Interactions of cowpea mosaic virus and *Vigna unguiculata* (cowpea) protoplasts

by

Alan G. Smith

A Thesis Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
MASTER OF SCIENCE

Department: Microbiology
Major: Microbiology (Virology)

Iowa State University
Ames, Iowa

1981

TABLE OF CONTENTS

INTRODUCTION

The specific attachment of virus to a component of the host cell, has been shown to play a major role in the determination of a virus's host cell range in the animal and bacterial systems (Luria and Darnell, 1967; Dales, 1965). The attachment of virus to cell is the first step in virus infection and is one of the major determinants of virus pathogenicity. Specific interaction of a plant virus to its host cell has not been shown to occur. Moreover, the mechanism by which a plant virus contacts and infects a cell is not defined and attachment could play a role in the determination of a cell's susceptibility.

Plant viruses can be transmitted by abrasion, insects, nematodes, fungi, grafting, parasitic plants, pollen and seeds. Most of these methods involve some type of damage to the plant before infection can occur and all involve the infection of only a very small percentage of the plant's total number of cells. These factors make it very difficult to study early events in the plant virus replication cycle. The advent of plant tissue culture, in particular the use of plant protoplasts in plant virology, has allowed the infection of large numbers of cells simultaneously. This has enabled researchers to examine, for the first time, the early events of plant virus infection. In this study,

a protoplast-plant virus interaction was used to research the attachment of plant viruses.

Vigna unguiculata (cowpea) protoplasts and cowpea mosaic virus (CPMV) were chosen as a model system to study the importance of virus attachment in determining a cell's resistance. Several resistant cultivars of V. unguiculata are known to exist with one of the cultivars having protoplasts which are resistant (Beier et al., 1977 and 1979). In addition, the techniques for protoplast isolation and inoculation have been determined (Beier and Bruening, 1975; Hibi et al., 1975) and poly-L-ornithine is not essential for infection (Beier and Bruening, 1975; Hibi et al., 1975). The lack of an absolute requirement for poly-L-ornithine during inoculation of cowpea protoplasts with CPMV eliminates a complicating factor found with most methods of protoplast inoculation.

To determine whether attachment of CPMV to the cell does function as the mechanism for cell resistance, the ability of resistant and susceptible protoplasts to bind virus was compared. The comparisons were made using iodinated CPMV in several environmental conditions.

LITERATURE REVIEW

Cowpea Mosaic Virus

Cowpea mosaic virus is the type member of the comovirus group of viruses (Gibbs and Harrison, 1976). The comoviruses are icosahedrol particles which contain single stranded RNA in a bipartite genome. The particles can be separated into three components: top (T), middle (M) and bottom (B) by their differing sedimentation in sucrose gradients. Top component particles contain no RNA and have a sedimentation value of 58 S. The middle component particles contain 1.37×10^6 molecular weight RNA and have a sedimentation value of 95 S. Bottom component particles contain 2.02×10^6 molecular weight RNA and have a sedimentation value of 115 S (Reijnders et al., 1974). The capsid is composed of two proteins with molecular weights of 44,000 and 22,000. Each particle is made up of 60 molecules of each protein (Geelen et al., 1972). Both the middle and bottom components are necessary for infection (Van Kammen and Van Griensven 1970). Rottier (1980) reported the presence of 11 proteins in CPMV infected cowpea protoplasts which were not present in mock infected protoplasts. Rottier (1980) also described the B-RNA as coding for replicase activity and other early functions in the CPMV replication cycle. Middle component codes for the two viral capsid proteins

(Rottier, 1980). In vitro synthesis and ribosome binding studies, using M-RNA, suggest that there are two initiation sites for protein synthesis on the M-RNA (Pelham, 1979). Using natural isolates differing in symptoms Thongmeearkom and Goodman (1978) assigned B-RNA to symptom production. However, Wood (1972) reported that an interaction of M- and B-RNA control lesion type.

Electrophoretic heterogenicity within the three components of CPMV has been reported (Agrawal, 1964; Semancik, 1966). A fast and slow-migrating form of each component can be separated by cellulose polyacetate or density gradient electrophoresis (Niblett and Semancik, 1970). It was shown that a host proteolytic enzyme removed amino acids from the capsid proteins to convert a slow-migrating form which predominated early in infection to a fast-migrating form that predominated late in infection (Lee et al., 1975). Niblett and Semancik (1970) correlated the conversion of slow to fast-migrating forms to an increase in relative infectivity.

The two RNA species of CPMV have a protein covalently bound to their 5' end (Stanley et al., 1978 and Daubert et al., 1978). The protein bound to the 5' end of CPMV RNA is coded for by the B-RNA (Stanley et al., 1980). Rottier (1980) hypothesized that this protein might function to anchor the RNA species to the cell membrane

during replication and provide a matrix to coordinate synthesis and assembly of the capsid proteins. Thus, the RNA-protein complex would be a part of the virus-specific cytopathic structure seen in CPMV infected cells and reported to be involved in viral RNA replication (de Zoeten et al., 1974 and Hibi et al., 1975). At the 3' end of both RNA species, is a sequence of polyodenylic acid.

From studies using actinomycin and other inhibitors, it was shown that the early events of CPMV infection require expression of a specific part of the host DNA (Rottier et al., 1979; Rottier, 1980).

Plant Protoplasts

A protoplast is simply a plant cell which has had its cell wall removed, leaving the plasmalemma exposed to the environment.

Prior to the isolation and infection of plant protoplasts, the study of plant viruses was limited to the use of whole plants and their vectors. Using intact plants to study plant viruses, has several disadvantages. The first is that only a very small percentage of the cells are infected initially. Secondly, synchrony of infection and replication is not possible since the virus is continually being spread from the point of

inoculation to other areas of the plant. Plant protoplasts can be used to obtain large numbers of simultaneously infected cells in which the virus is replicating in a synchronous fashion. They are also used in many other types of investigations outside the area of plant virology (Fowke and Gamborg, 1980).

With the cell wall completely removed, protoplasts are an excellent means to study how cell wall components are synthesized, transported and assembled (Cocking, 1970). Once the cell wall has been regenerated many types of protoplasts can begin cell division and ultimately form whole plants, making it possible to study DNA replication, cell division and cell differentiation in a controlled environment. Since the plasmalemma is exposed, protoplasts can be used to study the structure and components of the plasmalemma as well as the mechanisms of transport of molecules into the cell (Fisher, 1979). Many substances which could not be introduced into the cell because of the cell wall acting as a barrier will be readily taken up by protoplasts. Thus, the effects of many substances on the cell can be examined. Protoplasts afford a gentle means to isolate many cellular organelles without damage to the organelle (Quail, 1979).

One of the more interesting applications of plant protoplasts is their ability to fuse to create somatic

hybrids between two plants (Fowke and Gamborg, 1980). In this procedure, membrane fusion between two types of protoplasts is enhanced by the use of a fusion agent such as polyethylene glycol. The fate of a fusion product may be highly variable depending on the type of plant protoplasts being used, but the technique offers a chance of genomic mixing otherwise impossible.

Plant protoplasts have been isolated from a wide variety of plants. Many of these protoplasts have been used in plant virus studies (Takebe, 1975; Zaitlin and Beachy, 1974; Fowke and Gamborg, 1980). Protoplasts were first isolated by Cocking (1960) from tomato. Later it was shown that protoplasts could be infected and synchronous replication occurred (Cocking, 1966; Cocking and Pojnar, 1969; Aoki and Takebe, 1969; Takebe and Otsuki, 1969). Rapid expansion of the use of plant protoplasts for studying plant viruses has led to the development of many model systems of protoplast isolation and virus inoculation.

Vigna unguiculata protoplasts were first isolated by Hibi et al. (1975) and Beier and Bruening (1975). Although their isolation and inoculation procedures differed slightly, they were both able to isolate large numbers of viable protoplasts and show infection by CPMV. Both researchers concluded that poly-L-ornithine

was not an essential component of the inoculation medium. However, poly-L-ornithine did stimulate infection.

There are several advantages to using cowpea protoplasts relative to other plant protoplast systems. One advantage is that 9 to 11-day-old plants can be used (Hibi et al., 1975; Beier and Bruening, 1975), which is in contrast to the tobacco system that uses 60 to 80-day-old plants (Takebe et al., 1968). Also, the method described by Beier and Bruening (1975) eliminates the tedious task of peeling the epidermis from the leaves to expose the mesophytic tissue to cell wall degrading enzymes. In this method, the epidermis is abraded by lightly brushing with carborundum. An important advantage of using cowpea protoplasts to study CPMV is that the polycation poly-L-ornithine is not essential for infection. In most other protoplast-plant virus systems studied, there has been an absolute requirement for the use of a polycation during infection (Takebe, 1975; Zaitlin and Beachy, 1974; Burgess et al., 1973a). It is interesting to note that poly-L-ornithine has also been found to be nonessential for infection of cowpea protoplasts by cucumber mosaic virus (Koike et al., 1977) but essential for infection by alfalfa mosaic virus (Albas and Bol, 1977) and clover yellow mosaic

virus (Rao and Hiruki, 1978). The possible functions of poly-L-ornithine in virus uptake will be discussed in the next literature review section.

Cowpea protoplasts have been used to study several aspects of CPMV infection. Beier et al. (1977) surveyed the susceptibility of intact plants and protoplasts of 1031 lines of cowpeas. Of the 1031 lines, 65 were found to be resistant to CPMV by mechanical inoculation of whole plants. Fifty-five of the 65 resistant lines were used to isolate protoplasts; only one of the lines had protoplasts which were also resistant to CPMV infection. In a later study (Beier et al., 1979), the mechanism of immunity of the intact plants and protoplasts was examined. It was reported that the line of resistant protoplasts would support CPMV replication but only at 1% of that achieved in the line of susceptible protoplasts. Several hypotheses accounting for the differences in whole plant and protoplast susceptibility to CPMV were given but no conclusion was drawn. Rottier (1980), Rottier et al. (1979), and Rezelman et al. (1980) used cowpea protoplasts to study many aspects of CPMV infection, including protein synthesis, effects on host cell, and individual component expression.

Plant Virus Attachment to Protoplasts

In order to initiate infection of a plant cell, a virus must first come in contact with the cell and release its genome. In some of the animal systems studied, the specific attachment of the virus to the cell membrane determines whether the cell is resistant or susceptible to infection (Dales, 1965). Susceptible cells have specific sites for virus attachment, whereas resistant cells lack these sites. Therefore, virus can not absorb to the cell membrane to initiate infection. It should be noted that there are other factors besides attachment, such as inhibition of uncoating, transcription or translation which can be determinants of susceptibility to viral infection.

Attachment studies have been performed using whole plants (de Zoeten, 1981; Cocking, 1970). Results indicated that virus attachment to the plants involved the interaction of virus with trichomes damaged during the mechanical inoculation process. Kontaxis and Schlegel (1962) reported the accumulation of tobacco mosaic virus particles on the basal septa of broken trichomes of tobacco leaves. However, Herridge and Schlegel (1962) indicated that damage associated with trichomes may have a role in tobacco mosaic virus infection but are not factors in determining host susceptibility. The ectodesmata

have also been implicated as sites for virus infection. By treating tobacco leaves in specific ways, Brants (1964) was able to increase the number of visible ectodesmata and correlate this increase with an increase in the number of local lesions on the leaves after mechanical inoculation with tobacco mosaic virus. Thomas and Fulton (1968) were also able to correlate the number of ectodesmata with the susceptibility of tobacco to tobacco mosaic virus and concluded that ectodesmata serve as infection sites. However, Merkens et al. (1972), in an electron microscopy study, could not identify either tobacco mosaic virus particles or RNA at the ectodesmata and concluded that they do not serve as infection sites but may serve as sites for transport of uncoated viral RNA to the plasmalemma.

The use of inactivating agents has allowed researchers to develop a tentative time course of events during virus infection. By determining when during infection a virus is susceptible to an agent, Mink (1976) was able to identify four distinct phases of infection of cowpea leaf epidermal cells by peanut stunt virus. He proposed that the virus was confined in the epidermus for 3.5 hours prior to moving into the mesophylic tissue. In a study of cucumber mosaic virus and peanut stunt virus infection of cowpea leaf epidermal cells, Ehra and Mink (1980) concluded that the virus became attached immediately

to some stationary cell component and that the epidermal cells can distinguish between the two particles and their RNAs.

To determine if attachment of plant viruses to the host plays a role in determining susceptibility, investigators have compared the ability of virus to attach to host and nonhost plants. Takagi and Takahushi (1972) used two host and two nonhost plants to compare relative amounts of tobacco mosaic virus attachment. They found no difference in attachment between host and nonhost plants which were mechanically inoculated or in attachment to cell debris. Shaw (1969) reported that tobacco mosaic virus uncoating occurs in two distinct steps and that the earliest stage occurs in both host and nonhost plants. Gaard and de Zoeten (1979) also concluded that end-on virus attachment and uncoating occurs in both host and nonhost plants of tobacco rattle virus. This indicates that virus attachment may be a nonspecific interaction of virus and cell wall. Further evidence for a nonspecific virus host interaction was found by Niblett (1975) who studied the attachment of four viruses to host and nonhost plants. He concluded that virus attached to membrane sites of both host and nonhost plants and that this attachment was due to the relative surface charge of the virus particle. These results

agree with those reported by Shaw (1972a) that brome mosaic virus, with a relatively greater positive charge than cowpea chlorotic mottle virus, was retained to much greater degree by both host and nonhost plants.

There has also been evidence which supports the hypothesis of specific virus host cell interaction. Novikov and Atabekov (1970) studied the interference of infection between tobacco mosaic virus and barley stripe mosaic virus. They found that homologous virus was able to interfere with infection of Chenopodium amaranticolor but the heterologous virus was not able to interfere. Kiho (1974) was able to isolate a membrane fraction from tobacco plants which seemed to adsorb tobacco mosaic virus in a specific manner. This adsorption was inhibited by homologous virus and cucumber green mottle virus, but not by potato virus X or rice dwarf virus. However, he also found membrane fractions from non-hosts which could absorb tobacco mosaic virus.

Studies of plant virus attachment to protoplasts have been complicated by the presence of the polycation poly-L-ornithine during protoplast inoculation. In the development of methods to infect protoplasts with plant virus it was noted that poly-L-ornithine enhanced or was essential for infection and that poly-L-ornithine stimulated the number of virus particles bound to a

protoplast (Wyatt and Shaw, 1975; Zhuravlev et al., 1975; Shaw, 1972b). The first reports indicated that poly-L-ornithine increased infection by stimulating pintocytosis (Cocking and Ponjar, 1969; Zhuravlev et al., 1976). However, other investigations hypothesized that damage to the plasmalemma and virus aggregation were important effects of poly-L-ornithine stimulation of virus infection (Burgess et al., 1973b; Kubo et al., 1976; Kassanis et al., 1977). To date the exact mechanism remains unresolved.

Polyethylene glycol has also been used to enhance infection of protoplasts with plant viruses (Maule et al., 1980, Cassells and Barlass, 1978). The mechanism of increased infection using polyethylene glycol would appear to be completely different from that of poly-L-ornithine. Cassells and Barlass (1978) and Cassells and Cocker (1980) have reported that polyethylene glycol increases infection by trapping virus particles between the fusing plasmalemma of two protoplast. Thus, polyethylene glycol acts as a physical agent to obtain virus infection.

It would appear that plant virus attachment may not play a major role in determining host resistance. However, the area of plant virus attachment is not well-understood and some studies have indicated that specific attachment may occur. The use of protoplasts to

study the early events of plant virus infection should clarify some of the questions which still exist.

MATERIALS AND METHODS

Virus Purification

Cowpea mosaic virus was obtained from C. L. Niblett Department of Plant Pathology, University of Florida, Gainesville. Virus was propagated by mechanically inoculating 12 day-old Vigna unguiculata cv. California Blackeye (Calif. Bl.) after light dusting with 600 mesh carborundum. Virus was purified from systemically infected tissue 10 days post infection. Infected tissue was harvested and stored at -20°C overnight. One kg of tissue was homogenized for two min in a Waring Blender with 1.5 of 0.1 M potassium phosphate buffer pH 7.0. The infectious sap was squeezed through two layers of cheesecloth. The filtrate was clarified by the slow addition of a chloroform-butanol mixture (1:1 v/v) at a ratio of 1:1.5 (v/v) filtrate to chloroform-butanol. The emulsion was placed at 4°C for 15 min and then broken by centrifugation in a Sorvall RC2B refrigerated centrifuge (5,000 rpm for 7 min in a GSA rotor). The aqueous phase was collected and centrifuged at 10,000 rpm for 30 min in a GSA rotor. Virus was precipitated from the supernatant by the addition of polyethylene glycol, molecular weight 6,000, to 4% (w/v) and NaCl to 0.2 M. All further virus purification steps were carried out at 4°C. The

mixture was stirred for one hr after which time virus was collected by centrifugation for 15 min at 10,000 rpm in a GSA rotor. Virus pellets were dissolved overnight with gentle shaking in 0.25 M potassium phosphate buffer pH 7.0. Resuspended virus was homogenized in a glass Dounce Homogenizer and further clarified by centrifugation at 10,000 rpm in a Sorvall SS34 rotor. The supernatant was concentrated by high speed centrifugation in a Beckman Model L2-65B Ultracentrifuge (40,000 rpm for two hr in a Ti45 rotor. The pellet was resuspended in 0.2 M potassium phosphate buffer and homogenized in a glass Dounce Homogenizer. The resuspended virus was centrifuged 10 min at 10,000 rpm in an SS34 rotor. The virus containing supernatant was further purified by three cycles of the following differential centrifugation procedure. Virus was pelleted by high speed centrifugation at 40,000 rpm in a Ti45 rotor for 2 hr and resuspended in 0.02 M potassium phosphate buffer pH 7.0. The resuspended virus was homogenized in a glass Dounce Homogenizer and clarified by centrifugation at 10,000 rpm in an SS34 rotor. The differential centrifugation cycle was then repeated. The purification procedure was a modification of the procedure used by Van Kammen (1967).

Virus concentration was determined by absorbance at 260 nm using an extinction coefficient reported for

cowpea mosaic virus three component mixtures of 8.0 cm^2 mg^{-1} (Niblett and Semancik, 1969).

Media and Reagent Preparation

Water used in the preparation of all media and reagents was distilled and passed through a Super-Q Pure Water System (Millipore Co., Bedford, Mass.). When distilled water was forced through this system, organic ions were removed by activated charcoal filters, inorganic ions, by mixed bed ion-exchange resin filters and particulate material by a 0.8 μm filter. Water had a resistivity of 18 meghom-cm and was termed deionized distilled water (D-D H_2O).

Ouchterlony double diffusion tests

Ouchterlony double diffusion tests were used in 100 x 15 mm petri dishes for examining reactions between antigen and antisera. The following ingredients were dissolved by heating to 100°C with constant stirring:

4.5 g Noble Agar

4.25 g NaCl

0.5 g NaN_3

and enough water to make 500 ml.

Chromatography buffer

Chromatography buffer was used in the preparation of fluorosceinisothiocyanate conjugated antisera and fluorescent staining of protoplasts. It was adjusted to pH 7.2 by addition of 1 M NaOH or HCl. The composition was:

17.6 g NaCl

2.0 g Na_2HPO_4

0.68 g $NaH_2PO_4 \cdot 1\ H_2O$

and enough water to make 2 liters of solution.

Phosphate buffered saline (PBS)

PBS used in the ELISA system was prepared by mixing solution A and B until a pH of 7.2 was reached. The two solutions contained

A

2.76g $NaH_2PO_4 \cdot M_2O$

8.5 g NaCl

and enough water to make 1 liter of solution,

B

5.67 g Na_2HPO_4

17.0 g NaCl

and enough water to make 2 liters of solution.

PBS-Tween 20-Polyvinylpyrrolidone-Egg Albumin (PBS-Tween-PVP-OVA)

PBS-Tween-PVP-OVA was used in the enzyme-linked-immunosorbent-assay (ELISA) system and contains:

0.5 ml Tween-20 (Sigma, St. Louis, Mo.)

20.0 g Polyvinyl pyrrolidone (Sigma, Molecular Weight 40,000)

2.0 g Egg Albumin (Sigma, Grade II)

and enough PBS to make 1 liter of solution.

PBS-Tween 20 (PBS-Tween)

PBS-Tween was used for the dilution of antigen, antisera and enzyme-conjugated antisera for the ELISA system. It was prepared as PBS-Tween-PVP-OVA solution without polyvinylpyrrolidone or egg albumin.

Hoagland's growth solution

Hoagland's growth solution (Dunn and Arditti, 1968) was used for the hydroponic growth of plants prior to use for protoplast isolation. A concentrated solution was prepared as follows and diluted to single strength by the addition of 1:8.3 (v/v) concentrated Hoagland's solution to DD-H_2O

5.58 g $Na_2C_{10}H_{14}O_8N_2 \cdot 2H_2O$

6.81 g KH_2PO_4

25.28 g KNO_3

59.04 g $Ca(NO_3)_2 \cdot 4H_2O$

12.04 g $MgSo_4$

2.50 g $FeSO_4$

0.15 g H_3BO_3

0.09 g $MnCl_2 \cdot 4H_2O$

0.01 g $ZnSo_4 \cdot 7H_2O$

0.01 g $CuSO_4 \cdot 5H_2O$

0.01 g $Na_2MoO_4 \cdot H_2O$

and enough $DD \cdot H_2O$ to make 6 liters of solution.

Enzyme solution

Enzyme solution (Beier and Bruening, 1975) was used for the one step digestion of cowpea plant tissue to free protoplasts. It contained:

0.50 g Macerase (Calbiochem-Behring Corp., Bedford, Mass., 3,000 units/gm)

1.50 g Cellulysin (Calbiochem-Behring Corp., Bedford, Mass., 10,000 units/gm)

0.50 g Bovine serum albumin (Sigma, Fraction V)

10.93 g D-mannitol

and enough $DD \cdot H_2O$ to make 100 ml of solution. To increase

the solubility of the enzymes the pH was adjusted to 9.0 with 1.0 M NaOH and then back to 5.6 with 1.0 M HCl. The solution was sterilized by passage through a 0.22 µm millipore filter and used immediately.

Culture medium

Culture medium (Rottier, 1980) was used for the culture of protoplasts during CPMV replication. This medium was described by Rottier (1980) and contains:

109.32 g Mannitol

27.22 g KH_2PO_4

0.10 g KNO_3

0.12 g $MgSO_4$

1.47 g $CaCl_2 \cdot 2H_2O$

0.25 mg $CuSO_4 \cdot 5H_2O$

0.001 g 2, 4-dichlorophenoxyacetic acid

0.002 g Loridine (Eli Lilly & Co.)

and enough $D\text{-}DH_{20}$ to make 1 liter of solution. The pH was adjusted to 5.4 by the addition of 1 M NaOH or 1 M HCl and the solution was sterilized by passage through a 0.22 µm millipore filter.

Wash medium

Wash medium (Rottier, 1980) was used to remove unwanted material from protoplast suspension. It contained:

109.32 g D-mannitol

1.47 g $CaCl_2 \cdot 2H_2O$

and enough DD-H_2O to make 1 liter of solution. The solution was adjusted to pH 5.2 by the addition of 1 M NaOH or 1 M HCl autoclaved.

Inoculation medium

Inoculation medium was used for the infection of protoplasts and in virus-protoplast attachment studies. It was the base medium for several types of inoculation media formed by the incorporation of 7 different additives. It contained:

109.32 g D-mannitol

3.24 g $K_3C_6H_5O_7 \cdot H_2O$

and enough DD$\cdot H_2O$ to make 1 liter of solution. Inoculation medium was adjusted to pH 5.2 by the addition of 1 M NaOH or 1 M HCl.

Alkaline hydrolyzed gelatin

Alkaline hydrolyzed gelatin was used to control nonspecific reactions of FITC conjugated antisera and protoplasts during fluorescent staining (Bohol and Schmidt, 1968). It contained

2.0 g Bacto-gelatin (Difco)

and enough water to make 100 ml of solution. The pH was adjusted to 10.5 with 1 M NaOH and then autoclaved

for 10 min at 121°C. The solution was cooled to 25°C and the pH readjusted to 10.5.

Buffered glycerol

Buffered glycerol (Thomason and Wells, 1971) was used to control autoflourescence of fluorescent stained protoplasts. Buffered glycerol contained

2.28 g $K_2HPO_4 \cdot 3H_2O$

90 ml glycerol

10 ml DD-H_2O.

Antisera Production and Cross-absorption

Antisera against CPMV was prepared in New Zealand White rabbits by intramuscular injection into the hind leg of one ml of an emulsion of equal volumes of CPMV (0.5 mg/ml) and Freund's incomplete adjuvant (Difco, Detroit, Mich.). Four booster inoculations were given at one week intervals using CPMV concentrations of 0.5, 1.0, 1.0 and 2.0 (mg/ml). Blood was collected by cardiac puncture one week after the last booster injection. Serum was collected by removal of clotting factors from the blood. The serum was titered by a microprecipitin test (Ball, 1974), and stored at -30°C until used. Protein concentrations of antisera preparations were determined by the Biuret reaction (Gornell et al., 1949).

Antisera were cross-absorbed as described by McLaughlin et al. (1980) by the addition of lyophilized healthy cowpea tissue to antisera. For the cross-absorption of 10 ml of antisera, 40 g of healthy Calif. Bl. tissue from 22 day-old plants was homogenized in 40 ml of 0.05 M sodium borate buffer, pH 7.2 in a Waring blender. The sap was expressed through two layers of cheesecloth and dialyzed overnight at 4°C against 0.01 M sodium borate buffer pH 7.2. The sap was quick frozen in a dry ice acetone bath and placed on a Virtis lyophilizer until all water had been removed from the tissue. The lyophilized tissue was divided into four equal portions. One portion of lyophilized tissue was added to 10 ml of serum. The mixture was stirred at 4°C for 12 hr. Antisera-plant tissue complex and unreacted plant tissue was removed by high speed centrifugation at 15,000 rpm in an Ti21 rotor. The three remaining portions of lyophilized tissue were used in the same manner. Cross-absorbed antisera was titered by the microprecipitin test (Ball, 1974) and stored at -30°C.

To determine if all immunoglobulins reacting with healthy plant tissue had been removed, Ouchterlony double diffusion tests were performed. Twenty-two ml of dissolved agar were poured into a 100 x 15 mm petri plate and allowed to solidify. Using a Grafar gel punch

assembly (Grafar Corp., Detroit, Mich.) 4 mm diameter wells were cut in the agar in a pattern of six evenly distributed wells around a single center well (all wells were 5 mm apart). The center well was charged with the antisera, the six outer wells were divided into pairs and charged with healthy Calif. Bl. Sap, infected Calif. Bl. sap, and purified CPMV (1.0 mg/ml). Healthy Calif. Bl. sap was prepared from uninoculated 22 day-old Calif. Bl. primary and secondary leaves which had been stored frozen and ground with a mortar and pestle in 0.02 M potassium phosphate buffer (1:1 w/v). The sap was then squeezed through two layers of cheesecloth. Infected Calif. Bl. sap was prepared in the same manner except plants where mechanically inoculated with CPMV 10 days after planting. Plates were incubated in a moist chamber at 25°C for 10 days. The plates were observed daily with indirect light to detect any formation of precipitin bands.

FITC Conjugation with Antisera

Cross-absorbed antisera was conjugated with fluorosceinisothiocyanate by the method of Lewis et al. (1964). The major portion of albumin was removed from the antisera by precipitation with $(NH_4)_2SO_4$ (Campbell et al., 1970). One-third volume of saturated

$(NH_4)_2SO_4$ was added slowly with stirring to two-thirds volume of antisera. The mixture was stirred for 15 min at 25°C and then centrifuged at 2,000 rpm in an SS34 rotor. The supernatant was discarded and the pellet redissolved in a small amount of chromatography buffer. The $(NH_4)_2SO_4$ precipitation procedure was repeated. FITC was dissolved in 0.1 M potassium phosphate buffer pH 10.5 (0.625 mg/ml). Two ml of FITC solution was added, dropwise with stirring, to 2.5 ml of cross-absorbed-antisera (10 mg/ml). The pH of the solution was adjusted to 10.5 with 0.1 M NaOH and the mixture allowed to incubate for 6 to 10 hr at 25°C without mixing. The FITC conjugated antisera was removed from free FITC by gel filtration (Curtain, 1961). The FITC-antisera mixture was applied to a 20 x 2.5 cm column of Sephadex G-25 (Pharmacia) and eluted with chromatography buffer. Conjugate fractions were collected and stored at -30°C until used.

Assay of Cultivar Susceptibility

A cowpea cultivar susceptible to CPMV infection (Calif. Bl.) was obtained from the W. Altee Burpee Co., Clinton, Ia. Five immune cultivars (P.I. numbers 293467, 293514, 293453, 293582, and 364495) and one local lesion cultivar (P.I. number 194207) were selected from the survey of cowpea cultivar susceptibility to CPMV

infection (Beier et al., 1977) for use in this study. These cultivars were obtained from the U.S. Department of Agriculture, Regional Plant Introduction Station, Experiment, Georgia.

To determine cultivar susceptibility to CPMV infection, five 12-day-old plants of each cultivar were mechanically inoculated with purified CPMV (250 µg/ml) in 0.02 M potassium phosphate buffer by lightly dusting leaves with 600 mesh carborundum and rubbing each primary leaf with CPMV solution. Noninoculated control plants were treated in the same manner except 0.02 M potassium phosphate buffer was substituted for CPMV solution. Primary and secondary leaves of all five plants from each cultivar were harvested 10-days post infection. Prior to harvesting any symptoms due to CPMV infection were recorded. The leaves were then ground in 0.02 M potassium phosphate buffer pH 7.0, at a ratio of 1:1 (w/v), and the sap squeezed through two layers of cheesecloth. The sap was then used for Ouchterlony double diffusion tests and inoculation of Calif. Bl. indicator plants. Ouchterlony plates were prepared as described previously. The center well was charged with cross-absorbed antisera. The three pairs of outer wells received undiluted sap, and 1:2 and 1:4 dilutions of sap. Dilutions were made with 0.02 M potassium

phosphate buffer pH 7.0. The plates were incubated at 25°C for 10 days in a moist chamber and observed daily for the development of precipitin bands. To detect CPMV present in the sap at concentrations below the sensitivity of the Ouchterlony test, susceptible 12-day-old Calif. Bl. plants were inoculated. Any virus present in the sap would infect the susceptible plants and increase in concentration. These inoculated plants were observed for symptoms and assayed for presence of CPMV by ouchterlony tests as described.

ELISA Procedure

A solid phase enzyme-linked immunosorbent assay (ELISA) using polystyrene beads was used to ascertain if antigenic differences existed between ^{125}Iodine (^{125}I) labelled CPMV and nonlabelled CPMV. Before the test was used, the optimal conditions for ELISA were determined.

Optimal concentrations of coating antibody and enzyme conjugate were determined by varying the concentrations of either coating antibody or enzyme conjugate as described in the ELISA system of Chen (1981). Cross-absorbed antisera and alkaline phosphatase (E.C. No. 3.1.3.1., Sigma Type VII, 5 mg/ml in a suspension of 3.2 M ammonium sulfate, pH 7.0, containing 0.001 M magnesium chloride

and 0.0001 M zinc chloride) were conjugated by the addition of enzyme to 2.0 ml of antisera (1.0 mg/ml) in a ratio of 2:1 (w/w) enzyme:antisera. This mixture was dialyzed 12 hr at 4°C against PBS. Aqueous glutaraldehyde (25%) was added to a final concentration of 0.2% (v/v) and the mixture was dialyzed 48 hr at 4°C against PBS.

ELISA was performed using 6.5 mm polystyrene beads (Precision Plastic Ball Co., Chicago, Ill.), sensitized to cross-absorbed antisera. The optimum concentration of coating antibody used to sensitive beads was determined by incubating 20 polystyrene beads in 10 ml of cross-absorbed antisera at concentrations from 0 to 10 (µg/ml) diluted in 0.05 M $NaCO_3$ buffer pH 9.6 at 25° for 6 hr with gentle agitation. Beads were washed three times with PBS-Tween-PVP-OVA, by aspirating the liquid and refilling with PBS-Tween-PVP-OVA. The beads were then incubated in PBS-Tween for 1 hr. The sensitized beads were placed into Falcon 12 x 75 mm plastic test tubes which had been precoated for 1 hr at 25°C with PBS-Tween plus 2.0% (w/v) egg albumin. One ml of purified CPMV (5.0 µg/ml) diluted in PBS-Tween was added. The tubes were capped and incubated for 12 hr at 25°C with gentle shaking. Virus solution was removed and the beads were washed three times with PBS-Tween-PVP-OVA. The beads were transferred to new precoated Falcon tubes

and each received 0.5 ml of enzyme conjugate diluted 1:1000 with PBS-Tween. The tubes were capped and incubated at 37°C for 6 hr. Excess conjugated antisera was removed by three washings with PBS-Tween-PVP-OVA. Beads were transferred to 10 x 75 mm glass test tubes and 0.5 ml of alkaline phosphatase substrate (p-nitrophyenylphosphate 1.0 mg/ml, in 10% diethanolamine pH 9.8) was added. Beads were incubated at 25°C for 2 hr after which the reaction was stopped by the addition of 50 µl of 3.0 M NaOH. The reaction was read by absorbance of the solutions at 405 nm.

The optimum concentration of conjugated antisera to be used in the ELISA system was determined by using dilutions of the conjugate from 1:50 to 1:10,000 in the standardized system previously described. Cross-absorbed antisera at a concentration of 0.5 µg/ml was used for coating beads used in the optimization of conjugate concentration.

The sensitivity of the ELISA system was determined by varying the virus concentrations used from 0.0 to 95 µg/ml while using a coating antibody concentration of 0.5 µg/ml and conjugate dilution of 1:1,000.

Local Lesion Assay for CPMV

To determine the amount of infective virus, a local lesion assay using V. unguiculata C.V. Chinese Red X Iron (P.I. number 194207) was developed. Beier and Bruening (1975) reported a local lesion method for the detection of infective CPMV in protoplast extracts. The combination of the Beier and Bruening method with that of de Jager (1976) led to the development of a local lesion assay which gave a significant correlation between number of local lesions and CPMV concentration.

Vigna unguiculata plants were grown in a soil mixture in 10 cm pots with four plants per pot. Plants to be used in the local lesion assay were germinated and grown under controlled conditions. Plants were maintained in a Modutrol control system (Percival Co., Boone, Ia.) having a day cycle of 14,000 ft candles and 28°C and a night cycle at 23°C. Half leaves were marked by placing a small notch at either the right or left hand side of the leaf near the stem. The half leaves with the notch were inoculated with a standard amount of purified CPMV (2.5 µg/ml) in 0.02 M potassium phosphate buffer by lightly rubbing the leaf with the index finger moistened with the virus solution. Care was taken to give each half leaf 5 one-way strokes with the index finger. A series of purified CPMV dilutions were inoculated onto the other

half leaves with each dilution being used on at least 8 half leaves. The local lesions on each half leaf were counted 15 days post inoculation. Relative infectivity was calculated by dividing the total number of local lesions on a set of dilution half leaves, by the total number of local lesions on the corresponding half leaves inoculated with the standard amount of CPMV and multiplying by 100.

^{125}Iodine Labeling of Virus

Cowpea mosaic virus was labelled by a procedure using glucose oxidase and lactoperoxidase immobilized on hydrophilic spheres. When glucose is added to the enzyme mixture and the protein being labeled, glucose oxidase produces a small steady amount of hydrogen peroxide. Lactoperoxidase then catalyzes the peroxide oxidation of labeled iodide to iodine, which in turn reacts with the tyrosine residues of any protein present to produce iodinated protein. Since the amount of oxidizing agent (H_2O_2) in this system is controlled, there is very little damage done to the protein being labeled (Karonen et al., 1975; Tower et al., 1977). Additionally, the iodinated product formed is more stable than iodinated proteins formed by other radioiodination procedures, because less iodinated by-products were formed and less

double iodinated tyrosines were formed (Karonen et al., 1975; Tower et al., 1977).

Cowpea mosaic virus was iodinated by the addition of 25 µl of 1% beta-D-glucose to a solution containing: 50 µl of 0.2 M potassium phosphate buffer pH 7.2, 25 µl of CPMV (4.0 mg/ml in sodium azide free 0.02 M potassium phosphate buffer pH 7.0), 10 µl of $Na^{125}I$ (New England Nuclear, Boston, Mass., low pH, high concentration containing 1.0 m Ci of activity), 50 µl of Enzymobead Reagent (Pharmacia, enzymobeads were rehydrated at least one hr prior to use by the addition of 0.5 ml of deionized-distilled water). The mixture was allowed to react for 25 min, after which the reaction was stopped by separating enzymobeads and free $^{125}I_2$ from iodinated CPMV (I_2^{125}-CPMV) by applying the mixture to an 0.8 x 30 cm column containing Sephadex G25 (Pharmacia, Boston, Mass.). Ten drop fractions were collected on I_2^{125}-CPMV was eluted with 4°C 0.02 M potassium phosphate buffer pH 7.2. The radioactivity of each fraction was determined by counting the fraction in a Beckman DPM-100 liquid scintillation system using LSC Gamma Vials (Research Products International Corp.). The absorbance at 260 nm of each fraction was determined using a Beckman-DB spectrophotometer. The fractions containing both radioactivity and absorbance at 260 nm were pooled and again applied to an 0.8 x 30 cm

column containing Sephadex G25. Iodinated-CPMV was eluted and pooled as in the first gel-filtration. The protein concentration of the pooled fractions was determined spectrophotometrically using an extinction coefficient of 8 cm^2 mg^{-1} at 260 nm.

The specific activity of each $^{125}I_2$-CPMV mixture was determined by removing a 25 µl sample from the pooled fractions and counting. Three-tenths ml of unlabeled CPMV (4.0 mg/ml) was added to the sample as a carrier protein. Protein in the sample was precipitated by the addition of 0.9 ml of cold trichloroacetic acid (10% v/v) and incubating at 4°C for 30 min. The mixture was centrifuged at 5,000 rpm in an SS34 rotor and both the pellet and supernatant were counted. Total counts per minute per ml (cpm/ml) were determined by dividing the cpm of the 25 µl sample by 0.025 ml. The fraction of $^{125}I_2$ bound to CPMV was determined by dividing the cpm of the pellet by the sum of the cpm of the pellet and the supernatant. Specific activity was calculated by dividing the product of total cpm/ml and fraction of $^{125}I_2$ bound by the protein concentration of the $^{125}I_2$-CPMV mixture.

Antigenicity and Relative Infectivity of Labeled Virus

The potential change in CPMV during the radioiodination procedure was determined by comparing the antigenicity

and relative infectivity of $^{125}I_2$-CPMV and unabeled CPMV. Since $^{125}I_2$-CPMV was used to determine the ability of CPMV to attach to cowpea protoplasts, any change in the capsid proteins that could occur during the radioiodination procedure might affect the binding properties of CPMV.

Any differences in antigenicity between $^{125}I_2$-CPMV and unlabeled CPMV were determined by the ELISA system. Three sets of $^{125}I_2$-CPMV from three separate radioiodination reactions were diluted to 5.0 µg/ml in PBS-Tween. Unlabeled CPMV was also diluted to 5.0 µg/ml and used as a control. These dilutions were then used as antigens in the ELISA system described previously. The optimum concentrations of coating antibody (0.5 µg/ml) and enzyme conjugate (1:1,000 dilution) were used. The antigenicity of labeled CPMV was compared to that of unlabeled CPMV by the ability to react with homologous antisera. The ability to react with antisera is directly correlated with the absorbance (405 nm) of the substrate after reacting with the complex bound to the polystyrene bead.

Differences in infectivity of labeled and unlabeled CPMV were determined by their ability to cause local lesions. The three sets of labeled CPMV and unlabeled CPMV were diluted to 2.5 µg/ml. Local lesion assays were performed on each set of labeled CPMV and the unlabeled CPMV. Each $^{125}I_2$-CPMV and unlabeled CPMV was

inoculated onto eight half leaves as described for the local lesion assay of CPMV. The relative infectivities were calculated for each set of $^{125}I_2$-CPMV and compared to that calculated for unlabeled CPMV.

Growth of Plants for Protoplast Isolation

The physiological state of plants used for the isolation of plant protoplasts is very important (Rottier, 1980). To consistently isolate large numbers of undamaged protoplasts the plants used in the isolation must be grown under highly controlled conditions. The conditions for the growth of cowpeas to be used for protoplast isolation were first reported by Beier and Bruening (1975) and Hibi et al. (1975), and further modified by Rottier (1980).

Cowpea seeds were germinated in trays containing vermiculite and D-DH_2O 50% (v/v) at 30°C. Forty to 48 hr later seedlings were removed from the vermiculite and placed in Hoagland's growth solution. The seedlings were grown in a growth chamber with a day cycle of 28°C and 1,400 ft candles light intensity and a night cycle of 23°C for three days. The light intensity was then increased to 1,600 ft candles during the day cycle with the same growth temperature for the last five days of growth. Light intensities were measured with a Weston

Model 756 Sunlight Illumination Meter (Western Instrument, Inc., Newark, NJ). Ten days after seeding, primary leaves were fully expanded and used for protoplast isolation.

Isolation, Inoculation, and Culture of Protoplasts

Hibi et al. (1975), Beier and Bruening (1975) and Rottier (1980) have all described methods for the isolation of protoplasts from cowpeas. The method used in this study used a combination of all three methods but was greatly influenced by the method described by Rottier (1980).

Protoplasts were isolated from the primary leaves of 10-day-old cowpea plants grown as described earlier. The leaves were excised and washed in 70% ethanol and rinsed immediately in four changes of sterile DD-H_2O. To allow enzyme penetration, the lower epidermis of the leaf was abraded by lightly brushing 320 mesh carborundum (Fisher Scientific Co.) over the surface with a sterile, soft brush. The leaves were again washed four times in sterile DD-H_2O to remove all carborundum present. The midribs of the leaves were removed and the leaves were placed lower surface down into 0.6 M D-mannitol pH 5.6 for 1 hr at 25°C. The 1 hr treatment with osmoticum prior to enzyme treatment plasmolyzed the cells so that enzyme penetration into the cells was kept to a minimum.

The osmoticum was decanted from the leaves and 10 ml of enzyme solution was added for each one g of leaf tissue present. The tissue was incubated with enzyme for 1.5 hr with gentle agitation at 30°C. Undigested tissue was removed from the protoplasts by filtration through two layers of cheesecloth. Protoplasts were washed three times by centrifugation at 154 x g in a Model CL International clinical centrifuge (International Equipment Co., Cleveland, Ohio) for 2 min and resuspended in wash medium. The concentration of protoplasts was determined by counting the number of cells in a Neubauer hemacytometer. Enough protoplast solution to give a total of 5×10^6 protoplasts was centrifuged at 154 x g to pellet the protoplasts. These protoplasts were resuspended directly in 10 ml of inoculation medium containing 5 µg/ml CPMV. The protoplasts were incubated in this solution for 15 min at 25°C, with one agitation of the cell suspension 7 min post infection. Excess CPMV was removed by washing the protoplasts three times in wash medium as described previously. After the third centrifugation, protoplasts were resuspended in 10 ml of culture medium. Protoplasts were cultured at 25°C in 25 ml erlenmeyer flasks under constant light intensity of 500 ft. candles from two fluorescent bulbs. Control

cultures were treated in the same manner except no CPMV was included in the inoculation medium.

Assay of Protoplast Infection

The amount of virus produced by protoplasts at various times post inoculation was determined by the amount of infective virus present in the protoplast suspension and the amount of virus antigen present. The amount of infective CPMV present in infected protoplasts was determined by the local lesion assay using V. unguiculata cv. Chinese Red X Iron. The amount of CPMV antigen present in the inoculated cells was determined by staining the protoplasts with FITC conjugated antisera.

The amount of infective virus in protoplast suspensions was determined at 0, 12, 24, 36, 48, and 60 hr post inoculation. The protoplasts from culture flasks containing 10 ml of 5×10^5 protoplasts/ml were collected by centrifugation at 154 x g for 2.0 min. The protoplasts were washed three times by centrifugation as previously described. The pellet from the last centrifugation was resuspended in 3.0 ml of 0.02 M potassium phosphate buffer pH 7.0. This solution was homogenized in a glass Dounce Homogenizer and stored at -20°C until assayed. To determine relative infectivity, the solution was thawed and assayed on 8 half leaves of V. uniguiculata cv. Chinese Red X Iron as described previously.

The amount of CPMV antigen was also determined at 0, 12, 24, 36, 48, and 60 hr by staining the protoplasts with FIIC conjugated antisera. One ml of protoplast suspension was removed from a culture and centrifuged for 2 min at 154 x g. The protoplasts were washed by centrifugation as previously described. The pellet from the last centrifugation was resuspended in 0.2 ml of wash medium. One drop of the protoplast slurry was placed on a clean glass slide and allowed to air dry. The protoplasts were fixed to the slide by immersion in 95% ethanol for 30 min. The slides were removed, air dried and washed 30 min by immersion in chromatography buffer. To control nonspecific antisera absorption, a thin layer of alkaline hydrolyzed gelatin was applied over the fixed protoplasts and allowed to air dry. The area of protoplasts was covered with a 1:2 dilution of FITC conjugated antisera (dilution made with chromatography buffer). The slide was incubated for 15 min in a moist chamber at 37°C. Excess conjugate was removed by dipping the slide in chromatography buffer and then immersion of the slide in fresh chromatography buffer for 1 hr. Salts from the chromatography buffer were removed by dipping the slide in D-DH_2O. The slide was air dried and viewed with a Labophot fluorescent microscope (Nikon Inc.). To control autofluorescence 1 drop of

buffered glycerol pH 8.5 was placed on the slide prior to addition of the cover glass. The fluorescence of each sample was rated on a basis of 0 to 4 with 0 being background fluorescence and 4 maximum fluorescence.

Determination of Virus Attachment to Protoplasts

Protoplasts used in attachment studies were isolated by the method previously described. To conserve $^{125}I_2$-CPMV a smaller number of protoplasts were used for the binding studies than were used in infection studies. A binding study compared the attachment of CPMV to the 6 cowpea cultivars at 25°C and 4°C using the inoculation media previously described. Design of another binding study compared the ability of CPMV to attach to the 6 cultivars using 7 different additives to the inoculation media. Other binding experiments were designed to determine if pretreatment of the protoplast cultivar with the inoculation medium and additive had an effect on the attachment of CPMV to protoplasts. The ultimate goal of the binding studies was to determine if there were any differences in the attachment of CPMV to resistant and susceptible protoplasts.

The relative differences between CPMV attachment to protoplasts derived from the 6 cowpea cultivars were

determined at two temperatures. After the concentration of the protoplast suspensions were determined, enough of each suspension was removed to provide 2.5 x 10^5 protoplasts. The protoplasts were centrifuged at 154 x g for 2 min and the pellet resuspended in inoculation medium with $^{125}I_2$-CPMV (5 µg/ml) at either 4°C or 25°C. Inoculation was carried out at the prescribed temperature for 15 min with a single agitation after 7 min. The protoplasts were then washed 8 times, (after which the supernatants contained background radioactivity), by centrifugation at 154 x g for 2 min and resuspended in wash media which was at the same temperature as the inoculation media used. The pellet from the final centrifugation was not resuspended. The radioactivity associated with each pellet was determined. The amount of $^{125}I_2$-CPMV that 10^6 protoplasts could bind at each temperature was calculated for each cultivar. Control binding assays were treated in the same manner except protoplasts were omitted.

The amount of $^{125}I_2$-CPMV attached to each of the protoplast cultivars using 8 different inoculation media was determined as previously described in the first binding study. Only the 25°C inoculation temperature was used. The inoculation media contain 5 µg/ml CPMV and one or none of the following 7 additives:

1. 2 μg/ml poly-L-α-ornithine HBr (PLO) (Sigma, Type 1-C, molecular weight 1,200,000).
2. 2 μg/ml poly-L-lysine hydrobromide (PLL) (Sigma, Type 1-B, molecular weight 90,000).
3. 80 mg/ml polyethylene glycol (PEG) (Sigma, molecular weight 6,000).
4. 75 μg/ml protamine sulfate (PS) (Sigma, Grade X from Salmon).
5. 5 μg/ml bovine serum albumin (BSA) (Sigma, Fraction V).
6. 5 μg/ml turnip yellow mosaic virus (TYMV), (purified TYMV was provided by P. R. Desjardins, Univ. Calif.-Riverside).
7. 5 μg/ml CPMV.

The 7 additives were also used as a pretreatment before the addition of $^{125}I_2$-CPMV to the protoplasts. The same procedure was used during pretreatment as without pretreatment. The inoculation medium plus additive was added and allowed to incubate 15 min prior to the addition of $^{125}I_2$-CPMV. After this incubation $^{125}I_2$-CPMV was added at a final concentration of 5 μg/ml. This mixture was allowed to incubate for an additional 15 min with a

single agitation after 7 min. Washing steps and calculation of amount of virus attached was as previously described.

To facilitate comparisons between studies the results were reported as μg of CPMV bound to 10^6 protoplasts. This was calculated by dividing the radioactivity associated with each pellet (cpm) by the specific activity of the $^{125}I_2$-CPMV used and multiplying by 4 (the multiplication by 4 was necessary since only 2.5×10^5 protoplasts were used in each assay). The radioactivity of each pellet was calculated from the cpm associated with the pellet after subtracting background radioactivity and the radioactivity associated with the corresponding controls that did not receive protoplasts.

RESULTS

Virus Purification

The method described for purification of CPMV resulted in yields of 15 to 20 mg of CPMV per kg of infected tissue harvested. The virus was infectious for both whole plants and protoplasts, and served as antigen for antisera production.

Antisera Production and Cross-absorption

Antisera had titers from 1:256 to 1:1024. Normal sera had no titer to CPMV as measured by the microprecipitin test. The cross adsorption of antisera resulted in a two fold decrease in titer of the antisera. Prior to cross-absorption some serum preparations showed precipitin lines between the antisera and healthy cowpea tissue in Ouchterlony double diffusion tests. After the cross-absorption of antisera was completed, no precipitin bands could be seen in the Ouchterlony double diffusion plates where healthy sap was used. Precipitin lines did form where purified CPMV or infected sap was used.

The FITC conjugation of cross-absorbed antisera did not significantly change the titer of the antisera but some dilution did occur during the gel filtration procedure. The conjugate retained its activity over a one year period of storage at -30°C.

Cultivar Susceptibility to CPMV

The assay of cultivar susceptibility agreed well with the survey performed by Beier et al. (1977). All of the lines which showed immunity in their survey were also immune in this assay. The cultivars were defined as immune if no virus was detected in plant tissue 10-days-post inoculation by the Ouchterlony double diffusion test and no virus symptoms occurred in Calif. Bl. indicator plants. Five cultivars with P.I. numbers 293467, 293582, 293514, 293453, and 364495 were immune (Table 1). The Calif. Bl. cultivar was determined to be susceptible and P.I. number 194027 was determined to be a local lesion host for CPMV (Table 1).

Optimization of ELISA

The standardized ELISA system of Chen (1981) was optimized for the detection of CPMV. The optimum concentration of coating antibldy was 0.5 µg/ml (Figure 1). No increase in sensitivity was seen by using higher concentrations of coating antibody. The optimum dilution of enzyme-conjugated antisera for use in the ELISA system was 1:1,000 (1×10^{-3}) (Figure 2). This concentration provided adequate detection of CPMV and conserved the enzyme-conjugate. A ratio of absorbance at 405 nm (A_{405}) with CPMV and without CPMV (v/c ratio) for each

Table 1. Assay of cultivar susceptibility

	Cultivar inoculations		Calif. Bl. back inoculations	
Cultivar	Symptoms	Virus Present[a]	Symptoms	Virus Present[a]
Calif. Bl.	Mosaic	+	Mosaic	+
194207	Local Lesions	+	Mosaic	+
293467	None	-	None	-
293582	None	-	None	-
293514	None	-	None	-
293453	None	-	None	-
364495	None	-	None	-
Control	None	-	None	-

[a]A + or - indicates the presence or absence of precipitin bands between antisera and plant sap.

Figure 1. Optimization of coating antibody concentration for detection of purified CPMV by the ELISA system. Data also presented in Table 7 (Appendix)

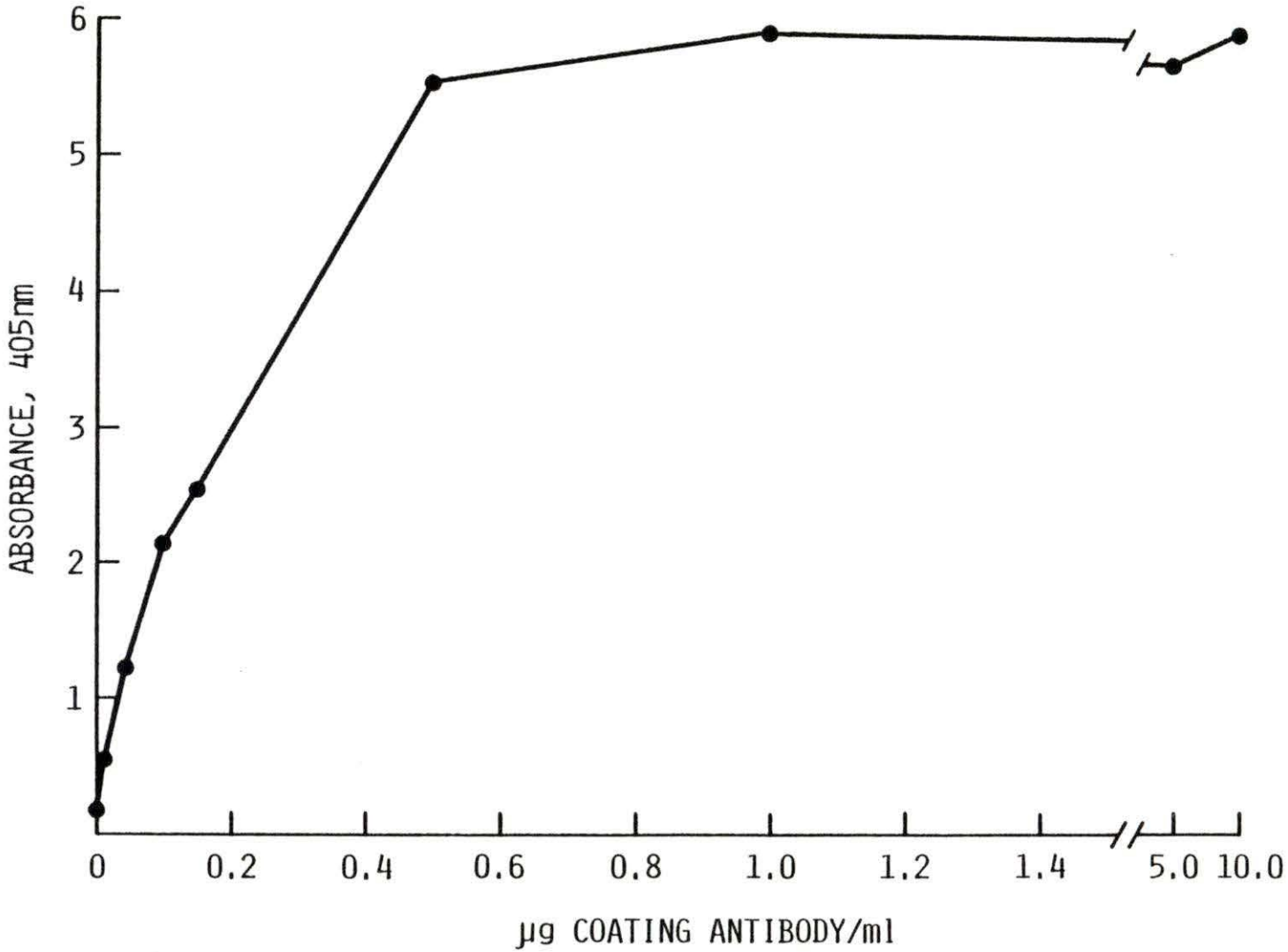
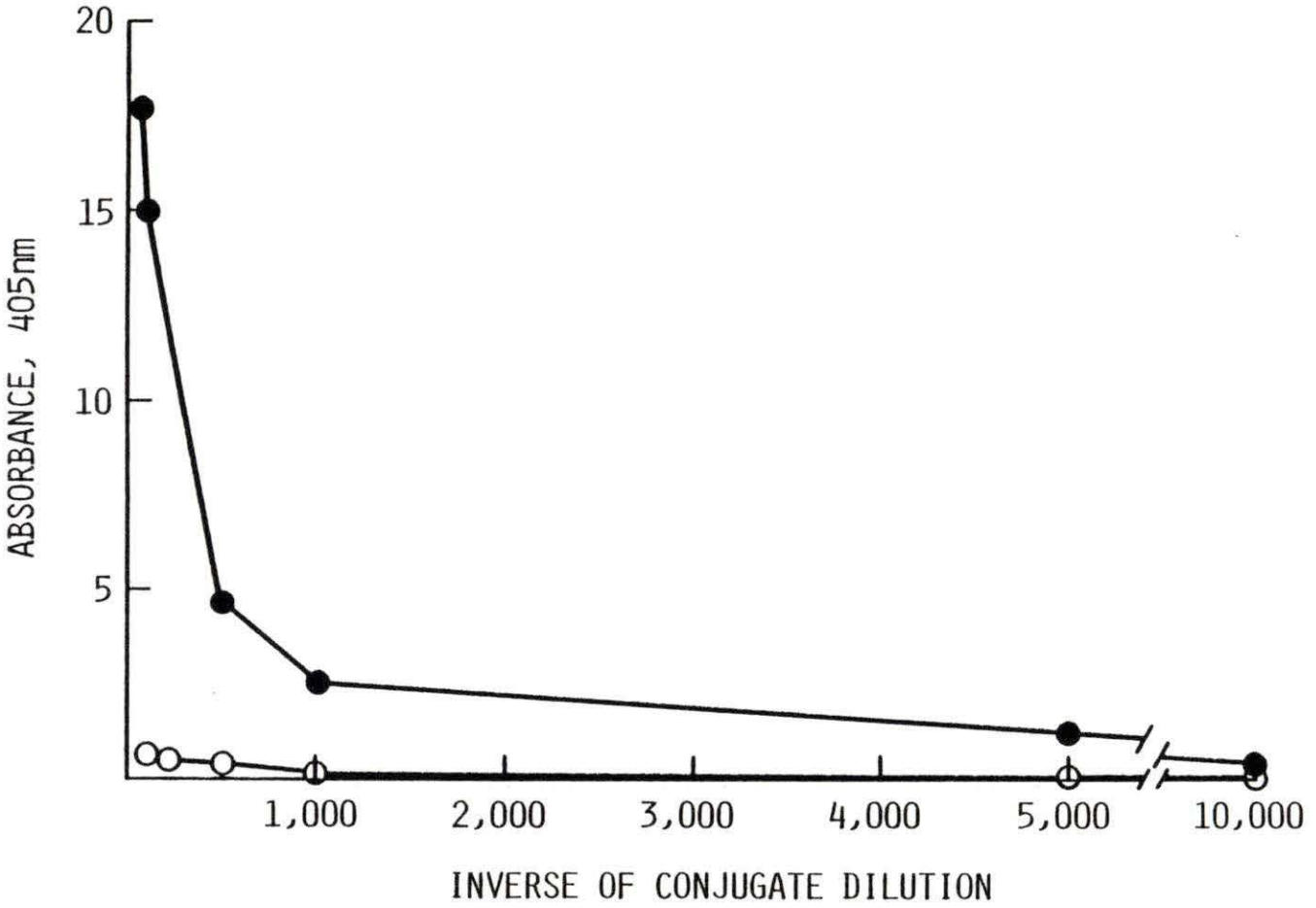

ABSORBANCE, 405nm
6
5
4
3
2
1
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
5.0
10.0
µg COATING ANTIBODY/ml

Figure 2. Optimization of enzyme-conjugated antisera dilution for detection of purified CPMV by ELISA, (●-●). Absorbance at 405 nm for PBS-Tween is also shown (o-o). Data is also presented in Table 3 (Appendix)

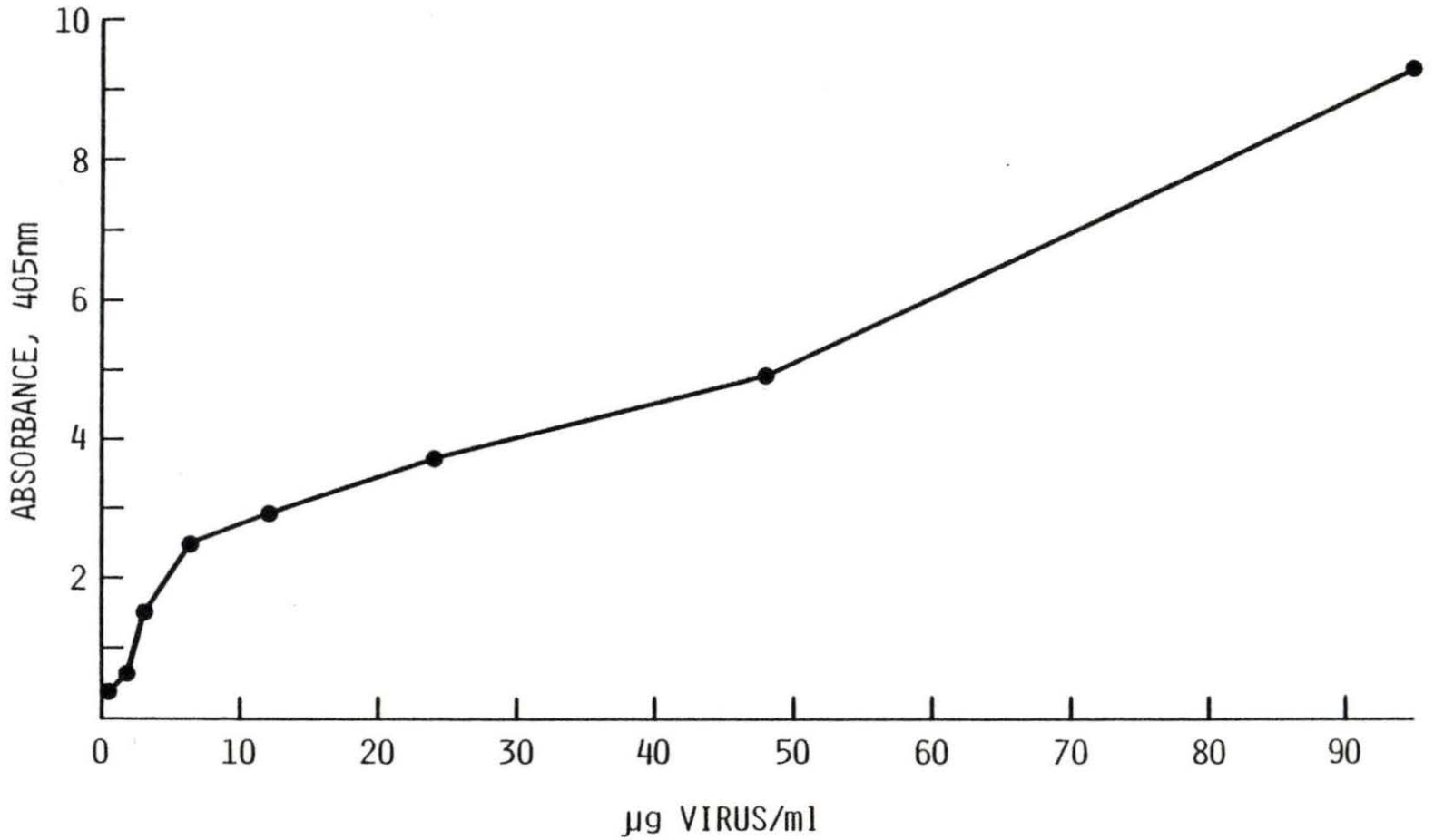

20
15
10
5
ABSORBANCE, 405nm
1,000
2,000
3,000
4,000
5,000
10,000
INVERSE OF CONJUGATE DILUTION

dilution of conjugate used, was calculated by dividing the A_{405} of the substrate solution with antigen present (5 µg/ml CPMV) by the A_{405} of the substrate with no antigen present. PBS-Tween was substituted for CPMV, (Table 8, Appendix). The sensitivity of the optimized ELISA systems for the detection of CPMV was at least 0.75 µg/ml of CPMV. This concentration of CPMV gave an A_{405} value that was approximately 10 times higher than the A_{405} value for the PBS-Tween control. These data are presented in Figure 3 and Table 9 (Appendix).

Local Lesion Assay for CPMV

Experiments demonstrated that V. unguiculata cv. Chinese Red X Iron (P.I. number 194207) could be used to determine the relative infectivity of CPMV containing solutions. The correlation coefficients for two separate local lesion assays were calculated to be 0.79 for experiment number 1 and 0.61 for experiment number 2. The calculated values of t for the correlation coefficients for experiments 1 and 2 were 6.51 and 3.08 respectively. Since both the calculated values of t were greater than the tabular t values, (6.51 is greater than 2.571 (5 degrees of freedom) and 3.08 is greater than 2.776 (4 degrees of freedom)), the correlation between number of local lesions and virus concentration was considered

Figure 3. Sensitivity of ELISA for the detection of purified CPMV. Data also presented in Table 9 (Appendix)


ABSORBANCE, 405nm
10
8
6
4
2
0
10
20
30
40
50
60
70
80
90
µg VIRUS/ml

significant. Data for the two local lesion assays are presented in Figure 4.

^{125}I Labeling of CPMV

Gel filtration of $^{125}I_2$-CPMV and the radioiodination mixture provided a rapid means to separate $^{125}I_2$-CPMV from all other radioactive material. A typical elution profile for the second gel filtration is shown in Figure 5. The specific activities of the three radioiodinations were: 1.16×10^5; 5.81×10^4; and 4.68×10^3 (cpm/μg). The antigenicity and relative infectivity of $^{125}I_2$-CPMV as compared to unlabeled CPMV are shown in Table 2. The differences were small, indicating that CPMV was not changed during the radioiodination procedure.

Protoplast Susceptibility to CPMV Infection

The growth of cowpea plants used in protoplast isolation was very important. The method described provided adequate numbers of protoplasts for use in the infection and attachment studies. The assay of protoplast infection (data presented in Figure 6, Table 3 and Table 10 (Appendix) determined that protoplasts from cultivar Calif. Bl., 293467, 293582, 293514, and 364495 were susceptible to infection by CPMV and that protoplasts from cultivar 293453 were resistant to infection by CPMV. Resistant

Figure 4. Local lesion assay for CPMV using V. unguiculata cv. Chinese Red X Iron. Relative infectivity was calculated from total lesions on 8 half leaves as described in the text. Data are shown for two experiments, 1 (●-●) and 2 (o-o)

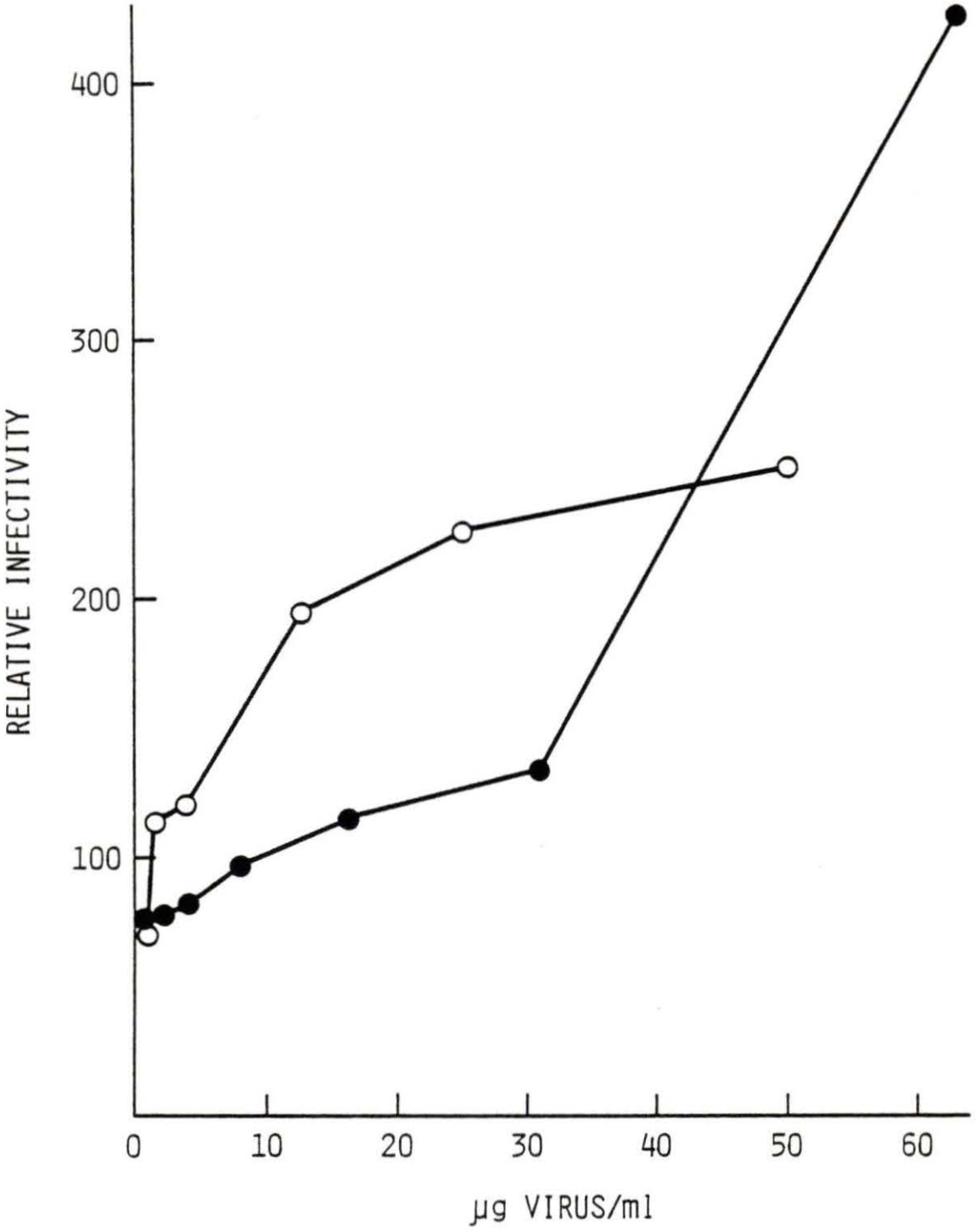

RELATIVE INFECTIVITY
400
300
200
100
0
10
20
30
40
50
60
µg VIRUS/ml

Figure 5. Elution profile of second gel filtration of the radioiodination reaction mixture showing separation of free $^{125}I_2$ and $^{125}I_2$-CPMV. Absorbance at 260 (o-o); and the counts per minute (●-●) for each fraction are shown

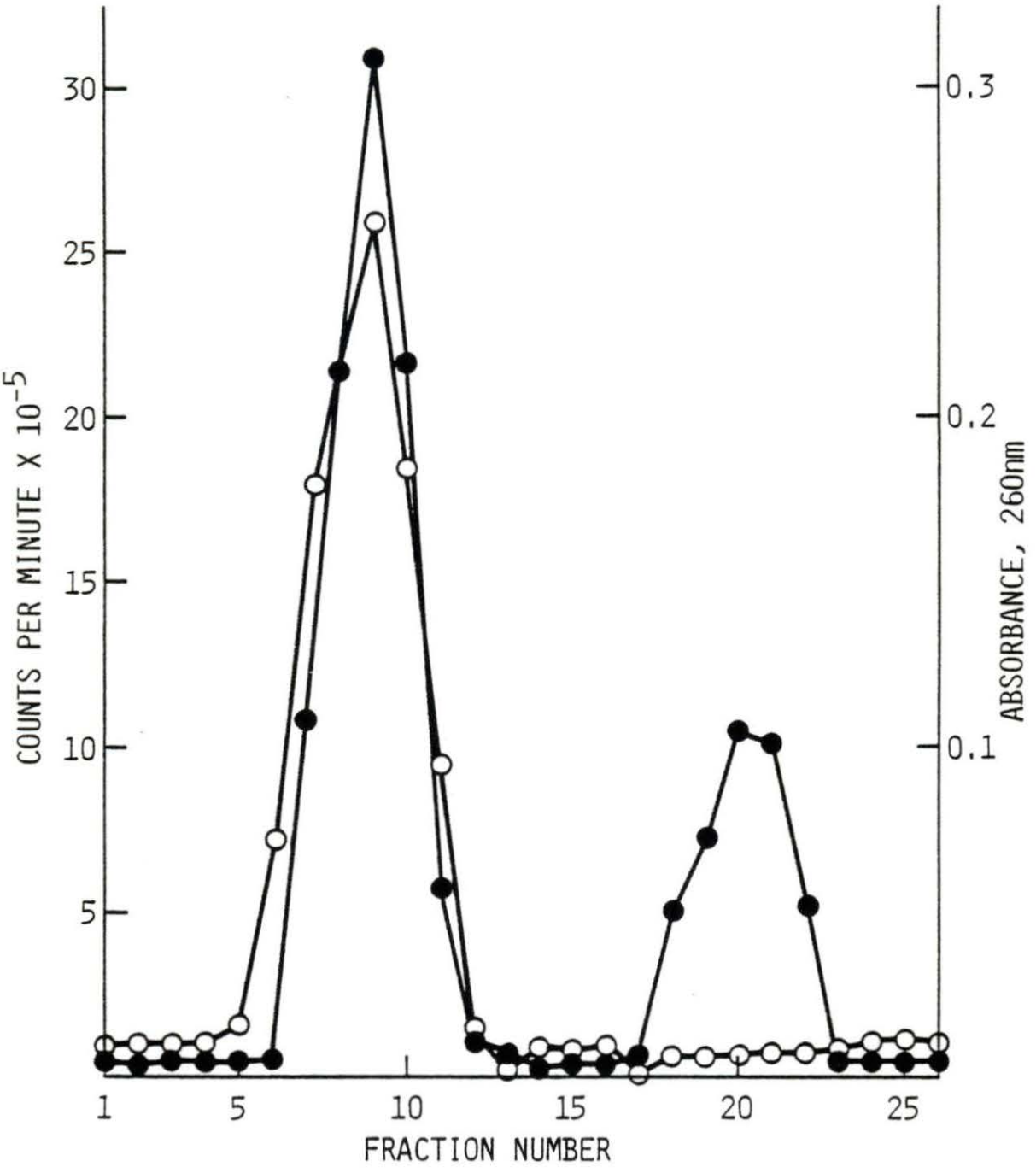

COUNTS PER MINUTE X 10^{-5}
ABSORBANCE, 260nm
FRACTION NUMBER
30
25
20
15
10
5
0.3
0.2
0.1
1
5
10
15
20
25

Table 2. Comparison of antigenicity and relative infectivity of ^{125}I-CPMV and unlabeled CPMV

CPMV preparation	Antigenicity	Relative infectivity
Unlabeled	2.07[a]	135[b]
$^{125}I_2$ Label 1	1.69	115
$^{125}I_2$ Label 2	2.07	107
$^{125}I_2$ Label 3	2.06	---[c]
No CPMV	0.07	0

[a]Average of duplicate samples in ELISA; coating antibody concentration at 0.5 µg/ml; conjugated-enzyme antisera used at 1:1,000 dilution; PBS-Tween was used as the control. Cowpea mosaic virus used at 5.0 µg/ml.

[b]Value calculated from total lesions on 8 half leaves as described in text. 0.02 M potassium phosphate buffer was used as the control. Cowpea mosaic virus used at 25 µg/ml.

[c]Not determined.

Figure 6. Relative infectivity of protoplast extracts from cultivars: P.I. number 293453 (●-●); P.I. number 293467 (△-△); P.I. number 293514 (■-■); P.I. number 364495 (□-□) Calif. Bl. (▲-▲); and control extracts of Calif. Bl. (○-○) mock inoculated without CPMV; at various times post infection. Data are also shown in Table 10 (Appendix)

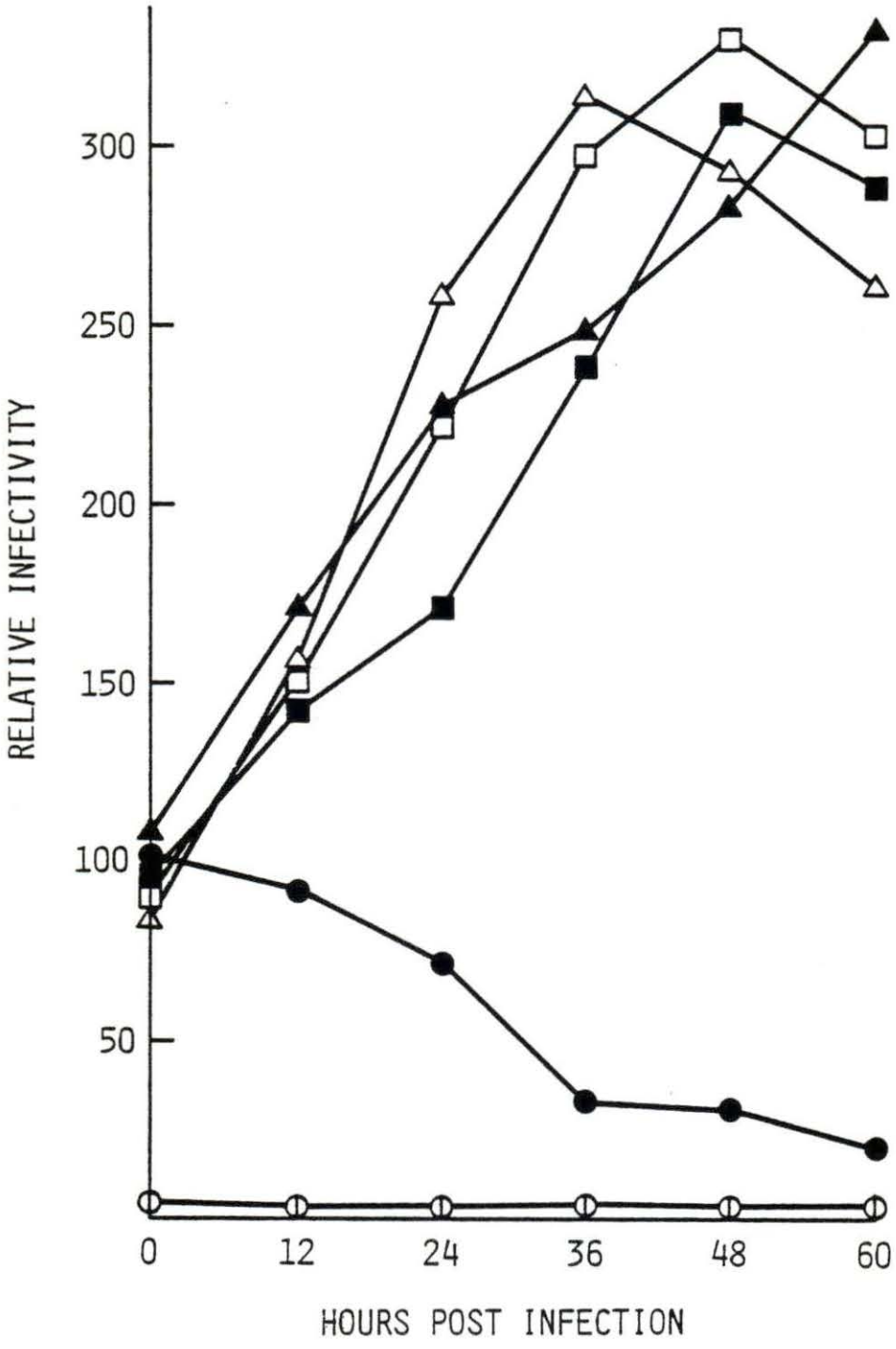

RELATIVE INFECTIVITY
300
250
200
150
100
50
0
12
24
36
48
60
HOURS POST INFECTION

Table 3. Comparison of CPMV antigen content of protoplast cultivars by fluorescent staining of protoplasts at various times post inoculation

Cultivar (P.I. number)	Antigen Content: Hours post inoculation 0	12	24	36	48	60
293453	0[a]	0	0	0	0	0
293467	0	1	2	4	4	4
293514	0	1	2	4	4	3
364495	0	1	2	4	4	4
293582	0	1	2	4	4	4
Calif. Bl.	0	1	2	4	4	4
Calif. Bl. Control	0	0	0	0	0	0

[a]Fluorescence of fixed protoplasts was rated on a basis of 0 to 4 (0 no fluorescence, 4 maximum fluorescence) by viewing stains in a Nikon Labphotfluorescence microscope.

protoplasts were defined as having no detectable level of CPMV by FITC-conjugated antisera staining and no increase in relative infectivity after inoculation. The relative infectivity seen at 0 time post infection (Figure 6) is due to residual virus from the inoculation procedure. Protoplasts from cultivar 293453 showed low levels of relative infectivity 60 hr post inoculation.

This is due to a very small amount of replication of CPMV in these protoplasts. Similar results were reported by Beier et al. (1979). The data in Figure 6 and Table 10 (Appendix) show most virus production is completed after 36-48 hr post inoculation. The fluorescent staining of protoplasts at various times post inoculation agrees with these results and is presented in Table 3.

The data comparing CPMV attachment to protoplasts from immune and susceptible cultivars at 4°C and 25°C are shown in Table 8. The effect of various additives to the inoculation medium on attachment of CPMV to protoplasts derived from the 6 cultivars is shown in Table 5. Results of the effect of pretreating the cultivars with the 7 inoculation media on attachment of CPMV are shown in Table 6. None of the binding studies showed a difference in the binding of CPMV to resistant and susceptible protoplasts. Differences were seen in attachment of CPMV due to the type of inoculation media but not between protoplasts that were resistant or susceptible.

Table 4. Attachment of $^{125}I_2$-CPMV to protoplast cultivars at 4°C and 25°C

Cultivar (P.I. number)	Temperature during inoculation	
	4°C	25°C
Calif. Bl.	0.48[a]	0.47
293467	0.47	0.47
293582	0.48	0.48
293514	0.47	0.47
293453	0.48	0.48
364495	0.48	0.48

[a]Average of duplicate assays expressed as µg $^{125}I_2$-CPMV bound per 10^6 protoplasts. All values are corrected for background and nonspecific binding to assay tubes (see text for calculation procedure).

Table 5. Attachment of $^{125}I_2$-CPMV to protoplast cultivars in differing inoculation media

Cultivar (P.I. number)	Additive used in inoculation medium[a]							
	PLO	PLL	PEG	PS	BSA	TYMV	CPMV	None
Calif. Bl.	0.49[b]	0.50	0.49	0.51	0.49	0.39	0.45	0.47
293467	0.54	0.50	0.49	0.51	0.48	0.38	0.45	0.47
293582	0.50	0150	0149	0.51	0.49	0.37	0.46	0.48
293514	0.49	0.49	0.49	0.51	0.49	0.38	0.45	0.47
293453	0.49	0.49	0.49	0.51	0.49	0.38	0.45	0.46
364495	0.50	0.50	0.49	0.51	0.49	0.39	0.45	0.46

[a]2 µg/ml poly-L-ornithine (PLO), 2 µg/ml poly-L-lysine (PLL), 80 mg/ml polyethylene glycol (PEG), 75 µg/ml protamine sulfate (PS), 5 µg/ml bovine serum albumin (BSA), 5 µg/ml turnip yellow mosaic virus (TYMV), 5 µg/ml cowpea mosaic virus (CPMV), no additive present in inoculation medium (None).

[b]Average of duplicate assays expressed as µg $^{125}I_2$-CPMV bound per 10^6 protoplasts. All values are corrected for background and nonspecific binding to assay tubes (see text for calculation procedure).

Table 6. Attachment of ^{125}I-CPMV to protoplast cultivars after pretreatment in differing inoculation media

Cultivar (P.I. number)	Additive used in inoculation medium[a]							
	PLO	PLL	PEG	PS	BSA	TYMV	CPMV	None
Calif. Bl.	0.53[b]	0.56	0.39	0.52	0.48	0.39	0.38	0.47
293467	0.48	0.54	0.42	0.54	0.39	0.39	0.29	0.48
293582	0.46	0.56	0.43	0.52	0.36	0.40	0.33	0.47
293514	0.49	0.54	0.45	0.55	0.42	0.46	0.39	0.44
293453	0.46	0.52	0.46	0.54	0.45	0.40	0.38	0.47
364495	0.46	0.53	0.42	0.53	0.48	0.39	0.33	0.47

[a]2 µg/ml poly-L-ornithine (PLO), 2 µg/ml poly-L-lysine (PLL), 80 mg/ml polyethylene glycol (PEG), 75 µg/ml protamine sulfate (PS), 5 µg/ml bovine serum albumin (BSA), 5 µg/ml turnip yellow mosaic virus (TYMV), 5 µg/ml cowpea mosaic virus (CPMV), no additive in inoculation medium (None).

[b]Average of duplicate assays expressed as µg ^{125}I-CPMV bound per 10^6 protoplasts. All values are corrected for background and nonspecific binding to assay tubes (see text for calculation procedure).

DISCUSSION

Antisera prepared against purified CPMV and cross-absorbed with lyopholized healthy cowpea tissue proved to be an adequate source of antibodies for use in Ouchterlony double diffusion tests, fluorescent staining of protoplasts and the ELISA detection of purified CPMV.

The assay of cowpea cultivar's susceptibility to CPMV infection by symptoms of cultivar, detection of CPMV antigen and the presence of CPMV by inoculating indicator cowpeas agreed with the survey of Beier et al. (1977). All immune lines selected from their survey were also found to be immune. These immune and susceptible cultivars were the basis for studying one possible mechanism of cultivar immunity to CPMV.

The proposed mechanism of cultivar immunity involved CPMV attachment or lack of attachment to the plasmalemma of resistant cells. It was hypothesized, that as in some animal systems studied, the lack of attachment of virus to some component of the cell membrane might distinguish the resistant cell from the susceptible cell. Protoplasts were chosen to examine the attachment of CPMV to the plasmalemma. Protoplasts provide an excellent system because the cell wall is completely removed, leaving the plasmalemma fully exposed.

Before cowpea protoplasts could be used to study attachment as a mechanism of resistance, the susceptibility of each cultivar's protoplasts to CPMV infection had to be determined. This was performed by inoculating the protoplasts with CPMV and assaying for virus production of various times post-infection. Fluorescent staining of protoplasts using FITC-conjugated antisera was a simple and fast method to detect CPMV antigen in protoplasts. However, the method did have some inherent problems. One problem involved the auto-fluorescence of protoplasts. When washed, unfixed and unstained protoplasts were viewed with 460 nm light (the wave length of light which excites FITC), the protoplast appeared a brilliant red color. The auto-fluorescence was partially reduced by the fixation in ethanol. The ethanol fixed the protoplasts to the slide and extracted some of the material responsible for the auto-fluorescence. Increased fixation time and the use of buffered glycerol when viewing the protoplasts decreased auto-fluorescence to a dull yellow-orange which contrasted with the bright apple-green fluorescence of FITC-conjugated antisera. The local lesion assay for CPMV using V. unguiculata cv. Chines Red X Iron P.I. number 194207, gave a significant correlation between CPMV concentration and local lesion numbers. Two other local lesion hosts were tested prior to 194207. Both

Chenopodium amaranticolor and Phaseolus vulgaris cv. Pinto (W. Altee Burpee Co.) did not give a significant correlation between CPMV concentration and local lesions (data not shown). The local lesion assay and fluorescent staining of protoplast complemented each other very well. The local lesion assay determined the amount of infectious virus present but took several weeks from start to completion. The fluorescent staining of protoplasts was very rapid but only detected CPMV antigen.

Only one of the 6 cultivars assayed had protoplasts which were resistant to infection by CPMV. Resistance of this cultivar and susceptibility of the other cultivars were confirmed by both local lesion assay and fluorescent staining. The local lesion data (Figure 6) revealed the virus replicative cycle was complete after 48 hr of incubation, except for Calif. Bl. which was still increasing at 48 hr. With the exception of Calif. Bl., the length of the replication cycle agrees well with that reported by Beier and Bruening (1975), Hibi et al. (1975), and Rottier (1980). The relative infectivity at 0 hr post infection was residual CPMV associated with the protoplasts. With the susceptibility to CPMV infection of each protoplast known, it was possible to compare attachment of virus to resistant and susceptible protoplasts.

To determine the amount of virus attached to a protoplast, CPMV was labelled with $^{125}I_2$ as a radiotracer. During the radioiodination procedure, the virus is exposed to two enzymes, oxidizing conditions, and the radiation from $^{125}I_2$ decay (γ rays). Since any major change in the capsid proteins of CPMV could affect the binding of virus to protoplasts, the antigenicity and relative infectivity of $^{125}I_2$-CPMV and unlabeled CPMV were compared by ELISA and local lesion assay, respectively. If any major changes to the capsid proteins did occur during the radioiodination procedure the antigenicity and, or relative infectivity should change also.

Before ELISA could be used to detect antigenic difference between labeled and unlabeled CPMV, the system's components were optimized. The optimum coating antibody for detection of purified CPMV was determined to be 0.5 µg/ml (Figure 1). Use of higher coating antibody concentrations did not increase the sensitivity of the system. A dilution of 1:1,000 of the enzyme-conjugated antisera was shown to give good sensitivity and low background absorbances (Figure 2). The detection limit of the optimized ELISA system was determined to be at least 0.75 µg/ml of purified CPMV. This ELISA system as well as the local lesion assay were then used to

detect any changes in CPMV due to the radioiodination procedure.

There were no detectable antigenic differences in labeled and unlabeled CPMV when used as antigens in the ELISA system. The largest antigenic difference was shown in label preparation number one. This difference was due to the fact, that the $^{125}I_2$-CPMV was the least concentrated, therefore it received the least dilution with PBS-Tween during the ELISA procedure. The relative infectivities of the two $^{125}I_2$-CPMV preparations were also very close to that of unlabeled virus. From these data, it was concluded that the radioiodination procedure did not significantly change CPMV and provided an adequate method for tracing CPMV in attachment studies.

When the attachment of $^{125}I_2$-CPMV to protoplasts from the 6 cultivars was examined, no difference was seen between the resistant protoplast (P.I. number 293453) and the other protoplasts which were susceptible to infection (P.I. numbers 293467, 293582, 293514, 364495, and Calif. Bl.). No difference in attachment was observed between any of the cultivars at 4°C or at 25°C. There was also very little difference in attachment between the two inoculation temperatures. These data (presented in Table 4) would indicate that CPMV attaches equally well to both resistant and susceptible protoplasts. Similar results were found

by Gaard and de Zoeten (1979) when the attachment of tobacco rattle virus particles to the host Nicotiana tabacum L. var. Xanthi-nc and the nonhost Zea mays L. was studied after leaf panel infiltration. In their study, it was concluded that end-on attachment and uncoating of tobacco cattle virus occurred after a nonspecific interaction with the cell wall of both host and nonhost plants. Maule et al. (1980), studied the mechanism of resistance of cucumber cultivars to cucumber mosaic virus. Protoplasts derived from a resistant cultivar retained their resistance to cucumber mosaic virus infection, when inoculated with intact cucumber mosaic virus particles or infectious RNA. When binding of cucumber mosaic virus to resistant and susceptible protoplasts was compared, no significant differences could be seen. They concluded that the resistance mechanism operates at the transcription or translation level. These data of this study seem to suggest attachment of CPMV to the cell does not play a role in resistance or susceptibility of that cell to infection.

However, the results of Niblett (1975) and Shaw (1972a) indicated that nonspecific attachment was a result of the surface charges on virus and cell. If this type of interaction was occurring between the protoplasts of cowpea and CPMV, the putative nonspecific

interaction might be masking any differences in the specific attachment of CPMV to resistant and susceptible protoplasts. Therefore, several additives were used in the inoculation medium to try to neutralize the potential nonspecific interactions between cell and virus. With the nonspecific attachment decreased, it was thought that differences in the specific attachment between protoplast cultivars could be discerned. To neutralize any charge affects PLO, PLL, PEG, and PS were used as separate additives to the inoculation medium. BSA, TYMV, and CPMV were also used to neutralize any nonspecific protein attachment sites. BSA was chosen as a protein to absorb to nonspecific attachment sites because it is completely unrelated to CPMV. TYMV was chosen because it is a small icosahedrol virus particle with an isoelectric point very similar to CPMV (TYMV and CPMV have isoelectric points of 3.5 and 3.4 to 4.5, respectively (Mattheus, 1970; Van Kammen, 1971) but it has a different host range from that of CPMV. The attachment studies showed relative differences between inoculation media but no difference between protoplast cultivars within a specific inoculation medium.

The polycations in the inoculation medium increased equally the amount of CPMV attaching to both resistant and susceptible protoplasts. The increase in attachment

is presumably due to the decrease in electrostatic repulsion between virus and protoplast. It has been reported that cowpea protoplasts have a net negative surface charge due to the presence of phosphate groups on the plasmalemma and that treatment of the protoplast with poly-L-ornithine, poly-L-lysine or protamine sulfate neutralize the negative charge (Nagata and Melchers, 1978). However, the surface charge on the cowpea protoplasts was less than that of Nicotinana, Petunia, or Brassica. It has also been shown that virus particles having a negative surface charge require poly-L-ornithine for infection (Motoyoshi et al, 1974; Otsuki et all, 1974; Okuno and Furusawa, 1978). This could explain the fact that poly-L-ornithine is not essential for CPMV infection of cowpea protoplasts but does stimulate infection.

If 5.09×10^6 g/mole is used as an average molecular weight for the three components of CPMV (Rottier, 1980) one µg of CPMV contains approximately 1.2×10^{11} particles. Therefore, in the inoculation procedure, 3.9×10^8 CPMV particles/protoplast were used. After washing, an average of 5.6×10^4 CPMV particles were retained by protoplasts in the inoculation medium without additives. The protoplasts inoculated in the presence of protamine sulfate (the polycation which gave the greates stimulation of CPMV attachment) retained 6.1×10^4 particles/protoplast

after washing. Inclusion of BSA in the inoculation medium gave a very slight increase in the amount of CPMV retained after washing. These results are in agreement with Zhuravlev et al. (1976) who reported that bovine serum albumin and casein hydrolysate did not appreciably affect tobacco mosaic virus retention by tobacco protoplasts but did decrease virus yields.

The inclusion of heterologous virus (TYMV) and homologous virus (CPMV) decreased the amount of CPMV attaching to protoplast relative to there absence. The result was surprising since the heterologous virus was able to decrease attachment more than the homologous virus. A possible explanation of this phenomenon is that this particular CPMV preparation had an isoelectric point at the lower region reported for CPMV which would give TYMV a higher isoelectric point relative to TYMV. The greater positive surface charge of TYMV, would cause increased attachment of the TYMV virions relative to CPMV.

Since the inclusion of additives in the inoculation media did not affect attachment of CPMV to resistant and susceptible protoplasts within a particular treatment when added simultaneously with virus, the protoplasts were given a 15 min pretreatment with inoculation medium plus additive prior to addition of $^{125}I_2$-CPMV.

The polycations stimulated $^{125}I_2$-CPMV attachment to both resistant and susceptible protoplasts equally. The pretreatment with polycations caused a greater number of particles to attach than when added simultaneously with virus, except for PEG. Polyethylene glycol showed an inhibition of CPMV attachment when used as a pretreatment. This is presumably a reflection of the putative difference in infection mechanism when using PEG rather than the other polycations (mechanisms described previously). The pretreatment with homologous and heterologous virus decreased the amount of CPMV attachment. However, when used as a pretreatment the homologous virus showed an increased inhibition over the heterologous virus. The difference in inhibition level depending on pretreatment or addition of treatment with virus inoculum, suggests differences in relative reaction rates. TYMV may be able to bind to protoplasts faster but not to the same extent as CPMV.

Although pretreatment of protoplasts with various inoculation media revealed differences between the inoculation media themselves, no differences were observed in the attachment of CPMV between resistant and susceptible cultivars within a specific treatment. Therefore, attachment of CPMV to protoplasts does not appear to be the determining factor in a protoplast's susceptibility

to CPMV infection. The resistance mechanism may be at the transcription or translation stage of virus replication as previously discussed for cucumber protoplast resistance to cucumber mosaic virus (Maule et al., 1980).

It is conceivable that in some plant virus--host cell interactions, the attachment of the virus (either to the cuticle, the cell walls or plasmalemma of epidermal or mesophylic tissue) may play a role in determining a plant's susceptibility. Perhaps attachment of virus to protoplasts is a completely artificial interaction. The treatment of plant cells with cell wall degrading enzymes may remove the specific components necessary for virus attachment by removing the cell wall itself or reacting with plasmalemma components. However at this point in the investigation of plant virus attachment studies, the initial interaction of virus with plant cells appears to be nonspecific.

SUMMARY

The susceptibility of several V. unguiculata cultivars to infection by CPMV was determined. Cultivar Calif. Bl. was shown to be a systemic host for CPMV. Cultivars of P.I. numbers 293467, 293582, 293514, 293453, and 364495 defined as immune to CPMV infection. The cultivar with number 194207 was a local lesion host for CPMV. The ability of CPMV to infect protoplasts from the 5 resistant cultivars was compared to that for the susceptible cultivar. The cultivar with P.I. number 293453, was the only cultivar that showed a resistance to CPMV at the protoplast level.

The ability of CPMV to attach to the resistant and susceptible protoplasts was compared. Before attachment was examined, CPMV was radioiodionated with $^{125}I_2$ using an immobilized preparation of lactoperoxidase and glucose oxidase. To detect any changes to the virus during the radioiodination procedure, the antigenicity and relative infectivity were compared by ELISA and local lesion assay (using V. unguiculata c.v. Chinese Red X Iron, P.I. number 194207). The antigenicity and infectivity of the virus was not changed during the radioiodination procedure. This $^{125}I_2$-CPMV preparation was used to reveal

any differences in the attachment of CPMV to resistant and susceptible protoplasts.

Attachment of CPMV was assayed by the amount of $^{125}I_2$-CPMV remaining bound to a protoplast suspension after inoculation. The binding of CPMV to the protoplast cultivars under several different conditions were examined. Binding at 4°C and 25°C using a potassium citrate and D-mannitol inoculation medium showed no difference in binding between cultivars or between temperatures. The ability of protoplasts to bind $^{125}I_2$-CPMV in inoculation medium plus several different additives was compared. The additives were either polycations (poly-L-ornithine, poly-L-lysine, polyethylene glycol, or protamine sulfate) or protein (bovine serum albumin, turnip yellow mosaic virus or cowpea mosaic virus). It was thought that the use of these additives during the inoculation of protoplasts would neutralize any nonspecific binding of CPMV to the protoplasts. