Polyethylene Glycol for Purification of Potato Yellow Dwarf Virus

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Supported in part by grants from the National Science Foundation (GB 20915) and the National Institutes of Health (AI 6392).

Part of this work was submitted by the senior author in partial fulfillment of the requirements for the Ph.D. degree from the University of Illinois, Urbana.

Accepted for publication 14 December 1972.

ABSTRACT

Polyethylene glycol of 6,000 molecular weight (PEG 6,000) was used to improve the purification of potato yellow dwarf virus. Optimal conditions of precipitation were determined to retain maximal amounts of the initial infectivity in the final preparation. Separation of green host pigment from virus was better in PEG-treated preparations than in controls in which virus was concentrated by differential centrifugation. PEG concentrations of 7 and 4% were optimal for the *sanguinolenta* and *constricta* varieties of the virus, respectively, but 0.1 M NaCl and pH 7.0 were optimal for both.

Phytopathology 63:692-696

Density-gradient centrifugation has been widely employed as a method of purification of virus and other biological entities since Brakke (5, 6) first invented it in his work with potato yellow dwarf virus (PYDV). It has been employed since then as the principal technique for the purification of PYDV and has been preceded by conventional centrifugation to sediment and concentrate the virus (4). Since Brakke's (7) work on the importance of protective solutions for the suspension of the partially purified virus, a solution of 0.1 M glycine and 0.01 M $MgCl_2$ has been an important solution for suspension of the virus.

The concentration and purification of various viruses in aqueous polyethylene glycol-dextran sulfate two-phase systems developed by Albertsson & Frick (1) has been applied to many viruses. However, special procedures must be employed for removal of the dextran sulfate and Hebert (13) introduced the use of polyethylene glycol (PEG) alone in precipitation studies with four plant viruses. Since then plant viruses (8, 10, 12, 21, 22), phages (24), and certain animal viruses (11, 18, 20, 23) have been concentrated by its use with minimal loss of infectivity. The present work was initiated principally because of the results on PEG precipitation of wound tumor virus by Reddy & Black (21) who showed that the concentration of PEG, NaCl and H^+ ions was each of considerable importance for optimal yields. Consequently, each has been studied in the following work. We report attempts to purify two varieties of PYDV with polyethylene glycol, mol wt 6,000 (PEG 6,000) in conjunction with density-gradient contrifugation. Preparations were handled aseptically when necessary and infectivity assays on vector cell monolayers (17) made it possible to evaluate various fractionations for infectious virus quickly and precisely.

MATERIALS AND METHODS.—Unless otherwise indicated details of materials and methods may be found in earlier contributions by Hsu (14) and Hsu & Black (15, 16, 17).

Two field varieties of PYDV: SYDV (for *sanguinolenta* yellow dwarf virus) and CYDV (for *constricta* yellow dwarf virus) (3) were used. Infected *Nicotiana rustica* plants were harvested at peak virus concentrations (17) and extracted in a protective (Gly-$MgCl_2$) solution of 0.1 M glycine and 0.01 M $MgCl_2$ brought to about pH 8.4 with NaOH. All procedures were carried out at about 4 C. After clarification and adjustment of the pH of the extract to 7.0, NaCl was stirred into the solution. After the NaCl had been completely dissolved, PEG was added and stirring was continued until solid PEG disappeared. The whole suspension was then left at 4 C for 60 min before the virus was pelleted in a Servall SS-1 rotor at 8,200 rpm for 5 min. The pellet was taken up in Gly-$MgCl_2$ solution at pH 7.0 unless otherwise stated. Sucrose gradient columns were prepared in a Gly-$MgCl_2$ solution. The resuspended virus was given one cycle of rate and quasi-equilibrium sucrose density-gradient centrifugation in an SW 25.1 rotor. Aseptic procedures were not used where they were not needed. This meant that where infectivities were determined only on purified preparations, asepsis was initiated only when virus was collected from the quasi-equilibrium zone. However, when infectivities were required on crude extracts, as when yields of purified virus were measured, asepsis was initiated with the surface sterilization of selected pieces of stem with 70% alcohol. Virus was collected from quasi-equilibrium columns by means of a sterile needle and sugar was removed by dialysis against Gly-$MgCl_2$ solution at pH 6.0 for SYDV and pH 6.5 for CYDV. For infectivity assays, further dilutions were made in a His-$MgCl_2$ solution of 0.1 M histidine and 0.01 M $MgCl_2$ (19) at pH 5.9 for SYDV and pH 5.3 for CYDV. Infectivities were assayed on vector cell monolayers, i.e., SYDV on AS cells and CYDV

