1-1-1986

Inoculation of tobacco protoplasts with potato virus Y.

Cheng-Rei R. Lee
University of Massachusetts Amherst

INOCULATION OF TOBACCO PROTOPLASTS WITH POTATO VIRUS Y

A Dissertation Presented

By

Cheng-Rei Ruth Lee

Submitted to the Graduate School of the
University of Massachusetts in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

September 1986

Department of Plant Pathology

INOCULATION OF TOBACCO PROTOPLAST WITH POTATO VIRUS Y

A Dissertation Presented

by

Cheng-Rei Ruth Lee

Approved as to style and content by:

George N. Agrios

Dr. George N. Agrios, Chairperson of Committee

Peter L. Webster

Dr. Peter L. Webster, Member

Mark S. Mount

Dr. Mark S. Mount, Member

Mark S. Mount

Dr. Mark S. Mount, Department Head
Department of Plant Pathology

This Dissertation is Dedicated to
My Mother,

Chien-Chiu Chang

ACKNOWLEDGMENT

The author wishes to express appreciation to Dr. George N. Agrios, advisor and Chairman of the dissertation committee, for his continuous guidance and enthusiastic encouragement during all phases of this investigation. The constructive comments and time given by the dissertation committee members, Dr. Mark S. Mount and Dr. Peter L. Webster are gratefully acknowledged.

Thanks are given to Mr. Jenn-Dong Li for his technical help in reformatting the final manuscript. Thanks also go to all the personell of The Department of Plant Pathology, my fellow graduate students and friends. We shared so much together that will always be remembered.

Appreciation is given to the author's parents-in-law, Mr. and Mrs. Hung-Ao Lee, for their strong support. Finally, the author would like to thank her husband Yi-Chau Lee for his grateful support and encouragement during the final stage of the study. His understanding allowed the author to devote her full energy to completion of this study.

ABSTRACT

INOCULATION OF TOBACCO PROTOPLASTS WITH POTATO VIRUS Y

September 1986

Cheng-Rei Ruth Lee, B.S., Chung-Hsing University, Taiwan

M.S., Chung-Hsing University, Taiwan

Ph.D., University of Massachusetts

Directed by: Professor George N. Agrios

Nicotiana tabaccum cv. White Burley mesophyll protoplasts were inoculated with potato virus Y (PVY). Successful infection was proven by both enzyme-linked immunosorbent assay (ELISA) and by staining with fluorescein isothiocyanate (FITC)-conjugated antibody to the virus. Generally, less than 1% of the protoplasts became infected and the yield of PVY per 3-5 x 10^6 protoplasts was approximately 1 ug, which was equivalent to 10^5 PVY particles per infected protoplast. Infection of protoplasts was successful when 5 x 10^5 protoplasts/ml were inoculated with 1 ug/ml PVY in the presence of 0.01 M potassium citrate buffer, pH 4.6 containing 3 ug/ml poly-L-ornithine (PLO). The inoculation procedure included a 20 min preinoculation incubation of the virus with PLO and a 10 min inoculation incubation of the virus and PLO with the protoplasts, both at room temperature with gentle shaking. A serological blocking method, involving treatment of inoculated protoplasts immediately after postinoculation washes with guinea pig anti-PVY antiserum, was developed to reduce the interference of PVY particles adsorbed on the surface of protoplasts with the serological

determination of virus produced within infected protoplasts. The serological blocking step improved considerably the efficiency of detection and quantitative determination (ELISA only) of true infection of protoplasts by both FITC staining and ELISA tests. Higher calcium chloride concentration (10 mM or 0.2 M) in the washing medium facilitated removal of residual PVY particles adsorbed on the surface of inoculated protoplasts. However, increasing calcium chloride concentration levels from 1 mM up to 0.2 M inhibited the efficiency of the serological blocking treatment. Light (3,000 lux, 16 h/day) was essential for replication of PVY in inoculated tobacco protoplasts. In the presence of light, temperature became a critical factor for virus replication. Inoculated protoplasts cultured at 4°C exhibited greatly reduced PVY replication compared to replication at 25°C.

TABLE OF CONTENTS

LEGENDS TO TABLES

LEGENDS TO FIGURES

CHAPTER I

INTRODUCTION

In spite of its much longer history, plant virology lags far behind bacterial or animal virology in understanding of the process of virus replication. One of the reasons for this stems from the nature of plant cells which make the need for a wound imperative if plant viruses are to gain access to plant cells. Another reason had been the lack of plant single cell systems which would allow a more critical step-by-step study of synchronized infection and replication of viruses in plant cells (102,113). Both problems were solved in 1969 by the introduction by Takebe et al. (115) of techniques making possible the isolation of large numbers of plant protoplasts and their inoculation with viruses. To date, more than 30 plant viruses have been successfully introduced into protoplasts isolated from at least one plant species (42). However, viruses of the potyvirus group, by far the largest and economically most important group of viruses, have not yet been reproducibly inoculated into protoplasts although isolated RNA of at least two potyviruses, PVY and tobacco vein mottle virus (TVMV) has been used to establish infection in tobacco protoplasts (5,128). Attempts to inoculate tobacco protoplasts with intact virus particles, however, were not successful (5,128).

The lack of successful infection of protoplasts by potyviruses is probably due to two factors: (1) Potyviruses are not easily purified in an infectious state because of the low yield of purified virus (5-25 mg/l) and because of aggregation and fragmentation of the long, flexuous

particles during purification. (2) Failure to provide adequate conditions for protoplast inoculation by potyviruses, including a proper physiological state of protoplasts and a proper medium for inoculation of protoplasts with potyviruses.

Differential centrifugation and sucrose density gradient centrifugation are the main methods which have been employed for PVY purification. These methods, however, usually cause considerable aggregation and fragmentation of PVY particles. As a result, the specific infectivity of purified virus is low. Since the quality of purified virus is important for inoculation of protoplasts, column chromatography has been used in the present study as a substitute for ultrahigh speed and sucrose density gradient centrifugations. Column chromatography has provided purified PVY preparations of higher yield and specific infectivity in a simpler and faster way.

Attempts have also been made to determine the proper conditions for inoculation of protoplasts with potyviruses (PVY); several series of variables have been tested involving precise combinations of culture medium, correct osmoticum, different buffers and buffer pHs, different fusagens and fusagen concentrations, different virus concentrations, presence of divalent ions, and different temperatures during inoculation. The role of some of the factors mentioned above on the rate of protoplast infection and virus replication was also studied when these factors were present in the washing medium or during culture of the inoculated protoplasts.

The main purpose of this study, then, has been to improve the infection of tobacco protoplasts with PVY by optimizing the inoculum and

the various steps and components of inoculation. A serological blocking treatment was also developed in order to reduce the interference of residual PVY adsorbed on the surface of protoplasts with the serological determination of virus produced within infected protoplasts.

CHAPTER II

REVIEW OF LITERATURE

The Virus

Potato virus Y (PVY) occurs worldwide in potato (24), pepper (89), tobacco (106), and tomato (26). Some plant species in Chenopodiaceae and Leguminosae are also hosts of PVY (44). Early infection of susceptible host plants can result in 70% yield reduction in tobacco (59) and 80% in potato (23). PVY can be transmitted by sap inoculation, by grafting, and by aphids in the nonpersistent manner (24). Three groups of PVY strains known as PVY^O (common strains), PVY^N (tobacco veinal necrosis strains) and PVY^C (stipple streak strains), can be distinguished by the severity of systemic symptoms in potato, tobacco or other diagnostic hosts (23). PVY^O strains are the most ubiquitous, inducing mainly severe systemic crinkle symptoms, rugosity or leaf-drop streak in potato, and systemic mottling in tobacco (24).

PVY is the type member of potyviruses. The virus particles are flexuous filaments about 11 nm wide and 740 ± 10 nm long (77). Delgado-Sanchez and Grogan (1966) reported a value of 684 nm as the most typical length of purified PVY. The nucleic acid of PVY is an infectious single-stranded RNA. The estimated molecular weight of PVY RNA is 3.1×10^6 (70) and comprises 5.4% of the particle mass (107). The molecular weight of PVY is approximately 6×10^7.

Purification of Potato Virus Y (PVY)

Numerous investigations concerning PVY purification have been conducted (7,22,26,47,61,67,70,107,119,122). The continuing efforts at purification are an indication of the difficulties and unsatisfactory results obtained from earlier methods of PVY purification. As a rule, the protocol of these methods includes ultracentrifugation and centrifugation through a sucrose density gradient (SDG) or a sucrose cushion. However, high speed centrifugation and even SDGC contributes to particle fragmentation (22,47) and aggregation (107). Delgado-Sanchez and Grogan (1966), by using 0.1 M borate buffer pH 8.2 containing either ethylenediaminetetraacetic acid (EDTA) or sodium diethyldithiocarbamate (Na-DIECA), obtained purified PVY with reduced particle aggregation (26). Damirdagh and Shepherd included 0.5 M urea in the resuspending medium which helped substantially in reducing particle aggregation, but breakage of the filamentous particles continued to be a problem (22). Venekamp and Mosch purified PVY and a few other plant viruses by passing clarified sap through a cellulose column using a polyethylene glycol (PEG) gradient solution as eluent (122). PVY was eluted in solutions containing 1% PEG but, again, particle aggregation was so serious that quantitative recovery was impossible (122). Steere and Ackers (108,109) and others (14,107,119) used agar gel filtration for purification of plant viruses. Cěch (14) has studied the behavior of plant sap constituents and virus on agar gel columns. He indicated that the elution pattern obtained with sap from different plant species and with viruses of different morphology

remained approximately the same -- one virus peak followed by a second peak of plant material. Stace-Smith and Tremaine used passage through a Sephadex column instead of a second high-speed centrifugation to purify PVY and concluded that both ways are equally effective (107). Abstracts of investigations using molecular sieving for purification of plant viruses have been published (16,61,66,67) but no details of the methods or comparative studies were available.

Isolation of Protoplasts

Takebe and co-workers (1968) were the first to enzymatically release tobacco mesophyll protoplasts from Nicotiana tabacum cv. Bright Yellow leaves to a yield high enough (50-90% of total cells) for further biochemical studies. Protoplasts have been isolated from at least 17 species of tobacco (30). Although protoplasts could be isolated from many plant tissues, leaf tissue is used most frequently (29) because protoplasts can be isolated from leaves more readily than from any other plant part (113). Takebe and Nagata (1984) claimed a yield of 10^7 protoplasts of palisade cells from 1 g of fresh leaf tissue (i.e. over 90% of the total cells) while 30% of cell suspension cells were converted into protoplasts (117). Protoplasts isolated from cell suspension or callus cultures are aseptic and more homogeneous physiologically (121). However, unstable chromosome numbers resulted in cultured cells used as donor material for protoplast isolation (28).

Among the factors responsible for isolation of protoplasts of sufficient viability and yield for further studies to be made, the

quality of the donor plants is the most important (13,28,125). Almost all researchers engaged in protoplast isolation use precise growth conditions (54,105,116,126). In general, fast and uninterrupted growth of plants is recommended, begining with early separation of young seedlings (102). Source plants should be well nourished, and only leaves that have just reached full size should be collected (103). Sander and Martes (1984) considered light intensity and relative humidity as the most crucial factors for successful protoplast isolation (102). Tobacco plants, grown for 12 hrs at 25°C in light at 10,800 lux alternating with 12 hrs at 20°C in darkness, were reliable sources for mesophyll protoplasts (54). Increase or decrease of the light period decreased the stability of protoplasts (54). Optimum relative humidity is between 40 to 70% (102,105). It was suggested that watering of plants ought to be avoided 24 hrs prior to harvesting (116). Under such conditions a better yield of protoplasts could be achieved. Cassells and Cocker (1982) found that the leaf shape index (LSI= LL/LW x LA where LL is the leaf length, LW is the leaf width, and LA is the leaf area) values are an indication of protoplast viability under greenhouse conditions (13). However, the physiological basis for the stability of protoplasts is still poorly understood. Therefore, the optimum conditions of plant growth for protoplast isolation have been determined mostly empirically (113).

Age of the source plants is the second factor to be considered. Most researchers prefer leaves which have just reached full expansion from plants 40 to 80 days old (86,102,116,125). The disadvantages of using older leaf tissue for protoplast isolation included longer

enzymatic digestion time (116), production of fragile protoplasts, and release of large numbers of small protoplasts which failed to sediment at the centrifugal forces normally used for pelleting protoplasts (125). Too young plants (<40 days) are no better than too old ones (125). Protoplasts from too young leaves do not survive subsequent inoculation or culture (113).

Two methods have been used for enzymatic isolation of tobacco protoplasts. The two-step method involves sequential treatment of leaf tissue with pectinase and with cellulase after removal of the lower epidermis. Pectinase releases mesophyll cells from leaf tissue, while cellulase digests the cell walls of released cells, thus releasing protoplasts (116). In the one-step method, leaf tissue, from which the lower epidermis has been removed, is incubated in an enzyme mixture containing both pectinase and cellulase (49). The one-step method is simpler, is less affected by the season when the plants are grown, and does not require addition of dextran sulfate to facilitate the release of stable tobacco mesophyll protoplasts. However, the one-step method yields a heterogeneous population of mesophyll protoplasts since it does not allow separation of palisade from spongy parenchyma cells. Moreover, protoplasts prepared by the one-step method appear to be less readily infected with viruses than are protoplasts obtained by the two-step method (113).

Inoculation of Protoplasts with Plant Viruses

In 1969, scientists from two laboratories reported tobacco mosaic

virus (TMV) infection of protoplasts from tomato fruits (18) and tobacco leaves (115). To date at least 30 plant viruses have been used to infect plant protoplasts of at least one plant species each (42).

Poly-L-ornithine (PLO)-mediated inoculation

Buffer and pH. Both pH and the dind of buffer used for inoculation can influence the level of infection of protoplasts by viruses (113). Potassium citrate buffer was the first used for infection of plant protoplasts (115) and is still the one used the most (6,53,84,92,96). Infection of turnip protoplasts with cauliflower mosaic virus (CaMV) was more efficient in citrate buffer than in phosphate buffer (38). Sodium citrate buffer at pH 5.2 was used for infection of tobacco protoplasts with cowpea chlorotic mottle virus (CCMV) (83). When potassium citrate buffer was used, pH 4.7 and pH 5.2 were the best for infection of tobacco protoplasts with CCMV (84). For infection of tobacco protoplasts with pea enation mosaic virus (PEMV) and brome mosaic virus (BMV) V5, pH 4.7 was the most effective (79,82). Inoculation of cowpea protoplasts with cowpea mosaic virus (CPMV) gave the highest infection rate at pH 4.8 (43). In barley yellow dwarf virus (BYDV)-cereal leaf protoplast and turnip yellow mosaic virus (TYMV)-*Brassica* leaf protoplast systems, potassium citrate buffer at pH 5.2 was the most effective (6,97). At PLO and BMV concentrations of 0.2 and 5 ug/ml, respectively, pH 4.2 was superior to a pH range from 4.5 to 5.1 for infection of barley protoplasts (90). Infection of protoplasts of five plant species by BMV increased with a pH decrease from 6.9 to 4.5 (37). The same was true for the turnip protoplast-CaMV system (38).

Generally, citrate buffer at lower pH seems to allow a greater frequency of infection of protoplasts by viruses (43). However, in potato virus X (PVX)-tobacco protoplast and clover yellow mosaic virus (CYMV)-cowpea protoplast systems , pH 5.8 to 6.0 yielded the highest percentage of infection (94,96). The ionic strength of citrate buffer used for inoculation is usually from 1 to 20 mM but 0.1 mM has been used sometimes (37,90), and in one protoplast-virus system, it was the best of all (37). At buffer molarity of 10 mM, infection of maize and oat protoplasts by BMV was completely inhibited (37).

Potassium phosphate buffer is the second most commonly used buffer in protoplast inoculation (1,3,48,56,62,95). Phosphate buffer at pH 5.5-9.0 was more efficient for infection of tobacco protoplasts with raspberry ringspot virus (RRV) than citrate buffer at pH 5.0-5.5 (3). In the CPMV-soybean protoplast system, phosphate buffer at pH 6.3 was superior to potassium citrate at pH 5.5, Tris-HCl at pH 8.0, or MES at pH 6.3 (48). The optimum pH in the bean pod mosaic virus (BPMV)-soybean protoplast system was 5.6 (62). For tobacco rattle virus (TRV)-tobacco protoplasts, phosphate buffer at pH 6.0 is more effective than citrate buffer at pH 5.2 (56). Phosphate buffer at 10 mM was used in the CPMV-soybean protoplast system (48) and was optimum in the BPMV-soybean protoplast system (62). However, a range of buffer molarities from 50 mM to 1.5 mM made no difference in the infection rate (3). Increase of buffer molarity to 0.1 M prevented any infection (3). In the absence of PLO, 0.1 M phosphate buffer pH 7.0 was superior to phosphate buffer of 0.05 and 0.025 M, as well as of 0.02 M phosphate citrate buffer at pH 5.2 (129). Infection of barley protoplasts with BMV in the presence of

0.05-0.1 M phosphate buffer at pH 8.8 also gave the highest infection rate (90).

Tris-HCl buffer has been used only occasionally in protoplast infection with viruses (48,78,80,100). For infection of turnip leaf protoplasts with turnip rosette virus (TRosV), Tris-HCl at pH 7.0 was superior to potassium phosphate buffer pH 6.8, and potassium citrate buffer at pH 6.0 and 7.0 (78). Tris-HCl buffer at pH 8.0 was also better than potassium phosphate buffer at pH 6.3 and potassium citrate buffer at pH 5.2 in promoting infection of soybean protoplasts by southern bean mosaic virus (SBMV) (48). However, in the Cymbidium ringspot virus (CyRSV)-cowpea protoplast system, Tris-HCl at pH 7.6-7.8 was as effective as either potassium citrate at pH 5.2-5.6 or potassium phosphate at pH 6.0-6.4 (100). Tris-HCl buffers were used at a molarity range of 10 to 50 mM.

Buffer systems other than the three mentioned above have also been studied regarding the enhancement of infection efficiency (48,52).

Russo and Gallitelli (1985) concluded that the buffer anion was more important in stimulating infection than the pH value. This conclusion seemed to apply to infection of soybean protoplasts with CPMV where at pH 6.3, potassium phosphate buffer induced 66% infection compared to only 2% infection induced by MES buffer (48). On the contrary, at a buffer pH range from 7.0 to 8.0, the percent infection of soybean protoplasts by SBMV was not affected by the kind of buffer used (48). At pH 5.7, potassium phosphate, potassium citrate, or potassium acetate also made no difference on PVX-infection of potato protoplasts (95).

The buffer used to prepare inocula also influenced the optimum concentration of protoplasts required to be inoculated to reach the highest infection rate (75). Inocula prepared in citrate buffer were generally much less dependent on protoplast concentration (42,75). For instance, infection of tobacco protoplasts by TRV in the presence of phosphate or Tris-HCl, but not citrate buffer, was favored by lower concentration of protoplasts during inoculation (75). In the presence of Tris-HCl buffer at pH 7.0, turnip protoplasts at lower concentrations (0.2×10^5 protoplasts/ml) facilitated infection with TRosV (78). Infection of soybean protoplasts by BPMV decreased with an increase in protoplast concentration above 1×10^5 /ml in the presence of 10 mM potassium phosphate buffer pH 5.6 (62). On the contrary, cucumber protoplasts at concentrations lower than 0.5×10^6/ml gave poor infection by cucumber mosaic virus (CMV) (73). Infection of protoplasts may be influenced by culture conditions of the plant and position of the leaves used as source of protoplasts (54,83). Kubo et al. (1974) stated that this influence could be minimized by using phosphate buffer for TRV-tobacco protoplasts (55). The sharp pH dependence of protoplast infection by viruses, as well as the drastic difference between the optimum pH for mechanical leaf inoculation and for protoplast inoculation, implies that in protoplast inoculation buffer and pH not only affect the virus but the protoplasts as well (37,92,97). Inclusion of buffer in the preinoculation incubation gave maximum levels of infection of protoplasts by viruses (57,62).

Poly-L-ornithine. The efficiency of TMV infection of tobacco mesophyll protoplasts was dependent upon the presence of PLO, a linear

polymer of a basic amino acid (115). The necessity for the presence of PLO for infection of protoplasts was also proven in many other virus-protoplast systems (1,3,92,94,95,97), although there are some exceptions. For examples, 4 to 9% infection was achieved in the absence of PLO in the cowpea protoplast-CYMV system (96). CyRSV infection of cowpea protoplasts at pHs ranging from 5.6 to 7.6 was PLO-independent (100). Forty five percent as opposed to 77% of tobacco protoplasts became infected by BMV V5 without PLO (82). A method was developed by Zhuravlev et al. (1980) to infect tobacco protoplasts with TMV in the absence of PLO (129). In all cases, although the presence of PLO was shown not to be crucial for infection, it nevertheless enhanced the infection rate substantially. The infection of maize, oat, barley, wheat, and radish protoplasts by BMV was neither PLO-dependent nor PLO-stimulatory (37).

Molecular weight of PLO greatly influences its ability to promote infection efficiency. PLO preparations with a M.W. of 130,000 were the most effective as compared with preparations of lower M.W.-- 90,000 and 16,000 (112). PLO of M.W. 13,000 induced little TRV infection when substituted for PLO of M.W. 120,000 (42). However, the survival rate of inoculated protoplasts was inversely related to the increase in M.W. of PLO (95). Batches of PLO varied in their effectiveness (8), phytotoxicity (114), homogeneity in molecular size, and electrophoretic behavior in polyacrylamide gels (112). Therefore, PLO from different manufacturers may behave differently in their ability to enhance infection of protoplasts (113). The optimum concentration of PLO for infection of protoplasts varies from 0.2 ug/ml (6) to 5 ug/ml (15), 1

ug/ml being the most commonly used (3,53,78,81,90,92,97). PLO was more or less deleterious to tobacco protoplasts at concentrations higher than 1 ug/ml (113). At concentrations higher than 1.5 ug/ml, PLO inhibited infection of soybean protoplasts by BPMV (62). Other than M.W. and optimum concentration of PLO, the effectiveness of PLO relies on a preinoculation incubation with buffer and the virus for a certain period of time before it is added to the protoplasts (38,83,94). For the majority of virus-protoplast systems, an incubation period of 10 (78,95,96) to 20 min (56,90,94) was sufficient to achieve the maximum level of infection. The preinoculation incubation is usually carried out at 25°C. However, in the alfalfa mosaic virus (AlMV)-cowpea protoplast and CaMV-turnip protoplast systems, incubation at 0° C was more stimulatory than 25°C (1,38).

Polycations other than PLO have also been used in successful infection of plant protoplasts with viruses (8,45,52,64,80,83,94). Polyanions such as poly-L-glutamic acid (M.W. 18,000) and sodium dextran sulphate (M.W. 500,000) in citrate buffer pH 5.0 were inhibitory to infection of barley protoplasts by BMV (90).

Virus and PLO/virus ratio. Levels of virus infection of protoplasts are primarily a function of the concentration of virus inoculum within a certain range (3,38,56,79,80,94,96,113). Frequency of infection decreases when concentration of virus is higher than optimum (1,3,52,78,80,112). Optimum inoculum concentration for protoplast inoculation in the presence of PLO can vary from 0.2 ug/ml (97) to 20-50 ug/ml (15,90) but it generally ranges between 1 and 5 ug/ml (20,57,73,79,80,90,94,96). The PLO/virus ratio also contributes to the

percentage of infection of protoplsts by viruses (73). For infection of cucumber protoplasts with CMV, Maule et al. (1980) state that a 1:1 ration of PLO and CMV concentrations was the best. At the 1:1 (PLO:CMV) ratio, changes in concentration of PLO and CMV also influenced the infection rate. A ten-minute contact (inoculation incubation) of the inoculum (buffer+PLO+virus) with protoplasts was usually enough to induce the maximum infection rate (52,94,96).

Divalent cation. The percentage of infected soybean protoplasts greatly increased when BPMV or CPMV inoculum was supplemented with calcium chloride (48,62); on the other hand, infection of both cowpea and tobacco protpoplasts by CPMV and infection of tobacco protoplasts by TMV did not require $CaCl_2$(43,46,52). Infection of protoplasts from five plant species by BMV was actually inhibited by increasing $MgCl_2$ or $CaCl_2$ salts in the inoculum (37). A concentration of 0.5 to 1 mM $CaCl_2$ gave the highest percentage of BPMV infection (62) while the percentage of infection by CPMV was independent of either the concentration of calcium or the anion content of calcium salts (48). Calcium chloride and PLO were found to have a synergetic effect in enhancing infection of soybean protoplasts with BPMV, especially at low virus concentrations (62). The infection efficiency of barley protoplasts by BMV (90) increased with increasing concentration of calcium chloride in the solution used to wash protoplasts after inoculation (post-inoculation wash). Exposure of protoplasts to $CaCl_2$ after inoculation, i.e. during the wash, did not increase the infection rate either in the CCMV-tobacco protoplast (83) or the CPMV-soybean protoplast (48) system. However, exposure of protoplasts to $CaCl_2$ before inoculation significantly

reduced the level of infection (48,83,90). Magnesium salts in the inoculum also facilitated the infection of soybean protoplasts by CPMV but to a lesser extent than calcium salts (48,62). The mechanism(s) of enhancement of the infection by divalent cations were thought to be : (1) stabilization of the virus particle (62); (2) stabilization of the plasma membrane (90).

Other factors. Inoculation incubation at 0°C was found stimulatory for virus infection of protoplasts in several virus-protoplast systems including AlMV-cowpea protoplasts (1), CMV-cucumber protoplasts (73), and PVX-potato protoplasts (95). With CCMV and tobacco mesophyll protoplasts, inoculation at 0°C gave almost as much infection as that at 25° C (83). Infection of soybean protoplasts by BPMV was also temperature-independent (62). However, the percentage of protoplasts infected by CPMV was reduced considerably at temperatures below 13 to 15 °C (48). Increase in virus yield was observed with increasing temperature of the inocula (5 -35°C) in the TMV-tobacco protoplast system (129). The susceptibility of healthy tobacco protoplasts to TMV (49), BMV (124) and CCMV infection (83,124) decreased with time in culture before inoculation.

Increasing the mannitol concentration from 0.5 M to 0.85 M immediately before or at the time of inoculation (but not after inoculation) enhanced infection of barley protoplasts by BMV (91). The change in osmotic strength of TMV inoculum brought about by 0.1-0.2 M sucrose increased the efficiency of protoplast infection (129). This stimulatory effect on infection efficiency was not demonstrated in inoculation of cucumber protoplasts by CMV RNA (74).

Factors affecting replication of virus. Inoculated protoplasts are generally incubated in a conical flask or Petri dishes under diffuse light (about 3,000 lux) at a temperature between 25 and 28°C (103). Light was found to be essential for active replication of both TYMV in Brassica leaf protoplasts (97) and TMV in tobacco protoplasts (49). Barker and Harrison (1977) found that protoplasts incubated for 2 to 3 days at 18 or 24°C yielded substantially less RRV than those kept at 20 or 22°C (20% at 48 hrs and 30% at 72 hrs of those at 20-22°C).

The ingredients of the culture medium also affect virus replication. An increase from 0.74 to 1.07 M mannitol decreased by about half the amount of TMV that accumulated in tobacco protoplasts (104). Mesophyll protoplasts do not require a rich medium for the production of virus (113). The presence of $CaCl_2$ in both washing and culture media was required for TMV replication in tobacco protoplasts and could be partially replaced by magnesium (49). Kassanis and White (1974) found that $MgSO_4$ at 0.1 mM was superior to 1 mM in stimulating TMV multiplication in tobacco protoplasts. They also state that a 5-fold increase in the concentration of N, P and K in the culture medium gave no increase in TMV production in tobacco protoplasts. The addition of sucrose, 2,4-D and 6-BA did not improve the percentage of infection or yield of CCMV in tobacco protoplasts (83). Loebenstein et al. (1980) showed that TMV multiplication was lower in protoplasts of tobacco cultivars carrying the N gene in the absence of 2,4-D in the culture medium. In contrast, omission of 2,4-D from the medium used to culture protoplasts of a tobacco cultivar lacking the N gene had no effect, or increased TMV multiplication (63). Barker and Harrison

(1984) found that 2,4-D had little or no effect on infection of potato protoplasts with either PVY RNA or TMV RNA nor on the quantity of antigen produced per infected protoplast (5). The effect of antibiotics in culture medium on virus replication was studied by Motoyoshi et al. (1974b) as well as some other researchers (1,9,43,50,83). Yet, no general conclusion could be reached.

Polyethylene glycol (PEG)-mediated inoculation

The method of protoplast inoculation with viruses using PEG was fist introduced by Cassells and Barlass (1978). PEG-mediated inoculation of tomato protoplasts with TMV resulted in 60% infection (11) compared to over 90% infection rate achieved by PLO-mediated inoculation of tobacco protoplasts with the same virus (113). Similar results were obtained upon inoculation of turnip protoplasts with CaMV (72), cowpea protoplasts with CyRSV (100) and sonchus yellow net virus (SYNV) (118), tobacco protoplasts with TMV (12), BMV, and CCMV (101), inoculation of cucumber protoplasts with CMV (14), potato protoplasts with TMV and tomato black ring virus (TomBRV) (4), and inoculation of protoplasts from Chenopodium quinoa with CPMV and CyRSV (25).

Buffer composition and pH of the PEG preparation were found important in the infection of cowpea protoplasts by SYNV (118). PEG, the molecular weight of which depends on degree of polymerization, is an agent (fusagen) used for protoplast fusion (19). Most PEG-mediated inoculations of protoplasts were carried out using PEG of 6,000 molecular weight (11,12,72,74,118), but some (101) used PEG of 1,500 M.W. Both the percentage of protoplasts infected with TMV and the mean

virus production per infected protoplast increased with an increase in PEG concentration (11). The presence of calcium in the inocula was not essential but was stimulatory for PEG-mediated infection of protoplasts by SYNV (118). Preincubation of tomato protoplasts in 5, 10, or 50 mM $CaCl_2$ solutions in mannitol did not change the infection rate (11). Washing inoculated protoplasts with high calcium (60 mM)/high pH (10.5) solution had no effect on either percentage of infection or virus production (11).

All PEG-mediated inoculations of protoplasts with virus particles mentioned above were carried out at room temperature. Inoculation at 20 ^{o}C was the best for cowpea protoplast infection by SYNV and for survival of the protoplasts (118).

The relationship between protoplast clumping and virus infection of tobacco protoplasts was studied by Cassells and Cocker (12). They suggested that initiation of virus infection in protoplasts in the presence of PEG could be separated into two stages, i.e., virus precipitation on the protoplast surface followed by interprotoplast fusion leading to infection. Therefore, the sequence of addition of virus inoculum and PEG preparation was critical (12). The significance of rapid dilution of PEG in PEG-mediated infections, a factor common to all inoculation procedures, is unclear (74). When the PLO- and PEG-mediated inoculation of protoplasts were systematically compared, PEG-mediated inoculations were less efficient than (4), as efficient as (25,101), or more efficient than (100) PLO-mediated inoculations. If protoplasts can be infected by viruses in the presence of PEG they can also be infected in the presence of PLO.

Liposome-mediated inoculation

Liposomes, or so-called phospholipid vesicles, were originally reported by Bangham et al in 1965 (2). A liposome is now defined as a closed vesicular structure which is formed spontaneously upon hydration of a dry layer of phospholipid deposit with aqueous buffer (31). The different liposome preparation techniques can be classified into two groups: the techiques forming large multilamellar or large unilamellar vesicles, and those forming small unilamellar vesicles (69). The different types of liposomes are referred to by a three-letter acronym (111), e.g. multilamellar vesicle (MLV), large unilamellar vesicle (LUV) and small unilamellar vesicle (SUV). A fourth type of liposomes, named reverse-phase evaporation vesicle (REV), was developed by Szoka and Papahadjopoulos in 1978 (110). According to their report, REVs have aqueous volume to lipid ratio 30 times higher than sonicated preparations and 4 times higher than MLVs. MLVs were considered best for investigators without previous experience with liposomes due to the simplicity of their preparation and minimal requirements for laboratory equipment (111). The capture volume of MLVs is relatively small, however (110).

Although liposomes had been used in many fields of biology, they were first used as carriers of foreign nucleic acid to be introduced into plant protoplasts for genetic modification in 1978 (10). Fukunaga et al (1981) reported the first successful case of liposome-mediated infection (80%) of protoplasts with TMV RNA (34). This success was further explored in other virus-protoplast systems by several research groups (32,88,98,99,123,128). However, only one publication reported

successful infection of protoplasts by liposome-mediated inoculation with intact virus particles (98). The virus (TRosV) is a monopartite isometric virus, 28-30 nm in diameter and 6.6×10^6 in M.W. (71). From 0.7 to 8% of the virions could be encapsulated into MLVs without apparent loss of infectivity. In the presence of 20% PEG and 60 mM $CaCl_2$, 30% of the protoplasts became infected at room temperature with the aid of neutral liposomes containing L-α-phosphotidylcholine (PC) and β-sitosterol (SS) (98). Positively charged MLVs containing PC and sterylamine (SA) could be associated with larger amount of virus particles but they were very toxic to turnip protoplsts (98) as well as to cowpea and carrot protoplasts (68). When Vinca rosea protoplasts were incubated with LUVs made from phosphotidylserine (PS), the protoplast/liposome samples fixed at the end of the 10 min incubation in the presence of PEG revealed that liposomes were associated with protoplasts in three ways : (1) adhering to the surface of protoplasts; (2) in association with an invaginating plasmalemma; and (3) within the cytoplasm, enclosed in vesicles apparently of cellular origin (35). These observations suggest that liposomes enter plant protoplasts via endocytosis rather than fusion.

CHAPTER III

MATERIALS AND METHODS

The Virus

Source and maintenance of potato virus Y (PVY)

The virus used throughout these investigations was obtained from Dr. Merritt R. Nelson, Department of Plant Pathology, University of Arizona at Tucson. The virus had been originally isolated from pepper (Capsicum annuum) and had been identified as PVY-common strain. In our laboratory, the virus has been maintained on Nicotiana tabacum cv. Samsun NN. The rate of PVY replication in Samsun NN was monitored by indirect Enzyme-Linked-Immunosorbent Assay (ELISA). Three leaf disks, each 1.1 cm in diameter, were randomly harvested with a #6 cork borer from inoculated, uninoculated, and top leaves of mechanically inoculated plants. The leaf disks were stored in a -20°C freezer until use.

Purification of PVY

Partial purification. Inoculated leaves of Samsun NN were harvested 30 to 40 days after inoculation. Leaf tissue was squeezed through stainless steel roller bars with 3 volumes 0.2 M sodium phosphate buffer (Na-PB), pH 7.6, containing 1% 2-mercaptoethanol (2-ME) and 0.01 M ethylenediaminetetraacetic acid (EDTA). The pulp was filtered through 8 layers of cheesecloth and the filtrate was emulsified with 8% cold n-butanol. The emulsion was stirred overnight at 4°C and was subsequently centrifuged first at 5,000 g and then at 10,000 g each for 15 min. The

supernatant was then filtered through Miracloth. Polyethylene glycol (PEG) was added to the supernatant while stirring to a final concentration of 4%. After the PEG had dissolved completely, the mixture was kept at 4°C for 80 min without stirring. PEG-precipitated PVY was collected by centrifuging at 12,000 g for 25 min. The pellet was resuspended with 0.1 M Na-PB, pH 7.6, containing 0.5 M urea [1.5 ml/100 g fresh tissue for column purification or 10 ml/100 g fresh tissue for purification by sucrose density gradient centrifugation (SDGC)] and was then centrifuged at 8,000 g for 10 min.

Further purification. For column purification, 1.5 ml partially purified PVY was placed on top of a 56-cm high bed of Sepharose CL-4B packed in a 0.9 x 60 cm (K9/60) column according to the procedures recommended by the manufacturer. Na-PB 0.1 M, pH 7.6, without additives, was used as eluent, with a flow rate of 5 to 6 drops per minute. The effluent was collected in 1 ml fractions.

For SDGC, the virus was first concentrated by high speed centrifugation at 55,000 g for 60 min and then resuspended overnight at 4°C in 1 ml 0.1 M Na-PB, pH 7.6, containing 0.5 M urea. Resuspended PVY was centrifuged at 5,000 g for 10 min and then layered on top of a linear 10 to 40% sucrose gradient in resuspending buffer. The gradient was centrifuged at 55,000 g in SW40 Ti rotor for 3 hrs. The virus band formed in the gradient was recovered with a syringe equipped with an L-shaped needle and was dialysed overnight against 0.1 M Na-PB, pH 7.6, with one change of buffer.

Assay of purified virus

UV spectrum and yield determination. The UV spectrum of purified virus preparations was obtained by scanning purified virus samples in a Gilford 260 Spectrophotometer. A260/280 and A260/245 ratios were calculated to compare with the published UV absorption pattern of PVY. Yields of the purified virus were estimated by using an extinction coefficient for PVY of A260 = 2.9 (Stace-Smith, 1970).

Specific infectivity assay. PVY preparations purified by either SDGC or column were adjusted by dilution with 0.1 M Na-PB, pH 7.6, to the same A260 value and were then diluted further to make parallel serial dilutions. The specific infectivity of the virus was assayed on Chenopodium amaranticolor. Half-leaf comparisons were employed to compare the specific infectivities of column- and SDGC-purified PVY preparations of the same A260 values. Local lesions were counted 14 days after inoculation.

Electron microscopy and particle length measurement. Both column- and SDGC-purified PVY preparations were diluted with distilled water to a concentration of 0.05 mg/ml (A260 = 0.145). The virus was negatively stained with 1% ammonium molybdate, pH 7.0. Particle length was determined by measuring, with the help of a flexible wire, the length of 250 particles for each treatment on photographs obtained through the electron microscope.

The Protoplasts

Source and isolation of protoplasts

Nicotiana tabacum cv. White Burley leaves were used as the main source of protoplasts. Seedlings about 6-cm tall (40-50 days after seeds were sown) were transplanted into 10-inch pots and maintained in the greenhouse without any insecticide spray. In the summer, the greenhouse was shaded by spraying it with Kool Ray liquid white shade (Continental Product Co., Euclid, Ohio). The plants were fertilized once every 10 days with complete fertilizer.

Fully expanded tobacco leaves were harvested 50 to 70 days after transplantation, washed with mild hand soap and rinsed thoroughly with running tap water. Leaves were surface-sterilized by soaking in 2% commercial Bleach in sterile distilled water containing 0.5% Tween-20 for 10 min. The leaves were then washed with sterile distilled water 3 times and finally by dipping them briefly in 70% ethanol for 10 seconds. The surface-sterilized leaves were allowed to dry in a laminated air flow hood for 30 min. The lower cuticle of the leaves was removed with a pair of fine-tip tweezers and the leaves were then placed in a Petri dish with lower (cuticle-less) side in contact with 10 ml 0.65 M mannitol. After the cuticle-peeling process was completed, 10 ml of 0.5% Macerase and 1% Cellulysin (Calbiochem) enzymes in 0.65 M mannitol replaced the 0.65 M mannitol. Complete release of protoplasts was achieved within 2 hrs at room temperature (r.t.) with shaking (80 rev/min).

Viability test

The viability of tobacco protoplasts was ascertained by the fluorescein diacetate (FDA) staining method (58).

Maintenance of Protoplasts

Inoculated protoplasts were cultured in the medium developed by Aoki and Takebe (1969) except that streptomycin at a concentration of 100 ug/ml was substituted for rimocidin (10 ug/ml). Protoplast cultures were maintained at 16 hrs day photoperiod at a light intensity of 3,000 lux in a 25°C incubator.

Virus Assay Techniques

Antisera to PVY were produced in both rabbit and guinea pig by two consecutive sets of subcutaneous injections with 1 mg of purified PVY in 1 ml distilled water mixed well with an equal volume of Freund's complete adjuvant. Each set of injections was made on 6 sites of the animal's back. The two sets of injections were administered 3 weeks apart. Rabbits received, in addition, an intravenous booster injection through the ear vein a week later. Antisera were collected 10 days after the last injection and the antiserum titer was determined by the microprecipitin test. The anti-PVY antisera were cross-absorbed 3 times with acetone-extracted healthy tobacco protein to remove nonspecific antibodies. The γ-globulin used for ELISA and for fluorescein isothiocyanate (FITC) staining was purified from cross-absorbed rabbit anti-PVY antiserum by 18% Na_2SO_4 precipitation and DEAE 22 column chromatography. The purified γ-globulin preparation was adjusted to a concentration close to 1 mg/ml (OD at 280 = 1.35) and was stored in the refrigerator. NaN_3 at 0.02% was added as preservative.

ELISA

Indirect ELISA conditions were similar to the double sandwich ELISA procedure described by Clark and Adams (1977) except the initial coating was with antigen in 200 ul of coating buffer. The optimum concentration of rabbit anti-PVY γ-globulin and of goat anti-rabbit IgG conjugated with alkaline phosphatase were each determined in preliminary ELISA tests to be 1 ug/ml. Known concentrations of purified PVY and healthy sap diluted 1:20 in coating buffer (H-sap) were used as positive and negative controls, respectively. The protoplast pellet and leaf tissue were ground in 1.2 ml coating buffer in a glass tissue homogenizer before use in ELISA.

The sensitivity of our ELISA system for PVY detection was determined by two experiments. The first one involved serial dilution of purified PVY either ing coating buffer or in H-sap. The dilution-end-point was then determined by comparing with the ELISA value of the negative control. In the second experiment, protoplasts isolated from PVY-infected Samsun NN leaf (PVY protoplasts) at a concentration of 1 x 10^6/ml were dilute 10-fold with healthy protoplasts at the same density to give preparations containing 10^5, 10^4, 10^3, 10^2, and 10^1 PVY protoplasts in a total number of 1 x 10^6 protoplasts, respectively. Parallel dilutions of PVY protoplasts in 1:20 dilution of healthy sap were also prepared. Again, dilution-end-points of PVY protoplasts in both diluents were determined by ELISA. FITC staining was also performed in the second experiment in order to determine the minimal percentage of infected tobacco protoplasts in 1 x 10^6 protoplasts needed to give a positive ELISA reading.

FITC staining

One drop of a thick protoplast suspension was applied onto slides freshly coated with albumin fixative (Fisher). The protoplast-carrying slides were allowed to dry for 10-15 min under a stream of hot air and were then placed in acetone for 20 min. The acetone-fixed protoplasts were stained with 1:40 diluted rabbit anti-PVY γ-globulin (A280 = 1.35) and then with 1:200 diluted FITC-conjugated goat anti-rabbit antiserum (Calbiochem) both in phosphate buffered saline (PBS) containing 0.02% NaN_3. The protoplasts on the slides were washed in PBS after each of the above steps.

Bioassay on local lesion host

Chenopodium amaranticolor was used as the local lesion host for PVY. Different treatments were compared by using a Latin Square Design (L.S.D.). All mechanical inoculations were carried out in the presence of carborundum and 0.1 M Na-PB, pH 7.6.

The sensitivity of C. amaranticolor to detect PVY was tested by determining the dilution-end-point of purified PVY in 0.1 M Na-PB, pH 7.6. The infectivity of PVY in PVY protoplasts at densities of 5×10^6, and 1×10^6 /ml was also tested on G. amaranticolor in 10-fold dilutions. The last bioassay experiment in conjunction with FITC staining and ELISA results, provided a mechanism for comparing the three assay methods primarily used throughout this study.

Starch-iodine lesion assay

Samsun NN tobacco plants at the 8 to 10 leaf stage were mechanically inoculated with a purified PVY preparation of known virus concentration by rubbing six times with an L-shaped glass rod dipped in the inoculum. Four to six days later, the inoculated leaves to be harvested were wrapped in aluminum foil overnight and the following morning they were detached from the plants and decolorized by placing in 95% ethanol at 55 C for 2 hrs. The decolorized leaves were stained by placing in an I_2KI solution (10 g I_2, 30 g KI, 1500 ml H_2O) for 30 to 60 seconds and were subsequently washed with tap water for several minutes. The starch lesions were viewed by placing the treated leaves on a Glow Box. When more than one inoculum were studied at the same time, each leaf was marked to give cross sections with a width of 3.5 cm. Each inoculum was applied onto one of the leaf sections.

Bioassay on a systemic host by using the ELISA technique

Leaves of Samsun NN tobacco plants at the 8 to 10 leaf stage were each marked to give 3 cross sections of equal width and each section was inoculated as above with 100, 10, or 1 ug/ml PVY. The inoculated leaves were detached immediately after inoculation and were placed in a 800 ml beaker containing 200 ml MS medium, with the patiole sunk in the medium. The beakers were covered with a plastic bag and were placed in an incubator at 25^{o}C, in light (16 hrs/day). Beginning on day 2 and daily up to day 7 after inoculation, 6 leaf disks each 0.6 cm in diameter were punched out randomly with a # 3 cork borer from each leaf section and were kept in a freezer until use. For the ELISA test, each

6-disk sample was ground in 1.2 ml coating buffer. The ELISA results were analyzed statistically.

Inoculation of Protoplasts with PVY

Poly-L-ornithine (PLO)-mediated inoculation

About 15 x 10^6 freshly isolated tobacco protoplasts were resuspended in 0.65 M mannitol to a concentration of 1 x 10^6 protoplasts per ml and were then mixed with an equal volume of inoculum. The inoculum consisted of 0.02 M potassium citrate buffer (K-CB), pH 4.6, in 0.65 M mannitol containing PLO in 0.65 M mannitol and column-purified PVY in 0.1 M Na-PB, pH 7.6 in a final concentration of 6 ug and 2 ug per ml, respectively. The inoculum was incubated at room temperature for 20 min with shaking (80 rev/min) before it was used to inoculate protoplasts (preinoculation incubation). The freshly resuspended protoplasts and the inoculum were then mixed and incubated at r.t. for 10 min with shaking (80 rev/min) (inoculation incubation). The inoculated protoplasts were then washed 3 times with 0.65 M mannitol containing 1 mM $CaCl_2$ (post-inoculation wash), to remove residual inoculum from the surface of the inoculated protoplasts, and were then placed in culture medium. Samples of inoculated protoplasts were usually harvested 48 hrs after inoculation; changes in virus titer in inoculated protoplasts were monitored mainly by ELISA, and occasionally by FITC or bioassay.

Buffer and pH effect. Three buffers (K-CB, K-PB, Tris-HCl), all having a molarity of 0.02, were tested as inoculation buffers. The pH

range tested for each buffer was : for K-CB, 4.6-6.0 at 0.4 unit increments; for K-PB, 6.0-8.0 at 0.4 unit increments; and Tris-HCl at pH 8.0 only. PLO and PVY concentrations in the inoculum were held at 6 and 10 ug/ml, respectively.

PLO/PVY ratio effect. The standard protoplast inoculation buffer of 0.02 M K-CB, pH 4.6, was supplemented with PLO and PVY at the following ratios (PLO:PVY, ug per ml), 10:20, 10:10, 6:20, 6:10, 6:2, 2:20, 2:10, and 2:2.

Virus concentration effect. When testing the virus concentration effect, the concentration of PLO was kept at 6 ug per ml inoculation medium while the PVY concentration was changed to give 10, 5, 2, 1, and 0.5 ug per ml of inoculation medium.

Washing medium effect. Three washing media containing increasing concentrations of $CaCl_2$ -- 1 mM or 10 mM in 0.65 M mannitol and 0.2 M in water -- were used to compare their effects on minimizing zero time (0 time) background PVY titer and on stimulation of viral replication in protoplasts. PLO and PVY concentrations in inoculation medium were held at 6 and 2 ug per ml, respectively. Inoculated protoplasts were divided into three equal parts and each part received three washes with one of the three washing media.

Blocking with antiserum to eliminate-0 time PVY titer. Three anti-PVY antisera developed in different animal species (guinea pig, rabbit, and sheep) were employed to treat inoculated protoplasts immediately after postinoculation washing for 60 min at r.t. All anti-PVY antisera were tested at 1:50 dilution. Rabbit anti-PVY antiserum was followed by treating protoplasts with anti-rabbit serum developed in

goat without enzyme marker for another 60 min at r.t. For all serological blocking experiments, inoculated protoplasts were resuspended in 5 ml 0.65 M mannitol containing 1 mM $CaCl_2$. Post-blocking washing was done twice with 0.65 M mannitol containing 1 mM $CaCl_2$.

Culture temperature and light effects. PVY-inoculated protoplasts were partitioned into four equal parts. Each part received one of the four light and temperature combinations : 4°C in dark, 4°C with light, 25°C in dark, 25°C with light. In several experiments, an additional treatment was included, in which the inoculated protoplasts were first incubated at 4°C in dark for 24 hrs and then transfered to 25°C with light for another 24 hrs. The light source in the 4°C incubator was a incandescent lamp with a light intensity of 3,500 lux. The 25°C incubator was equipped with both fluorescent and incandescent light of 3,000 lux. The protoplasts were sampled both immediately after inoculation (without and with block treatment) and 24 or 48 hrs after inoculation. All samples harvested were stored in freezer (-20°C) until use for ELISA.

Polyethylene glycol (PEG)-mediated inoculation

Cassells and Cocker's (1980) method was adopted. To 8 X 10^6 freshly isolated protoplasts, suspended in 3.2 ml of 0.65 M mannitol in a 50 ml conical centrifuge tube, was added 0.8 ml purified PVY (0.5 mg/ml) in 0.1 M Na-PB, pH 7.6, and the mixture was swirled gently. Immediatly afterwards, 3.2 ml of 83.3 mM PEG (M. W. 6000, Sigma) in 0.65 M mannitol was added, followed by the addition of 9.6 ml 0.65 M mannitol in 0.05 M Na-PB, pH 7.6. After each addition, the mixture was swirled gently to

ensure even distribution and was then incubated at 25°C for 60 min in dark. Inoculated protoplasts were pelleted, washed 3 times with 0.65 M mannitol containing 1 mM $CaCl_2$ and subjected to blocking treatment with antiserum prior to culture.

Liposome-mediated inoculation

Preparation of liposomes. The procedure used to prepare liposomes was basically that developed by Rollo and Hull (1982). Multilamellar vesicles (MLVs) were prepared by placing 3.8 uM phosphotidylcholine (PC, Sigma) and 0.4 uM β-sitosterol (SS, Sigma), both in chloroform-methanol (9:1, V/V), into a 50-ml round bottom glass tube. The solvent base was removed while swirling the tube slowly to generate a thin layer of lipid film at the bottom. The dry lipid film was resuspended in 0.2 ml 10 mM Tris-HCl buffer, pH 7.2 containing 0.1 M NaCl by vortexing the tube for 5 min at full speed of a Vortex. The MLVs thus produced were left to equilibrate at room temperature for 60min. Equilibrated MLVs were washed 3 times with the same buffer and finally resuspended in 0.4 ml 0.65 M mannitol. Evan's Blue at a final concentration of 0.05% was used as additive to check the intact membrane structure of produced MLVs. To determine fusion of protoplasts with MLVs, the resuspension buffer was supplemented with 0.01% FDA. PVY was added at a rate of 0.5 mg per ml resuspension buffer to obtain PVY-carrying MLVs. To examine the size and structure of MLVs thus formed, freshly prepared MLVs in resuspension buffer were negatively stained with 2% ammonium molybdate, pH 5.5 and examined with the electron microscope.

Detection of PVY carried by MLVs. PVY was extracted by disru-

pting PVY-carrying MLVs with 0.1 ml chloroform, followed immediately by the addition of 0.6 ml either carbonate buffer (coating buffer) for ELISA or 0.1 M Na-PB, pH 7.6, for bioassay. The emulsion was placed in a 1.5 ml Eppendorf tube and spinned in an Eppendorf centrifuge 5414 for 2 min. The upper phase was saved.

Fusion of MLVs with tobacco protoplasts. Freshly pelleted 3×10^6 protoplasts were resuspended with 0.4 ml liposomes in 0.65 M mannitol at a concentration of 3.8, 1.9, 0.95, or 0.47 uM PC content. The mixture was incubated for 10 min at room temperature before adding 0.4 ml of protoplast culture medium containing 20% PEG and 60 mM $CaCl_2$. After an additional 10 min incubation at room temperature, the protoplasts were resuspended to a final 6 ml volume with 0.65 M mannitol and were washed with high calcium (60 mM) high pH (10.5) buffer (51).

CHAPTER IV

RESULTS

Purification of PVY

PVY, partially purified by the PEG precipitation procedure, usually appeared opalescent to pale green. Such preparations generally gave satisfactory results in subsequent purification steps. Large tissue quantities (> 300 g) tended to be harder to clarify than smaller (100-200 g) samples.

After partially purified PVY was run through sucrose density gradient centrifugation (SDGC), the virus in the centrifuge tube appeared as a broad single band 0.7 to 1.5 cm wide. Occasionally, continuously distributed light-scattering materials were present in the gradient after centrifugation and no band could be recognized. A pellet always appeared at the bottom of the centrifuge tubes at the end of SDGC. When the pellet was examined under the electron microscope, clumps of aggregated particles could be seen.

When purification was carried out by passing partially purified virus preparations through a Sepharose CL-4B column, PVY usually came out in the fractions No. 12 through 15, with the highest virus concentration at fraction number 13 (Fig. 1). The material in these fractions was proven to be PVY by its UV absorption spectrum and its A260/280 ratio, by bioassay on both systemic and local lesion hosts, and by ELISA. A second peak which also absorbed UV light, appeared at fractions 30 to 37. The ingredients of these fractions consisted of materials of

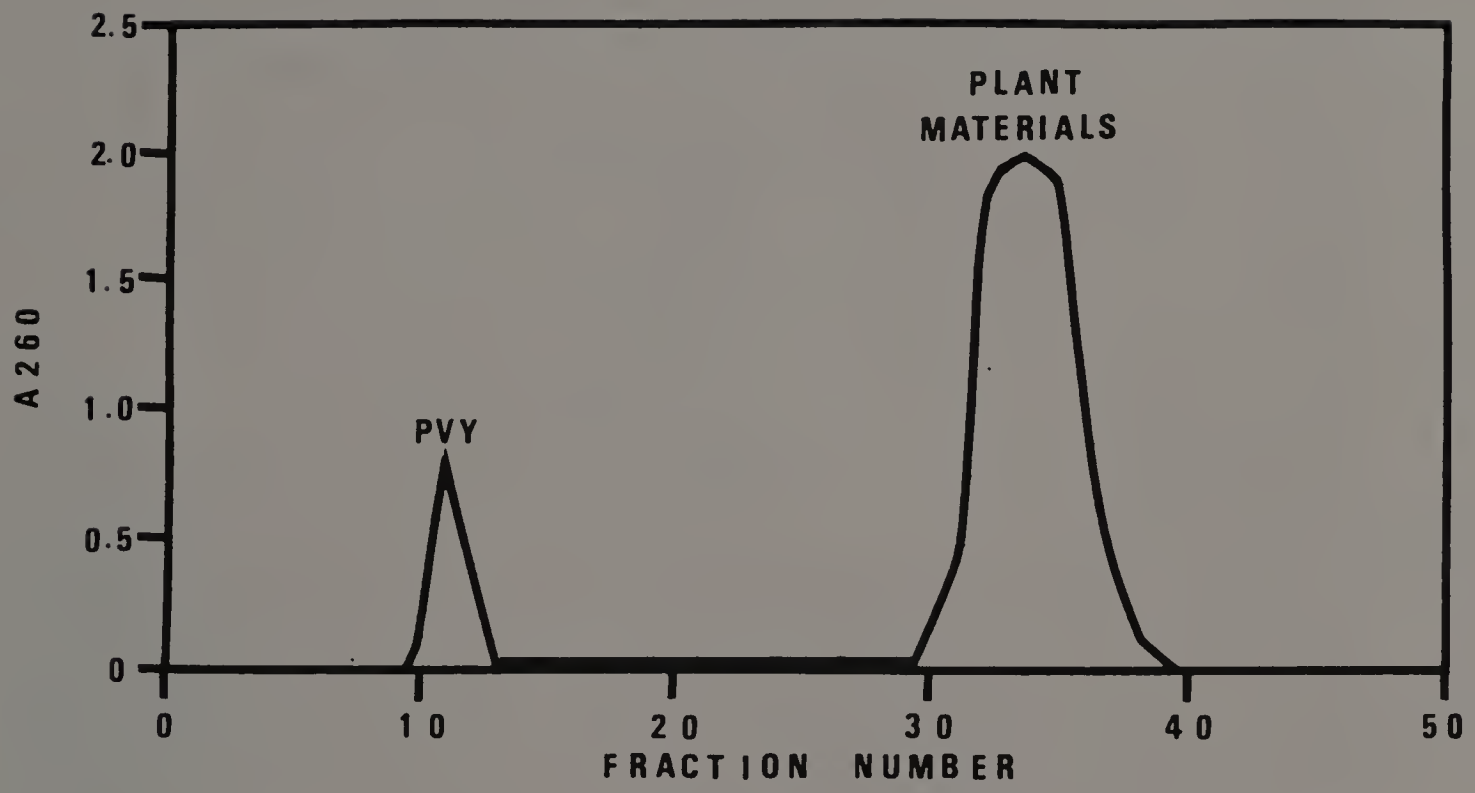


Figure 1. Elution profile of a typical PVY preparation from Sepharose CL-4B column.

low molecular weight and lacked infectivity. The elution profile of the preparation could shift left or right depending on the bed volume of the column. If the virus was concentrated by ultracentrifugation, a broader virus peak (tailing) was obtained indicating a preparation of greater particle length heterogeneity.

Both column- and SDGC-purified virus preparations showed the typical UV spectrum of PVY with maximum absorbance at 260-261 nm and minimum absorbance at 245-247 nm (Fig. 2). The A260/280 and A260/245 ratios of both preparations were in the range of 1.21-1.29 for A260/280, and 1.09-1.17 for A260/245, respectively.

The purity of PVY purified by both methods was also assessed by EM. When a preparation with a typical UV pattern was examined, no recognizable contaminants were present regardless of the method of purification (Fig. 3).

Yields of PVY in column purification experiments ranged from 0.9 to 2.5 mg/100 g fresh leaf tissue with an average of 1.5 mg (Table 1). Yields of PVY in SDGC purification experiments ranged from 0.2 to 0.9 mg/100 g fresh leaf tissue with an average of 0.5 mg. Thus, PVY yields obtained by column purification were on the average two to five times greater than PVY yields obtained by SDGC. Actually, in later column purification trials, PVY yields as high as 6.4 mg /100 g fresh leaf tissue were obtained.

Three concentrations (0.05, 0.01, 0.002 mg/ml) of column- and SDGC-purified PVY preparations were inoculated onto half-leaves of Chenopodium amaranticolor. The numbers of local lesions induced by column-purified PVY preparations were always greater than those induced

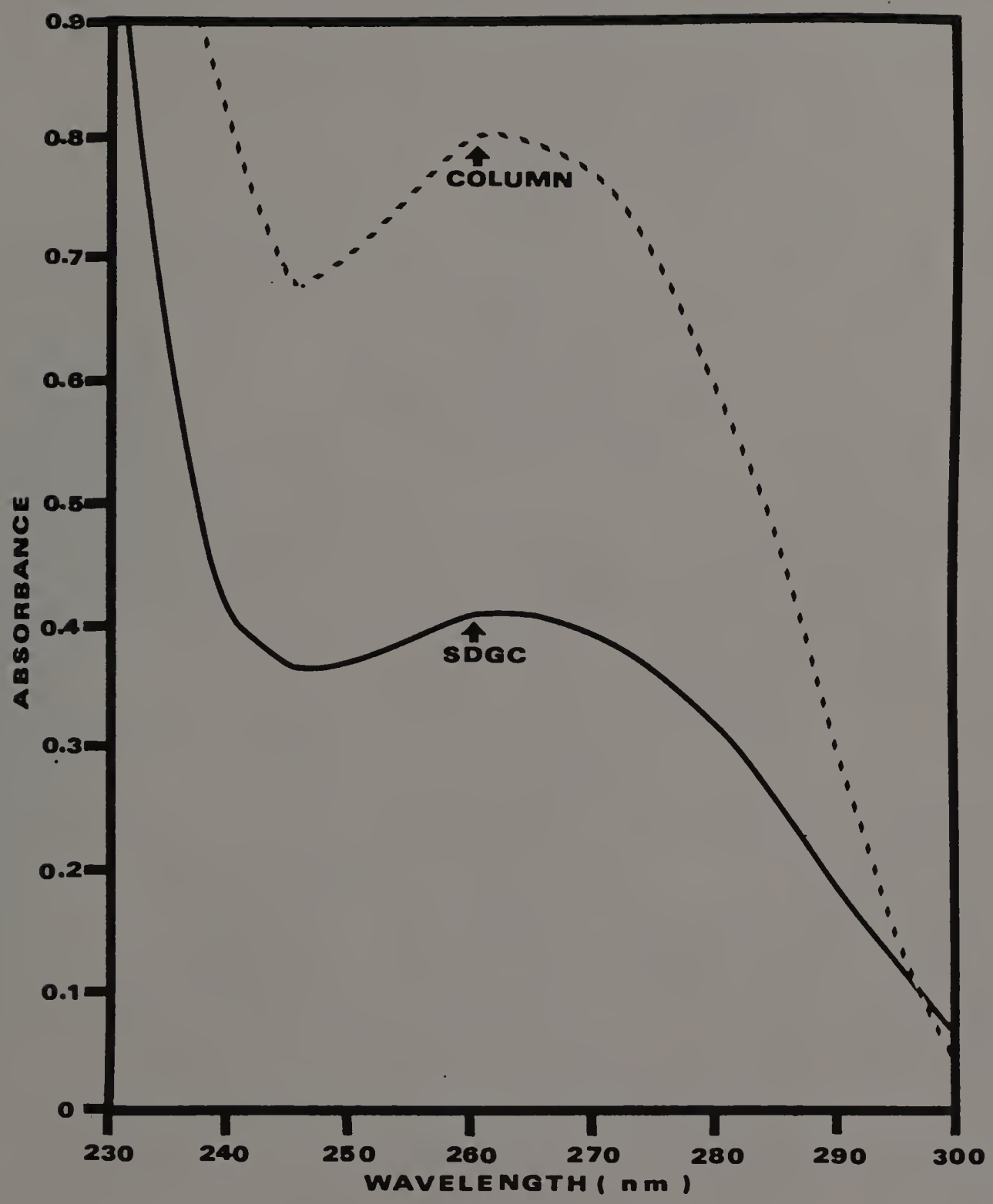


Figure 2. UV spectrum of column- and sucrose density grdient centrifugation (SDGC)-purified PVY.

Figure 3. Electron microscopy of (a) column- and (b) sucrose density gradient centrifugation (SDGC)-purified PVY. (20.000 X)

Table 1. PVY yields obtained from 100 g fresh leaf tissue after clarified virus was further purified either by sucrose density gradient centrifugation (SDGC) or by passing through a Sepharose CL-4B column.

EXPERIMENT NUMBER	COLUMN PURIFIED (mg)	SDGC PURIFIED (mg)
1	0.91	0.22
2	0.94	0.31
3	2.40	0.90
4	1.07	0.40
5	2.22	0.70
6	1.46	0.54
AVERAGE	1.50	0.50

by SDGC-purified PVY preparations of equal concentration (Fig. 4), although the numbers of local lesions produced varied among the paired infectivity comparison experiments.

SDGC-purified PVY preparations generally showed more severe aggregation and fragmentation than column-purified PVY (Fig. 3). Actually, a few SDGC-purified PVY preparations showed so much aggregation that particle length measurement in EM photographs was impossible. The most common particle length of PVY purified by either column or SDGC was about 720 nm. In column-purified PVY, more than 66% of the particles had lengths in the range of 650 to 750 nm, while only 40% of the particles of SDGC-purified PVY had lengths in that range (Fig. 5). Less than 15% of the virus particles in column-purified preparations had a length shorter than 600 nm. On the other hand, more than 30% of SDGC-purified PVY particles had lengths shorter than 600 nm -- an indication of the fragmentation of particles purified by that method.

Protoplast Isolation

In the majority of the experiments performed, protoplasts were completely released from leaf tissues after 1.5 to 2 hrs treatment with enzymes (Fig. 6). A preliminary measure of the quality of the protoplasts was given by the synchronization of protoplast release, the degree of protoplast breakage, and by the roundness of the protoplasts. Good preparations were clean, contained perfectly rounded protoplasts and were released within 2 hrs. Protoplast preparations which did not round up within 2.5 hrs from cells were generally not satisfactory. The

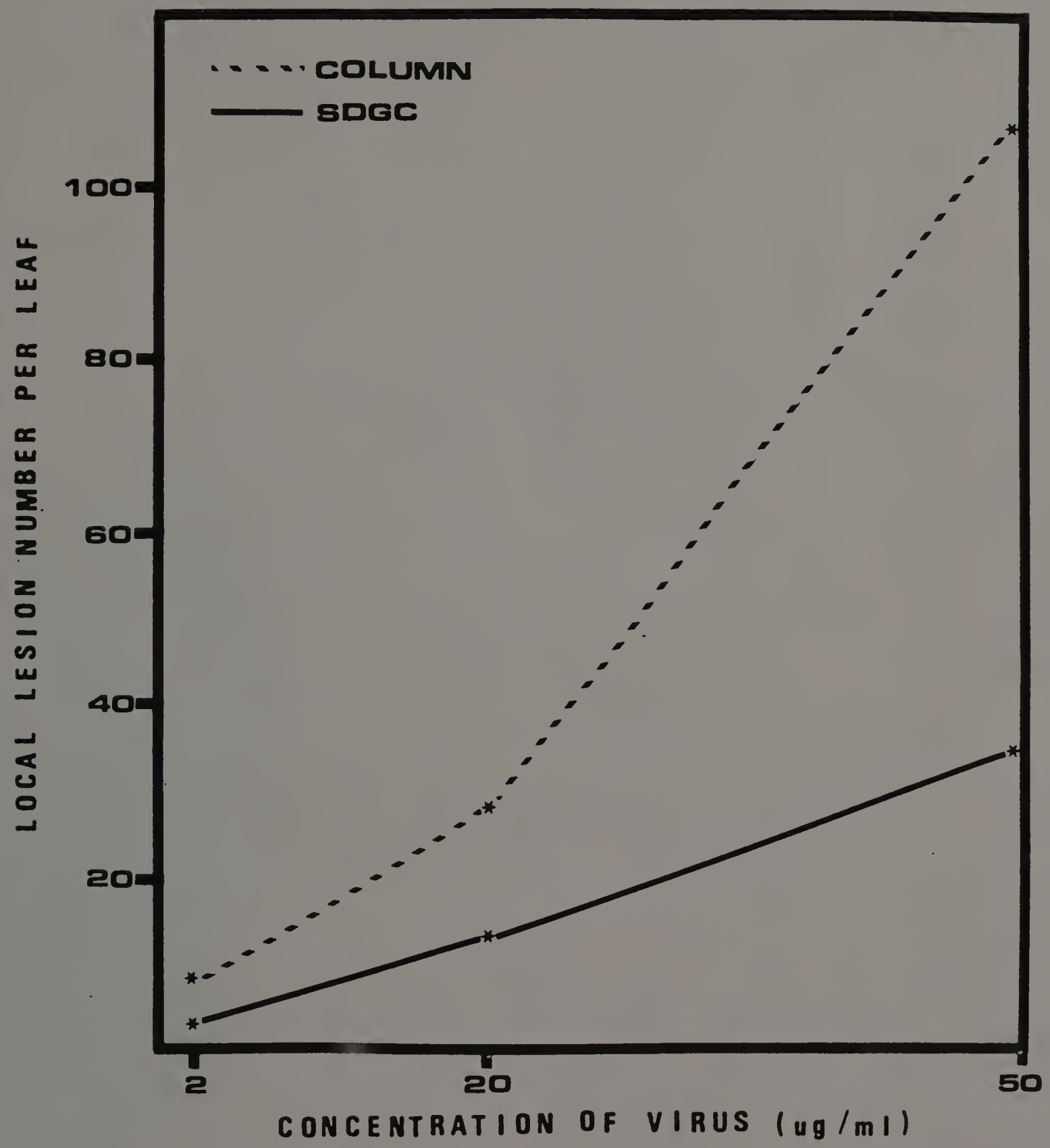


Figure 4. Local lesion assay on Chenopodium amaranticolor of PVY purified by either sucrose density gradient centrifugation (SDGC) or through column.

