicated that no lower molecular weight antigens were produced as a result of the treatment.

d) Incorporation of the degrading compound in the immunodiffusion system.

In these double diffusion tests, each of the degrading compounds under investigation was incorporated into the agar solution at the appropriate dilution to give the desired final concentration in the agar. Identical tests were set up in each quadrant of a petri dish, using antisera PVY-MC, PVY-NC, PVY-NB and normal serum in the central wells. Three antigen wells were placed 4-5 mm from each central well, in a triangular fashion so that the distances between each antigen well were equal. Purified PVY (1.0 mg/ml) and

crude sap of infected and healthy tobacco were used as the antigens. Plates were incubated at room temperature and observed at regular intervals for 24 hours. Results (Table 7) indicate that precipitin lines against both purified PVY and PVY in crude sap were formed in immunodiffusion systems containing the detergents LSA or SDS, whereas only purified PVY exhibited precipitin lines in gels containing Igepon T-73. Antisera PVY-MC and PVY-NC were identical in their reactions; neither antiserum PVY-NB nor normal serum reacted in an observable manner to the PVY antigens.

Table 7. Serological reactions between antiserum PVY-MC and various antigens in immunodiffusion tests with the degrading compound incorporated in the agar.

Degrading agent and concentration in gel.	Purified PVY (1.0 mg/ml)	Sap of PVY-infected tobacco	Sap of healthy tobacco
0.2% SDS	+[a]	+	-[b]
2.5% Pyrrolidine	-	-	-
2M Guanidine-HCl	-	-	-
1% LSA	+	+	-
4M Urea	-	-	-
25M ETA pH 10.5	-	-	-
30% Pyridine	-	-	-
0.1% 2-DMAE	-	-	-
0.25% Igepon T-73	+	-	-
0.5M Glycine-NaOH pH 12.0	-	-	-
Control (plain agar)	-	-	-

[a](+) indicates the presence of a precipitin line
[b](-) indicates the absence of a precipitin line

It should be observed that in these tests only the three detergents were effective in producing diffusible viral fragments. Even Igepon T-73, which previously had not provided evidence for being an effective degrading agent for PVY, caused the formation of precipitin lines, albeit only between purified PVY and its antigens.

With the realization that the experiments were by no means exhaustive and that changes in pH and concentration of the degrading compounds could possibly render them more effective, the results were nevertheless accepted as an indication that the detergents SDS and LSA, when incorporated in the agar, were potentially the most effective of the compounds investigated in their ability to facilitate the detection of PVY in crude expressed sap of infected tobacco.

e) Immunodiffusion tests with crude extracts of PVY-infected tobacco leaves.

Incubation in small glass tubes of crude expressed sap of PVY-infected tobacco with the various degrading compounds for 4 hours at 25°C and 44 hours at 3°C and subsequent use of these antigens in the immunodiffusion system, did not generally result in the formation of reproducible precipitin lines. Occasionally diffuse lines were formed between antisera PVY-MC or PVY-NC and SDS or LSA-treated sap.

In an experiment to demonstrate that the formation of precipitin lines is dependent on the amount of diffusible PVY antigen in the sap, purified virus was added to expressed

sap of healthy tobacco prior to exposure to the degrading compound. Serial two-fold dilutions were made using crude sap as the diluent. The final dilution contained 0.03 mg/ml of PVY prior to the addition of an equal volume of the degrading compound.

Results in Table 8 indicate that the degrading compounds which had been found effective in producing diffusible fragments of purified PVY (Chapter IV, C), were also effective in degrading purified PVY which had been added to crude sap. It is clear that the formation of precipitin lines depends on the concentration of the virus in the sap. Evidently then, the low concentration of PVY in infected tobacco prevents detection by this method.

In another experiment with crude sap of PVY-infected and healthy tobacco, 2.0g samples of leaf tissue were triturated with 1 ml of the degrading compound under investigation. The strained homogenate was used directly in the antigen well of the immunodiffusion system.

Inexplicable precipitin line formation resulted with all immune sera as well as with normal rabbit serum. Precipitin bands even formed between antigen wells or at other places in the immunodiffusion system. No explanation was sought for these phenomena and the method was not investigated further.

Table 8. Immunodiffusion tests with PVY-MC antiserum and sap from healthy tobacco to which purified PVY had been added.

Degradation[a] treatment	Antigens							
	Crude sap from infected tobacco	Crude sap from healthy tobacco	Crude sap of healthy tobacco containing added PVY at indicated concentrations (mg/ml)					
			0.5	0.25	0.125	0.06	0.03	0.015
0.2% SDS	±[d]	-[c]	+[b]	+	±	-	-	-
2.5% Pyrrolidine	-	-	+	±	-	-	-	-
2M Guanidine-HCl	-	-	-	-	-	-	-	-
1% LSA	±	-	+	±	±	-	-	-
4M Urea	-	-	+	±	-	-	-	-
25M ETA pH 10.5	-	-	+	±	-	-	-	-
30% Pyridine	-	-	-	-	-	-	-	-
0.1% DMAE pH 10.2	-	-	-	-	-	-	-	-
0.25%Igepon T-73	-	-	-	-	-	-	-	-
0.5M Glycine-NaOH pH 12.0	-	-	+	±	-	-	-	-

[a] 4 hrs. at 25°C and 44 hrs. at 3°C
[b] (+) indicates formation of precipitin line.
[c] (-) indicates absence of precipitin line.
[d] (±) indicates occasional formation of precipitin line.

2. Sucrose Density Gradient Analyses of Partially Purified Preparations of PVY, Degraded with Sodium Dodecyl Sulphate or Ethanolamine-HCl Buffer pH 10.5

a) Effect of the concentration of the degrading compound on the depolymerization of the virus.

Purified preparations of PVY were subjected to degradation with increasing concentrations of SDS or ETA pH 10.5, and analyzed after sucrose density gradient centrifugation. Figs. 11 and 12 illustrate that exposure to the higher concentration of SDS even for one minute resulted in more complete degradation of the virus. A final concentration of 0.5% SDS was found to be the minimum concentration required to effect degradation in 4 hours at 3°C. A concentration of 0.1% SDS had no apparent effect on the virus (Figs. 11A and 12A) and even seemed to reverse aggregation, although upon further investigation this could not be confirmed. Figs. 11D and 12D show that only partial degradation resulted from exposure of the virus to 0.2% SDS for one minute or 4 hours.

Sucrose density gradient centrifugation profiles of degraded PVY suspensions which had been stores at 3°C for 8 days following dialysis, were almost identical to those shown in Fig. 12, thereby providing proof that termination of the degradation process through removal of the degrading compound results in a relatively stable nucleoprotein suspension, consisting of viral fragments or subunits with a physical heterogeneity determined by the

Figure 11. Sucrose density gradient centrifugation profiles of PVY, treated with various concentrations of SDS for one minute at 3°C, prior to dialysis with 0.02M borate buffer, pH 7.8.

10-40% sucrose gradients in 0.02M borate pH 7.8, centrifuged in a SW 40 rotor at 40,000 rpm for 90 minutes.

A. Treated with 0.1% SDS.
B. Control. Untreated PVY, recently purified, at 1.0 mg/ml.
C. Treated with 0.5% SDS.
D. Treated with 0.2% SDS.

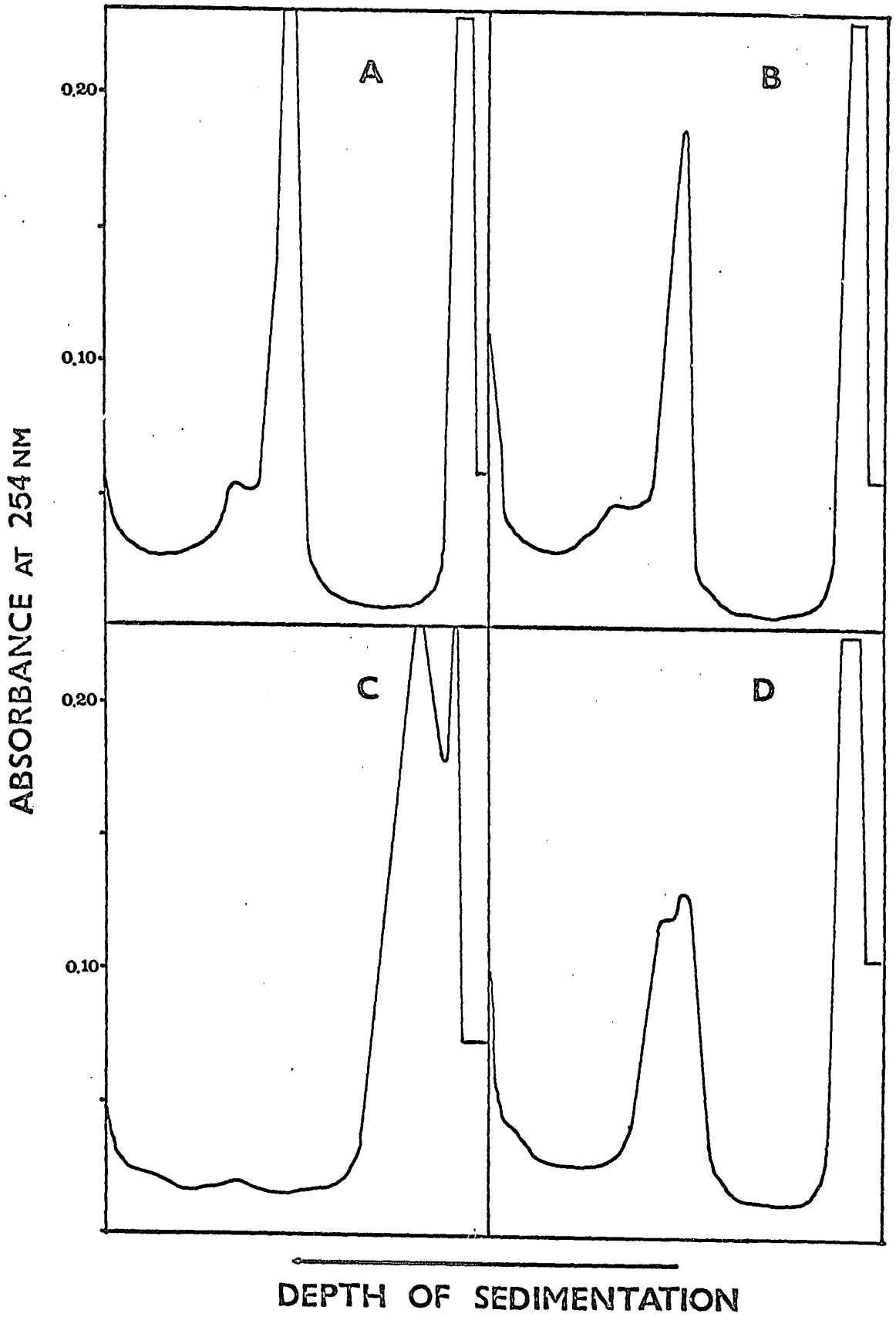
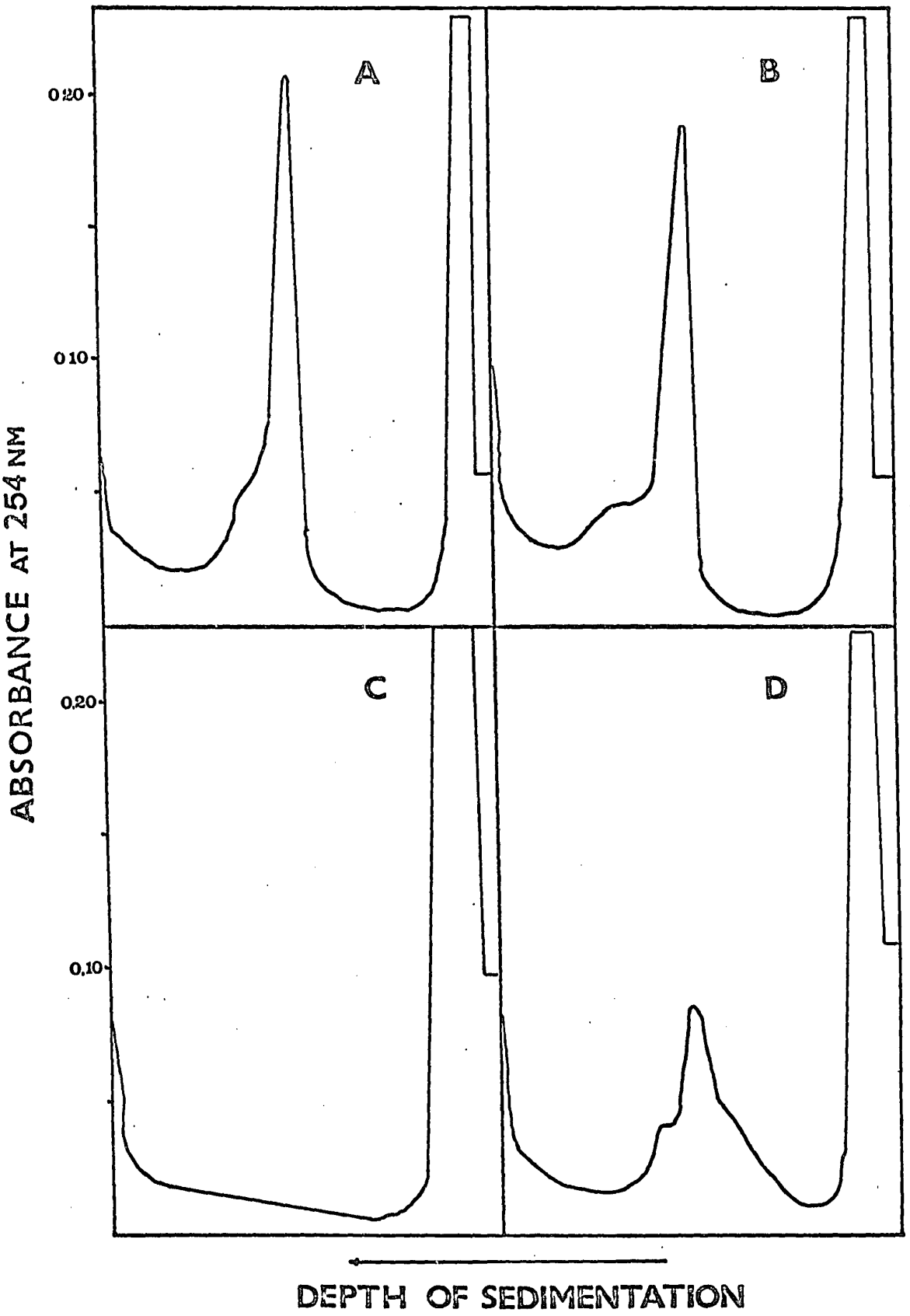

A
B
C
D
0.20
0.10
0.20
0.10
ABSORBANCE AT 254 NM
DEPTH OF SEDIMENTATION

Figure 12. Sucrose density gradient centrifugation profiles of PVY, treated with various concentrations of SDS for 4 hours at 3°C, prior to dialysis with 0.02M borate buffer, pH 7.8.

10-40% sucrose gradients in 0.02M borate pH 7.8, centrifuged in a SW 40 rotor at 40,000 rpm for 90 minutes.

A. Treated with 0.1% SDS.
B. Control. Untreated PVY, recently purified at 1.0 mg/ml.
C. Treated with 0.5% SDS.
D. Treated with 0.2% SDS.


A
B
C
D
0.20
0.10
0.20
0.10
ABSORBANCE AT 254 NM
DEPTH OF SEDIMENTATION

concentration of the degrading compound, and the temperature.

Similar observations were made with virus preparations degraded at 3°C with increasing concentrations of ETA pH 10.5. However, the high pH of the solution resulted in a much faster rate of degradation and sucrose density gradient centrifugation profiles of PVY suspensions previously degraded with 0.1M, 0.5M and 1.0M ETA pH 10.5, showed that all UV absorbance was at or near the meniscus of the gradient with only minor variations due to concentration, exposure time and temperature. A low concentration of ETA (0.1M) combined with a minimum exposure time at 3°C, resulted in a profile with an extremely disperse area of absorbance between the meniscus and the position to which intact PVY normally sediments.

All UV-absorbing fractions from the gradients reacted positively in microprecipitin tests with PVY-MC antiserum. When these same fractions were tested in double diffusion tests, only the SDS-degraded virus produced precipitin lines, which were of varying intensity. The sharpest line was produced by viral material in the fraction collected from the meniscus of the gradient tube containing PVY treated with 0.5% SDS for 4 hours. No lines were formed with fractions containing virus treated with 0.1% SDS.

b) Effect of time of exposure to the degrading compound on the depolymerization of the virus.

A comparison of profiles C and D in Figures 11 and 12, shows that degradation of the virus had progressed

further in the sample which was exposed to SDS for 4 hours prior to the commencement of dialysis. Presumably time of exposure of the virus to ETA pH 10.5 had similar consequences, although little evidence of this could be obtained through sucrose density gradient centrifugation analysis. Profiles of PVY treated with ETA for 4 hours or 1 minute showed intense absorbance at or near the meniscus depending on the initial ETA concentration.

c) The effect of temperature on degradation.

Evidence was obtained that degradation of PVY could be accomplished at a much faster rate at room temperature than 3°C. Virus preparations exposed to 0.2% SDS at 25°C for 1 minute, followed by dialysis, were completely degraded since sucrose density gradient profiles showed an intense zone of absorbance at the meniscus. Even exposure of the virus to a 0.1% solution of SDS at 25°C resulted in partial degradation since an additional broad absorbance zone could be resolved in the gradient above the position corresponding to intact PVY.

D. SPECTROPHOTOMETRIC ANALYSIS OF SDS-DEGRADED PVY AND PVY-PROTEIN

Figure 13 shows the ultraviolet absorption spectra of intact PVY, SDS-degraded PVY and PVY-protein, with additional data provided in Table 9. It was known from previous experiments and spectrophotometric analyses of PVY, that the purified suspension contained some host impurities.

Figure 13. Ultraviolet absorption spectra of preparations of PVY, SDS-degraded PVY and PVY-protein in 0.02M borate-NaOH buffer pH 7.8.

A. SDS-degraded PVY.

B. PVY-protein.

C. Partially purified PVY.

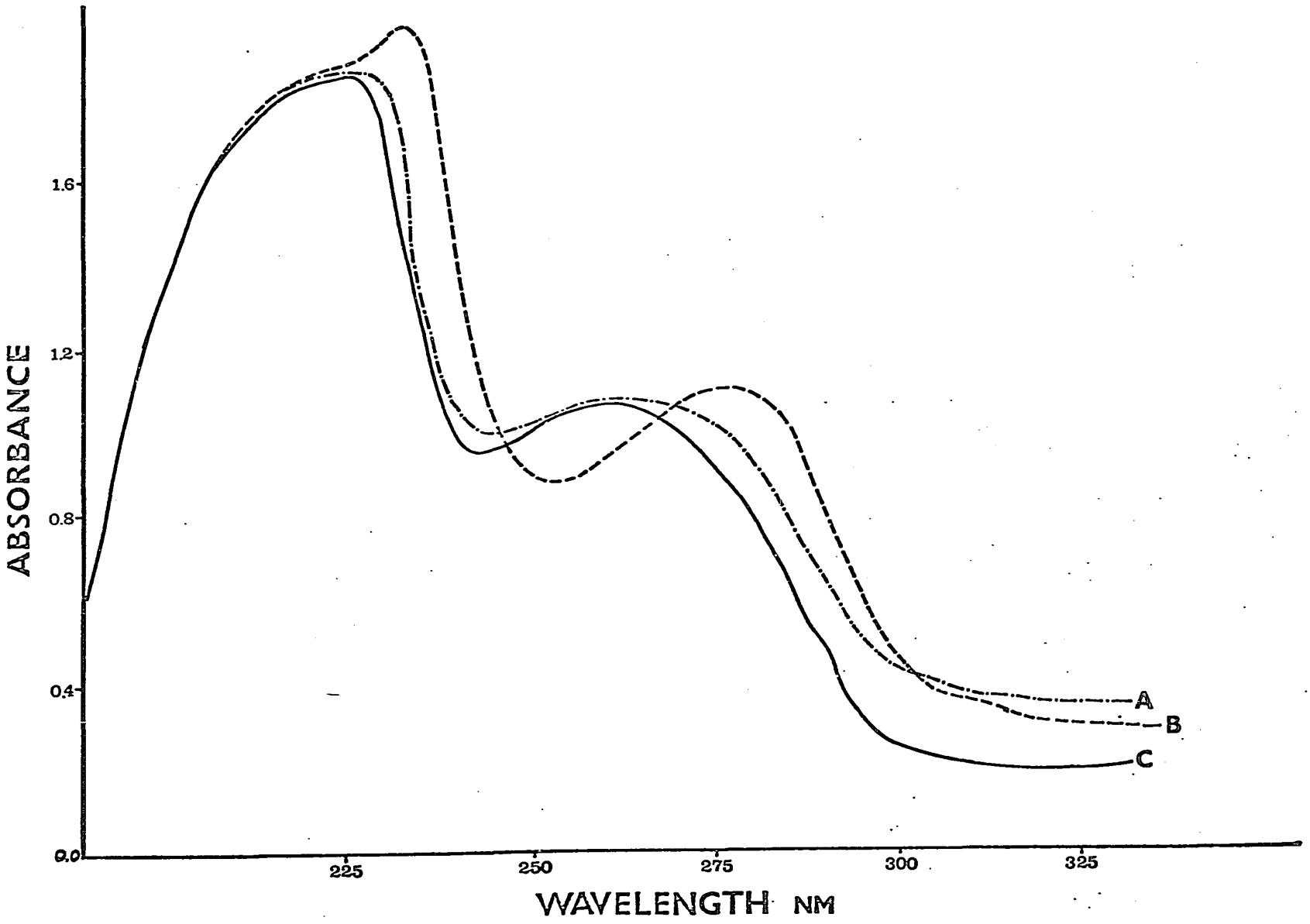

ABSORBANCE
1.6
1.2
0.8
0.4
0.0
225
250
275
300
325
WAVELENGTH NM
A
B
C

Table 9. Comparative spectrophotometric data of suspensions of PVY, SDS-degraded PVY and PVY-protein.

Preparation	Absorption (min.) nm	Absorption (max.) nm	Ratio A280/A260	% Nucleic Acid[a]
PVY	244	259	0.81	6.25%
SDS-degraded PVY	244-246	261-265	0.86	5.25%
PVY-protein[b]	250	278	1.70	0.15%

[a]Demoss and Bard (1957).

[b]Prepared with glycine-NaOH buffer, pH 12.0

This is also indicated by the low A280/A260 ratio, which reflects a rather high nucleic acid content of the suspension (Demoss and Bard, 1957). Degradation with SDS resulted in only a slight loss of nucleic acid and for the purpose of calculating the total nucleoprotein content of a suspension of SDS-degraded PVY, the extinction coefficient for PVY, i.e., 2.8, was routinely used.

For PVY protein preparations, which generally showed a nucleic acid content of 0.15%, with a maximum absorption at 278 nm, the value 1.0 was used as the extinction coefficient.

E. PRODUCTION AND ANALYSIS OF ANTISERA

1. Schedule of Injection

The amount of virus administered to each rabbit is given in Tables 10, 11 and 12. Antiserum to intact PVY

was prepared in two rabbits and antisera to SDS-degraded PVY and PVY-protein were prepared in one rabbit each.

2. Titration of Antisera

a) Titration by microprecipitin test.

Because flexuous rod-shaped viruses have a strong tendency to aggregate, the efficacy of this method to give reproducible results has been questioned (Matthews, 1970). However, after initial experimentation with various buffer solutions reproducible results could be obtained by using 0.05M Tris-HCl pH 7.1 as the diluent. In every test it was imperative to include normal antiserum and buffer controls, for non-specific precipitates sometimes occurred. Homologous and heterologous antiserum titers are tabulated in Table 13. The term "heterologous" is used here to denote any antigen reacting with an antiserum which was not produced as a result of immunization with that particular antigen. Antiserum prepared against PVY-protein had a very low antibody level against homologous and heterologous antigens. Antisera to PVY and SDS-degraded PVY showed a high antibody level to homologous and heterologous antigens except PVY-protein.

b) Titration by double diffusion in agar gel.

The immunodiffusion titer of PVY-and SDS-degraded PVY antiserum was found to be only 1:4 and 1:2 respectively (Table 13). Clear precipitin lines were produced when the

Table 10. Injection schedule of PVY antigen into two rabbits for the preparation of PVY antiserum.

Time of Injection	Route	Amount (ml) of PVY-adjuvant emulsion injected		Concentration[a] of PVY injected (mg/ml)	
		Rabbit A	Rabbit B	Rabbit A	Rabbit B
Day 1	intravenous[b]	1	1	1.3	3.5
	intramuscular	3	3	1.3	1.2
day 7	intramuscular	3	2	1.3	2.0
14	intramuscular	4	2	1.1	1.0
21	intramuscular	3	2	1.1	1.0
55	intramuscular	-	3	-	1.1
69	intramuscular	4	-	1.0	-

[a]Concentration of virus injected intravenously or after emulsification with adjuvant in case of intramuscular injection.

[b]No adjuvant used.

Table 11. Injection schedule of SDS-degraded PVY antigen into a rabbit for the preparation of SDS-degraded PVY antiserum.

Time of injection	Route	Amount (ml) of PVY-adjuvant emulsion injected	Concentration (after emulsifying) of PVY injected (mg/ml)
Day 1	intramuscular	2.6	6.5
7	intramuscular	3.0	1.0
14	intramuscular	3.4	1.1
21	intramuscular	3.4	1.0

Table 12. Injection schedule of PVY-protein antigen into a rabbit for the preparation of PVY-protein antiserum.

Time of injection	Route	Amount (ml) of PVY-adjuvant emulsion injected	Concentration (after emulsifying) of PVY injected (mg/ml)
Day 1	intramuscular	4.0	1.25
8	intramuscular	3.0	0.65
15	intramuscular	2.2	0.75
38	intramuscular	3.0	1.45

Table 13. Determination of homologous and heterologous antiserum titers by micro-precipitin and immunodiffusion tests.

	Microprecipitin titer			Immunodiffusion titer[a]		
	Antigens			Antigens		
Antisera	PVY	SDS-degraded PVY	PVY-protein	PVY[b]	SDS-degraded PVY	PVY-protein
PVY	512	512	16	4	2	1
SDS-degraded PVY	512	256	8	2	2	1
PVY-protein	8	8	8	0	0	0

[a]Titers expressed as the reciprocal of the highest reactive antiserum dilution.

[b]0.2% SDS and 0.1% sodium azide was incorporated into the immunodiffusion system.

antiserum was used undiluted but the intensity of the lines was reduced drastically with each successive antiserum dilution. No reaction could be observed when PVY-protein antiserum was used. Of interest is the fact that an antiserum with a minimum microprecipitin titer of 1:256 is required to give an observable reaction in immunodiffusion. This was already pointed out in Section C, 1, a of this chapter when it was demonstrated that PVY-NB antiserum, which had shown a microprecipitin titer of 1:128 did not react with PVY antigens in an immunodiffusion system containing 0.2% SDS and 0.1% sodium azide. PVY-NC, with a microprecipitin titer of 1:512, had an immunodiffusion titer of 1:4, identical to the titer shown by the PVY antiserum analyzed here.