on AC cells at optimal pHs for inoculation. The direct fluorescent antibody technique (9) was employed for detection of PYDV infection 40-48 hr after inoculations. Infectivity measurements were each based on the number of infected cells in two diametral zones perpendicular to each other in each of two coverslip monolayers. The infected cells, stained with fluorescent antiserum, were counted under a 40X objective lens (Leitz NPl 40/0.65).

RESULTS.–*Effect of PEG 6,000 on the recovery of PYDV.*–In the following experiments SYDV was always investigated before CYDV because the latter is considerably more difficult to work with. A Waring Blendor was used to disintegrate acutely infected *N. rustica* plants in a pH 8.4 Gly-$MgCl_2$ solution in the ratio of 1 g of plant tissue to 3 ml of solution. The extract was clarified and adjusted to pH 7.0 with NaOH. NaCl was added to a concentration of 0.5 M. The extract was then equally distributed into several beakers. To each beaker a different amount of PEG 6,000 was added.

The use of 7% PEG 6,000 gave the best yield of infective SYDV but in the case of CYDV 4% PEG 6,000 was best (Fig. 1). SYDV and CYDV, though closely related, require quite different PEG concentrations for optimal results.

Effect of NaCl on the recovery of PYDV.–Virus extract was prepared in the same way as in the previous section. After adjustment to pH 7.0 the extract was divided into several equal parts. To each part a different amount of NaCl was added while stirring. After complete solution of NaCl, PEG 6,000 was added to each of the SYDV and CYDV extracts in optimal amounts. After the PEG 6,000 had completely disappeared, the remainder of the procedure was carried out as before. The effects of various concentrations of NaCl on recovery of infective virus are shown in Fig. 2. For both SYDV and CYDV the optimal concentration of NaCl for viral recovery was 0.1 M.

After finding that 0.1 M NaCl gave better recovery of PYDV than 0.5 M NaCl, the effect of PEG concentration on the recovery of PYDV was re-examined in the presence of 0.1 M NaCl.

The optimal concentration of PEG 6,000 for the recovery of SYDV and CYDV was still the same; i.e., 7% for SYDV and 4% for CYDV (Fig. 3).

Effect of pH on the recovery of PYDV.–A clarified extract from infected plants was prepared with the use of a pH 8.4 Gly-$MgCl_2$ solution. NaCl was added to the extract to give a concentration of 0.5 M. One part of extract was added to four parts of a His-$MgCl_2$ solution of a certain pH to obtain each desired pH at a concentration of 0.1 M NaCl. PEG 6,000 was then added to give a concentration of 7%