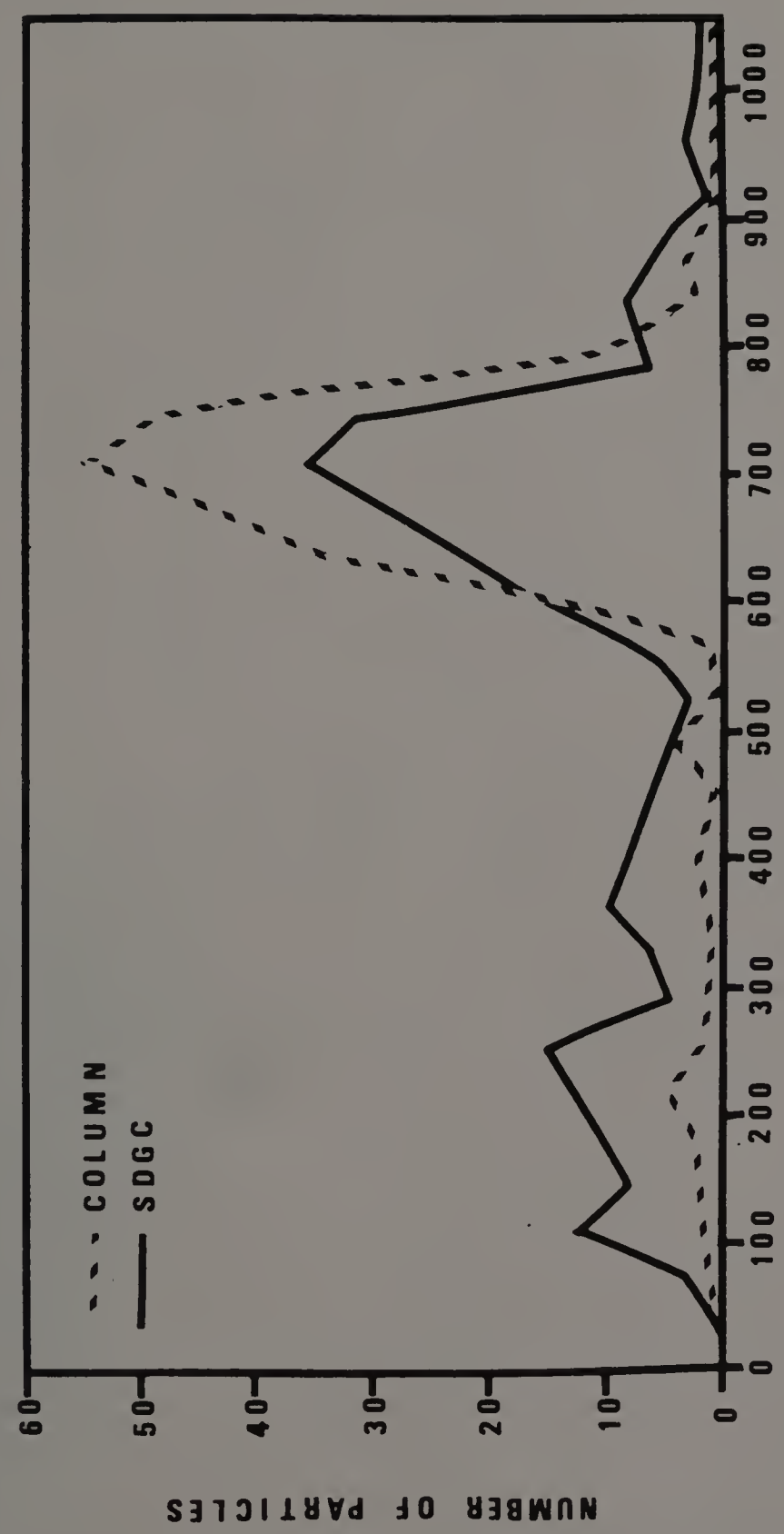
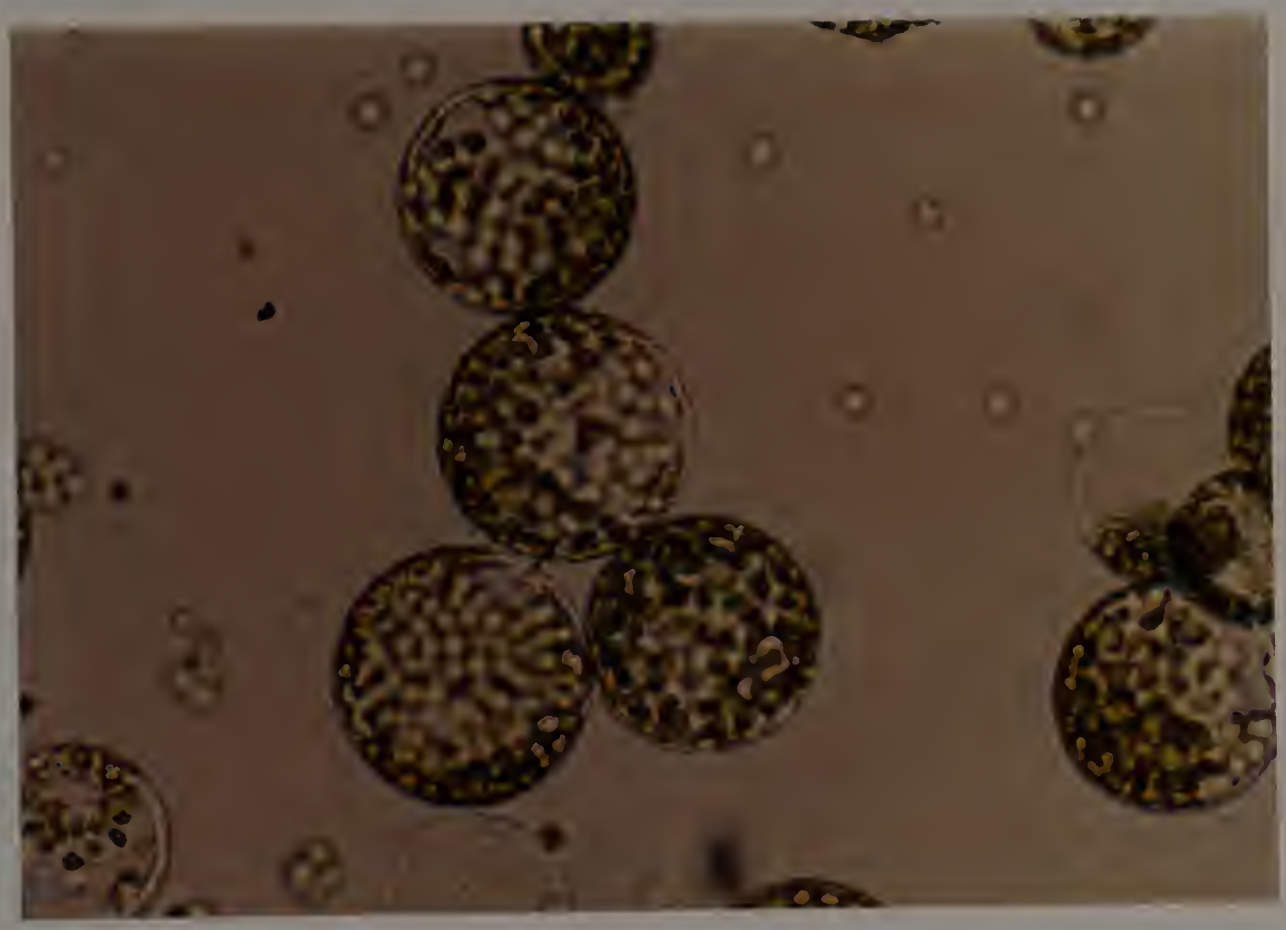


Figure 5. Length distribution of PVY particles purified by sucrose density gradient centrifugation (SDGC) and through column.



700 X

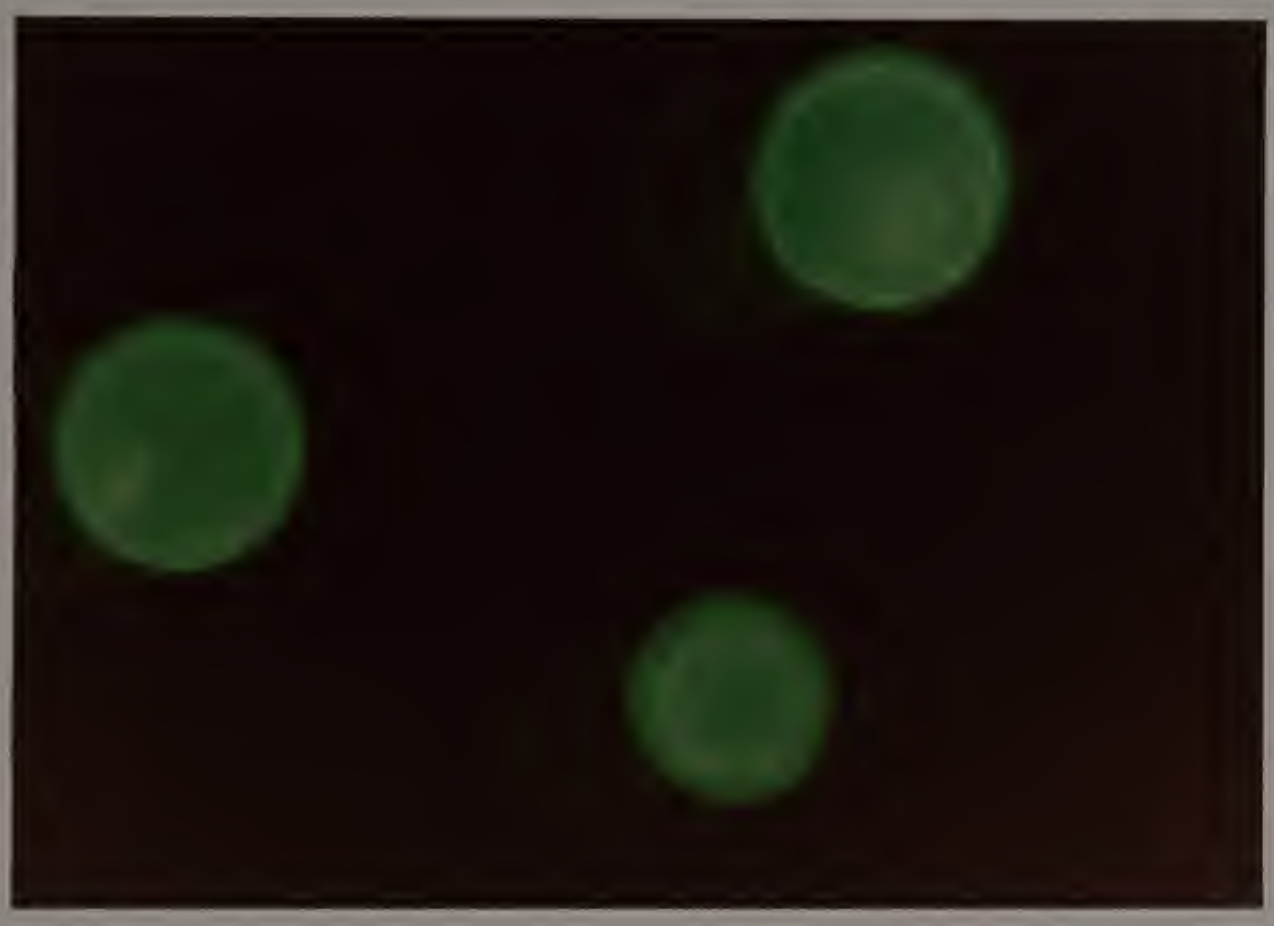
Figure 6. Mesophyll protoplasts from Nicotiana tabacum cv. White Burley released from leaves after treatment with enzymes.

quality of protoplasts was further evaluated by observing their behavior during the washing process following enzyme treatment. Presence of large numbers of chloroplasts among the protoplasts indicated unceasing breakage of protoplasts during the wash. This usually took place at higher frequency when leaves used for protoplast isolation were harvested from plants less than 25 days after transplantation and was attributed to unstable physiological properties and/or unstable physical structure of the membrane of protoplasts from juvenile plants.

White Burley tobacco leaves yielded, on the average, about 2.0 (0.76-3.83) x 10^6 protoplasts per gram of fresh leaf tissue. Such yields were approximately 20% of the yields of palisade protoplasts isolated from Xanthi tobacco (113). The size of isolated protoplasts ranged from 19 to 55 um, with an average of 34 um, in diameter (dia.). Specifically, in three experiments, 5.7% of the protoplasts were less than 20 um in dia., 26.4% were 21-30 um in dia., 47.1% were 31-40 um in dia., and 21% were 41 to 55 um in dia. Older leaves tended to contribute a larger proportion of small protoplasts and/or protoplasts containing sparse chloroplasts.

All freshly isolated protoplasts, including the misshaped ones, fluoresced after FDA staining (Fig. 7).

During the PLO-mediated virus inoculation process, variation in the physiological properties of protoplasts was further noticed. The variation expressed itself either as different degrees of aggregation of protoplasts during inoculation incubation, or as different survival rates of the inoculated protoplasts. Macroscopically, aggregation of protoplasts into tiny clusters during inoculation incubation was a sign



700 X

Figure 7. Physiologically active tobacco protoplasts fluorescing green after treatment with 0.01% fluorescein diacetate (FDA).

of subsequent poor survival of inoculated protoplasts, especially if the inoculated protoplasts stayed as clumps and did not resuspend well after being centrifuged. When such clumps were examined under the microscope, a part or the majority of the protoplasts in the clumps were either broken or misshapen. Sometimes, even well-shaped inoculated protoplasts died after overnight incubation, especially if protoplasts were obtained from leaves of a young plant (< 40 days after transplantation). Protoplasts obtained from leaves of older plants (> 70 days after transplantation) tended to aggregate severely during inoculation incubation.

Survival rates of inoculated protoplasts varied considerably. In some of the best protoplast preparations, 30% of the inoculated protoplasts survived 120 hrs whereas in other preparations only 5% of the protoplasts survived 48 hrs. Generally, in more than 50% of the experiments, 30 to 50% of inoculated protoplasts survived more than 48 hrs.

Sensitivity of ELISA in Detecting PVY

When purified PVY was diluted 10-fold in coating buffer, the presence of even 1 ng PVY/ml (the lowest concentration tested) was sufficient to yield a positive ELISA result. On the other hand, if a 1:20 dilution of healthy Samsun NN leaf sap (H-sap) in coating buffer was used as diluent for the purified PVY, more than 10 ng, but less than 100 ng, PVY per ml was required to yield a positive result under the same conditions (Table 2). Therefore, when healthy sap rather than coating buffer was present with PVY, ELISA became nearly 100-fold less

Table 2. Minimum PVY concentration detectable by ELISA when the virus was diluted either in buffer or in 1:20 dilution of healthy sap.

EXPERIMENT NUMBER	CONCENTRATION OF PVY (ng/ml) 10,000	1,000	100	10	1	1:20 H-SAP
1(x)	1.844(z)	1.072	0.306	0.188	0.162	0.213
1(y)	OVER	OVER	OVER	0.943	0.184	
2(x)	OVER	0.914	0.298	0.154	0.131	0.106
2(y)	OVER	OVER	OVER	1.286	0.395	
3(x)	OVER	1.454	0.334	0.190	-	0.180
4(x)	1.947	1.004	0.420	0.189	0.107	0.075
AVERAGE(x)	OVER	1.111	0.340	0.180	0.133	0.143
(y)	OVER	OVER	OVER	1.114	0.289	

(x): Diluted in a 1:20 dilution of healthy sap.
(y): Diluted in coating buffer.
(z): ELISA values -- absorbance at 405 nm.

sensitive. This was also true when sap extracted from PVY-infected leaves (PVY-sap) was tested instead of purified PVY.

In a later experiment, in which 2-fold dilutions were tested (Fig. 8), it was shown that the ELISA dilution-end-point of purified PVY in a 1:20 dilution of healthy sap was 39 ng/ml. A 2-fold increase in PVY concentration resulted in an increase of 0.2 to 0.3 units of A405 value.

When 1×10^6 protoplasts isolated from a PVY-infected Samsun NN leaf (PVY protoplasts) were diluted in healthy protoplasts prior to grinding or in a 1:20 dilution of healthy sap after the protoplasts had been ground, the resulting ELISA values at the same dilutions were essentially the same (Fig. 9). In most preparations of PVY protoplasts 20 to 50% of the protoplasts were infected with PVY, as judged by FITC staining. When 1×10^6 PVY protoplasts were diluted 10-, 100-, and 1000-fold in 1:20 dilution of healthy sap, they gave 0.777 (0.608-1.100), 0.205 (0.154-0.342), and 0.110 (0.082-0.185) ELISA values, respectively, as compared to 0.135 (0.107-0.203) for a 1:20 dilution of healthy sap. Meanwhile, 10-, 100-, and 1000-fold dilutions of 1×10^6 PVY protoplasts with healthy protoplsts, so that the total number of 1×10^6 protoplasts per ml was maintained, gave ELISA values of 0.630 (0.337-0.989), 0.098 (0.082-0.115), and 0.052 (0.032-0.101), respectively, as compared to 0.050 (0.032-0.075) ELISA value for 1×10^6 healthy protoplasts. PVY protoplasts diluted 10^4- and 10^5-fold with healthy protoplasts gave ELISA values lower than 0.050 (0-0.081).

These results indicate that ELISA failed to detect the presence of PVY if PVY-infected protoplasts accounted for less than 0.2% (100-fold

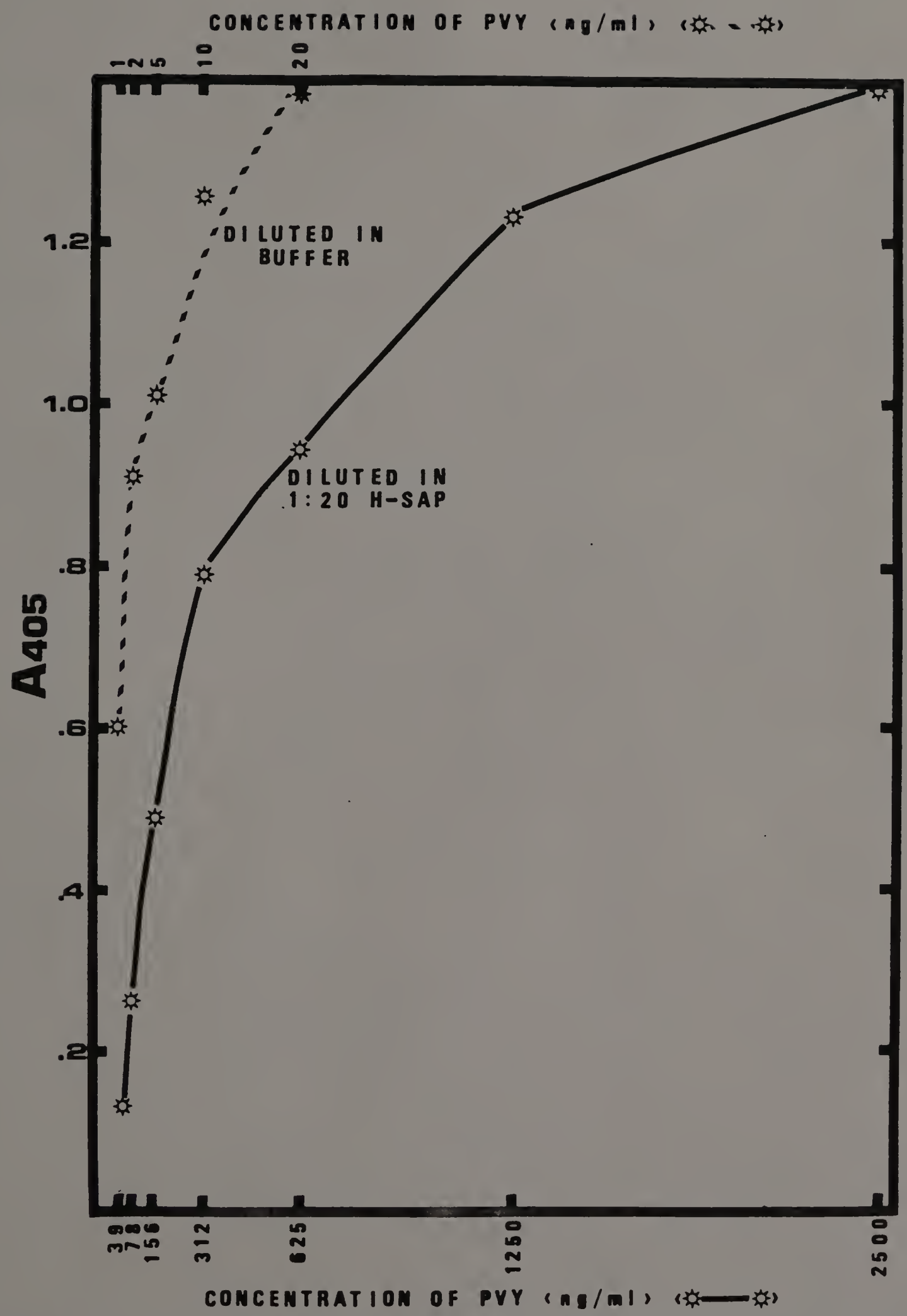


Figure 8. ELISA reading at 405 nm of two-fold dilutions of purified PVY diluted either with coating buffer or with a 1:20 dilution of healthy sap.