F. IMMUNODIFFUSION STUDIES WITH SODIUM DODECYL SULPHATE (SDS) AND SODIUM DIBUTYL NAPHTHALENESULPHONATE (LSA)

1. Evidence for a Change in Antigenic Identity of PVY upon Prolonged Storage in Borate Buffer

The antisera to intact PVY and SDS-degraded PVY could both be reliably used undiluted in double diffusion tests to detect the presence of PVY in crude extracts of infected tobacco. No lines formed when PVY-protein antiserum was used (Fig. 14). Initially, precipitin-line formation was obtained when 0.5% SDS and 1.0% sodium azide (Gooding and Bing, 1970) were incorporated in the agar, but other concentrations of SDS or LSA, were found equally or more suitable (see section F, 2). Occasionally, multiple

precipitin lines formed between SDS-degraded PVY antiserum and purified preparations of the virus (Fig. 14A, quadrant 2) but since this was not a regular phenomenon it was not further investigated.

Normally, precipitin lines originating from adjacent antigen wells containing purified virus and crude sap of PVY-infected tobacco showed complete coalescence, indicating identical antigens were present in each well (Fig. 14B). However, when using purified PVY which had been stored in 0.02M borate buffer pH 7.8 at 3°C for an extended period of time (10 days), spur formation always occurred with freshly purified PVY or with expressed sap of PVY-infected tobacco or potato (Figs. 15, 16, 17). This indicates that during storage in the low molarity buffer solution the virus loses some of its antigenic determinants. The same phenomenon was observed between "old" and "fresh" preparations of PVY when SDS-degraded PVY antiserum was used (Fig. 18).

2. Factors Influencing the Formation of Precipitin Lines in the Agar Gel Double Diffusion System.

a) Effect of the concentration of the detergent and of the electrolyte.

It was observed that suitable reactions could be obtained with various concentrations of SDS but that an increase or decrease in the concentration of the detergent required an increase or decrease in the concentration of the

Figure 14. Double diffusion with PVY antigens and antiserum prepared against intact PVY, SDS-degraded PVY and PVY protein.

Immunodiffusion system: 0.8% agar-Noble in distilled water
0.5% SDS
1.0% sodium azide

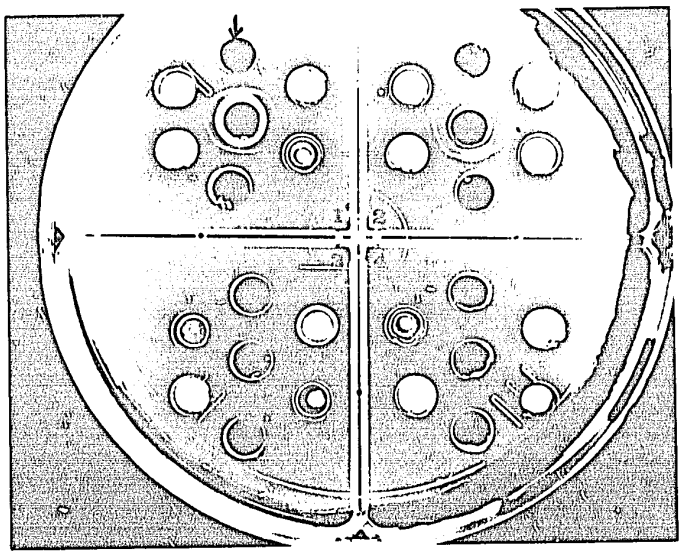
A. Peripheral wells (clockwise from arrow in all quadrants) contained the following antigens:

1 and 4. Purified PVY at 0.5 mg/ml.
2 and 5. Crude sap of PVY-infected tobacco.
3 and 6. Crude sap of healthy tobacco.

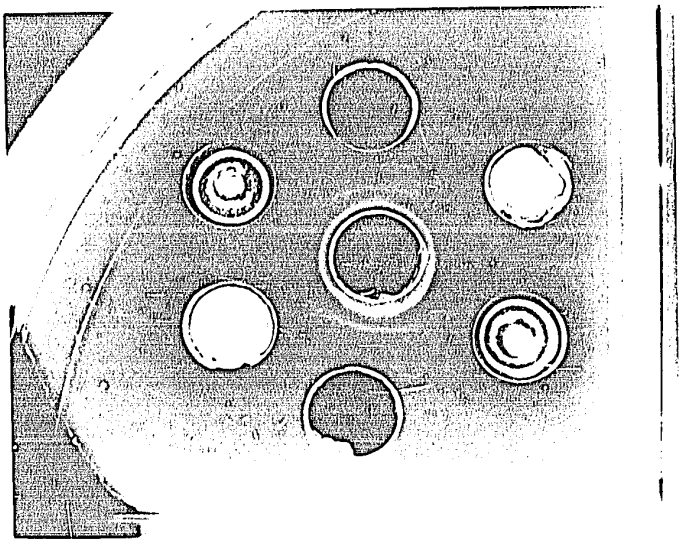
Central wells contained the following antisera:

Quadrant 1: PVY-MC
2: PVY-NC
3: PVY-NB
4: Normal serum

B. 2 x enlargement of quadrant 1 of A.



A



B

electrolyte, sodium azide. No precise quantitative relationship could be demonstrated between SDS and sodium azide, but it was empirically determined that 0.2% SDS required a sodium azide concentration of 0.1% while 0.5% SDS required 1.0% sodium azide. Either combination gave reliable and reproducible results although differences in intensity of precipitin lines could usually be observed (Fig. 15). Often, a better resolution of precipitin lines was shown in the immunodiffusion system containing 0.2% SDS and 0.1% sodium azide but at these concentrations a white crystalline precipitate formed in the agar, which on occasion made the reading of the tests difficult (Fig. 19 C and D). Such precipitates were never observed in plates containing 0.5% SDS and 1.0% sodium azide. Sodium chloride was not required in immunodiffusion plates which contained SDS and sodium azide.

LSA facilitated the detection of PVY when incorporated in the agar at concentrations of 0.75%, 1.0% or 1.5%. Optimum results were achieved with 1.0% LSA, 0.85% sodium chloride and 0.04% sodium azide (Fig. 16). The addition of both of the latter reagents was not critical but resulted in considerable improvement and a better resolution of precipitin lines.

Although occasionally spurious precipitin lines were obtained in all or any of the immunodiffusion plates, results were reproducible within limits of variability. Studies depicted in Fig. 19 A-F indicate that some of the

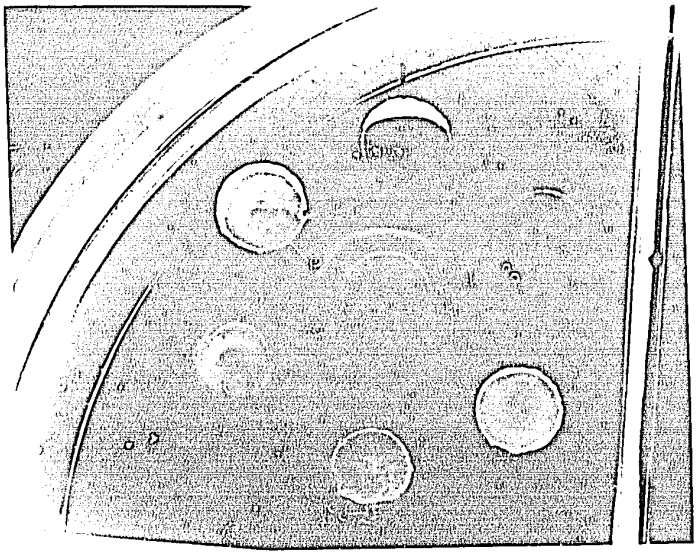
Figure 15. Double diffusion with PVY antigens and antiserum prepared against intact PVY, showing spur formation between recently purified PVY and purified PVY stored for 3 weeks at 3°C. Comparison between immunodiffusion systems containing different concentrations of SDS and electrolyte.

A. Immunodiffusion system: 0.8% agar-Noble in distilled water
0.5% SDS
1.0% sodium azide

B. Immunodiffusion system: 0.8% agar-Noble in distilled water
0.2% SDS
0.1% sodium azide

Peripheral wells (clockwise from arrow in A and B) contained the following antigens:

1. Recently purified PVY (0.5 mg/ml)
2. Purified PVY (0.5 mg/ml) after three weeks storage in 0.02M borate buffer, pH 7.8.
3. Infected tobacco
4. Infected potato cv. Green Mountain
5. Healthy potato cv. Green Mountain
6. Infected tobacco



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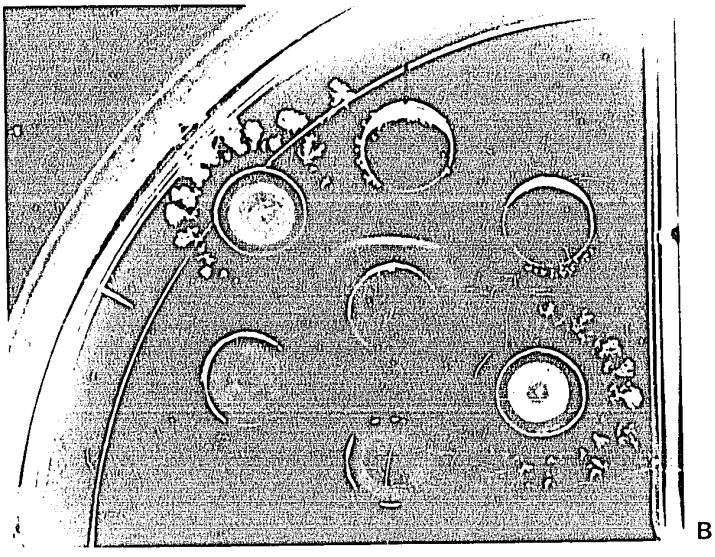


B

Figure 16. Double diffusion with PVY antigens and antiserum prepared against intact PVY, showing spur formation between recently purified PVY and purified PVY stored for 3 weeks at 3°C. Influence of time of incubation on spur formation.

A. Incubated at 24°C for 10 hrs.

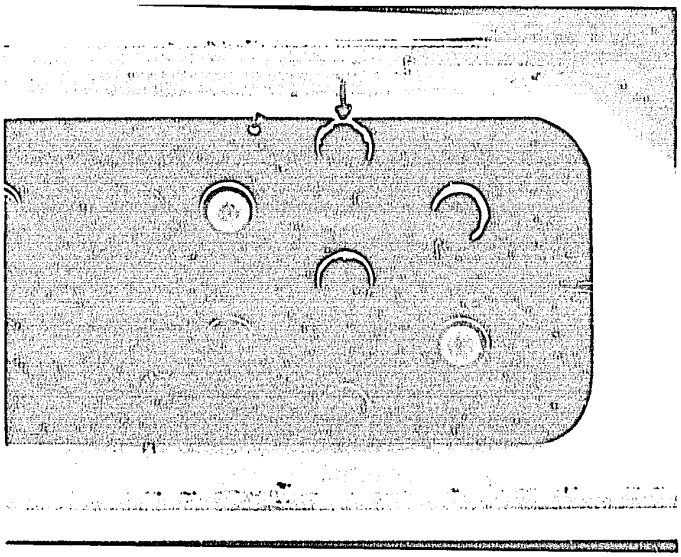
B. Incubated at 24°C for 22 hrs.

Immunodiffusion system in A and B;

0.8% agar-Noble in distilled water
1.0% LSA
0.85% sodium chloride
0.04% sodium azide

Peripheral wells (clockwise from the arrow in A and B) contained the following antigens:

1. Recently purified PVY (0.5 mg/ml)
2. Purified PVY (0.5 mg/ml) stored for 3 weeks at 3°C in 0.02M borate buffer pH 7.8.
3. Infected tobacco
4. Infected potato cv. Green Mountain
5. Healthy potato cv. Green Mountain
6. Infected tobacco



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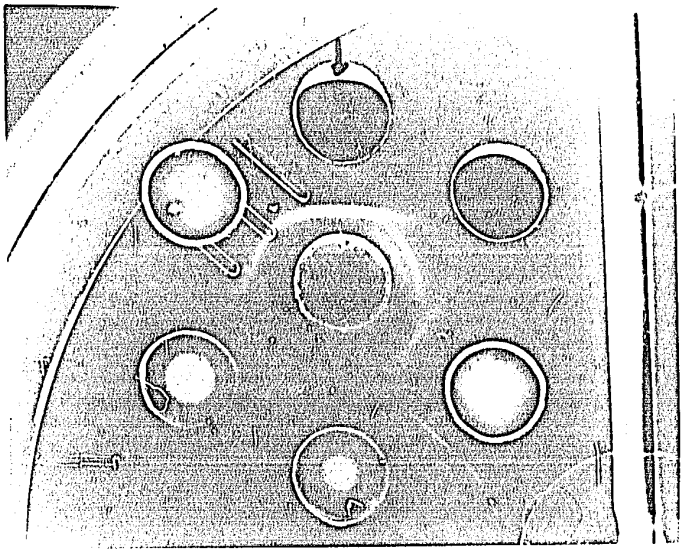


B

spurious precipitin lines may be due to qualitative differences in the antisera. PVY antiserum from rabbit B (Table 10) was shown to have a higher "avidity" (Van Slogteren, 1969) than antiserum from rabbit A, which had shown an identical antibody level in microprecipitin and immunodiffusion titration experiments. Similarly, SDS-degraded PVY antiserum seemed less effective than PVY antiserum from rabbit B. However, in numerous other immunodiffusion experiments no differences between the two PVY antisera and the SDS-degraded PVY antiserum could be observed.

b) Effect of pH of the immunodiffusion system.

The pH of the immunodiffusion system containing SDS or LSA in distilled water was 7.4 and in order to investigate whether the formation of precipitin lines could be improved by changing the pH, a series of identical tests was set up with the pH of the immunodiffusion medium ranging from 4.0 to 10.0 at intervals of one pH unit. Changes in pH were effected by adding drops of 1N HCl or NaOH to the warm agar prior to pipetting it into the diffusion chamber.

Results indicated that at pH 4.0 no precipitin lines were formed between PVY antiserum and purified PVY (0.5 mg/ml) or crude sap of PVY infected tobacco. Precipitin lines which formed at pH 5, 6, 9 and 10 were diffuse; sharper lines were produced at pH 7 and 8 but optimum results were produced in the control gel (pH 7.4).

c) Effect of temperature of incubation.

Normally, immunodiffusion plates were incubated at room temperature (24°C) which resulted in the formation of precipitin lines of maximum clarity in 10-12 hours. Upon continued incubation the lines became increasingly diffuse and eventually (5-7 days) disappeared. This process of a presumably continual degradation of the antigen, resulting in dissolution of the precipitate, could be arrested by incubation at 3°C. Precipitin lines could be preserved indefinitely at this temperature.

When plates were incubated at 3°C immediately after charging the wells, no lines developed. At 10°C precipitin lines appeared after 4 days. In plates incubated at 37°C precipitin lines developed within 6 hours, but lines were generally more diffuse than those that formed at room temperature and no crystalline complex precipitated in plates containing 0.2% SDS (see Chapter F,2,a).

It is clear that the formation of precipitin lines is greatly affected by the temperature presumably through a compound effect on the process of diffusion and degradation.

d) Effect of the concentration of the agar.

The concentration of the agar (0.6, 0.7, 0.8, 0.9 and 1.0%) did not noticeably affect the formation of precipitin lines. However, gelation of the agar was affected below concentrations of 0.7% and for routine purposes immunodiffusion systems were usually prepared with an agar

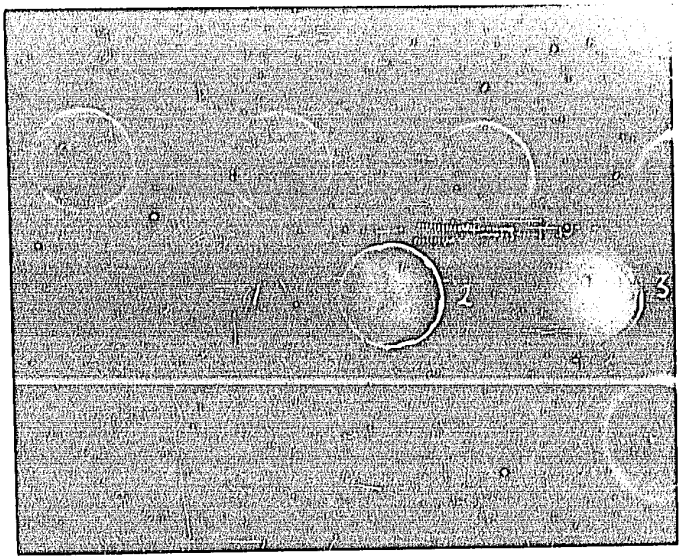
Figure 17. Double diffusion with PVY antigens and antiserum prepared against intact PVY, showing spur formation between recently purified PVY and purified PVY stored for 3 weeks at 3°C. Wells placed in alternate line pattern.

Immunodiffusion system: 0.8% agar-Noble in distilled water
1.0% LSA
0.85% sodium chloride
0.04% sodium azide

Top wells: Antiserum to intact PVY.

Middle wells:
1. Recently purified PVY at 0.5 mg/ml
2. Purified PVY at 0.5 mg/ml stored for three weeks at 3°C in 0.02M borate buffer pH 7.8.
3. Infected tobacco

Bottom wells: normal serum


1
2
3

concentration of 0.8% to facilitate the cutting of the wells.

e) Immunodiffusion on glass slides.

Results obtained with double diffusion tests on glass slides, were practically identical with those obtained in the standard quadrant type petri dish (Fig. 19 A-F), if the usual limits of variability were taken into consideration. Results with LSA seemed slightly better than those obtained with SDS.

The advantage of performing immunodiffusion tests on glass slides is that only minimum quantities of antiserum and antigens are needed to fill the wells. Slides are easily prepared and may be stored in a moist chamber at $3^{o}C$ for several weeks prior to use.

A disadvantage of the method is that manipulation of small quantities of plant sap or antiserum is difficult. Wells may easily be overcharged, thereby flooding the agar and possibly invalidating results of the entire test.

Fig. 19 A-F is representative of the variability that was commonly encountered in all immunodiffusion plates, throughout the course of this investigation. This variability occurred even when all conditions were kept constant. Nevertheless, on the basis of a great many immunodiffusion experiments, the least variable results were obtained using PVY antiserum (rabbit B) in gels containing 1% LSA.

Figure 18. Double diffusion with PVY antigens and antiserum prepared against SDS-degraded PVY, showing spur formation between recently purified PVY and purified PVY stored for three weeks at 3°C.

Immunodiffusion system: 0.8% Agar-Noble in distilled water
0.5% SDS
1.0% sodium azide

Peripheral wells (clockwise from arrow) contained the following antigens:

1. Recently purified PVY (0.5 mg/ml)
2. Purified PVY (0.5 mg/ml) stored for 3 weeks at 3°C in 0.01 M borate buffer pH 7.8
3. Infected tobacco
4. Infected potato cv. Green Mountain
5. Healthy potato cv. Green Mountain
6. Infected tobacco

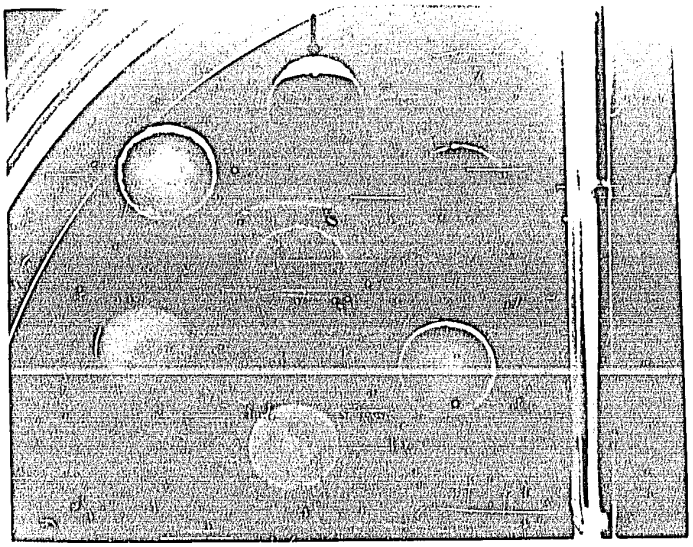


Figure 19 A-F Double diffusion tests with PVY antigens and antisera prepared against intact PVY and SDS-degraded PVY, showing variability in precipitin line formation.

Each six-well test pattern (clockwise from arrow) contained the same antigens.

1. Purified PVY (0.25 mg/ml)
2. Infected potato cv. USDA 41956
3. Healthy potato cv. USDA 41956, with added purified PVY (0.25 mg/ml)
4. Healthy potato cv. USDA 41956
5. Infected potato cv. Keswick
6. Infected tobacco

Center wells in each plate contained the following antisera:

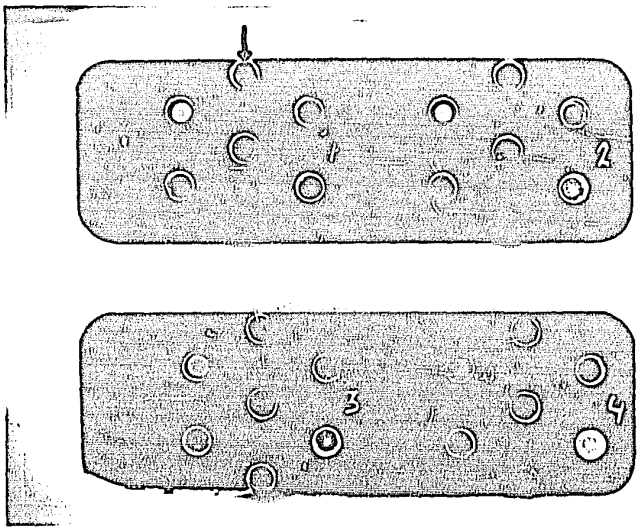
1: Antiserum to intact PVY (rabbit B)
2: Antiserum to SDS-degraded PVY
3: Antiserum to intact PVY (rabbit A)
4: Normal serum

Immunodiffusion systems:

A and B 0.8% agar-Noble in distilled water
0.5% SDS
1.0% sodium azide

C and D 0.8% agar-Noble in distilled water
0.2% SDS
0.1% sodium azide

E and F 0.8% agar-Noble in distilled water
1.0% LSA
0.85% sodium chloride
0.04% sodium azide



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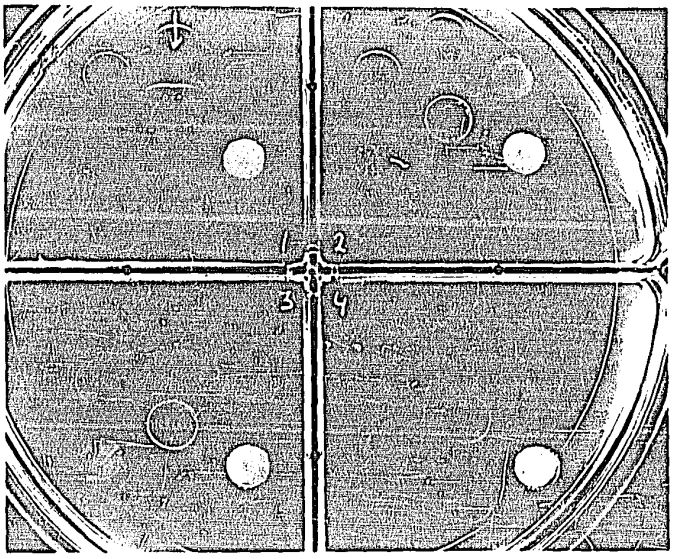

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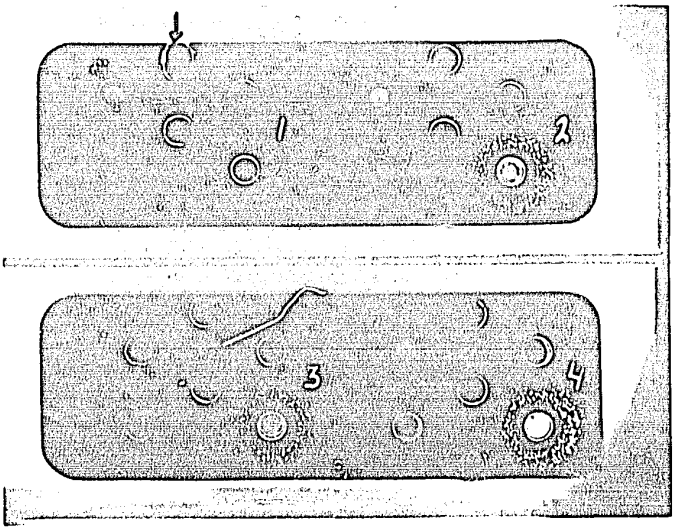
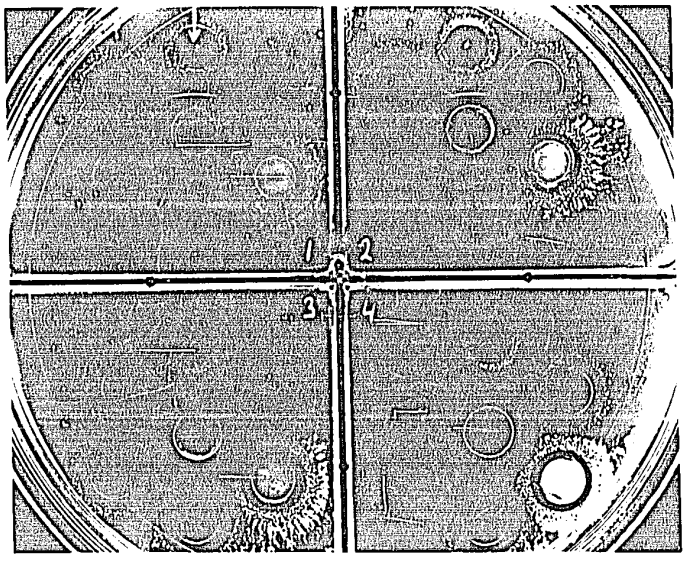
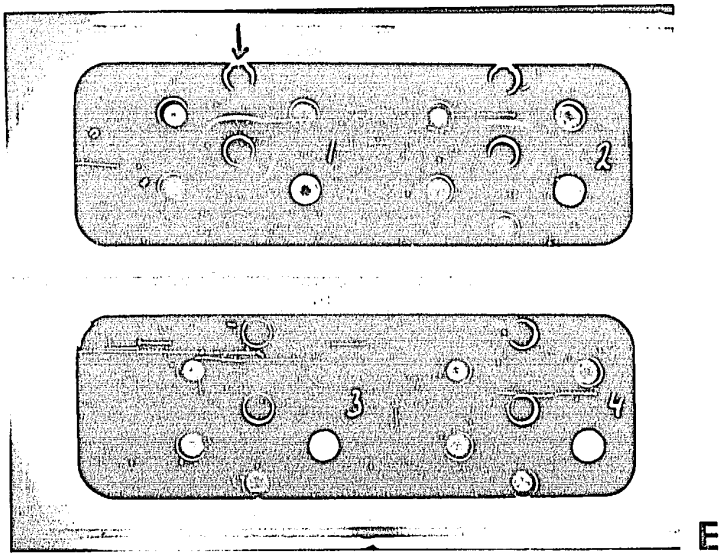


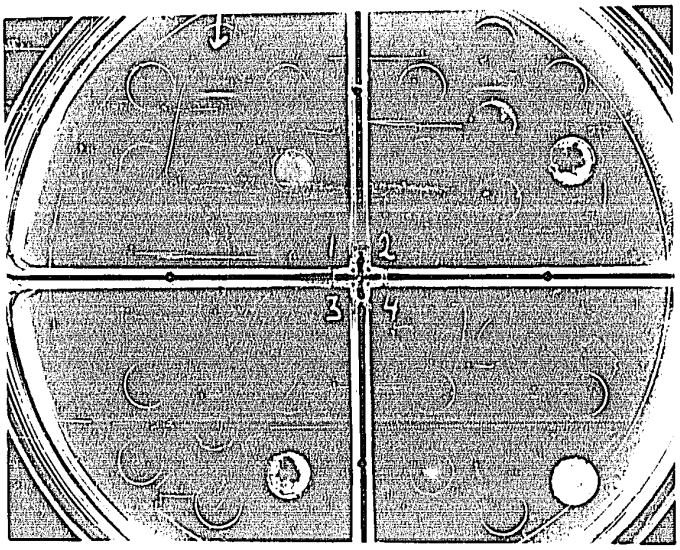
C



D



E



F

3. Minimum Amount of PVY Detectable with Antisera Prepared Against Intact PVY, SDS-degraded PVY and PVY-protein.

a) Detection by microprecipitin.