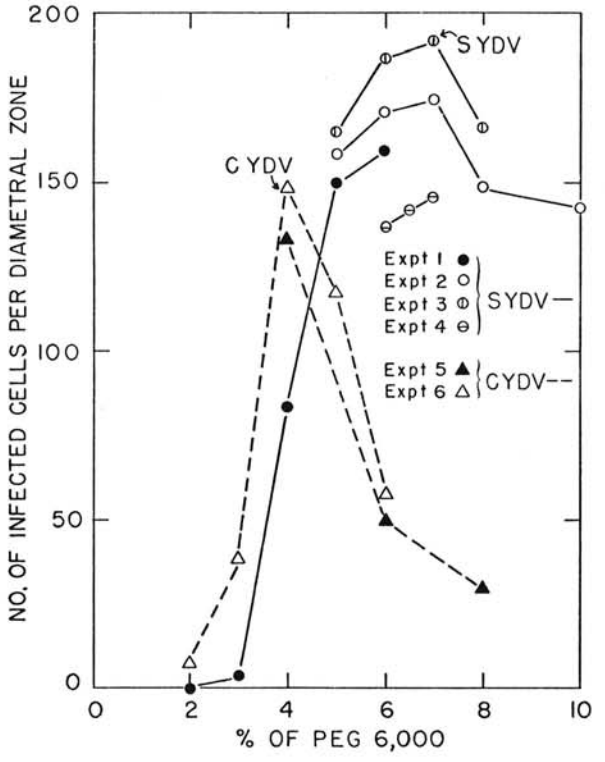

Fig. 1. Effect of various concentrations of polyethylene glycol M.W. 6,000 (PEG 6,000) on the precipitation of infective SYDV and CYDV from extracts at pH 7.0 and with 0.5 M of added NaCl. Each number of infected cells is an average from four diametral zones.

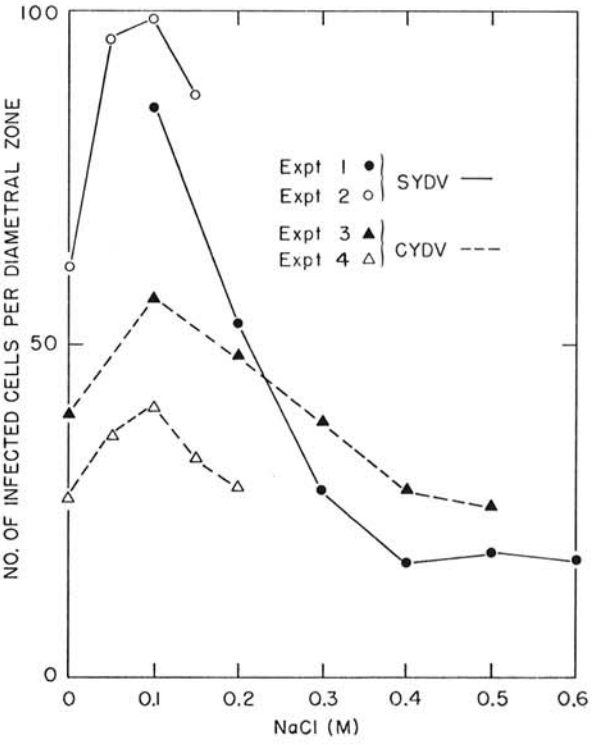

Fig. 2. Effect of various concentrations of NaCl, in conjunction with 7 and 4% PEG 6,000 for SYDV and CYDV, respectively, on the recovery of infective virus from clarified plant extracts at pH 7.0. Each number of infected cells is an average from four diametral zones.