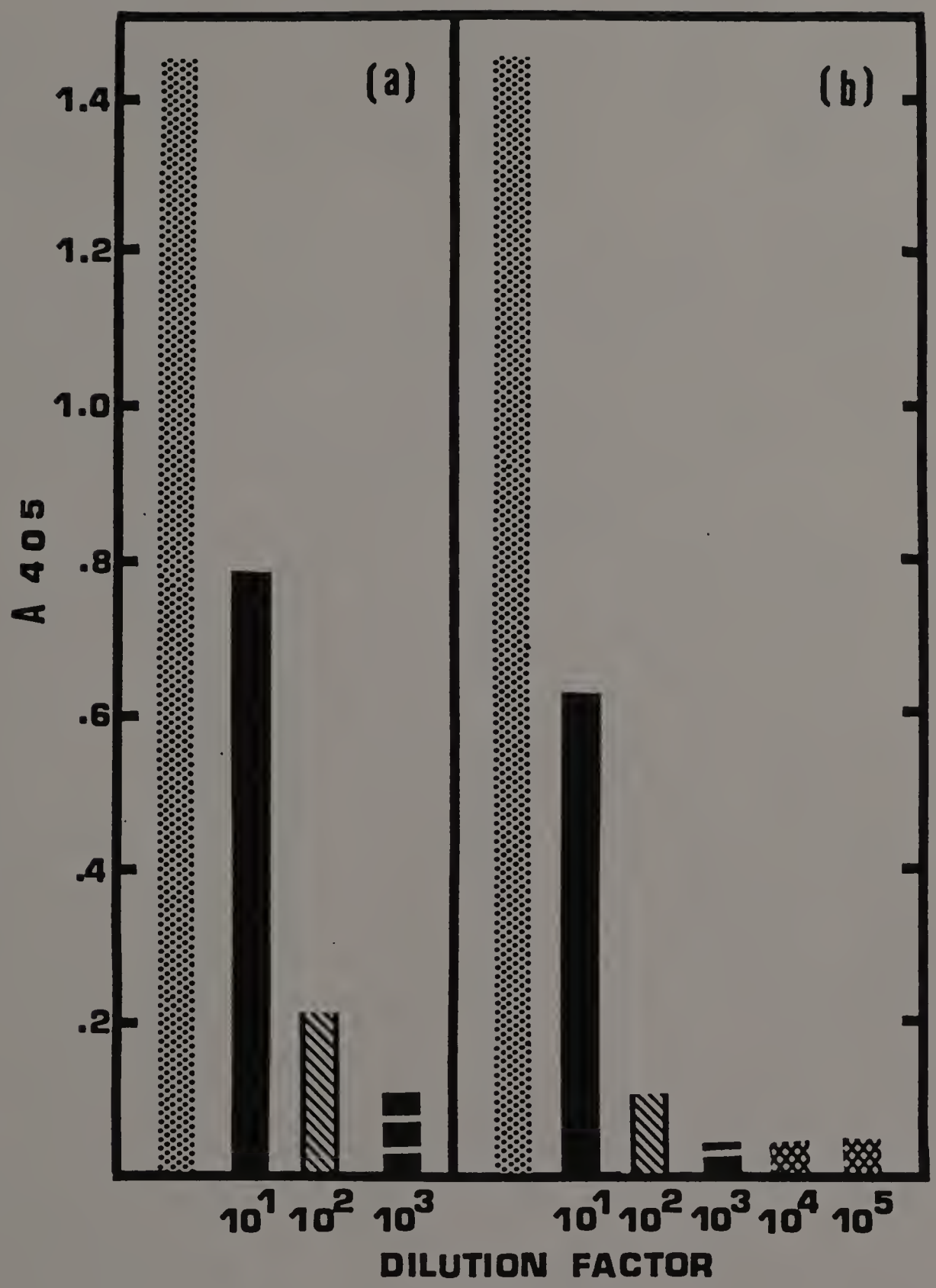


Figure 9. ELISA values of 1 x 10^6 protoplasts obtained from leaves of PVY-infected tobacco plants and diluted 10-fold either with with healthy protoplasts or in 1:20 dilution of healthy sap (H-sap).

dilution) of the population of the total (1×10^6) protoplasts.

Sensitivity of Bioassay on Local Lesion Host

When selected concentrations of different purified PVY preparations were inoculated on Chenopodium amaranticolor during a 6-month period, the numbers of local lesions produced consistantly indicated that the lowest amount of PVY detectable by C. amaranticolor was in the range of 0.1 to 0.5 ug/ml (Table 3). There was an excellent correlation between the concentration of PVY in inocula within the range of 10 to 0.5 ug/ml, and the number of lesions incited on C. amaranticolor. The bioassay data presented here are quite representative, although sometimes in other experiments 10 ug/ml of PVY gave as many as 100 local lesions per leaf. Such differences probably point out the existing variation in the specific infectivity of different preparations of purified PVY.

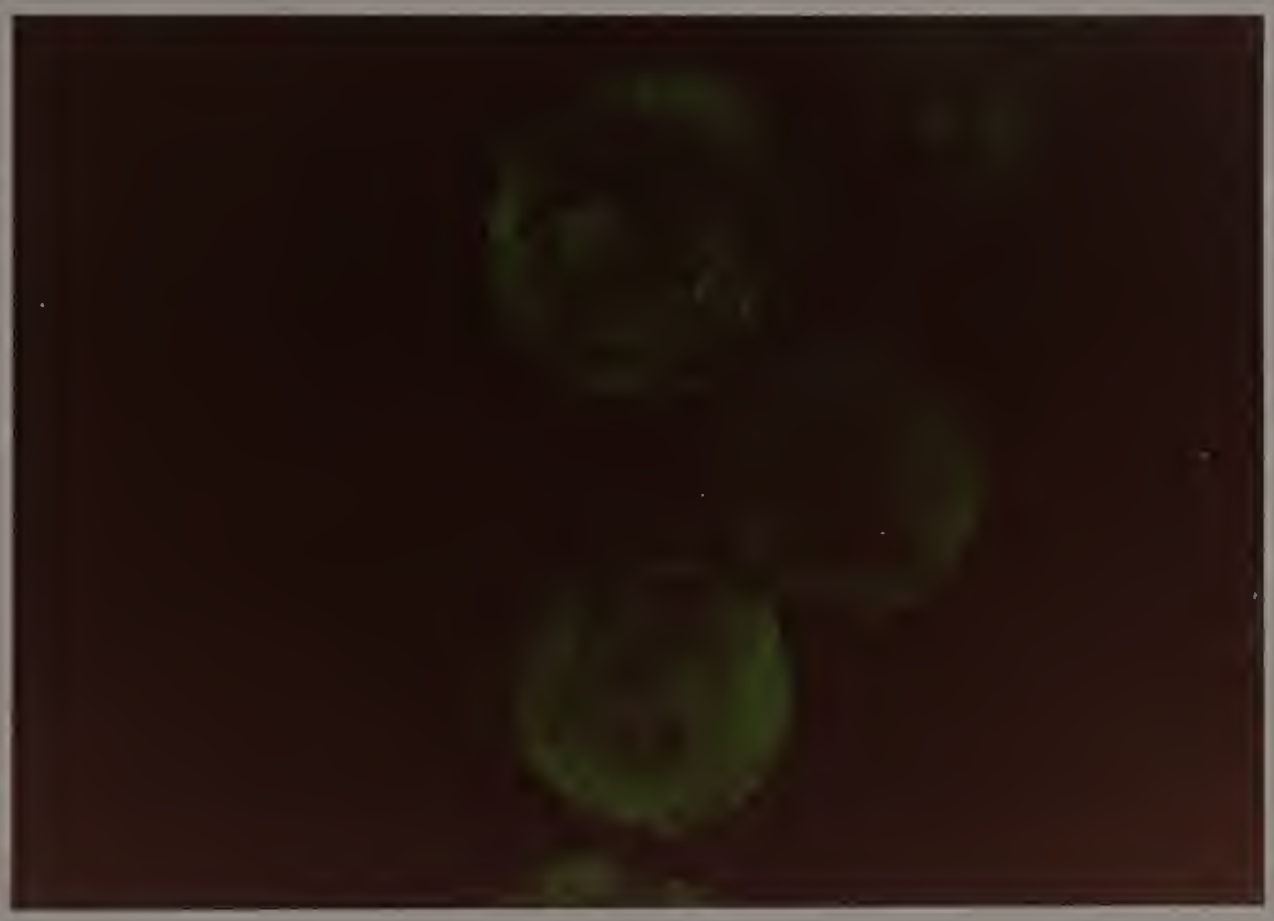
Fluorescent Antibody Staining

Protoplasts stained immediately after postblocking washes (in the case of PLO-mediated inoculation) showed very weak fluorescence homogeneously distributed all over the surface of protoplasts (Fig. 10). Meanwhile, protoplasts stained 48 hrs after inoculation showed much stronger fluorescence as big patches which distributed in an irregular pattern on the infected protoplasts (Fig. 11). Protoplasts which were inoculated in the presence of liposomes (MLVs), showed spotty fluorescence at time zero.

Table 3. Minimum PVY concentration detectable by sap inoculation of the local lesion host Chenopodium amaranticolor.

EXPERIMENT NUMBER	CONCENTRATION OF PVY (ug/ml)						
	10	5	1	0.5	0.1	0.05	0.01
1	26.75(x)	-	1.75	-	-	-	-
2	60.00	16.00	3.25	1.80	-	-	-
3	-	16.60	1.80	0.60	0	0	0
4	-	20.80	6.25	2.60	0.80	0	0
AVERAGE	43.37	17.80	3.26	1.70	0.40	0	0

(x): Number of local lesions/leaf.



700 X

Figure 10. Weak fluorescence associated with inoculated protoplasts at zero time after fluorescent antibody staining.

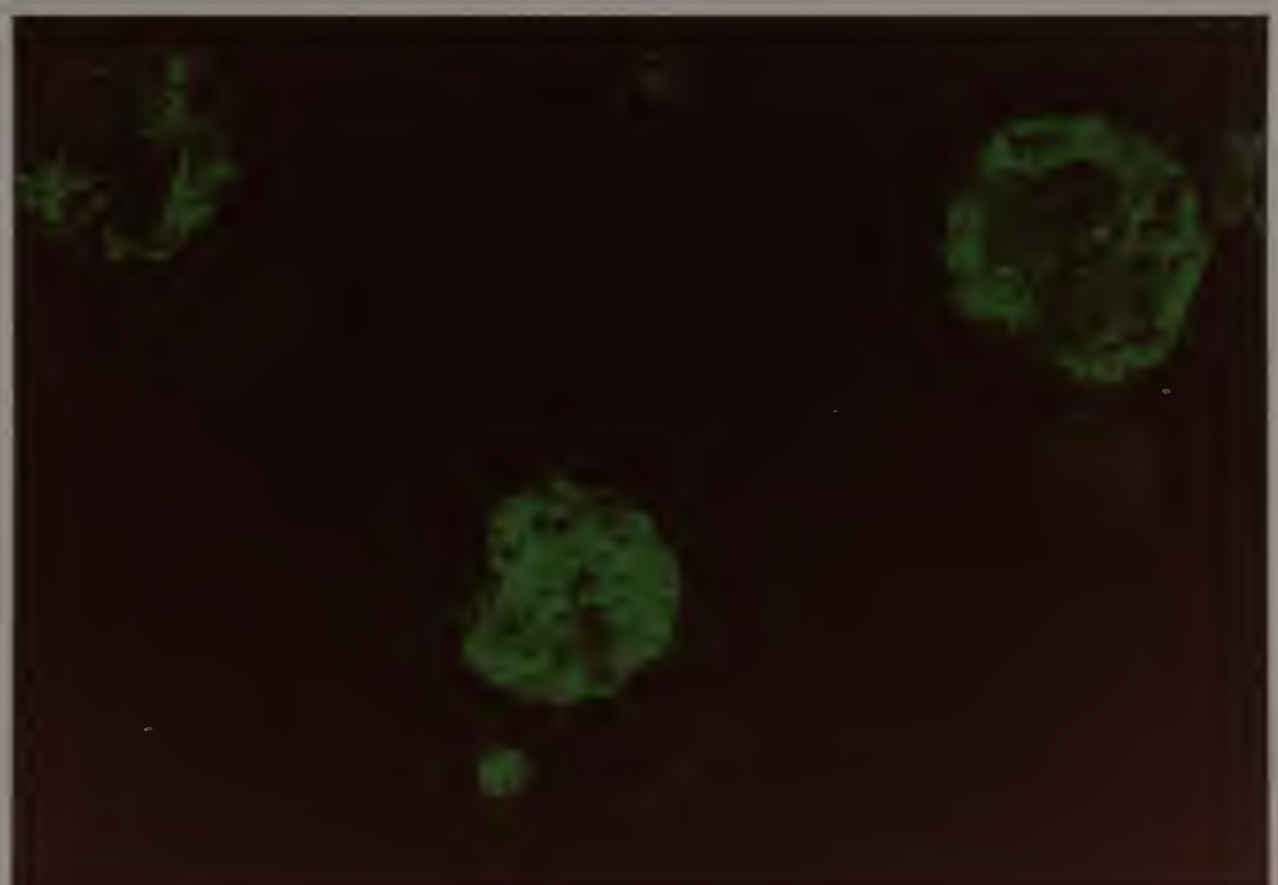


Figure 11. Strong fluorescence associated with inoculated protoplasts at 48 hrs after fluorescent antibody staining.

Relationship of Data Generated by the FITC Staining, Bioassay, and ELISA Methods

Protoplasts isolated from PVY-infected Samsun NN tobacco leaves were subdivided into three parts. Each part was then either stained with FITC-conjugated antibody to measure the percentage of infection, or assayed by ELISA, or bioassayed by inoculating *C. amaranticolor*. FITC staining revealed that only about 30% of the protoplasts were actually infected, i.e. contained sufficient PVY antigen to be detected by fluorescent antibody staining. The ELISA values of 10-fold dilutions of 5×10^6 PVY protoplasts corresponded to the ELISA value given by 10-fold dilutions of 10 ug/ml purified PVY (Table 4). The numbers of lesions incited by the same number of PVY protoplasts on *C. amaranticolor* were comparatively low (Table 4). When a similar comparison was made with 10-fold dilutions of 1×10^6 PVY protoplasts, the PVY content of the starting preparation appeared to be equivalent to between 1 and 10 ug. Since the percentage of infection and the PVY content in 5×10^6 PVY protoplasts, as well as the M.W. of PVY (6×10^7 dal.), are known, the average number of PVY particles per individual infected protoplast can be computed as follows:

$$\frac{[10\ (\text{ug/ml}) \times 1.2\ \text{ml}] \times \dfrac{6.02 \times 10^{23}\ (\text{particles/mole})}{6 \times 10^7\ (\text{g/mole})}}{30\% \times (5 \times 10^6\ \text{protoplasts})}$$

$$= 80{,}000\ (\text{particles/infected protoplast})$$

Table 4. Relationship of values obtained by ELISA and of numbers of local lesions obtained by bioassay in evaluating virus content in PVY-infected protoplasts.

DILUTION FACTOR	5×10^6 PVY PROTOPLASTS		1×10^6 PVY PROTOPLASTS		10 ug/ml PURIFIED PVY	
1(x)	1.618(y)	16(z)	1.561(y)	4(z)	1.844(y)	40(z)
10	1.197	4.5	0.626	0.5	1.072	7.5
100	0.447	1.5	0.176	0	0.306	1
1000	0.225	-	0.144	-	0.188	-
10000	0.180	-	0.178	-	0.162	-

(x): Protoplasts ground in 1.2 ml of coating buffer.
(y): ELISA values of samples diluted in a 1:20 dilution of healthy sap.
1:20 diluted healthy sap had an A405 value of 0.120.
(z): Bioassay results. Number of local lesions/leaf.

Starch-iodine Lesion Staining

Starch-iodine lesions appeared on PVY-inoculated Samsun NN leaves 4 days after inoculation as pale spots surrounded by a purple background (Fig. 12). The lesions continued to enlarge on day 5 and by day 6 they coalesced and lost their individuality. No starch-iodine lesions could be recognized the first three days after inoculation with PVY. In 6 experiments of starch-iodine lesion staining, only one experiment gave descending numbers of lesions that correlated well with 10-fold dilutions of the inoculum (230, 135, and 23 lesions for 100, 10, and 1 ug/ml of PVY, respectively). In the other five experiments only the two inocula at higher PVY concentration could be differentiated from the third inoculum by starch-iodine lesion counting (200, 189, and 41 lesions, on the average, for 100, 10, and 1 ug/ml of PVY, respectively). Occasionally, faint chlorotic spots could be seen on unstained PVY-inoculated Samsun NN leaves. Both the number and distribution of the chlorotic spots coincided with those of starch-iodine lesions after staining.

Bioassay on a Systemic Host by Using the ELISA Technique

ELISA values of leaf sections inoculated with 100 ug/ml of PVY began to increase by day 3 after inoculation and the values continued to increase for several days afterwards (Fig. 13). Replication of PVY in leaf sections inoculated with either 10 or 1 ug/ml of PVY had a lag phase of 4 days as determined by ELISA (Fig. 13). According to Anova

Figure 12. Starch-iodine lesions present on a Samsun NN leaf inoculated with PVY and treated with I_2-KI 4 days after inoculation.

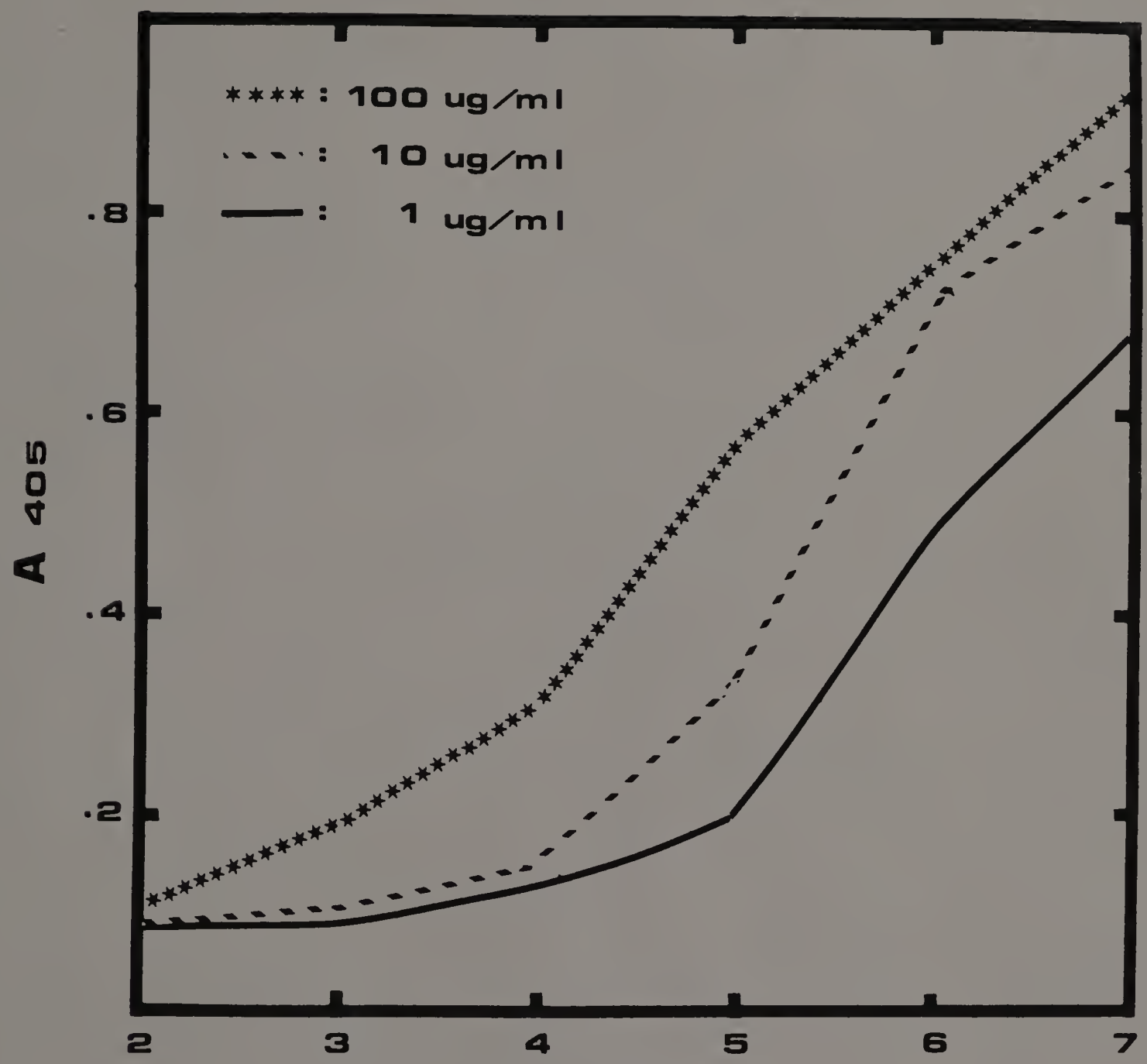


Figure 13. A time study on the accumulation of PVY in Samsun NN leaf sections inoculated with 1, 10, or 100 ug/ml of PVY as determined by ELISA.

tests of the data, there was no significant difference between any pair of sample means of inocula of different concentrations of PVY harvested at the same time, except for the pair of 100 and 1 ug/ml of PVY inocula harvested 5 days after inoculation (PROB = 0.0344 < 0.05).

The correlation between PVY concentrations in the inocula and ELISA values of inoculated leaf sections of the systemic host becomes more apparent when the differences are plotted on a daily basis (Fig. 14). The curve pattern in Figure 14 is divided into 2 nearly symmetric parts by the curve of day 5. A405 values at day 2 were the same as uninoculated control for all treatments regardless of PVY concentration in inoculum. Treatments with 100 ug/ml of PVY in the inoculum resulted in earlier increases in PVY titer and maintained the fastest PVY replication up to day 5. After that day, virus replication in the two treatments with lower PVY concentration in the inoculum caught up to that in the highest PVY concentration in the inoculum.

PLO-Mediated Inoculation of Protoplasts with PVY

Evidence for infection of protoplasts by PVY

Infection of protoplasts by PVY was confirmed by both FITC staining (Fig. 10,11) and ELISA (see also Table 14). Of the 19 protoplast inoculation experiments considered positive by ELISA, 8 had 1% and 6 had 0% protoplasts infected with PVY, as ascertained by FITC staining. However, no positive infection was obtained upon bioassay of inoculated protoplasts on *C. amaranticolor*.

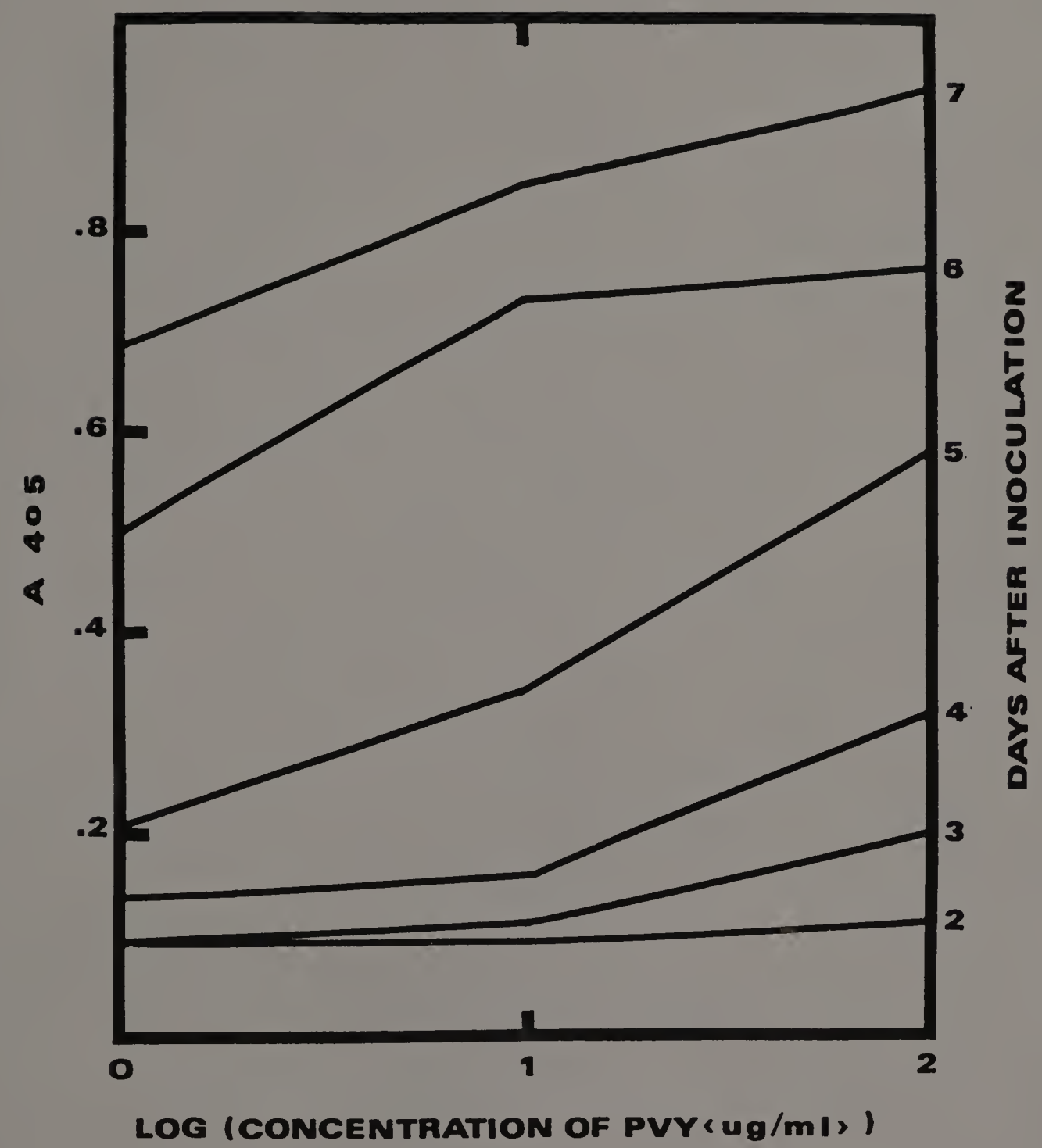


Figure 14. Values of the daily differences in PVY titer among leaf sections inoculated with three different concentrations of PVY as determined by ELISA.