Dilution titration experiments, using two-fold dilution series of the various antisera and of clarified sap of PVY-infected tobacco, indicated that the virus could be detected in a 1:32 sap dilution at an optimum PVY-antiserum dilution of 1:8. SDS-degraded PVY antiserum at an optimum dilution of 1:8 detected PVY in sap dilutions not exceeding 1:16. PVY-protein antiserum was the least sensitive because PVY could only be detected in a 1:4 sap dilution at an antiserum dilution of 1:4. For accurate observations all tests needed to be controlled with normal serum and sap of healthy tobacco. In an experiment with purified virus at an initial concentration of 0.5 mg/ml, results were similar to those obtained with clarified sap. PVY-antiserum at a 1:8 optimum dilution detected PVY at concentrations not lower than 0.032 mg/ml. A 1:4 dilution of PVY-protein antiserum detected PVY at 0.12 mg/ml.

b) Detection by immunodiffusion.

Antiserum titration experiments had indicated that the immunodiffusion titer of the PVY antiserum was 1:4. However, at this dilution, diagnostic results became extremely unreliable (Fig. 20).

Figure 20. Double diffusion with PVY antigens and antiserum prepared against intact PVY, showing antiserum dilution end-point reactions in immunodiffusion plates containing different concentrations of SDS.

Peripheral wells (clockwise from arrow in all quadrants) contained the following antigens:

1. Infected tobacco
2. Healthy tobacco
3. Infected tobacco
5. Purified PVY (0.5 mg/ml), stored for three weeks at 3°C.
6. Healthy potato cv. USDA 41956.

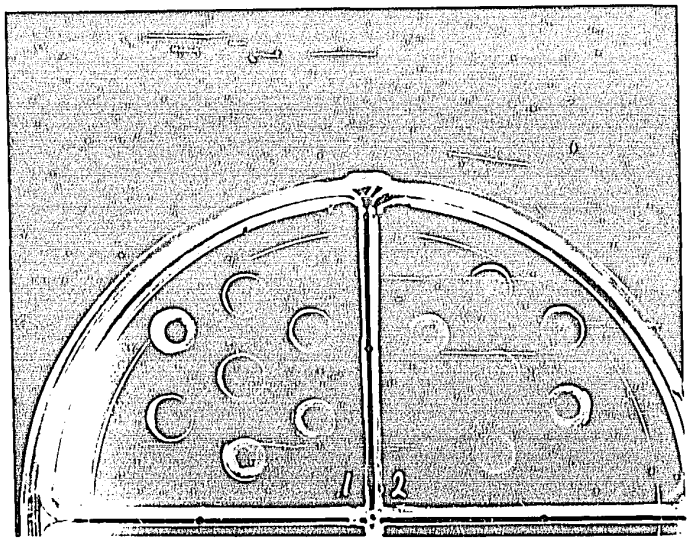
Central wells in A and B contained the following antisera:

Quadrant 1. Antiserum to PVY
2. Antiserum to PVY, diluted 1:4

Immunodiffusion systems:

A. 0.8% agar-Noble in distilled water
0.5% SDS
1.0% sodium azide

B. 0.2% agar-Noble in distilled water
0.2% SDS
0.1% sodium azide



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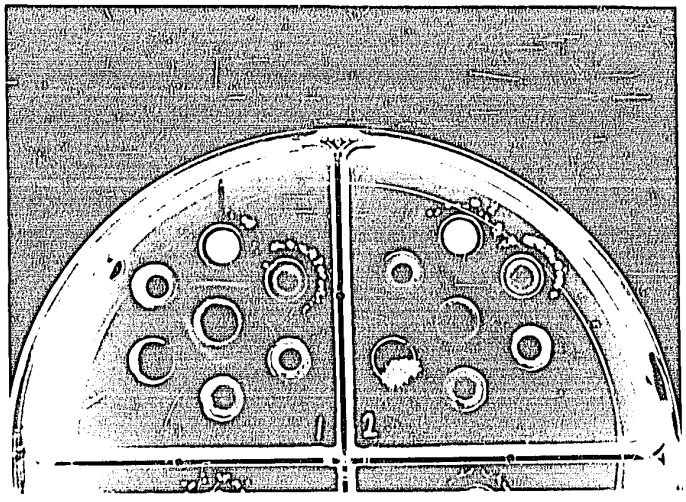


B

Figure 21. Double diffusion titration with PVY antigens and antiserum prepared against intact PVY, showing minimum amount of detectable virus in crude sap of tobacco.

Immunodiffusion system: 0.8% agar-Noble
0.2% SDS
0.1% sodium azide

Antigens deposited in central well of each quadrant:

Quadrant 1. Crude sap of healthy tobacco containing purified PVY at 0.12

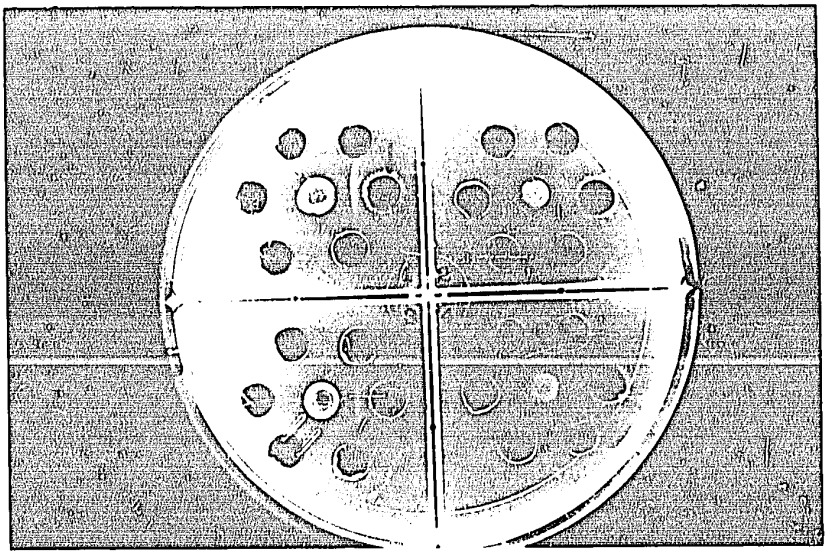
2. As in quadrant 1, with purified PVY at 0.01 mg/ml

3. As in quadrant 1, with purified PVY at 0.06 mg/ml

4. As in quadrant 1, with purified PVY at 0.03 mg/ml

Peripheral wells (clockwise from arrow in all quadrants) contained antiserum at the following dilutions:

1. undiluted PVY antiserum
2. PVY antiserum diluted 1:2
3. " " " 1:4
4. " " " 1:8
5. " " " 1:16
6. " " " 1:32



Using undiluted PVY antiserum, the dilution endpoint of PVY in crude sap of infected tobacco was found to be 1:16 in an immunodiffusion system containing 0.2% SDS and 0.1% sodium azide. Purified PVY at 0.5 mg/ml could be detected at a dilution of 1:32 or 0.16 mg/ml. When purified PVY was added to crude sap of healthy tobacco (final concentration, 0.5 mg/ml), the presence of the virus could be demonstrated in dilutions not exceeding 1:16. Antiserum to SDS-degraded virus detected PVY in 1:8 dilutions.

In another quantitative immunodiffusion experiment serial dilutions of crude sap from healthy tobacco containing purified PVY (0.12 mg/ml) were placed in the center well. Peripheral wells were filled with serial dilutions of PVY antiserum. Fig. 21 shows that precipitin bands only formed with undiluted antiserum. The minimum detectable amount of PVY was 0.03 mg/ml.

c) The use of the immunoglobulin fraction from PVY antiserum.

The immunoglobulin fraction was used to investigate whether other serum components were responsible for the spurious reactions which sometimes occurred in the immunodiffusion system. The original volume of the antiserum was also reduced fivefold in an attempt to raise the antibody titer. Microprecipitin titration of the globulin fraction showed a titer of 1/512, identical to the titer of the original antiserum. However, subsequent use of the fraction in double diffusion did not result in the formation of

precipitin lines.

4. Detection of PVY by Single Radial Diffusion.

The usefulness of this potentially rapid and sensitive immunodiffusion procedure was thoroughly investigated but precipitin ring formation could only be obtained when high concentrations of PVY were deposited in the antigen wells, i.e., 0.5 mg/ml (Fig. 22). Occasionally, very narrow precipitin rings were obtained with crude sap of PVY-infected tobacco in immunodiffusion systems containing 1% LSA, 0.85% NaCl and 0.04% NaN_3. The efficacy of the detergents was investigated at concentrations ranging from 0.2-2% at 0.2% intervals. Sometimes precipitin rings were formed with purified healthy tobacco antigens or crude sap of healthy tobacco.

5. Detection of PVY in Foliage of Infected Potato Varieties

a) Diagnosis of PVY in the potato seedling USDA 41956.

Tubers of this PVY-immune seedling were obtained from the Uihlein Farm of Cornell University, Ithaca, New York, courtesy of Dr. W. F. Rochow, Department of Plant Pathology. Equal halves of a tuber were planted separately in large containers, 18 inches in diameter, to allow for tuber formation. Three weeks after sprouting the plants were tested for the presence of PVY, PVX and PVS by the microprecipitin test. All were found to be infected with PVS. Antisera to PVS and PVX were supplied by Dr. R. H. Bagnall, Canada Department of Agriculture,

Figure 22. Single radial diffusion with PVY antigens. Antiserum to intact PVY was incorporated in the immunodiffusion system.

Immunodiffusion systems:

Quadrant 1: 1.0% agar-Noble and PVY antiserum (1:1)
0.2% SDS
0.1% sodium azide

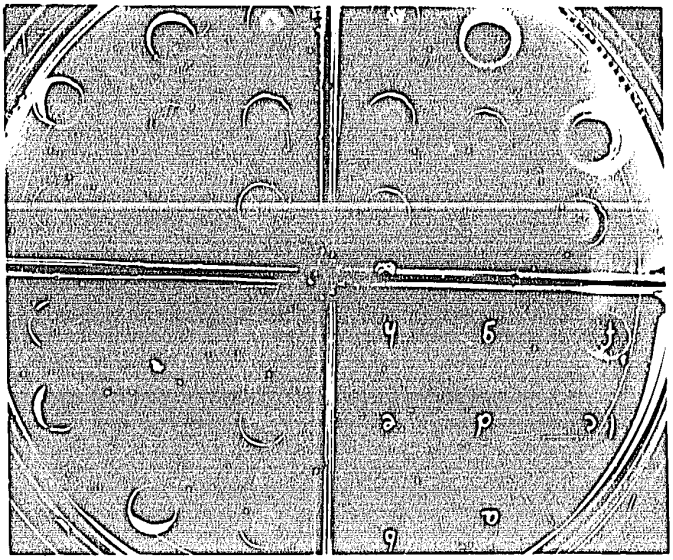
2: 1.0% agar-Noble and PVY antiserum (1:1)
0.5% SDS
1.0% sodium azide

3: 1.0% agar-Noble and PVY antiserum (1:1)
1.0% LSA
0.85% sodium chloride
0.04% sodium azide

4: 1.0% agar-Noble and normal serum (1:1)
1.0% LSA
0.85% sodium chloride
0.04% sodium azide

Antigens in all quadrants:

a. Purified PVY (0.5 mg/ml)
b. Infected tobacco
c. Purified PVY (1.0 mg/ml)
d. Infected potato cv. USDA 41956
e. Healthy tobacco
f. Healthy potato cv. Keswick
g. Infected potato cv. Keswick
h. Healthy tobacco sap, containing 0.25 mg/ml PVY



Fredericton, N.B. and Dr. R.I. Hamilton, of Macdonald College, respectively. Immediately after this diagnosis one plant was mechanically inoculated with the PVY isolate. The other plant was used as the "healthy" control.

Five days after inoculation local necrotic lesions appeared on the inoculated leaves, followed at about 15 days by sytemic necrotic flecks on one of the upper leaves. Soon after, necrotic symptoms appeared on other leaves, affecting small veins and petioles first but eventually progressing to severe necrosis and death of the entire leaflet, which drooped and fell off. During the course of symptom development, composite and specific leaf samples were collected and diagnosed for the presence of PVY by double diffusion. (Figs. 19 A-F and 20.)

Results indicated that reliable diagnosis was difficult, presumably due to the low virus concentration in the plant and perhaps because of the unequal distribution of the virus. PVY was never diagnosed in severely necrotic and chlorotic leaves or in leaves which did not show symptoms. However, the virus could be diagnosed in leaves showing severe rugosity and distortion, but only weak precipitin lines were formed.

The possibility of the presence in potato sap of factors which would inhibit or interfere with the antigen-antibody reaction, was considered. Purified PVY at 0.5

mg/ml was added to crude sap of healthy potatoes and serially diluted with potato sap. The virus could be reliably detected in concentrations of 0.03 mg/ml by immunodiffusion.

As a point of interest it should be noted that there was a large difference in tuber yield between the healthy and the PVY-infected plant, 110 days after planting. Characteristically, the infected plant yielded small-sized tubers with a total weight of 253 g. Weight of the tubers from the healthy plant was 460 g.

Evidence for an unequal distribution of the virus in the tubers was demonstrated when a single tuber, harvested from an infected plant, cut in equal halves and planted in separate plots, yielded an infected plant and a plant which did not show any symptoms of PVY infection (Fig. 23). PVY was reliably detected in the shoots of the plant with secondary infection, but precipitin lines were weak, presumably indicating that the virus occurred in low concentrations in this variety.

b) Diagnosis of PVY in several commercail potato varieties.

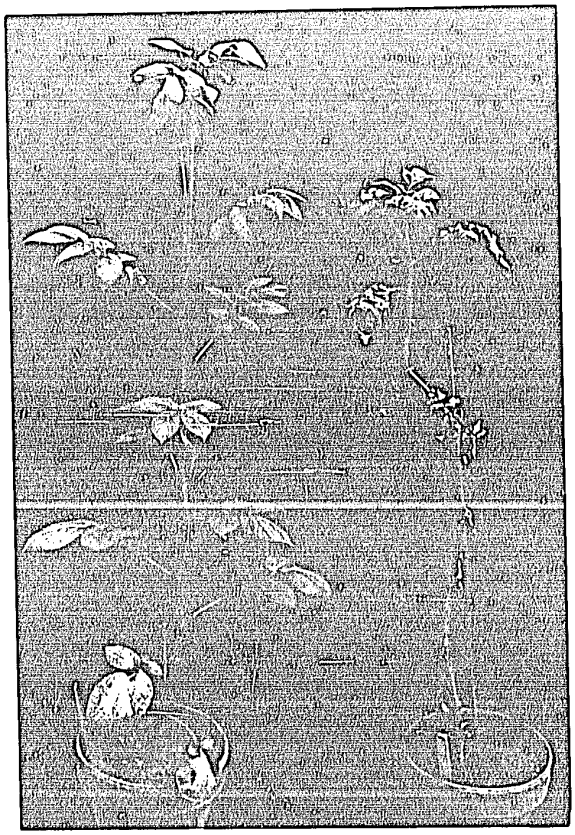
Virus-free tubers of six commercial potato varieties, i.e., Green Mountain, Irish Cobbler, Katahdin, Kennebec, Keswick and Sebago were received from Dr. N.S. Wright, Canada Department of Agriculture, Vancouver, B.C.

One tuber of each variety was cut into two parts each of which was planted in a clay pot (10" dia.), to produce multiple-stem plants. Three weeks after planting one

Figure 23. Five week old potato plants, cv. USDA 41956, grown from equal halves of a PVY-infected tuber.

left: apparently healthy plant

right: plant showing symptoms of secondary PVY infection.



stem of one plant of each variety was mechanically inoculated with the PVY isolate, while the other plant served as a healthy control. In another experiment, one tuber of each variety was cut into four parts, each of which was planted in a clay pot (7" dia.), to produce single-stem plants. After three weeks, one plant of each variety was inoculated with the PVY isolate. The second plant was double inoculated with PVY and PVX in order to observe whether the presence of PVX would enhance or suppress the detection of PVY by immunodiffusion. The PVX isolate was supplied by Dr. R. I. Hamilton of Macdonald College. The third plant was inoculated with PVY recently isolated from a Quebec source of the potato variety Green Mountain by passage through the potato seedling USDA 41956 to eliminate PVX and by passage through tobacco to eliminate PVS. Tubers from the Green Mountain variety were received from Mr. Jacques Laganiere, Canada Department of Agriculture, La Pocatière, Quebec, and were known to be infected with PVY, PVX and PVS. The fourth plant of each variety served as a healthy control.

Symptom development in all plants was observed over a period of about 8 weeks. Top leaves of each stem of all multiple-stem plants were used six weeks after inoculation to determine the dilution end-point of the virus by microprecipitin, to be correlated with symptom development and the ability to detect the virus in crude sap by immunodiffusion. Analysis of all single-stem plants was restricted to observation of symptoms and diagnosis of the virus by immunodiffusion.

Results of the analysis of multiple-stem plants are summarized in Table 14 and indicate that there is no correlation between symptom expression and the ability to detect PVY by immunodiffusion. However, a high dilution end-point of the virus in clarified sap generally corresponded with an increased reliability of obtaining precipitin line formation in immunodiffusion. For example, the antigen dilution end point of the virus in the variety Sebago, as determined by microprecipitin test, was 1:8. The plant showed very severe necrosis six weeks after inoculation (Fig. 24A) but only weak precipitin lines were obtained after immunodiffusion with crude sap of top leaves showing distortion and severe mottling (Fig. 26). The variety Keswick showed only mild symptoms six weeks after inoculation (Fig. 24B) but the virus could be diagnosed in microprecipitin tests in a 1:32 dilution of clarified sap and was also reliably diagnosed in crude sap in immunodiffusion (Fig. 27). PVY produced local lesions on inoculated leaves of variety Kennebec. Eventually leaves became chlorotic and dropped off the plant. No systemic symptoms were observed and the virus could not be diagnosed by microprecipitin or immunodiffusion (Fig. 28).

The variety Green Mountain reacted with severe systemic necrosis to inoculation with PVY (Fig. 25) and the virus could be reliably diagnosed by immunodiffusion (Fig. 28). Symptoms on the variety Katahdin were less

Table 14. Correlation between symptom development, antigen dilution end-point(DEP) and ability to detect PVY by immunodiffusion, in multiple-stem potato plants, six weeks after inoculation with PVY.

Variety	Symptoms	DEP[a]	Immunodiffusion[b]	No. of stems infected
Green Mountain	severe systemic necrosis, mottling and leafdrop	1/8	+	5/5
Irish Cobbler	severe systemic necrosis, mottling and leafdrop	1/16	+	5/5
Katahdin	systemic necrotic spots	1/4	±	4/4
Kennebec	lesions and leafdrop of inoculated leaves no systemic symptoms	--	-	0/3
Keswick	rugosity of leaves mottle - no necrosis	1/32	+	4/4
Sebago	very severe necrosis of entire plant	1/8	±	5/5

[a]determined by microprecipitin tests

[b](±) indicates weak precipitin line

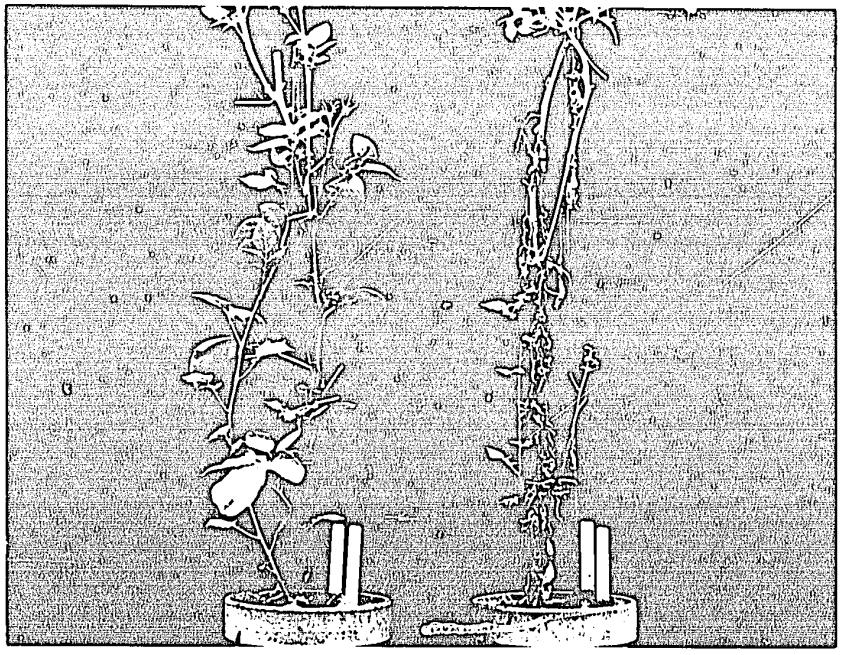
(+) indicates formation of precipitin line

(-) indicates absence of precipitin line.

Figure 24 Symptoms in the potato varieties Sebago and Keswick, six weeks after mechanical inoculation with PVY.

A. Sebago. Uninoculated plant on the left; inoculated plant on the right

B. Keswick. Uninoculated plant on the left; inoculated plant on the right



A

B

Figure 25. Symptoms in the potato variety Green Mountain, six weeks after inoculation with PVY.

Figure 26. Diagnostic immunodiffusion test with crude sap of healthy and PVY infected potato plants cv. Sebago and Irish Cobbler.

Central well: Antiserum to intact PVY.

Peripheral wells, clockwise from arrow:

1. Infected tobacco
2. Infected potato cv. Sebago
3. Infected potato cv. Irish Cobbler
4. Healthy tobacco
5. Healthy potato cv. Sebago
6. Healthy potato cv. Irish Cobbler

Immunodiffusion system: 0.8% agar-Noble in distilled water
1.0% LSA
0.85% sodium chloride
0.04% sodium azide

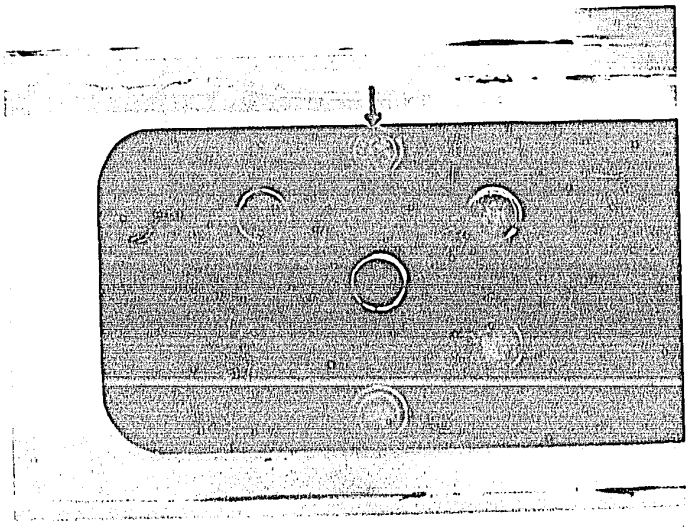


Figure 27. Diagnostic immunodiffusion test with crude sap of healthy and PVY-infected potato plants cv. Katahdin and Keswick.

Central well: Antiserum to intact PVY.

Peripheral wells, clockwise from arrow:

1. Infected tobacco
2. Infected potato cv. Katahdin
3. Infected potato cv. Keswick
4. Healthy tobacco
5. Healthy potato cv. Katahdin
6. Healthy potato cv. Keswick

Immunodiffusion system: 0.8% agar-Noble
in distilled water
1.0% LSA
0.85% sodium chloride
0.04% sodium azide

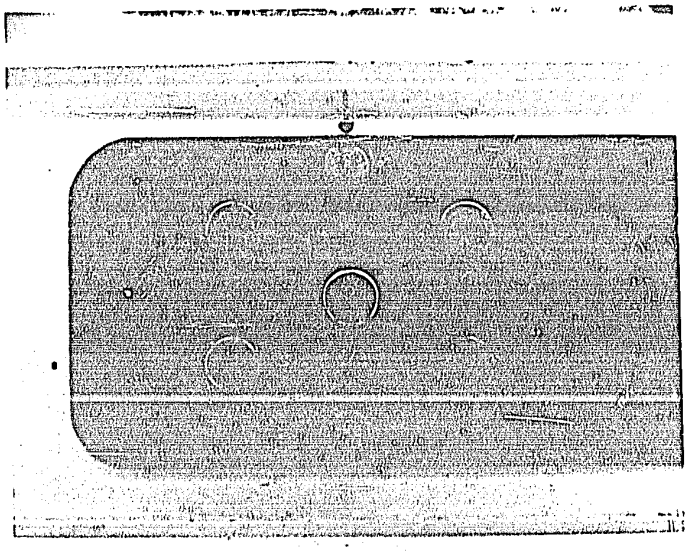


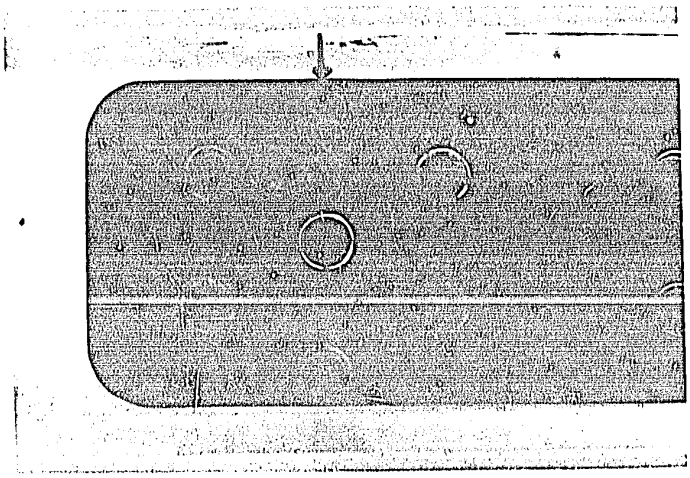
Figure 28. Diagnostic immunodiffusion test with crude sap of healthy and PVY-infected potato plants cv. Kennebec and Green Mountain.

Central Well: Antiserum to intact PVY.

Peripheral wells, clockwise from arrow:

1. Infected tobacco
2. Infected potato cv. Kennebec
3. Infected potato cv. Green Mountain
4. Healthy tobacco
5. Healthy potato cv. Kennebec
6. Healthy potato cv. Green Mountain

Immunodiffusion system: 0.8% agar-Noble in distilled water
1.0% LSA
0.85% sodium chloride
0.04% sodium azide



severe ranging from mild mottling and rugosity to necrotic blotches and the virus could not be reliably detected by immunodiffusion (Fig. 27).