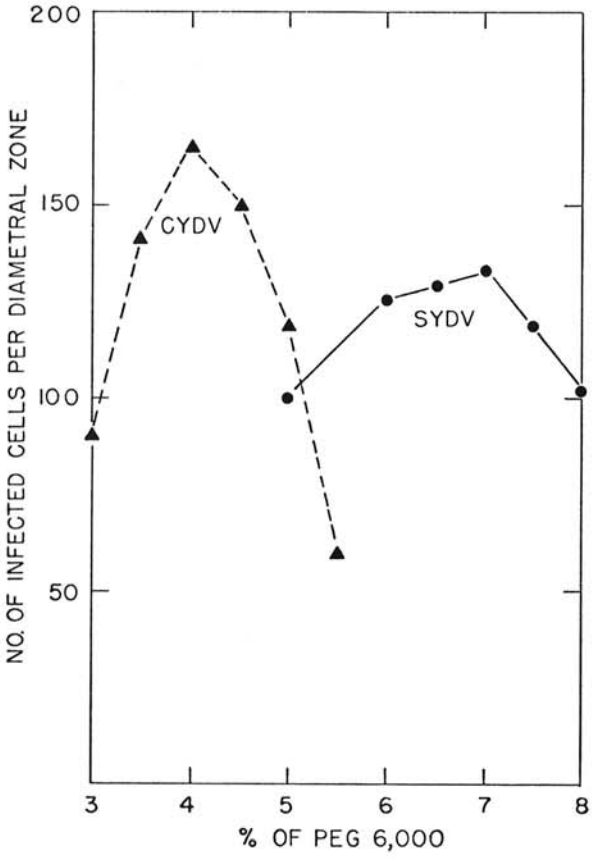

Fig. 3. Effect of various concentrations of PEG 6,000 in the presence of 0.1 M of added NaCl, on the recovery of infective SYDV and CYDV, at pH 7.0. Each number of infected cells is an average from four diametral zones.

for SYDV and 4% for CYDV. After complete solution of the PEG the remainder of the procedure was carried out as before.

The changes in the pH of extracts during the 60 min of PEG treatment were less than 0.01 pH. The pHs of extracts before and after pelleting of the virus also differed less than 0.01 pH. Therefore, only the pH of each supernatant after pelleting virus was measured and recorded. The results of the effect of pH during the PEG 6,000 treatment indicate that the optimal pH for PEG treatment of both SYDV and CYDV is about 7.0 (Fig. 4).

Yield of infective PYDV after purification.—Known weights of *N. rustica* stems acutely infected with either SYDV or CYDV were surface sterilized with 70% ethanol and washed with sterile distilled water followed by sterile Gly-$MgCl_2$ solution at about pH 8.4. They were triturated aseptically with a mortar and pestle and a small amount of sand. Gly-$MgCl_2$ solution at pH 8.4 was added to the plant material. The final volume of the preparation including plant debris was measured and all dilutions were expressed in terms of weight of starting plant material. After clarification in an SS-1 rotor at 8,200 rpm for 5 min the supernatant was removed and a sample was diluted in a suitable His-$MgCl_2$ solution for immediate inoculation. The remainder of the supernatant was equally divided into two parts. One received PEG 6,000 treatment under optimal conditions for virus recovery. The other one received high speed centrifugation (12,600 rpm in an SS-1 centrifuge) for 60 min to sediment the virus. Both virus pellets were resuspended in Gly-$MgCl_2$ solution and went through one cycle of rate and quasi-equilibrium centrifugation before the measurement of infectivities. The infectivities of the crude extract and of the two purified virus preparations were compared (Table 1).

With the use of PEG 6,000 about 25% of SYDV infectivity remained after purification. Only about 5% of SYDV infectivity, however, was recovered by use of differential centrifugation. In the case of CYDV, use of PEG 6,000 gave a recovery of about 10% to 15% infectivity after purification and only about half that amount by use of differential centrifugation.

A better separation of green host pigment from virus was observed in the PEG-treated preparations. Virus zones after rate zonal density-gradient centrifugation were free from green color in PEG-treated samples. Previously, after purification in which virus was concentrated by high speed centrifugation, a slight green color was associated with the virus even after quasi-equilibrium density gradient centrifugation.

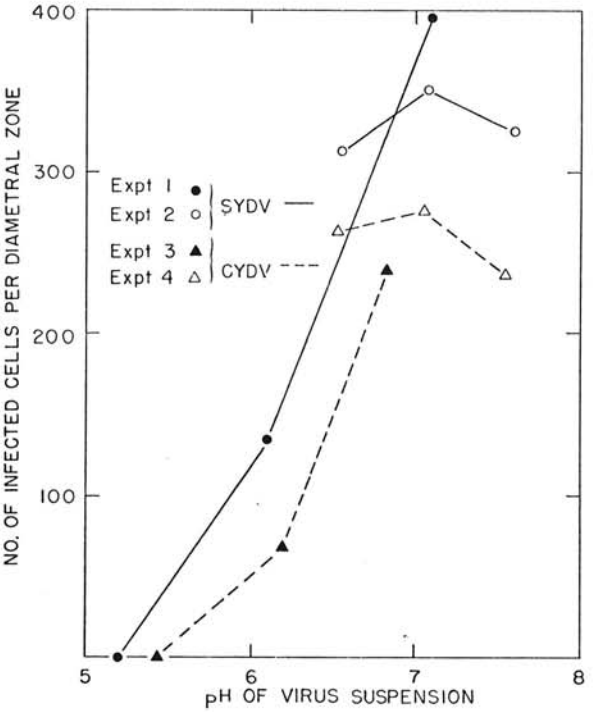

Fig. 4. Effect of various pHs on the recovery of infective SYDV and CYDV from plant extracts with optimal PEG and NaCl concentrations. Each number of infected cells is an average from four diametral zones.

TABLE 1. Comparison of infectivities of crude and purified preparations of PYDV[a]

Virus	Experiment 1				Experiment 2			
			Purified virus involving[b]				Purified virus involving[b]	
	Virus Dilution	Crude Extract	Differential Centrifugation	PEG 6,000 Precipitation	Virus Dilution	Crude Extract	Differential Centrifugation	PEG 6,000 Precipitation
SYDV	10^{-3}	484	41	156	10^{-3}	277	18	76
	10^{-4}	79	5	17	10^{-4}	47	2	9
	10^{-5}	7	0	1	10^{-5}	5	0	1
CYDV	10^{-2}		109	188	10^{-2}	216	14	21
	10^{-3}	154	17	31	10^{-3}	35	0	4
	10^{-4}	27	2	2	10^{-4}	3	0	0
	10^{-5}	4	0	0	10^{-5}	0	0	0

[a] Figures give the average number of infected cells per diametral zone, based on the perpendicular and horizontal zones of each of two coverslips (except in Expt. 1 on CYDV where three coverslips were used).

[b] Although comparisons are to be made between virus preparations concentrated by differential centrifugation or by PEG precipitation the infectivity assays were made after such preparations had been purified by both rate and quasi-equilibrium density gradient centrifugations.

Procedure for producing, purifying and storing infective PYDV.—PYDV is a very labile virus (2, 7). The present work with PEG and the demonstration of the extent of peaking of PYDV concentration in *N. rustica* (17) represent perhaps the most important improvements in the purification of PYDV since Brakke's work on density-gradient centrifugation (5, 6) and protective solutions for the purified virus (7). It is, therefore, desirable to describe in detail the complete procedure for obtaining and storing purified infective PYDV.

Because of the rise and fall of infective virus concentration in *N. rustica* plants it is very important to determine the time of peak concentration if a good source of virus is desired. *N. rustica* plants are mechanically inoculated with either variety of PYDV grown and maintained in a greenhouse at approximately 30 C. In order to have a satisfactory number of plants develop vein-clearing at about the same time, about 30 *N. rustica* plants are selected for inoculation. These should be about 3-6 cm from soil level to growing point stripped of leaves. The youngest newly expanded and newly unwrinkled leaf, having a leaf blade about 15- to 20-cm long, is most susceptible to inoculation. The top of the plant above this leaf is broken out, and the leaf inoculated with concentrated inoculum in 0.1 M phosphate buffer at pH 7.0. Better results have been obtained with CYDV by inoculating the next older leaf on each plant as well. The leaves to be inoculated are dusted with No. 320 Carborundum (or 1.5 Celite is added to the inoculum itself) and the leaf is rubbed three times with bare fingers to a point just less than that producing obvious inoculation injury of the leaf. It is necessary to obtain some hundreds of primary lesions on the inoculated leaf in order to approximate synchrony in the development of vein-clearing. Such synchrony is especially difficult to obtain with CYDV and usually requires a second passage with inoculum taken from the best plant in the first inoculated lot. About 10 days after inoculation with CYDV it is often desirable to break out the biggest axillary shoot in order to favor development of infection in the next oldest shoot. Peak virus concentrations are reached about 10 days after the first systemic symptom, vein-clearing on young leaves, is noticed. Acutely infected leaves and adjacent stem parts are placed in a cold room to cool to about 4 C. Stems are cut into pieces about 2-cm long and these pieces along with selected leaves are blended for 1 min with Gly-$MgCl_2$ solution at pH 8.4 (3 ml:1 g) in a Waring Blendor. The extract is squeezed through one layer of bandage gauze and centrifuged at 8,200 rpm in an SS-1 centrifuge for 5 min. The pellet is discarded and the supernatant adjusted to pH 7.0 with NaOH. Then NaCl is stirred in until the concentration of added NaCl is equivalent to 0.1 M. Optimal amounts of solid PEG 6,000, 7% for SYDV and 4% for CYDV, are added to the mixture and stirred until completely dissolved. The mixture is kept in a cold room for 60 min and then centrifuged at 8,200 rpm for 5 min. The pellets are resuspended in a volume of Gly-$MgCl_2$ solution (brought to pH 7.0 with NaOH) one-tenth the weight of the starting material. Five to 8 ml of the virus preparation is floated on top of each rate zonal density-gradient column (17) and centrifuged at 22,500 rpm in SW 25.1 or SW 25.2 rotors at 4 C for 35 min in a Spinco Model L2 centrifuge. Virus is collected from a visible zone located about 1.3 - 2.4 cm from the bottom of the tube. Five to 8 ml of the virus solution is floated on each of the standard quasi-equilibrium density-gradient columns and centrifuged at 22,500 rpm for 85 min. Virus is collected aseptically with a bent needle and a syringe from a visible virus zone located about 1.8 - 2.3 cm from the base of the tube. [Sometimes CYDV yields two infective virus zones in both rate and quasi-equilibrium centrifugations (17).] The collected zones may be diluted with Gly-$MgCl_2$ solution and the sugar removed by pelleting the virus in either R30

or R40 rotors at 25,000 rpm or 28,000 rpm, respectively, for 40 min. The pellets are taken up in a small amount of His-$MgCl_2$ solution at pH 6.0 for SYDV and 6.5 for CYDV. Such highly concentrated virus suspensions appear milky white. However, in most cases virus suspensions taken from quasi-equilibrium density columns are dialized overnight against Gly-$MgCl_2$ solution at pH 6.0 for SYDV and 6.5 for CYDV after transferring them aseptically to a preboiled dialysis tube. After dialysis, one end of the tube is dipped in boiling water for 30 sec before cutting and removing virus suspension from the tube with a 10-ml sterile pipette. These purified infectious virus preparations are free from microorganisms. Aliquots of the preparations, undiluted or aseptically diluted, are placed in sterile ampules (1.2 ml, Wheaton Glass Co., Millville, N.J.) and hermetically sealed. They are then frozen by rotating the ampules in a mixture of dry ice and 95% ethanol and stored in a deep freezer at −80 C for future use.

DISCUSSION.—Of those factors tested the only optimal condition that differed in the treatment of SYDV and CYDV was the concentration of PEG 6,000. The optimal PEG concentration of 7% for SYDV and 4% for CYDV show how different optimal conditions for PEG treatments may be for even closely related viruses (3).

In comparison with many earlier procedures for virus purification, the advantages of using PEG in concentrating and purifying viruses include the simplicity of the technique and the rapidity of precipitation (11, 12, 23, 24), less damage to the structure of virus particle (18), greater recovery of infectivity (8, 24), and less contamination by host components (12, 20, 23). Our results with PYDV support these observations because the use of PEG 6,000 for both varieties of PYDV purification gave cleaner preparations and a better recovery of infectivities than earlier procedures (4).

LITERATURE CITED

1. ALBERTSSON, P. A., & G. FRICK. 1960. Partition of virus particles in a liquid two-phase system. Biochim. Biophys. Acta 37:230-237.
2. BLACK, L. M. 1955. Concepts and problems concerning purification of labile insect-transmitted plant viruses. Phytopathology 45:208-216.
3. BLACK, L. M. 1970. Potato yellow dwarf virus. No. 35 (4 pages). *In* "Descriptions of Plant Viruses" Commonwealth Mycological Institute and Assoc. Appl. Biol., Kew, England.
4. BLACK, L. M., K. M. SMITH, G. J. HILLIS, & R. MARKHAM. 1965. Ultrastructure of potato yellow-dwarf virus. Virology 27:446-449.
5. BRAKKE, M. K. 1951. Density gradient centrifugation. A new separation technique. J. Am. Chem. Soc. 73:1847.
6. BRAKKE, M. K. 1953. Zonal separations by density-gradient centrifugation. Arch. Biochem. Biophys. 45:275-290.
7. BRAKKE, M. K. 1956. Stability of potato yellow-dwarf virus. Virology 2:463-476.
8. CLARK, M. F. 1968. Purification and fractionation of alfalfa mosaic virus with polyethylene glycol. J. Gen. Virol. 3:427-432.
9. COONS, A. H., H. J. GREECH, R. N. JONES, & E. BERLINER. 1942. The demonstration of pneumococcal antigen in tissues by the use of fluorescent antibody. J. Immunol. 45:159-170.
10. de BOKX, J. A. 1965. Hosts and electron microscopy of two papaya viruses. Plant Disease Rep. 49:742-746.
11. FREIDMANN, T., & M. HAAS. 1970. Rapid concentration and purification of polyoma virus and SV40 with polyethylene glycol. Virology 42:248-250.
12. GOODING, G. V., JR., & T. T. HEBERT. 1967. A simple technique for purification of tobacco mosaic virus in large quantities. Phytopathology 57:1285.
13. HEBERT, T. T. 1963. Precipitation of plant viruses by polyethylene glycol. Phytopathology 53:362.
14. HSU, H. T. 1971. Assay of potato yellow dwarf virus on leafhopper cell monolayers. Ph.D. Thesis. University of Illinois, Urbana, 65 p.
15. HSU, H. T., & L. M. BLACK. 1970. Effect of pH on viral inoculations of monolayers of insect vector cells. Phytopathology 60:1297.
16. HSU, H. T., & L. M. BLACK. 1971. Peak concentrations of potato yellow dwarf viruses in Nicotiana rustica revealed by infectivity assay on vector cell monolayers. Phytopathology 61:896.
17. HSU, H. T., & L. M. BLACK. 1973. Inoculation of vector cell monolayers with potato yellow dwarf virus. Virology 51:(In press).
18. KANAREK, A. D., & G. W. TRIBE. 1967. Concentration of certain myxoviruses with polyethylene glycol. Nature 214:927-928.
19. KIMURA, I., & L. M. BLACK. 1971. Some factors affecting infectivity assays of wound-tumor virus on cell monolayers from an insect vector. Virology 46:266-276.
20. MC SHARRY, J., & R. BENZINGER. 1970. Concentration and purification of vesicular stomatitis virus by polyethylene glycol "precipitation." Virology 40:745-746.
21. REDDY, D. V. R., & J. A. LESNAW. 1971. An improved method for purification of wound tumor virus. Phytopathology 61:907.
22. SUN, M. K. C., & T. T. HEBERT. 1972. Purification and properties of a severe strain of peanut mottle virus. Phytopathology 62:832-839.
23. WAGNER, G. G., J. L. CARD, & K. M. COWAN. 1970. Immunochemical studies of foot-and-mouth disease. VII. Characterization of foot-and-mouth disease virus concentrated by polyethylene glycol precipitation. Arch. Ges. Virusforsch. 30:343-352.
24. YAMAMOTO, K. R., B. M. ALBERTS, R. BENZINGER, L. LAWHORNE, & G. TREIBER. 1970. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. Virology 40:734-744.