Buffer and pH effect

In the presence of 0.02 M K-CB during inoculation incubation, A405 values of inoculated protoplasts decreased at both zero time and 48 hrs as the buffer pH was increased from 5.0 to 6.2 at 0.4 unit increments (Table 5). K-CB at pH 4.6 had a lower zero time ELISA reading than at pH 5.0, yet the subsequent increase in PVY titer was slightly greater in samples at pH 4.6. When 0.02 M K-PB was employed instead of K-CB, in 3 out of 4 protoplast inoculation experiments ELISA readings were lower than 0.1 for both zero time and 48 hr protoplasts (Table 6) and the results were, of course, considered negative. Same conclusion as for phosphate buffer was applied to inoculation of protoplasts in the presence of 0.02 M Tris-HCl, pH 8.0 (Table 7).

PLO/PVY concentrations effect

When PVY concentration was kept constant, the amount of PVY adhering to the membrane of protoplasts was approximately the same regardless of whether 10, 6, or 2 ug/ml of PLO was included in the inoculum. PVY titer at 48 hrs was the highest in treatments which contained PLO at 6 ug/ml regardless of whether 20, 10, or 2 ug/ml of PVY was included in the inoculum. The PLO/PVY concentrations at 6:20 and 6:10 ug/ml had the highest yield of PVY at 48 hrs while the 6:2 ug/ml ratio gave the lowest amount of virus adsorbed on protoplasts. PLO at a concentration of 10 ug/ml in the inoculum was detrimental to the survival of inoculated protoplasts (Table 8).

Table 5. Effect of pH of 0.02 M potassium citrate buffer at inoculation on PVY yield of tobacco protoplasts 48 hrs after inoculation as determined by ELISA.

pH OF 0.02 M K-CB(x)	A405 VALUES	
	ZERO TIME	48 HR
Exp.1		
4.6	0.306	0.566
5.0	0.528	0.653
5.4	0.369	0.286
5.8	0.156	0.149
6.2	0.121	0.094
Exp.2		
4.6	0.273	0.535
5.0	0.569	0.723
Exp.3		
4.6	0.500	0.796
5.0	0.856	1.027

(x): PLO and PVY concentrations were 6 and 10 ug/ml, respectively.

Table 6. Effect of pH of 0.02 M potassium phosphate buffer at inoculation on PVY yield of tobacco protoplasts 48 hrs after inoculation as determined by ELISA.

pH OF 0.02 M K-PB(x)	A405 ALUES ZERO TIME	48 HR
Exp.1		
6.0	0.043	0.074
6.4	0.051	0.056
6.8	0.040	0.047
7.2	0.031	0.035
7.6	0.016	0.054
8.0	0.018	0.043
Exp.2		
6.0	0.014	0.099
6.4	0.016	0.069
6.8	0.009	0.064
7.2	0.020	0.036
7.6	0.013	0.104
8.0	0.016	0.029
Exp.3		
6.4	0.322	0.338
7.2	0.247	0.329
8.0	0.154	0.204
0.02 M K-CB		
4.6	0.601	0.900
Exp.4		
6.4	0.066	0.149
7.2	0.064	0.136
0.02 M K-CB		
4.6(1)	0.097	0.265
4.6(2)	0.237	0.595

(x): PLO and PVY concentrations were at 6 and 10 ug/ml, respectively.

Table 7. Effect of 0.02 M Tris-HCl buffer at pH 8.0 on PVY yield of tobacco protoplasts 48 hrs after inoculation as determined by ELISA.

pH OF 0.02 M Tris-HCl(x)	A405 ALUES	
	ZERO TIME	48 HR
Exp.1		
8.0	0.010	0
Exp.2		
8.0	0.037	0.050
Exp.3		
8.0	0.051	0.017
0.02 M K-CB		
4.6	0.068	0.310

(x): PLO and PVY concentrations were at 6 and 10 ug/ml, respectively.

Table 8. Influence of various PLO/PVY concentration combina-tions on PVY yield of inoculated tobacco protoplasts as determined by ELISA.

PLO/PVY (ug/ml)	A405 VALUES	
	ZERO TIME	48 HR
10/20	0.788	0.777
10/10	0.412	0.657
6/20	0.850	0.993
6/10	0.556	0.873
6/2	0.090	0.365
2/20	0.672	0.843
2/10	0.432	0.736
2/2	0.169	0.307

PVY concentration effect

When PLO concentration in the inoculum was kept at 6 ug/ml, virus titer at 48 hrs actually increased as PVY concentration in the inoculum increased (Table 9). Inocula containing more than 2 ug/ml PVY tended to give a high zero time (background) ELISA reading.

Inocula containing either 2 or 1 ug/ml PVY were investigated further. Inoculum which contained 2 ug/ml PVY resulted in a greater net PVY yield at 48 hrs than inoculum containing 1 ug/ml PVY under a similar zero time PVY titer (Table 10).

Serological blocking of residual virus on the surface of inoculated protoplasts

When commercially produced sheep anti-PVY antiserum was used for serological blocking of PVY on the protoplast surface, the virus titer at zero time decreased by 60% (Table 11). When treatment of protoplasts with rabbit anti-PVY antiserum was followed by treatment with plain goat anti-rabbit antiserum (no enzyme marker), the level of surface virus blocking achieved was similar to that induced by sheep anti-PVY antiseum. The 50% of virus titer at zero time was substracted from the inoculated protoplasts after blocking treatment. Guinea pig anti-PVY antiserum was the most effective among the three antisera tested. It reduced the zero time virus titer by 96%.

In all the serological blocking experiments, both sheep and guinea pig anti-PVY antisera functioned in a consistent manner. The blocking effect provided by the combination of both rabbit anti-PVY antiserum and goat anti-rabbit antiserum was erratic and in some cases no blocking

Table 9. Effect of PVY concentration in the inoculum on PVY yield of inoculated tobacco protoplasts as determined by ELISA.

CONCENTRATION OF PVY (ug/ml)	A405 VALUES	
	ZERO TIME	48 HR
10	1.031	1.547
5	0.709	1.275
2	0.233	0.600
1	0.140	0.444
0.5	0.093	0.262

Table 10. Effect of PVY concentration in the inoculum on PVY yield of inoculated tobacco protoplasts as determined by ELISA.

CONCENTRATION OF PVY (ug/ml)		A405 VALUES ZERO TIME	A405 VALUES 48 HR
Exp.I	2	0.504	1.245
	1	0.354	0.617
Exp.II	2	0.275	0.914
	1	0.104	0.835
Exp.III	2	0.227	0.547
	1	0.175	0.238
AVERAGE	2	0.335	0.902
	1	0.211	0.563

Table 11. Effectiveness of various serological blocking methods on reducing A405 readings of inoculated protoplasts at time zero as determined by ELISA.

BLOCKING METHOD	A405 VALUES AT TIME ZERO	
	BEFORE BLOCKING	AFTER BLOCKING
1:50 Sheep anti-PVY antiserum	0.935	0.686
1:50 Rabbit anti-PVY antiserum + 1:50 Goat anti-rabbit antiserum	0.534	0.382
1:50 Guinea pig anti-PVY antiserum	0.872	0.130

effect could be detected.

Effects of $CaCl_2$ on virus adsorption and virus yields of inoculated protoplasts

Three molarities of $CaCl_2$ in the washing medium were tested to evaluate their effects on the removal of virus from the surface of inoculated protoplasts and on virus yields of inoculated protoplasts (Table 12). Increasing the concentration of $CaCl_2$ from 1 mM to 10 mM reduced the zero time ELISA reading of unblocked protoplasts from 1.155 to 0.810; 0.2 M $CaCl_2$ further decreased the ELISA reading to 0.706. Thus, $CaCl_2$ appeared to facilitate somewhat the removal of PVY residue from the surface of inoculated protoplasts during the postinoculation washing process.

The presence of high $CaCl_2$ concentrations in the washing medium did not enhance virus replication in inoculated protoplasts. Furthermore, after inoculated protoplasts had been washed with media containing either 10 mM $CaCl_2$ in 0.65 M mannitol or 0.2 M $CaCl_2$, the serological blocking treatment became less effective in blocking residual virus from the surface of inoculated protoplasts at zero time after inoculation. Calcium chloride at 0.2 M was toxic to protoplasts.

Effects of culture temperature and light on PVY yields in inoculated protoplasts

In all three experiments (Table 13), incubation of inoculated protoplasts at 4°C resulted in reduced PVY replication in the protoplasts. This is shown best by the differences of ELISA values of protoplasts

Table 12. Effect of calcium chloride concentration in the washing medium on zero time ELISA values and virus yields of inoculated tobacco protoplasts.

EXPERIMENT	A405 VALUES		
	1mM$CaCl_2$(x)	10mM$CaCl_2$(x)	0.2M$CaCl_2$(y)
I.0 Time w/o blocking	1.494	1.085	0.913
0 Time w/ blocking	0.138	0.174	0.357
48 hr	1.575	0.997	1.332
II.0 Time w/o blocking	0.486	0.420	0.764
0 Time w/ blocking	0.180	0.156	0.490
24 hr	0.647	0.774	0.890
III.0 Time w/o blocking	1.481	0.924	0.441
0 Time w/ blocking	0.023	0.044	0.130
24 hr	0.511	0.527	0.692
AVERAGE			
0 Time w/o blocking	1.155	0.810	0.706
0 Time w/ blocking	0.114	0.125	0.326
24 OR 48 hr	0.911	0.766	0.971

(x): Prepared in 0.65 M mannitol.
(y): Prepared in distilled water.

Table 13. Effect of light and temperature on PVY yield of inoculated tobacco protoplasts as determined by ELISA.

EXPERIMENT	A405 VALUES				
	4°C(D)	4°C(L)	4°C-25°C(DL)	25°C(D)	25°C(L)
I.0 Time	0.138				
48 hr	0.482	0.612	0.812	0.431	1.575
II.0 Time	0.180				
24 hr	0.312	0.392	-	0.307	0.647
III.0 Time	0.023				
24 hr	0.186	0.380	-	0.168	0.511
AVERAGE					
0 Time	0.114				
24 or 48hr	0.327	0.461	-	0.302	0.911

(D): Cultured in the absence of light.
(L): Cultured in the presence of light.
(DL): Cultured in the absence of light for 24hr at 4°C and then in the presence of light for another 24hr at 25°C.

kept at 25°C under light and at 4°C under light. The differences were 0.963, 0.255 and 0.131 for experiments I, II, and III, respectively. The average reading difference was 0.450 and it accounted for a 49% decrease in ELISA readings. The longer the inoculated protoplasts were cultured the greater the difference of the virus titer between 4°C and 25°C incubation in the presence of light [a greater difference (0.963) was obtained in experiment I, in which protoplasts were tested 48 hrs after inoculation, than that obtained in experiments II (0.255) and III (0.131), in which protoplasts were tested 24 hrs after inoculation]. In the absence of light, virus yields of inoculated protoplasts were very low and were the same regardless of culture temperature. In some other experiments, inoculated protoplasts kept for 48 hrs at 4°C in the dark gave ELISA readings similar to those given by protoplasts at zero time. When temperature was constant, light promoted PVY replication. In the presence of light, the higher temperature (25°C) stimulated PVY replication substantially.

Protoplasts survived much better at 4°C than at 25°C.

Yield of PVY in infected protoplasts

The PVY yield 48 hrs after inoculation was calculated by comparing the ELISA values of inoculated protoplasts with the ELISA values of the stndard dilution curve of purified PVY (in 1:20 dilution of healthy sap) established in each ELISA test. The amount of PVY produced by 3-5 x 10^6 inoculated protoplasts ranged from 0.1 to 1.0 ug (Table 14). The amount of PVY associated with the surface of the protoplasts (zero time) was less than or equal to 0.01 ug. Therefore, the yield of PVY at the end

Table 14. PVY yields of 3-5 x 10^6 inoculated tobacco protoplasts 48 hrs after inoculation as determined by ELISA.

EXPERIMENT NUMBER	PROTOPLASTS AT 0 TIME	PROTOPLASTS AT CULTURED	PURIFIED PVY (ug/ml) 10	1	0.1	0.01
1(48hr)	0.210(x)	0.410	OVER(x)	1.114	0.298	0.134
2(48hr)	0.121	0.890	,,	,,	,,	,,
3(48hr)	0.135	1.575	OVER	1.454	0.334	0.190
4(48hr)	0.180	0.650	,,	,,	,,	,,
5(48hr)	0.025	0.520	,,	,,	,,	,,
6(64hr)	0.060	0.660	1.844	1.072	0.306	0.190
7(64hr)	0.060	0.600	,,	,,	,,	,,
8(48hr)	0.030	0.290	OVER	1.482	0.565	0.264
9(48hr)	0.220	1.483	,,	,,	,,	,,
10(48hr)	0.060	0.900	,,	,,	,,	,,
11(48hr)	0.085	0.490	,,	,,	,,	,,
12(48hr)	0.150	0.980	,,	,,	,,	,,

(x): A405 values of PVY-inoculated protoplasts and of known amount of purified PVY diluted in a 1:20 dilution of healthy sap.

of 48 hrs was equivalent to a 10- to 100-fold increase in virus over the detectable amount of virus adsorbed onto the protoplasts. When the amount of PVY yielded by 3-5 x 10^6 inoculated protoplasts was converted into particle numbers, it was estimated that there were approximately 10^5 virus particles per infected protoplast .

PEG-Mediated Inoculation of Protoplasts with PVY

Tobacco protoplasts inoculated in the presence of PEG showed a slight increase in PVY titer at 48 hrs (Table 15). The 48-hr ELISA readings remained low (< 0.200) in 3 of 5 experiments. No fluorescent protoplasts could be detected after FITC-conjugated antibody staining. Sixty percent or more of the inoculated protoplasts survived at least 48 hrs.

Liposome-Mediated Inoculation of Protoplasts with PVY

Liposomes prepared according to the method of Rollo and Hull (1982) appeared as a solid pellet at the bottom of the centrifuge tube after 30 min centrifugation at 15,000 rpm. If the resuspension medium was supplemented with 0.05% Evan's Blue, the MLV pellet remained blue even after 3 washes and after overnight resuspension in resuspension buffer not containing Evan's Blue. EM photographs of MLVs showed round or nearly round vesicles 0.1 to 0.7 um in diameter with the majority (80%) of the vesicles having a diameter smaller than 0.3 um (Fig. 15). The multilamellar nature of the membrane of MLVs was clearly shown in the EM

Table 15. Results of polyethylene glycol (PEG)-mediated inoculation of tobacco protoplasts with PVY as determined by ELISA.

EXPERIMENT	A405 VALUES		
	0 TIME W/O BLOCK	0 TIME W/ BLOCK	48 HR
I	0.852	0.208	0.410
II	-	0.172	0.445
III	0.335	0.076	0.140
IV	0.475	0.048	0.127
V	0.285	0.067	0.170
AVERAGE	0.487	0.114	0.258

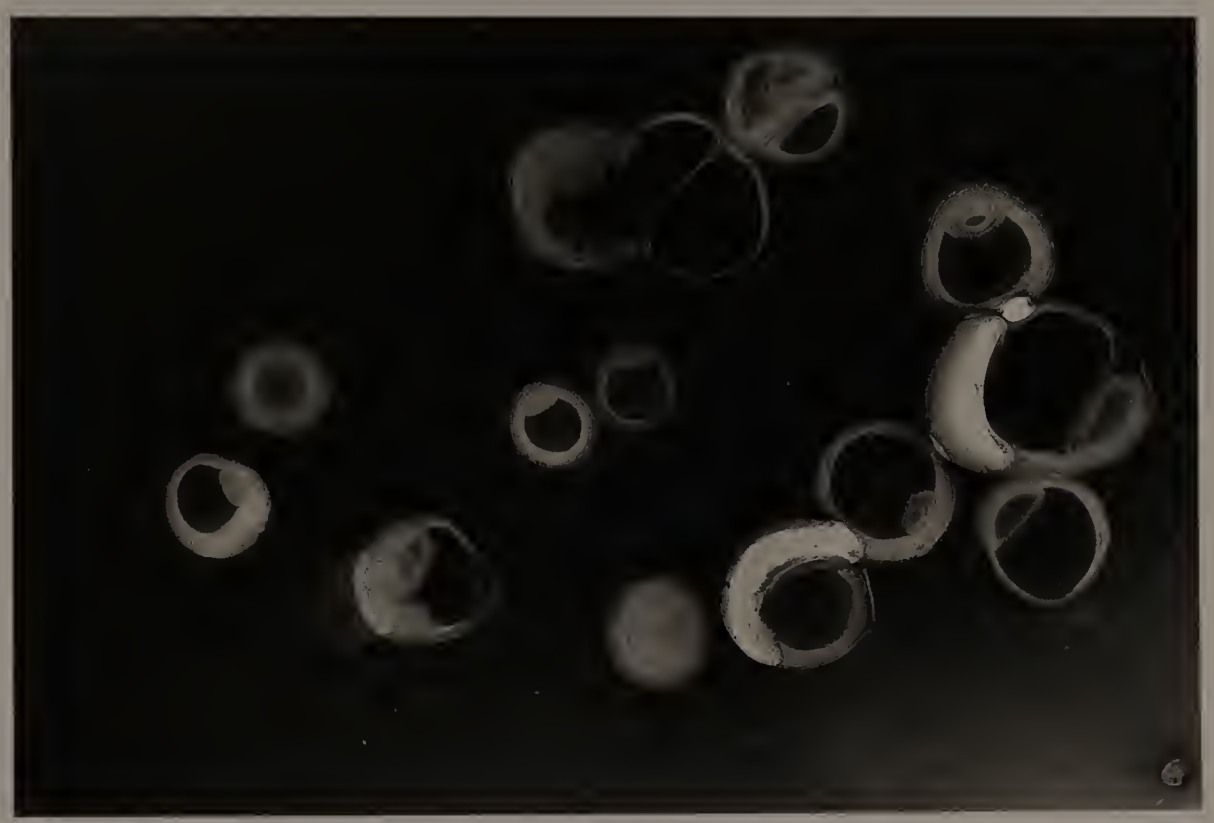


Figure 15. The multilamellar nature and compartmentalization of liposomes (MLVs) consisting of phosphotidylcholine (PC) and -sitosterol (SS) as revealed by electron microscopy.

photographs (Fig. 15). Partitioning of a single MLV into several small compartments was also common (Fig. 15).

When 0.01% FDA was added to the resuspension buffer and the resulting MLVs were mixed with preparations of healthy protoplasts, 88%, 80%, 70.6%, and 65% of the protoplasts exhibited fluorescence after fusion with FDA-containing MLVs prepared with lipid contents of 3.8, 1.9, 0.95, and 0.47 uM, respectively. Healthy protoplasts stained with 0.01% FDA in 0.65 M mannitol gave 100% fluorescent protoplasts. Sixty to eighty percent of the MLV-treated protoplasts survived for at least 48 hrs. No difference in survival rate was detected in protoplast preparations incubated with MLVs of varied lipid content.

The nature of PVY carried in MLVs was assessed by both bioassay and ELISA. When compared with the ELISA values of the standard curve of purified PVY in coating buffer, the amount of PVY carried by MLVs was estimated to be 2 ug/3.8 uM of lipid content. This was equal to about 1% of the virus present in resuspension buffer. However, if PVY was added after the MLVs had formed, the amount of PVY carried on the PVY-coated MLVs was similar to that of MLVs formed in the presence of PVY. On the average, 6 lesions per leaf were induced on C. amaranticolor inoculated with PVY-carrying MLVs (3.8 uM PC content dissolved in 0.6 ml of 0.1 M Na-PB, pH 7.6). The identity of the virus in these lesions was further ascertained by ELISA.

In the four liposome-mediated protoplast inoculation experiments performed, the ELISA readings at 48 hrs were the same or even lower than corresponding ELISA readings at time zero (Table 16). Two fluorescent protoplasts were observed in all the slides examined after staining of

Table 16. Results of tobacco protoplast inoculation with PVY mediated by liposomes at different phosphotidylcholine (PC) contents as determined by ELISA.

EXPERIMENT	CONTENT OF PHOSPHOTIDYLCHOLINE (PC)			
	3.8uM	1.9uM	0.95uM	0.47uM
I.0 Time	0.230(x)	0.300	0.289	0.450
48 hrs	0.200	0.190	0.150	0.160
II.0 Time	0.389	0.349	0.334	0.343
48 hrs	0.349	0.237	0.248	0.030
III.0 Time	0.160	0.078	0.108	-
48 hrs	0.207	0.179	0.133	
IV.0 Time	0.236	0.182	0.185	-
48 hrs	0.111	0.081	0.040	
AVERAGE				
0 Time	0.253	0.227	0.229	-
48 hrs	0.193	0.171	0.142	-

(x): All numbers are absorbance at 405 nm.

the protoplasts with FITC-conjugated antibody to the virus 48 hrs after inoculation.

CHAPTER V

DISCUSSION

Of the several published protocols for partial purification of PVY that were explored in this study, the one developed by Damirdagh and Shepherd (22), with minor modifications, was found superior to others for providing partially purified virus suitable for placing onto the column. Collection and UV-scanning of fractions containing the virus could be usually completed within 2 hrs. The entire column purification process, including the overnight incubation of sap with n-butanol, could be carried out in about 20 hrs, while purification by SDGC took about 60 hrs.

The advantages of column purification included not only savings in time required for high speed centrifugation and resuspension of the virus, preparation of sucrose density gradients, layering of the virus, centrifugation of the gradients, withdrawing the virus band and overnight dialysis, but also avoidance of particle fragmentation and aggregation and the loss of virus that usually occur in several of these steps. Also, gel filtration of viruses is a very mild separation process and may therefore, be useful for purification of viruses which are labile under traditional virus purification conditions.

One packing of the column with Sepharose CL-4B is generally good for eight to ten successive purification runs. The average cost of Sepharose CL-4B per run was estimated to be approximately one dollar. The problem of decreased flow rate with time could be sufficiently, although temporarily, solved by siphoning out the top 0.5-1.0 cm layer

of the gel where the contaminants responsiblefor the problem are trapped. Sepharose CL-4B gel has a molecular weight resolution range between 6 x 10^4 and 20 x 10^6 daltons. PVY has a molecular weight of 6 x 10^7 daltons. Thus, PVY was excluded by the gel pores and came out in the void volume.

The reported yield of PVY obtained by SDGC purification was 3-4 mg/kg (107) although yields of 10-25 mg/kg have been obtained by centrifugation at moderate centrifugal force (47) and as high as 40 mg/kg have been obtained by the more time-consuming and more expensive method of CsCl centrifugation (76). In the parallel comparison studies, column purification resulted in an average PVY yield of 15 mg/kg (with maximum yields of 25 mg/kg). This was approximately 3 times greater than the yields we obtained by SDGC in the parallel comparison experiments and those obtained through SDGC by Stace-Smith and Tremaine (107). The highest yield of PVY obtained throughout the entire investigations was 64 mg/kg.

The purity of column-purified PVY preparations, as determined by UV spectrophotometry and electron microscopy, was equal to that of SDGC purified PVY. Particle aggregation and fragmentation, determined by electron microscopy, showed that about 66% of column-purified and 40% of SDGC-purified PVY particles had lengths in the 650 to 750 nm range (Fig. 5). Various typical PVY particle lengths had been reported as 684 nm in purified preparations (26), 740 nm in partially purified preparations (77), and 730 nm in sap preparations. Our purified PVY preparations, both through column and by SDGC, had a typical particle length of 710-720 nm (Fig. 5). The decreased particle length homogeneity in SDGC-

purified PVY preparations was correlated with a reduction in specific infectivity (Fig. 4). It has been shown that the ratio of PVY infectivity to serological activity was often considerably reduced by the purification method used (7), presumably as a result of virus aggregation and fragmentation. The differences in local lesion numbers between experiments performed at different times could be explained by the differences in susceptibility of the assay host plant to PVY and/or differences in specific infectivity of the various batches of purified PVY.

Based on our data, column purification is highly recommended over SDGC for purification of small amounts of plant viruses that have elongated particles because it is faster and milder, and because it yields more virus of better particle length homogeneity and greater infectivity.

Source plants for protoplasts need proper growing conditions throughout their growth since any factors impeding the growth of the plants renders them unsatisfactory as sources of protoplasts useful for inoculation with viruses. Under our conditions, plants grown in a growth chamber (25°C-18°C/day-night, 16 hrs light at 3,000 lux) produced protoplasts of low yields and low quality, while plants grown in the greenhouse gave more and better protoplasts. The best protoplasts were produced by plants with fully expanded leaves from January to late May, before high temperatures became prevalent in the greenhouse. Insecticides sprayed on plants to control aphids and white flies drastically reduced efficiency of protoplast isolation and survival of protoplasts although the size and appearance of the plants seemed

normal. Therefore, only sanitation and physical barriers were used for insect control on these plants.

In our experiments, quantitative assay of PVY by ELISA and bioassay on C. amaranticolor proved satisfactory in terms of reliability and sensitivity (Tables 2 and 3). Starch-iodine lesion staining and bioassay on a systemic host followed by ELISA may be useful under some circumstances but were not as dependable as the first two methods. FITC detected viral antigen at the cellular level and was used to identify individual infected protoplasts. The intensity of fluorescence by FITC-stained protoplasts is, presumably, an indication of the amount of antigen present but it could not be measured quantitatively in these experiments. When PVY was diluted in coating buffer, ELISA was more than 500-fold more sensitive (DEP < 1 ng/ml) than bioassay on the local lesion host (DEP was 500 ng/ml). However, when PVY was diluted in a 1:20 dilution of healthy sap ELISA was only about 15 times more sensitive (DEP 39 ng/ml) than the bioassay. Gugerli (1978) compared the sensitivity of ELISA for PVY with a biological assay using A6 (Solanum demissum x S. tuberosum) leaves. The maximum dilution (either in buffer or 1:20-50 H-sap) of either potato or tobacco extracts in which PVY could be detected was 1,000 to 10,000 with ELISA and 20 to 200 with the A6 bioassay (41). Under our conditions, the DEP of sap extracted from PVY-infected Samsun NN tobacco leaves diluted with 1:20 dilution of healthy sap and tested by ELISA was 6,400. The ELISA test relies upon the sensitive detection of non-precipitating products of reactions, made possible by the use of enzyme-labelled antibodies (Clark and Adams, 1977). The decrease of ELISA readings in the presence of a 1:20

dilution of healthy sap as compared to parallel dilutions made in buffer was, presumably, caused by the interference from plant proteins in the healthy sap. Lommel et al (1982) reported a 0-50% decrease in ELISA values after addition of 0.06 to 200 ug/ml carnation protein or BSA into a purified CaMoV preparation in coating buffer (65). The interference was due to a competition between viral and nonviral protein for a finite number of binding sites on the solid phase. The same was true in experiments attempting to detect SMV in crude seed extracts (27). However, no interference was caused by 1:20 dilution of healthy sap in the bioassay of PVY on the local lesion host (data not presented). At a concentration of 1.5×10^6 tobacco protoplasts per ml, the protoplasts occupy about 1.5×10^{10} um^3 which corresponds to 1.5% of the total suspension volume (130). Therefore, 1:20 dilution of healthy sap was equivalent to approximately 5×10^6 protoplasts per ml -- the number of protoplasts used for ELISA and bioassay in our study. Quantification of virus content by ELISA has been used in plant virus (120) as well as in animal virus studies (21).

Even 10- to 100-fold differences in virus concentration in the inoculum used to infect leaves of a systemic tobacco host of PVY did not induce significant changes in ELISA values 48 hrs (day 2) after inoculation (Fig. 13). The attempt to develop a bioasssay method for PVY that might be more sensitive than the bioassay on C. amaranticolor failed, as judged by statistical analysis of the results. However, there was an obvious trend of increasing ELISA values with increasing virus concentrations in inocula. A major reason for the failure of such values to show significant differences may have been that the variations between

some experiments were so great that the differences caused by changes in virus concentration in the inocula were over-shadowed.

Before the serological blocking method was employed, inoculated protoplasts harvested at time zero and stained with fluorescent antibody showed such an intensive fluorescence that identification of infected protoplasts by the same staining method, even at 24 or 48 hrs after inoculation, was not possible. The same problem was encountered by Barker and Harrison (1984) in their attempt to infect protoplasts with PVY regardless of whether the PLO or PEG method was used. Protoplasts from Xanthi tobacco and from several cultivars of potato equally bound PVY during inoculation incubation. Since this phenomenon was also observed in protoplasts from White Burley, Samsun NN and Connecticut Broad Leaf Burley tobacco -- cultivars used in our laboratory -- it may be concluded that this characteristic is related to the virus rather than the host.

The amount of PVY retained by 15 x 10^6 tobacco protoplasts was calculated as 0.36 to 3.6 ug, accounting for approximately 1 to 10% of the virus content in the inoculum. This was equivalent to 300-3,000 PVY particles per protoplast. Other researchers reported that the number of TMV particles adhering to individual tobacco protoplasts ranged from 100 to 8,000 (93,115,127,130). Figures within a similar range were reported for CPMV on cowpea protoplasts (43) and BSMV on barley protoplasts (15). In the CCMV-tobacco protoplast system, the number of particles adsorbed per infected protoplast at time zero ranged from 760 to 12,000 (81). Motoyoshi et al. (1974) calculated a value of 11.8 (in the absence of PLO) and 26.4 ug (in the presence of PLO) of BMV V5 in 10^6 inoculated

protoplasts at one hour after inoculation. Such an amount of virus corresponded to about 130,000 particles per protoplast. Nevertheless, this high zero time virus titer was not detectable by immunofluorescence and did not interfere with the identification of infected protoplasts at 24 hrs after inoculation (82). Therefore, the high intensity of fluorescence at zero time in the PVY-protoplast system can not be readily explained as due to a greater number of PVY particles adsorbed onto protoplasts. It has been reported that CaMV forms a stable association with Brassica protoplasts that is resistent to repeated washing (72). However, the virus particles revealed by FITC staining immediately after post-inoculation washes disappeared by 24 hrs after inoculation and rendered identification of infected protoplasts feasible (72). In our system, the intensity of the fluorescence associated with zero time protoplasts without blocking treatment was reduced considerably after 24 hrs incubation. However, we could not be sure whether the protoplasts fluorescing at 24 or 48 hrs were truly infected or were merely exhibiting fluorescence that was carried over from zero time.

Infection of tobacco protoplasts by PVY in the presence of PLO was shown by both FITC staining and ELISA. Since FITC staining never revealed an infection rate higher than 1%, it was apparent that it could not be used to detect changes in infection rate caused by other treatments. Therefore, all further studies were monitored with ELISA. The failure of the infectivity assay to detect infection of protoplasts by PVY was probably due to the low yields of PVY in inoculated protoplasts and to the low sensitivity of the local lesion host (C. amaranticolor) to PVY (DEP 0.1 to 0.5 ug/ml).

Before the serological blocking treatment was developed, the effect of various treatments on the amount of PVY adsorbed on the surface of protoplasts at zero time and on the yield of PVY in inoculated protoplasts after incubation for certain period of time had to be considered in order to make a decision regarding further studies. A treatment would be considered superior to others if it induced a low zero time A405 reading and a high virus yield.

Three buffer systems with pH ranging from 4.6 to 8.0 were compared for their effects on yields of PVY in inoculated protoplasts (Tables 5, 6, and 7). The results indicated that the increase in PVY yield at 48 hrs was directly related to the decrease in pH value of the buffer used in the inoculation process. The same relationship was found between the buffer pH and the amount of virus adhering to the protoplast membrane immediately after inoculation (zero time ELISA readings). Therefore, a direct relationship could be established between the amount of PVY being retained on protoplasts at time zero (up to a point) and the yield of PVY at 48 hrs in the presence of PLO in the inoculum. The same was true for the CCMV-tobacco protoplast system (81), BSMV-barley protoplast system (15), TMV V5-tobacco protoplast system (82), and TMV-tobacco protoplast system (113,130). However, when only the net increase of virus in inoculated protoplasts was considered, only 0.02 M K-CB at pH 4.6 and 5.0 resulted in an increase in PVY titer at 48 hrs (Table 5) and no virus replication could be detected in the presence of inoculation buffers with pH higher than 5.0. The zero time A405 readings were lower at pH 4.6. Based on these observations, potassium citrate buffer at pH 4.6 was selected for further studies.

The increase in A405 values at time zero was directly related to the concentration of PVY but not that of PLO in the inoculum (Tables 8, and 9). PVY at 10 ug/ml appeared to give the largest net increase of PVY in inoculated protoplasts at 48 hrs at all concentrations of PLO tested. PLO at 2 and 6 ug/ml induced similar retention of PVY by protoplasts but more PVY was produced by 48 hrs in the presence of 6 ug/ml of PLO. Increased concentrations of virus and PLO in the inoculum were reported to enhance retention of virus by protoplasts in other virus-protoplast systems (130). Since PLO at 10 ug/ml in the inoculum was toxic to protoplasts, PLO was used at 6 ug/ml to further determine the optimum concentration of PVY for protoplast inoculation. Xu et al. (1984) reported serious losses of protoplasts when they were inoculated in the presence of 20 ug/ml PLO as suggested by Goffinet and Verhoyen (1979). In our experiments, serious losses of protoplasts occurred even in the presence of 5 ug/ml of PLO in the inoculum-protoplast mixture.

No conclusive relationship was observed between the PLO/PVY combination and the net increase of PVY at 48 hrs (Table 8). However, it appeared that higher PLO/PVY ratios may have lowered the zero time background ELISA readings since at the highest PLO/PVY ratio (6/2 ug/ml) the zero time ELISA value was the lowest (0.090).

When PLO concentration was held at 6 ug/ml, the net increase of PVY in inoculated protoplasts at 48 hrs increased as the PVY concentration in the inoculum increased up to 10 ug/ml (Table 9). PVY concentration at 2 and 1 ug/ml yielded smaller amounts of virus as compared with protoplasts inoculated with 5 and 10 ug/ml of PVY. However, the A405 values of protoplasts inoculated with either 2 or 1 ug/ml of PVY at time

zero were much lower than those inoculated with 5 or 10 ug/ml of PVY. Therefore, PVY concentrations at 2 and 1 ug/ml were selected over the others to perform further comparative studies. As shown in Table 10, the net increase of PVY in inoculated protoplasts at 48 hrs was much higher when 2 ug/ml of PVY was used in the inoculum under an over all similar zero time A405 background readings. Therefore, 6 ug/ml PLO and 2 ug/ml PVY in 0.02 M K-CB at pH 4.6 were used as the standard inoculum.

Before serological blocking was included as a procedure that followed inoculation, more than 60% (40 out of 59) of the protoplast inoculation experiments gave inconclusive results in regard to protoplast infection with PVY because the high zero time background ELISA reading interfered with the detection of infected tobacco protoplasts. No such problem is mentioned in any report concerning virus inoculation of protoplasts except in the report of Barker and Harrison (1984) who also tried to infect protoplasts with PVY. They noted that, because of high background PVY levels on the surface of protoplasts, they could not detect infected protoplasts after FITC staining. Since both FITC staining and ELISA are serological tests involving different markers, it was not surprising that ELISA was interfered with in a similar manner. Thus, the serological blocking treatment was used to minimize the interference and to enable us to detect protoplast infection. Actually, two types of blocking treatment were employed: direct blocking of the available antigenic determinants of adsorbed PVY by antibodies developed in an animal other than rabbit, thereby inhibiting the binding rabbit anti-PVY antibodies with PVY during ELISA; and indirect blocking of the available antigenic sites of rabbit immunoglo-

bulin by goat anti-rabbit antibodies without enzyme marker, thereby inhibiting the binding of goat anti-rabbit antibodies, which are conjugated with enzyme, with rabbit immunoglobulin during ELISA. Theoretically, both direct and indirect blocking methods should be equally effective in minimizing the A405 values and the intensity of fluorescence of inoculated protoplasts at time zero. In practice, however, direct blocking was more effective than indirect, especially when guinea pig anti-PVY antiserum was used as the blocking antiserum (Table 11).

Calcium chloride at 0.1 mM was usually included in 0.65 M mannitol in routine post-inoculation washes as suggested by Takebe and Otsuki (1969). However, Morris-Kusinich et al. (1979) found that $CaCl_2$ at 1 mM was better than 0.1 mM for stabilizing inoculated protoplasts. Okono and Furusawa (1978a) and Hibi et al. (1975) used calcium chloride at 10 mM in 0.65 M mannitol to wash protoplasts after inoculation. Luciano (1984) suggested the use of 0.2 M $CaCl_2$ for post-inoculation wash to remove residual virus from the surface of protoplasts. This concentration was effective in removing TVMV (a potyvirus) from inoculated tobacco protoplasts (personal communication). This information encouraged us to study the effect of calcium chloride concentration in the washing medium on zero time ELISA values as well as on the virus yields in PVY-inoculated tobacco protoplasts (Table 12). Both 1 mM and 10 mM $CaCl_2$ were prepared in 0.65 mannitol. Calcium chloride at 0.2 M has an osmotic pressure high enough to serve as osmoticum so that mannitol way be omitted. The effect of 1 and 10 mM $CaCl_2$ on survival of tobacco protoplasts was the same. When the $CaCl_2$ concentration reached

0.2 M, however, th protoplasts exhibited serious aggregation. High-$CaCl_2$-induced aggregation of protoplasts could be reversed by washing protoplasts several times in 0.65 M mannitol containing 1 mM $CaCl_2$, but the protoplasts had been damaged permanently. Virus titer at time zero was inversely related to the concentration of $CaCl_2$ in the washing medium (Table 12). Surprisingly, an inverse relationship was also observed between $CaCl_2$ concentration in the washing medium and the efficiency of the serological blocking treatment. The concentration of $CaCl_2$ in the washing media did not substantially affect the yield of PVY in protoplasts (Table 12).

Light and temperature have been reported to affect the infection and replication of virus in intact plants (71) as well as protoplasts (1,48,71,95,97,129). This was also true in the PVY-tobacco protoplast system. As in the TYMV-turnip protoplast system(97), light appeared to be required for replication of PVY in inoculated tobacco protoplasts. In the presence of light, temperature became the limiting factor for virus replication. PVY replicated much more efficiently in inoculated protoplasts cultured at 25°C than at 4°C (Table 13). The yields of PVY in inoculated protoplasts were in the range of 0.1 to 1.0 ug per 3-5 x 10^6 protoplasts of which no more than 1% were infected. Therefore, the number of PVY particles produced per infected protoplast was approximately 2.4 x 10^5. The reported yields of virus particles per infected protoplast range from 10^4 (79) to 10^5 (84) but are close to 10^6 in most cases (43,49,78,97,115). A rough time course of PVY replication in tobacco protoplasts could be drawn from the data in Table 12. In experiment III, inoculated protoplasts were harvested 24 hrs after

inoculation while in experiment I protoplasts were harvested 48 hrs after inoculation. These two experiments were performed in two consecutive days under identical inoculation conditions (including very similar age and physiological state of the protoplasts, quality of purified virus , chemicals used) and assay technique. The difference in PVY content at harvest between experiments I and III could be taken as the increase in PVY content between 24 and 48 hrs after inoculation. Thus, there was, approximately, a 10-fold increase in PVY titer during the 24 to 48 hrs incubation time period after inoculation. In another experiment, in which protoplasts from both White Burley and Connecticut Broad Leaf Burley were inoculated under the same conditions, the highest PVY yields were detected in both hosts at 96 hrs after inoculation (data not shown).

Even after inclusion of the serological blocking treatment, only 66% (54 out of 82) of the inoculation experiments were positive for PVY replication as determined by ELISA. The lack of consistancy could be attributed to a combination of the low efficiency of infection as well as the inevitable variations among inoculation experiments. Variation among experiments is not uncommon in infection of protoplasts with virus (46,49,54,84,85,118) since the entire inoculation process is rather long and involves several uncontrollable factors. These factors include: (1) variation in susceptibility to virus infection among the protoplasts isolated from plants cultured in greenhouse (49,54,85); (2) variation in the age of plants used for protoplast isolation (83); (3) variation in susceptibility to virus infection between leaves of different position on the plant (83); and (4) variation in virus specific infectivity among

batches of purified virus (73). If the virus used for inoculation is not freshly purified each time, a fifth source of variation could be added, i.e., variation among virus preparations used for protoplast inoculation caused by the decline in virus infectivity during storage (71).

Variation due to the physiological state of the protoplast source plants can be reduced by specifying the growth conditions of the plants and by standarizing the harvest protocol concerning the age and the position of leaves to be harvested. Purified preparations of plant viruses often contain noninfectious particles (36,71) that generally can not be separated from infectious ones. The specific infectivity of plant viruses is considered important for stimulating infection of protoplasts (73,84). This was one of the advantages of using column-purified PVY for protoplast inoculation since that procedure retained a higher specific infectivity of the virus (Fig. 4). Infection of protoplasts appeared to be most efficient when the inoculum contained 0.02 M K-CB at pH 4.6. Unfortunately, however, under such conditions, the infectivity of PVY was only 10% of that in 0.1 M Na-PB at pH 7.6. Matthews (71) reported complete loss of PVY infectivity at pH below 4.5. The conflict between optimum conditions for retention of the highest level of virus infectivity and optimum conditions for the most efficient infection of protoplasts could be the cause of failure in infection of protoplasts by certain plant viruses.

PEG-mediated inoculation was somewhat milder on protoplasts than PLO-mediated inoculation as revealed by the higher survival rate of inoculated tobacco protoplasts at 48 hrs (60% vs. 30-50%). However,

the efficiency of infection was lower in PEG-mediated inoculations.

Liposomes have been a useful tool for tansfering macromolecules, including drugs, into living cells. They have been found especially useful for introduction of nucleic acids into cells because of the protection they provide to encapsulated nucleic acid from digestion by cellular nucleases. The MLVs prepared in our study were neutral liposomes. Positively charged MLVs, containing PC and sterylamine (SA), somehow altered the chemical nature of the dyes (Evan's Blue and FDA), which were used as markers to follow the path of MLVs during protoplast-MLV fusion. The altered nature of these dyes was revealed by the change in the color of Evan's Blue (it appeared purple when added into the positively charged MLVs) and by the conversion of FDA to its fluorescent state in the absence of esterase. More importantly, no successful fusion with protoplasts was detected. Therefore, neutral MLVs containing PC and SS were used for inoculation. PVY was mostly bound on the surface of MLVs and/or trapped between MLVs in MLV clumps, as revealed by ELISA and electron microscopy. Actually, the diameter of most MLVs prepared in these experiments was much smaller (300 nm) than the particle length of PVY (720 nm). This could be the reason for lack of PVY infection of tobacco protoplasts. PVY carried by MLVs retained its infectivity. Neutral MLVs containing PC and SS had little or no effect on survival of tobacco protoplasts at all lipid concentrations tested. Liposome-mediated inoculation appeared to be milder than PLO-mediated inoculation. The zero time protoplasts, harvested after liposome-mediated inoculation with PVY, showed spotty fluorescence. The latter was, apparently, associated with the membrane of protoplasts and

was not distributed homogeneously as is the fluorescence of inoculated protoplasts at time zero after PLO-mediated inoculation. These fluorescent spots seemed to indicate the locations where the liposomes came in contact with the tobacco protoplasts.

LITERATURE CITED

1. Alblas, F., and Bol, J.F. 1977. Factors influencing the infection of cowpea mesophyll protoplasts by alfalfa mosaic virus. J. gen. Virol. 36:175-185.

2. Bangham, A., Standish, M., and Watkins, J. 1965. Diffusion of univalent ions across the lamellae of swollen phopholipids. J. Mol. Biol. 13:238-252.

3. Barker, H., and Harrison, B. D. 1977. Infection of tobacco mesophyll protoplasts with raspberry ringspot virus alone and togather with tobacco rattle virus. J. gen. Virol. 35:125-133.

4. Barker, H., and Harrison, B. D. 1982. Infection of potato mesophyll protoplasts with five plant viruses. Plant Cell Reports 1: 247-249.

5. Barker, H., and Harrison, B. D. 1984. Expression of genes for resistance to potato virus Y in potato plants and protoplasts. Ann. appl. Biol. 105:539-545.

6. Barnett, A., Hammond, J., and Lister, R. M. 1981. Limited infection of cereal leaf protoplasts by barley yellow dwarf virus. J. gen. Virol. 57:397-401.

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

8. Beier, H., and Bruening, G. 1976. Factors influencing the infection of cowpea protoplasts by cowpea mosaic virus RNA. Virology 72:363-369.

9. Boulton, M. I., Maule, A. J., and Wood, K. R. 1985. Effect of actinomycin D and UV irradiation on the replication of cucumber mosaic virus in protoplasts isolated from resistant and susceptible cucumber cultivars. Physiol. Plant Pathology 26:279-287.

10. Cassells, A. C. 1978. Uptake of charged lipid vesicles by isolated tomato protoplasts. Nature 275:760.

11. Cassells, A. C., and Barlass, M. 1978. The initiation of TMV infection in isolated protoplasts by polyethylene glycol. Virology 87:459-462.

12. Cassells, A. C., and Cocker, F.M. 1980. TMV inoculation of tobacco protopalsts in the presence of protoplast fusion agents. Z. Naturforsch. 35C:1057-1061.

13. Cassells, A. C., and Cocker, F. M. 1982. Seasonal and physiological aspects of the isolation of tobacco protoplasts. Physiol. Plant. 56:69-79.

14. Céch, M. 1962. Some notes on the use of agar gel filtration in the study of plant viruses. Virology 18:487-489.

15. Chiu, B. S., and Po, T. 1982. Infection of barley protoplasts with barley stripe mosaic virus detected and assayed by immunoperoxidase. J. gen. Virol. 58:323-327.

16. Chiu, R. J.,Lee, C-R. R., Chen, M. J., Chen,C. C., Hsu, Y. H., Lu, Y. T.,and Kou, T. T. 1981. Purification of rice transitory yellowing virus. Ann. Rep. of Acad. Sinica. July 1980-June 1981.

17. Clark, M.F.,and Adams,A.N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. gen. Virol. 34:475-483.

18. Cocking, E. C.,and Pojnar, E. 1969. An electron microscopic study of the infection of isolated tomato fruit protoplasts by tobacco mosaic virus. J. gen. Virol. 17:289-294.

19. Constabel, F. 1984. Fusion of protoplasts by polyethylene glycol (PEG). Pp. 414-422. In I.K. Vasil (ed.). "Cell Culture and Somatic Cell Genetics of Plants." Vol. I, Academic Press, Orlando, FL.

20. Coutts, R. H. A., Cocking, E. C., and Kassanis, B. 1972. Infection of tobacco mesophyll protoplasts with tobacco mosaic virus. J. gen. Virol. 17:289-294.

21. Crowther, J. R., and Abu-El Zein, E. M. E. 1979. Detection and quantification of foot and mouth disease virus by enzyme labelled immunosorbent assay techniques. J. gen. Virol. 42:597-602.

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

23. De Bokx, J.A. 1972. Viruses of potatoes and seed potato production. Pudoc, Wageningen, Netherlands. 233 p.

24. De Bokx, J. A., and Huttinga, H. 1981. Potato virus Y. CMI/AAB Descriptions of Plant Viruses No.242.

25. De Varennes, A. M., Russo, M., and Maule, A. J. 1984. Infection of protoplasts from Chenopodium quinoa with cowpea mosaic virus and Cymbidium ringspot virus. J. gen. Virol. 65:1851-1855.

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

27. Diaco, R., Hill, J. H.,Hill, E. K., Tachibana, H.,and Durand, D.P. 1985. Monoclonal antibody-based biotin-avidin ELISA for the detection of soybean mosaic virus in soybean seeds. J. gen. Virol.66: 2089-2094.

28. Evans, D. A., and Bravo, J. E. 1983a. Plant protoplast isolation and culture. Inter. Rev. of Cytology 16(Supl.):33-53.

29. Evans, D. A., and Bravo, J. E. 1983b. Protoplast isolation and culture. Pp.124-176. In D. E. Evans (ed.) "Handbook of Plant Cell Culture." Vol.I, MaCmillian, New York, NY.

30. Flick, C. E., and Evans, D. A. 1984. Tobacco. Pp.606-630. In W.R. Sharp et al.(eds.) "Handbook of Plant Cell Culture." Vol.II, MaCmillian, New York, NY.

31. Fraley, R. T. 1984. Liposome preparation and incubation with plant protoplasts. Pp.483-491. In I. K. Vasil (ed.) " Cell Culture and Somatic Cell Genetics of Plants." Vol.I, Academic Press, Orlando, FL.

32. Fraley, R. T., Dellaporta, S. L., and Papahadjopoulos, D. 1982. Liposome-mediated delivery of tobacco mosaic viru RNA into tobacco protoplasts: A sensitive assay for monitoring liposome-protoplast interactions. Proc. Natl. Acad. Sci. USA, 79:1859-1863.

33. Francki, R. I. B. 1972. Purification of viruses. P.295-335. In C. I. Kado, and H. O. Agrawal(eds.). "Principles and Techniques in Plant Virology." Van Nostrand Reinhold, New York.

34. Fukunaga, Y., Nagata, T., and Takebe, I. 1981. Liposome-mediated infection of plant protoplasts with tobacco mosaic virus RNA. Virology 113:752-760.

35. Fukunaga, Y., Nagata, T., Takebe, I, Kakehi, T,and Matsui, C.1983. An ultrastructural study of the interaction of liposome with plant protoplasts. Exp. Cell Res. 144:181-189.

36. Furumoto, W. A.,and Wildman, S. G. 1963. The specific infectivity of tobacco mosaic virus. Virology 20:53-61.

37. Furusawa, I., and Okuno, T. 1978. Infection with BMV of mesophyll protoplasts isolated from five plant species. J. gen. Virol.40:489 -491.

38. Furusawa, I., Yamaoka, N., Okuno, T., Yamamoto, M., Kohno, M., and Kunoh, H. 1980. Infection of turnip protoplasts with cauliflower mosaic virus. J. gen. Virol. 48:431-435.

39. Goffinet, D., and Verhoyen, M. 1979. Infection of tobacco protoplasts by potato virus Y. Parasitica 35:25-33.

40. Grout, B. W. W., and Coutts, R. H. A. 1974. Additives for the enhancement of fusion and endocytosis in higher plant protoplasts:An electrophoretic study. Pl. Sci. Letters 2:397-403.

41. Gugerli, P. 1978. The detection of two potato viruses by enzyme-linked immunosorbent assay (ELISA). Phytopath. Z. 92:51-56.

42. Harrison, B. D., and Mayo, M. A. 1983. The use of protoplasts in plant virus research. Pp.69-137.In J.P. Helgeson and B.J. Deverall (eds."Use of tissue culture and protoplasts in plant pathology." Academic Press, New York, NY.

43. Hibi, T., Rezelman, G.,and van Kammen, A.1975. Infection of cowpea mesophyll protoplasts with cowpea mosaic virus. Virology 64:308-318.

44. Hooker, W. J. (ed.). 1981. Compendium of potato disease. APS, St. Paul, MN.

45. Huber, R., Hontelez, J., and van Kammen, A. 1981. Infection of cowpea protoplasts with both common strain and the cowpea strain of TMV. J. gen. Virol. 55:241-245.

46. Huber, R., Rezelman, G.,Hibi, T., and van Kammen, A. 1977. Cowpea mosaic virus infection of protoplasts from Samsun tobacco leaves. J. gen. Virol. 34:315-323.

47. Huttinga, H. 1973. Properties of viruses of the potyvirus group.I. A simple method to purify bean yellow mosaic virus, pea mosaic virus, lettuce mosaic virus and potato virus Y. Neth. J. Pl. Path. 79:125-129.

48. Jarvis, N. P.,and Murakishi, H. H. 1980. Infection of protoplasts from soybean cell culture with southern bean mosaic and cowpea mosaic viruses. J. gen. Virol. 48:365-376.

49. Kassanis, B.,and White, R. F. 1974. A simplified method of obtaining tobacco protoplasts for infection with tobacco mosaic virus. J. gen. Virol. 24:447-452.

50. Kassanis, B., White, R. F., and Woods, R. D. 1975. Inhibition of multiplication of tobacco mosaic virus in protoplast by antibiotics and its prevention by divalent metals. J. gen. Virol. 28:185-191.

51. Keller, W. A., and Melchers, G. 1973. The effect of high pH and high calcium on tobacco leaf protoplasts fusion. Z. Naturforsch. 28c:737-741.

52. Kikkawa, H., Nagata, T., Matsui, C.,and Takebe, I. 1982. Infection of protoplasts from tobacco suspension cultures by tobacco mosaic virus. J. gen. Virol. 63:451-456.

53. Kluge, S., Kirsten, U., and Oertel, C. 1983. Infection of Dianthus protoplasts with carnation mottle virus. J.gen.Virol.64:2485-2487.

54. Kubo, S., Harrison, B. D., and Barker, H. 1975. Defined conditions for growth of tobacco plants as sources of protoplasts for virus infection. J. gen. Virol. 28:255-257.

55. Kubo, S., Harrison, B.D.,and Robinson, D. J. 1974. Effect of phosphate on the infection of tobacco protoplasts by tobacco rattle virus. Intervirology 3:382-387.

56. Kubo, S., Harrison, B. D., Robinson, D. J., and Mayo, M. A. 1975. Tobacco rattle virus in tobacco mesophyll protoplasts: Infection and virus multiplication. J. gen. Virol. 27:293-304.

57. Kubo, S., Robinson, D. J., Harrison, B. D., and Hutcheson, A. M. 1976. Uptake of tobacco rattle virus by tobacco protoplasts, and the effect of phosphate on infection. J. gen. Virol. 30:287-298.

58. Larkin, P. J. 1976. Purification and viability determinations of plant protoplasts. Planta 128:213-216.

59. Latorre, B. A., Florres, V.,and Marholz, G. 1984. Effect of potato virus Y on growth, yield, and chemical composition of flue-cured tobacco in Chile. Pl. Dis. 68:884-886.

60. Laurent, T. C., and Killander, J. 1964. A theory of gel filtration and its experimental verification. J. Chromatog. 14:317-330.

61. Lee, C.R., Lei, J.D., and Agrios, G.N. 1983. Purification of potyviruses by column chromatography. Phytopathology 73:369 (Abstr.).

62. Lesney, M. S.,and Murakishi, H. H. 1981. Infection of soybean protoplast from cell suspension culture with bean pod mottle virus. J. gen. Virol. 57:387-395.

63. Loebenstein, G.,Gera, A., Barnett, A., Shabtai, S., and Cohen, J. 1980.Effect of 2,4-dichlorophenoxyacetic acid on multiplication of tobacco mosaic virus in protoplasts from local-lesion and systemic responding tobaccos. Virology 100:110-115.

64. Loesch-Fries,L.S.,and Hull, T.C. 1980. Synthesis, accumulation and encapsidation of individual brome mosaic virus RNA components in barley protoplasts. J. gen. Virol. 47:323-332.

65. Lommel, S. A., McCain, A. H.,and Morris, T. J. 1982. Evaluation of indirect enzyme-linked immunosorbent assay for the detection of

plant viruses. Phytopathology 72:1018-1022.

66. Lu, Y. T. 1981. Purification of several plant viruses by molecular sieving chromatography. Plant Prot.Bull.(Taiwan) 23:263 (Abstr.).

67. Lu, Y.T., and Lu, C.H. 1982. Improvement of purification of potato virus Y-mosaic strain. Plant Prot. Bull.(Taiwan) 24:282 (Abstr.).

68. Lurquin, F., Sheehy, R.E.,and Rao, N.A. 1981. Quantitative aspects of nucleic acids sequestration in large liposome and their effects on plant proto plasts. FEBS Letters 125:183-187.

69. Maierhofer, G. 1985. Liposomes: Preparation and application. ACPR June:33-44.

70. Makkouk, K. M., and Gumpf, D. J. 1974. Isolation and properties of potato virus Y ribonucleic acid. Phytopathology 64:115-118.

71. Matthews, R. E. F. 1981. Plant virology.2nd ed., Academic Press, New York, NY. 897p.

72. Maule, A. J. 1983. Infection of protoplasts from several Brassica species with cauliflower mosaic virus following inoculation using polyethylene glycol. J. gen. Virol. 64:2655-2660.

73. Maule, A. J., Boulton, M. I., and Wood, K. R. 1980. An improved method for the infection of cucumber leaf protoplast with cucumber mosaic virus. Phytopath. Z. 97:118-126.

74. Maule, A. J., Boulton, M. I., Edmunds, C., and Wood, K. R. 1980. Polyethylene glycol-mediated infection of cucumber protoplsts by cucumber mossaic virus and virus RNA. J. gen. Virol. 47:199-203.

75. Mayo, M. A. 1978. The effect of protoplasts concentration during inoculation on the infection of tobacco protoplasts. Intervirology 9:184-188.

76. McDonald, J. G., Beveridge, T. J.,and Bancroft, J. B. 1976. Self-assembly of protein from a flexuous virus. Virology 69:327-331.

77. Moghal, S. M.,and Francki,R. I. B. 1981. Towards a system for the identification and classification of potyviruses.II.Virus particle length, symptomatology, and cytopathology of six distinct viruses. Virology 112:210-216.

78. Morris-Kusinich, B. A. M.,Hull, R., and Russo, M. 1979. Infection of turnip leaf protoplasts with turnip rosset virus. J.gen.Virol. 43:339-347.

79. Motoyoshi, F., and Hull, R. 1974. The infection of tobacco proto-plasts with pea enation mosaic virus. J. gen. Virol. 24:89-99.

80. Motoyoshi, F., and Oshima, N. 1975. The use of Tris-HCl buffer for inoculation of tomato protoplasts with tobacco rattle virus. J. gen. Virol. 32:311-314.

81. Motoyoshi, F.,Bancroft,J.B.,and Watts, J.W.1973. A direct estimate of the number of cowpeachlorotic mottle virus particle absorbed by tobacco protoplasts that became infected. J. gen. Virol. 21:159 161.

82. Motoyoshi, F., Bancroft, J.B.,and Watts, J.W. 1974a. The infection of tobacco protoplasts with a varient of brome mosaic virus. J. gen. Virol. 25:31-36.

83. Motoyoshi, F., Watts, J. W., and Bancroft, J. B. 1974b. Factors influencing the infection of tobacco protoplasts by cowpea chlorotic mottle virus. J. gen. Virol. 25:245-256.

84. Motoyoshi, F., Bancroft, J. B.,Watts, J. W., and Burgess, J. 1973. The infection of tobacco protoplasts with cowpea chlorotic mottle virus and its RNA. J. gen. Virol. 20:177-193.

85. Murakishi, H., Lesney, M., and Carlson, P. 1984. Protoplasts and plant viruses. Adv. in Cell Culture 3:1-55.

86. Nagata, T., and Ishii, S. 1979. A rapid method for isolation of mesophyll protoplasts. Can. J. Bot.57:1820-1823.

87. Nagata, T., and Takebe, I. 1970. Cell wall regeneration and cell division in isolated tobacco mesophyll protoplasts. Planta 92:301-308.

88. Nagata, T., Okada,K., Takebe, I, and Matsui,C. 1981. Diliveray of tobacco mosaic virus RNA into plant protoplast mediated by reverse phase evaporation vesicles (Liposomes). MGG 184:161-165.

89. Nelson, M. R., and Wheeler, R. E. 1978. Biological and serological characterization and separation of potyviruses that infect peppers. Phytopathology 68:979-984.

90. Okuno, T., and Furusawa, I. 1978a. Modes of infection of barley protoplasts with brome mosaic virus. J. gen. Virol. 38:409-418.

91. Okuno, T.,and Furusawa, I. 1978b. The use of osmotic shock for the inoculation of barley protoplasts with brome mosaic virus. J. gen. Virol. 39:187-190.

92. Otsuki, Y., and Takebe, I. 1973. Infection of tobacco mesophyll protoplasts by cucumber mosaic virus. Virology 52:433-438.

93. Otsuki, Y., Takebe, I., Honda, Y., and Matsui, C. 1972. Ultrastructure of infection of tobacco mesophyll protoplasts by tobacco

mosaic virus. Virology 49:188-194.

94. Otsuki, Y., Takebe, I., Honda, Y., Kakita, S.,and Matsui, C. 1974. Infection of tobacco mesophyll protoplasts by potato virus X. J. gen. Virol. 22:375-385.

95. Prakash, J., and Foxe, M. J. 1985. Optimization of conditions for infction of isolated potato protoplasts with potato virus X. Archieves of Virology 85:269-279.

96. Rao, D. V.,and Hiruki, C. 1978. Infection of cowpea mesophyll protoplasts with clover yellow mosaic virus. J.gen.Virol.38:303-311.

97. Renaudin, J., Bove, J.M., Otsuki,Y.,and Takebe, I. 1975. Infection of Brassica leaf protoplasts by turnip mosaic virus. MGG 141:59-68.

98. Rollo, F.,and Hull, R.1982. Liposome-mediated infection of turnip protoplasts with turnip rosset virus and RNA. J. gen. Virol. 60: 359-363.

99. Rouze, P., Deshayes, A.,and Caboche, M. 1983. Use of liposomes for the transfer of nucleic acids: Optimization of the method for tobacco mesophyll protoplasts with tobacco mosaic virus RNA. Pl. Sci. Letters 31:55-64.

100. Russo, M.,and Gallitelli, D. 1985. Infection of cowpea protoplasts with Cymbidium ringspot virus. J. gen. Virol. 66:2033-2037.

101. Sakai, F.,Dawson, J. R. O., and Watts, J. W. 1983. Interference in infection of tobacco protoplasts with two bromoviruses. J. gen. Virol. 64:1347-1354.

102. Sander, E., and Martes, G. 1984. Use of protoplasts and separate cells in plant virus research. Adv. Virus Res. 29:215-262.

103. Sarkar, S. 1977. Use of protoplasts for plant virus studies. Methods in Virology 6:435-456.

104. Shabtai, S., Gera, A., and Loebenstein, G. 1982. Partial suppression of tobacco mosaic virus multiplication in protoplasts at increasing mannitol concentration. Pl. Sci. Letters 24:157-161.

105. Shepard, J. F.,and Totten, R. E. 1975. Isolation and regeneration of tobacco mesophyll protoplasts under low osmotic conditions. Plant Physiol. 55:689-694.

106. Sievert, R. C. 1978. Effect of potato virus Y on cultivars and hybrids of burley tobacco. Phytopathology 68:974-978.

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

108. Steere, R. L. 1963. Tobacco mosaic virus: purifing and sorting associated particles according to length. Science 140:1089-1090.

109. Steere, R. L.,and Ackers, G. K. 1962. Purification and separation of tobacco mosaic virus and southern bean mosaic virus by agar-gel filtration. Nature 194:114-116.

110. Szoka, F., and Papahadjopolous, D. 1978. Procedure for preparation of liposomes with large internal aequeous space and high capture by reverse-phase evaporation. Proc. Natl. Acad. Sci. USA, 75:4194 4198.

111. Szoka, F.,and Papahadjopolous, D. 1981. Liposomes: Preparation and characterization. Pp.51-82. In C. G. Knight (ed."Research Monographs in Cell and Tissue Physiology." Vol. VII, Elsevier/North-Hollan, New York, NY.

112. Takebe, I. 1975. The use of protoplasts in plant virology. Ann. Rev. Phytopathol. 13:105-125.

113. Takebe,I.1977. Protoplasts in the study of plant virus replication .Comprehensive Virology 11:237-283.

114. Takebe, I.1984. Inoculation of protoplasts with plant viruses. Pp. 492-502. In I. K. Vasil (ed.). " Cell Culture and Somatic Cell Genetics of Plants. Vol.I, Academic Press, Orlando, FL.

115. Takebe,I.,and Otsuki,Y. 1969. Infection of tobacco mesophyll protoplasts by tobacco mosaic virus. Proc. Natl. Acad. Sci.USA, 64: 843-848.

116. Takebe, I., Otsuki, Y., and Aoki, S. 1968. Isolation of tobacco mesophyll cells in intact and active state. Plant and Cell Physiology 9:115-124.

117. Uchimiya, H., and Murashige, T. 1974. Evaluation of parameters in the isolation of viable protoplasts from cultured tobacco cells. Plant Physiol. 54:936-944.

118. Van Beek, N. A. M., Derksen, A. C. G.,and Dijkstra, J. 1985. Polyethylene glycol-mediated infection of cowpea protoplasts with sonchus yellow net virus. J. gen. Virol. 66:551-557.

119. Van Regenmortel, M. H. V. 1962. Purification of a plant virus by filtration through granulated agar. Virology 17:601-602.

120. Van Regenmortel, M. H. V., and Burckard, J. 1980. Detection of a wide spectrum of tobacco mosaic virus strains by indirect enzyme-linked immunosorbent assay (ELISA). Virology 106:327-334.

121. Vasil, I. K., and Vasil, V. 1980. Isolation and culture of protoplasts. Inter. Rev. of Cytology 11B(Supl.):1-18.

122. 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 polyethyleneglycol-containing solutions as solvents. Virology 23:394-402.

123. Watanabe, Y., Ohno, T., and Okada, Y. 1982. Virus multiplication in tobacco protoplasts inoculated with tobacco mosaic virus RNA encapsulated in large unilamillar vesicle liposomes. Virology 120:478-480.

124. Watts, J.W.,and Dawson, J.R.O. 1980. Double infection of tobacco protoplasts with brome mosaic virus and cowpea chlorotic mottle virus. Virology 105:501-507.

125. Watts, J. W., Motoyoshi, F.,and King, J. M. 1974. Problems associated with the production of stable protoplasts of cells of tobacco mesophyll. Ann. Bot. 38:667-671.

126. Wilson, H.M.,Styer, D. J.,Conrad, P. L.,Durbin, R.D.,and Helgeson, J.P. 1980. Isolation of sterile protoplasts from unsterilized leaves. Pl. Sci. Letters 18:151-154.

127. Wyatt, S. D., and Shaw, J. G. 1975. Retention and dissociation of tobacco mosaic virus by tobacco protoplasts. Virology 63:459-465.

128. Xu, Z. K., Luciano, C. S.,Ballard, S. T., Rhoads, R. E., and Shaw, J.G. 1984. Infection of tobacco protoplasts with liposome-encapsulated potyviral RNA. Pl. Sci. Letters 36:137-142.

129. Zhuravlev, Y. N., Yudakova, Z. S., and Pisetskaya, N. F. 1980. Infection of tobacco protoplasts with TMV in the absence of Poly-L-ornithine and the electroosmotic mechanism of virus entry. Phytopath. Z. 98:296-309.

130. Zhuravlev, Y. N., Pisetskaya, N. F., Shumilova, L.A., Musorok, T. I., and Reifmen, V. G. 1975. Attachment of labeled TMV to tobacco mesophyll protoplasts. Virology 64:43-48.