Except in the variety Kennebec, which is known to react mildly to primary infection with most common strains of PVY (MacLeod, 1962), the virus could be diagnosed in all stems of the infected plants.

Diagnostic immunodiffusion tests with crude expressed sap from the single-stem potato plants showed results similar to those obtained with the multiple-stem plants. The PVY isolate from Green Mountain was detected with the same degree of reliability as the normal PVY isolate. Precipitin lines obtained with the two isolates of PVY showed complete coalescence, indicating that they were antigenically identical. In plants doubly infected with PVX and PVY, the presence of PVX did not affect the detection of PVY. Doubly inoculated plants reacted with only slightly more severe symptoms, but presumably this severity is increased in succeeding generations. Unfortunately, because of the dormancy period of the tubers, there was no opportunity to test for the presence of PVY in sprouts and shoots of plants with secondary infection.

V. DISCUSSION

Based on recent concepts and theories put forward by several investigators (Purcifull and Shepherd, 1964; Hamilton, 1964; Shepard and Secor, 1969; Purcifull and Gooding, 1970; Gooding and Bing, 1970; Shepard and Shalla, 1970), this investigation was initiated to develop or adapt a rapid and reliable serodiagnostic method for the detection of PVY in crude expressed sap of infected potato plants.

Results reported herein indicate that PVY could be reliably detected in crude sap of several infected tobacco varieties in immunodiffusion systems containing SDS or LSA incorporated in the agar at specific concentrations. Although the test may be termed "reliable", its sensitivity does not compare favourable with other diagnostic methods, for PVY was not detected in sap dilutions exceeding 1:32. PVY could be detected in tobacco sap diluted 1:2000 by electron microscopy (DeBokx, 1969). Sampson and Taylor (1969) detected PVY by microprecipitin in sap dilutions not exceeding 1:50, a result which is similar to those obtained in these investigations.

Immunodiffusion titration experiments indicated that

the sensitivity of the method can probably be increased by using an antiserum with a titer higher than 1:512. Intensity of the precipitin lines decreased with each antiserum dilution and no lines formed with antiserum dilutions exceeding 1:4, regardless of the antigen concentration. Conversely, when undiluted antiserum (microprecipitin titer 1:512) was used, the intensity of the precipitin lines increased with an increasing antigen concentration up to 0.5 or 1.0 mg/ml. However, such high antigen concentrations evidently caused an imbalance in the antibody/antigen ratio, resulting in the occasional formation of multiple precipitin bands (Matthews, 1970).

Crowle (1961) stated that removal of some serum components such as albumin may significantly reduce the density of precipitin lines in immunodiffusion. Presumably, this explains why the immunoglobulin fraction of the PVY antiserum failed to give positive results in immunodiffusion tests reported here, in spite of the fact that the fraction had a microprecipitin titer of 1:512.

Unlike results obtained by Shepard and Shalla (1970), who found that antisera to PVX and PVX-protein contained relatively high homologous antibody titers but low levels of reactivity to the heterologous antigens, PVY-protein antiserum

failed to react in immunodiffusion and had low antibody levels against both homologous and heterologous antigens in micro-precipitin (Table 13). However, PVY-protein reacted weakly with intact-PVY antiserum in immunodiffusion, which does not exclude the possibility that the antigen had lost some of its antigenic determinants to intact PVY. However, because of the apparent poor immunogenicity and antigenicity of PVY-protein no reliable conclusions can be made about the relationship of PVY-protein to intact PVY and SDS-degraded PVY. The reasons for the poor immunogenicity and antigenicity of PVY-protein could perhaps be elucidated by immunizing a larger number of animals with PVY-protein prepared by different methods. Rabbits or any other animals may vary greatly in their immune response and degradation of PVY with glycine-NaOH buffer at pH 12.0, may yield viral fragments or subunits unable to elicit a proper immune response. Support for such a hypothesis was recently provided by Shepard (1970) who found that pyridine-degraded PVS was only useful as an immunizing antigen since it would not diffuse well through agar gels. Shepard et al. (1971) demonstrated that detection of potato virus M (PVM) by immunodiffusion was dependent upon the use of antibody specific for pyrrolidine-degraded PVM-protein. Production of the antiserum was more successful in

a goat than in rabbits, because goat antiserum exhibited much higher homologous antibody levels.

Although the results from this investigation do not allow for any conclusions regarding the degree of antigenic similarity or disparity between PVY-protein and intact PVY, they do suggest that intact PVY and SDS-degraded PVY are probably antigenically similar, because SDS-degraded PVY was readily detected in immunodiffusion tests with antiserum to intact PVY. Shepard and Shalla (1970) demonstrated that PVX-protein could not be reliably detected with antiserum to intact PVX as a consequence of antigenic disparity between PVX-protein and intact PVX. Shalla and Shepard (1970) provided evidence that this antigenic disparity was the result of conformational changes of monomeric or dimeric protein subunits upon depolymerization. It is reasonable to assume therefore that SDS-degraded PVY consisted of oligomeric protein subunits or perhaps viral fragments. By analogy to PVX, such oligomeric fragments retain the antigenic specificity of the intact viral nucleocapsid. This assumption is in line with the hypothesis put forward by Shepard and Shalla (1970) that the majority of serological cross-reactivity between degraded protein preparations and virus antiserum is provided through the retention or reformation of stable intermediate-sized

aggregates upon degradation of the virus.

In contrast with PVY-protein, SDS-degraded PVY was a good immunogen because it stimulated the formation of a high level of PVY-precipitating antibodies. Rabbits responded similarly to immunization with PVY and SDS-degraded PVY, producing antisera with comparable titers. It is possible that the difference in immunogenicity between PVY-protein and SDS-degraded PVY is a direct consequence of a difference in molecular weight between the two antigens and it is well documented that the subunits of the viral protein coat are much less efficient in stimulating specific antibody production than whole virus (Loor, 1967; Matthews, 1970). However, Marbrook and Matthews (1966) demonstrated that intact turnip yellow mosaic virus is substantially more immunogenic than the apparently identical protein capsid without RNA. Matthews (1970) hypothesized that the presence of RNA may stimulate cell-division nonspecifically in the appropriate antibody producing cells. In this respect it should be noted that very little RNA was present in the PVY-protein preparations while the SDS-degraded PVY preparation used for immunization contained almost the same percentage of RNA that was present in suspensions of intact PVY. Presumably, the RNA in the SDS-degraded PVY suspension was not encapsidated with the viral protein because experiments by Sreenivasaya and Pirie

(1938) indicated that SDS separated the nucleic from the protein. The presence or absence of RNA, then, could perhaps account for the difference in the relative immunogenicities of the viral antigens if these are not caused simply by differences in molecular weight.

The only evidence of antigenic disparity associated with PVY was obtained when purified virus, which had been stored for 3 weeks in 0.02M borate-NaOH buffer pH 7.8 at 3^{o}, was reacted in immunodiffusion with antiserum prepared to intact PVY. Precipitation patterns of partial identity formed with a recently purified preparation of PVY and with crude infected tobacco sap. This observation is similar to that of Shalla and Shepard (1970) who found that protein with the same antigenic specificity as PVX protein is released spontaneously from virus during prolonged storage in neutral buffers of low ionic strength. By analogy, then, PVY protein could be antigenically distinct from intact PVY and if so, it must be concluded that degradation with glycine-NaOH buffer pH 12.0 yields protein with significantly reduced immunogenic and antigenic properties.

The variability that was observed between results obtained with antisera from differnet rabbits or sometimes even with antiserum from the same rabbit, is apparently to be

expected in immunodiffusion studies. Crowle (1961) states that antibodies produced by a single animal against one antigen may have widely differing characteristics which affect immunodiffusion results. On occasion the problem of variability was compounded by the fact that spurious or non-specific precipitation occurred, a phenomenon which was also pointed out by others (Gooding and Bing, 1970; W. W. Bing, personal communication). Several suggestions as to the cause of non-specific precipitates have been made including the presence of lipids in the antiserum, especially in later bleedings (Matthews, 1957) and the presence of extraneous substances in crude plant sap (Van Slogteren, 1969). It is hypothesized here that the addition of crude sap from different host species and varieties to the immunodiffusion system may effect changes in the pH and introduce undesirable ions in the system. Sreenivasaya and Pirie (1938) pointed out that SDS may form insoluble complexes with dilute buffer solutions containing phthalates, phosphates or borates. It was observed in these experiments that insoluble complexes formed in immunodiffusion plates containing 0.2% SDS and 0.1% sodium azide but their formation could not directly be attributed to the presence of specific ions. Sreenivasaya and Pirie (1938) demonstrated that the rate of degradation of TMV depended on the temperature, concentration of SDS, and pH, factors which

also appeared to affect the degradation of PVY in the studies reported here. Apparently they were the first investigators to attempt preparation of an antiserum against disrupted TMV antigens, but their attempts were not successful.

Considering the fact that detection of PVY by immunodiffusion relies on a relatively high antigen concentration in the plant, results obtained with the detection of the virus by immunodiffusion of crude sap of various potato varieties are extremely encouraging. The virus was detected, if not always reliably, in all but one variety, i.e., Kennebec. This variety exhibited a hypersensitive response to inoculation with PVY and did not show any signs of systemic infection. Although symptom development could not be correlated with the ability to detect PVY by immunodiffusion, results from comparative dilution end-point determinations suggested that the virus concentration in the plant was dependent on the potato variety. A high dilution end-point could be correlated with a more intense appearance of precipitin lines in immunodiffusion.

The diagnostic tests reported in this thesis were performed on varieties with primary PVY infections, with the exception of USDA seedling 41956, which produced infected plants (secondary infection) from infected tubers. Detection of PVY in these plants was generally better than in plants of

the same variety with primary infections and it is likely that this applies to all other varieties. DeBokx (1964) demonstrated that the reliability of detecting PVY in potatoes with primary infection depended on the time of infection. Detection of PVY in leaves of plants with secondary infection was performed with a high degree of reliability. Beemster (1972) pointed out that in order to develop a system for virus diagnosis, information is needed on virus translocation in potato plants, e.g., how long will it take for a virus to reach the tubers from the initial site of infection. He focused attention on the fact that the rate of virus translocation differs with potato varieties and virus strains. The testing of tuber sprouts and plants with secondary infection would have led this investigation to its logical conclusion, but circumstances did not permit further experimentation.

In conclusion, the immunodiffusion experiments described here potentially provide a reliable and rapid means of diagnosing PVY in crude sap of tobacco and potatoes if the sensitivity of the test can be augmented. An essential requirement appears to be the use of high titered antiserum to whole virus, but the possibility that an effective antiserum to PVY-protein may increase the specificity of the

reactions should not be excluded. It does not appear that degradation with glycine-NaOH pH 12.0 produces antigens suitable for immunization but other degrading compounds may be more effective. SDS and LSA apparently degrade PVY into fragments or oligomeric subunits which have antigenic properties similar to intact PVY.

The need for increasing the sensitivity of the test is emphasized by the fact that the concentration of PVY in the potato plant varies with the variety. Of great practical importance is the question whether PVY can be reliably detected in sprouts of infected tubers or whether diagnosis should be carried out at a later stage of growth. Virus distribution in the tuber and translocation of the virus in general are other aspects that require further investigation.

VI. SUMMARY

A virus isolate from field-grown tobacco was identified as a normal strain of PVY by means of serology and by inoculation to a series of differential hosts. The virus was purified from infected tobacco, using high molarity sodium citrate and n-butanol for the initial clarification of homogenized tissue, followed by differential centrifugation and iso-electric precipitation of the virus at pH 5.6.

Several degrading compounds were investigated for their efficacy in degrading PVY and facilitating detection of the degraded products in immunodiffusion. PVY could be detected in 1:32 dilutions of crude infected tobacco sap in immunodiffusion systems containing the detergents sodium dodecyl sulphate (SDS) or sodium dibutylnaphthalene sulphonate (LSA), using undiluted antiserum prepared against intact PVY or SDS-degraded PVY. The antisera possessed an identical microprecipitin titer of 1:512 and immunodiffusion titers of 1:4 and 1:2, respectively. Antiserum prepared against PVY-protein did not react with degraded PVY antigens in immunodiffusion and possessed a microprecipitin titer of 1:8.

Evidence of antigenic disparity was demonstrated

between freshly purified PVY or PVY in infected plant sap and PVY which had been stored for a prolonged period of time in dilute borate buffer. Precipitation patterns of partial identity formed when these antigens were reacted in double diffusion tests with antiserum to intact PVY or SDS-degraded PVY. It is hypothesized that disparity between antigens may well be responsible for the low degree of cross-reactivity between PVY-protein antiserum and SDS-degraded viral antigens, but the possibility is considered that the PVY-protein immunogen simply failed to evoke a proper immune response in the rabbit. It is further suggested that reactions between SDS- or LSA-degraded virus and antisera prepared against intact PVY or SDS-degraded PVY are the result of the presence of intermediate sized stable viral fragments which possess antigenic determinants similar to those of the intact virus.

Dilution end-point titrations with clarified sap of PVY-infected potato leaves, by the microprecipitin procedure, indicated that the virus concentration in the leaves varied with the variety. Dilution end-point values could be correlated with the ability to detect the virus by immunodiffusion.

LITERATURE CITED

Aach, H.G. 1959. Serologische Untersuchungen zur Struktur des Tabakmosaikvirus. Biochim. Biophys. Acta 32, 140-146.

Atabekov, J.F.,Dementyeva, S.P., Schaskolskaya, N.D., and Sacharovskaya,G.N. 1968. Serological study on barley stripe mosaic virus protein polymerization. II. Comparative antigenic analysis of intact virus and some stable protein intermediates. Virology 36, 601-612.

Ball, E.M. 1961. Serological tests for the identification of plant viruses. Amer. Phytopath. Soc., Ithaca, N.Y. 16 p.

Ball, E.M. 1964. Serology: Techniques used in plant virus research. In: Plant Virology. M.K. Corbett and H. D. Sisler (eds.) Univ. Fla. Press, Gainesville, Fla. 527 p.

Bartels, R. 1957. Beitrag sum serologischen Nachweiss des Y-Virus in der Kartoffel. Phytopathol. Z. 30, 1-16.

Bawden, F.C. 1964. Plant viruses and virus diseases. 4th ed. Ronald Press Co., New York. 361 p.

Bawden, F.C. and Pirie, N.W. 1939. The purification of insect-transmitted viruses. Brit. J.Exp. Pathol 20, 322-329.

Beemster, A.B.R. 1967. Partial infection with potato virus Y^N of tubers from primarily infected potato plants. Neth. J. Plant Pathol. 73, 161-164.

Beemster, A.B.R. 1972. Virus translocation in potato plants and mature plant resistance. In: Viruses of potatoes and seed potato production. J.A. DeBokx (Ed.) Wageningen. 233 p.

Beemster, A.B.R. and Rozendaal, A. 1972. Potato viruses: properties and symptoms. In: Viruses of potatoes and seed-potato production. J.A. DeBokx (Ed.) Wageningen. 233 p.

Bercks, R. 1960. Serologischen Untersuchungen zur Differenzierung von Isolaten des Phaseolus-Virus 2 und ihrer Verwandtschaft mit Phaseolus-Virus 1. Phytopathol. Z. 39, 120-128.

Bercks, R. 1967. Methodische Untersuchungen uber den serologischen Nachweiss planzen-pathogener Viren mit dem Bentonit-Flockungstest, dem Latex-test und dem Bariumsulfat-test. Phytopathol. Z581-17.

Brakke, M.K. 1959. Dispersion of aggregated barley stripe mosaic virus by detergents. Virology 9, 506-521.

Brakke, M.K. 1967. Density gradient centrifugation. In: Methods in Virology. II. K. Maramorosch and H. Kowprowski (Eds.). Academic Press, N.Y.

Brandes, J. and Bercks, R. 1965. Gross morphology and serology as a basis for classification of elongated plant viruses. Advan. Virus Res. 11, 1-24.

Brenner, S. and Horne, R.W. 1959. A negative staining method for high resolution electron microscopy of viruses. Biochim. Biophys. Acta 34, 103-110.

Cremer, M.C. 1951. The preparation of antiserum against potato virus Y. p. 85-87. Proc. 1st Conf. Potato Virus Diseases. Lisse-Wageningen.

Crowle, A.J. 1961. Immunodiffusion. Academic Press, New York, London. 333 p.

Damirdagh, I.S. and Shepherd, R.J. 1970(a). Purification of the tobacco etch and other viruses of the potato virus Y group. Phytopathology 60, 132-142.

Damirdagh, I.S. and Shepherd, R.J. 1970 (b). Some of the chemical properties of the tobacco etch virus and its protein and nucleic acid components. Virology 40, 84-89.

DeBokx, J.A. 1964. Onderzoekingen over het aantonen van aardappel Y^N virus met behulp van toetsplanten. Dissertation. Wageningen.

DeBokx, J.A. 1969. Detection of elongated potato viruses by electron microscopy. Meded. Ryksfak. Landbouwwetensch. Gent. 34, 903-916.

DeBokx, J.A. and Waterreus, H.A.J.I. 1971. Serology of potato virus S after ultrasonic treatment. Meded. Ryksfak. Landbouwwetensch. Gent. 36, 364-375.

Delgado-Sanchez, S. and Grogan, R.G. 1966(a). Purification and properties of potato virus Y. Phytopathology 56, 1397-1404.

Delgado-Sanchez, S. and Grogan, R.G. 1966(b). Chenopodium quinoa, a local lesion assay host for potato virus Y. Phytopathology 56, 1394-1396.

Delgado-Sanchez, S. and Grogan, R.G. 1970. Potato virus Y. C.M.I./A.A.B. Descriptions of plant viruses No. 37. Kew, Surrey, England.

DeMoss, R.D. and Bard, R.D. 1957. Physiological and biochemical technics. In: Manual of Microbiological Methods. McGraw-Hill, New York. 315 p.

Green, A.A. and Hughes, W.L. 1955. Protein fractionation on the basis of solubility in aqueous solutions of salts and organic solvents. In: Methods in Enzymology 1, 67-90. S.P. Colowick and N.O. Kaplan (Eds.). Academic Press Inc., New York.

Gooding, G.V. and Bing, W.W. 1970. Serological identification of potato virus Y and tobacco etch virus using immunodiffusion plates containing sodium dodecyl sulphate. Phytopathology 60, 1293 (Abstr.).

Hamilton, R.I. 1964. Serodiagnosis of barley stripe mosaic facilitated by detergent. Phytopathology 54, 1290-1291.

Harrison, B.D., Finch, J.T., Gibbs, A.J., Hollings, M., Shepherd, R.J., Valenta, V. and Wetter, C. 1971. Sixteen groups of plant viruses. Virology 45, 365.

Hill, J.H. and Shepherd, R.J. 1972. Biochemical properties of turnip mosaic virus. Virology 47, 807-816.

Hollings, M. 1965. Disease control through virus-free stock. Ann. Rev. Phytopathol. 3, 367-396.

Horvath, J. 1966(a). Studies on strains of potato virus Y. 1. Strain C. Acta Phytopathologica 1, 125-138.

Horvath, J. 1966(b). Studies on strains of potato virus Y. 2. Normal strain. Acta Phytopathologica 1, 333-352.

Horvath, J. 1967(a). Studies on strains of potato virus Y. 3. Strain causing browning of midribs in tobacco. Acta Phytopathologica 2, 95-108.

Horvath, J. 1967(b). Studies on strains of potato virus Y. 4. Anomalous strain. Acta Phytopathologica 2, 195-210.

Horvath, J. 1967(c). Separation and determination of viruses pathogenic to potatoes with special regard to potato virus Y. Acta Phytopathologica 2, 319-360.

Kahn, R.P., Scott, H.A., Bozicevich, J. and Vincent, M.M. 1967. Detection of potato viruses X, M and S in dormant potato tubers by the bentonite flocculation test. Phytopathology 57, 55-61.

Kleczkowski, A. 1961. Serological behaviour of tobacco mosaic virus and of its protein fragments. Immunology 4, 130-141.

Koenig, R. 1969. Analyse serologischer Verwandschaften innerhalb der potato virus X-gruppe mit intakten Viren und Virusbruchstücken. Phytopathol. Z. 65, 379-386.

Loor, F. 1967. Comparative immunogenicities of tobacco mosaic virus, protein subunits and re-aggregated subunits. Virology 33, 215-220.

Maat, D.Z. 1970. Experiments on the use of latex, bentonite and water insoluable antiserum protein polymers in sensitive serological tests. Neth. J. Phytopath. 76, 79-92.

McLeod, D.J. 1962. Mosaic and streak viruses of the potato. Canada Dept. of Agriculture. Publication 1150. Ottawa. 80 p.

Marbrook, J. and Matthews, R.E.F. 1966. The differential immunogenicity of plant virus proteins and nucleoproteins. Virology 28, 219-228.

Matthews, R.E.F. 1957. Plant virus serology. Cambridge University Press. 128 p.

Matthews, R.E.F. 1970. Plant Virology. Academic Press. New York and London. 778 p.

McKinney, H.H., Silber, G. and Greeley, L.W. 1965. Longevity of some plant viruses stored in chemically dehydrated tissues. Phytopathology 55, 1043-1044.

Miki, T. and Oshima, N. 1972. On the size of the protein subunits in potato virus Y. J. Gen. Virol. 15, 179-182.

Milne, K.S. and Grogan, R.G. 1968. Identification of strains of watermelon mosaic virus by serology and other characteristics. Phytopathology 58, 1060 (Abstr.)

Ouchterlony, O. 1958. Diffusion-in-gel methods for immunological analysis. In: Progress in allergy. P. Kallos (ed.) S. Karger, Basel and New York.

Purcifull, D.E. 1964. Serological studies with several rod-shaped plant viruses. Ph.D. Dissertation. Univ. of California. Davis.

Purcifull, D.E. 1966. Some properties of tobacco etch virus and its alkaline degradation products. Virology 29, 8-14.

Purcifull, D.E. and Gooding, G.V. 1970. Immunodiffusion tests for potato Y and tobacco etch viruses. Phytopathology 60, 1036-1039.

Purcifull, D.E. and Shepherd, R.J. 1969. Preparation of the protein fragments of several rod-shaped plant viruses and their use in agar gel diffusion tests. Phytopathology 54, 1102-1108.

Rappaport, I. 1965. The antigenic structure of tobacco mosaic virus. Advan. Virus Res. 11, 223-275.

Rappaport, I., Siegel, A. and Haselkorn. 1965. Influence of the state of subunit aggregation on the antigenic specificity of tobacco mosaic virus and turnip yellow mosaic virus. Virology 25, 325-328.

Reichmann, M.E. 1960. Degradation of potato virus X. J. Biol. Chem. 235, 2959-2963.

Sampson, P.J. and Taylor, R.H. 1968. Comparison of the electron microscope, microprecipitin tests and indicator plants for the detection of potato viruses S, X and Y. Phytopathology 58, 489-493.

Schulz, J.T. 1963. Tetranychus telarius L. new vector of virus Y. Plant Disease Reptr. 47, 594-596.

Shalla, T.A. and Shepard, J.F. 1970 (a). An antigenic analysis of potato virus X and of its degraded protein. II. Evidence for a conformational change associated with the depolymerization of structural protein. Virology 42, 835-847.

Shalla, T.A. and Shepard, J.F. 1970(b). A virus induced soluble antigen associated with potato virus X infection. Virology 42, 1130-1132.

Shepard, J.E. 1970. A radial immunodiffusion test for simultaneous diagnosis of potato virus S and X. Phytopathology 60, 1669-1671.

Shepard, J.F. and Grogan, R.G. 1967. Partial purification, properties and serology of western celery mosaic virus. Phytopathology 57, 1104-1110.

Shepard, J.F., Jutila, J.W., Catlin, J.E., Newman, F.S. and Hawkins, W.M. 1971. Immunodiffusion assay for potato virus M infection. Phytopathology 61, 873-874.

Shepard, J.F. and Secor, G.A. 1969. Detection of potato virus X in infected plant tissue by radial and double diffusion tests in agar. Phytopathology 59, 1838-1844.

Shepard, J.F and Shalla, T.A. 1970. An antigenic analysis of potato virus X and of its degraded protein. I. Evidence for and degree of antigenic disparity. Virology 42, 825-834.

Shepherd, R.J. and Pound, G.S. 1960. Purification of turnip mosaic virus. Phytopathology 50, 797-803.

Sreenivasaya, M. and Pirie, N.W. 1938. CCXXII. The disintegration of tobacco mosaic virus preparations with sodium dodecyl sulphate. Biochem. J. 32, 1707-1710.

Spitnik-Elson, P. 1965. The preparation of ribosomal protein from Escherichia coli with lithium chloride and urea. Biophys. Res. Commun. 18, 557-562.

Stace-Smith, R. and Tremaine, J.H. 1970. Purification and composition of potato virus Y. Phytopathology 60, 1785-1789.

Takahasi, W.N. and Gold, A.H. 1960. Serological studies with X-protein, tobacco mosaic virus, polymerized X-protein and virus reconstituted from nucleic acid and X-protein. Virology 10, 449-458.

Tomlinson, J.A. and Walkey, D.G.A. 1967. Effects of ultrasonic treatment on turnip mosaic virus and potato virus X. Virology 32, 267-278.

Van Regenmortel, M.H.V. 1964. Purification of plant viruses by zone electrophoresis. Virology 23, 495-503.

Van Regenmortel, M.H.V. 1966 (a). Plant virus serology. Advan. Virus Res. 12, 207-271.

Van Regenmortel, M.H.V. 1966(b). The problem of contaminating plant antigens in virus preparations. In: Viruses, Wageningen. A.B.R. Beemster and J. Dykstra (Eds.). North-Holland Publishing Comp. Amsterdam.

Van Slogteren, D.H.M. 1954. Serological microreactions with plant viruses under paraffin oil. In: Proc. Second Conf. on Potato Virus Diseases. E. Streutgers et al. (Eds.). H. Veenman and Zonen. Wageningen.

Van Slogteren, D.H.M. 1969. Analytical serology of plant viruses. In: Analytical serology of microorganisms. Vol. I. J.B.G. Kwapinsky (Ed.). Interscience Publishers, New York.

Van Slogteren, D.H.M. 1972. Serology. In: Viruses of potatoes and seed potato production. J. A. Debokx (Ed.). Wageningen. 233 p.

Varma, A., Gibbs, A.J. and Woods, R.D. 1968. Some observations on the structure of the filamentous particles of several plant viruses. J. Gen. Virol. 2, 107-114.

Venekamp, J.H. and Mosch, W.H.M. 1964. Chromatographic studies on plant viruses. III. The purification of potato virus X, potato virus Y, tobacco mosaic virus and potato stem mottle virus by chromatography on cellulose columns with polyethylene glycol containing solutions as solvents. Virology 23, 394-402.

Wetter, C. 1960. Partielle Reinigung einiger gestreckter Planzenviren und ihrer Verwendung als Antigene bei der Immunisierung Mittels Freundschem Adjuvans. Arch. Mikrobiol. 37, 278-292.

Wetter, C. 1965. Serology in virus disease diagnosis. Annu. Rev. Phytopath. 3, 19-42.

Wiersema, H.T. 1961. Methods and means used in breeding potatoes with extreme resistance to viruses X and Y. Proc. 4th Conf. on Potato Virus Diseases. M. Veenman and Zonen. Wageningen.

Wright, N.S. and Stace-Smith, R. 1966. A comparison of the sensitivity of three serological tests for plant viruses and other antigens. Phytopathology 56, 944-948.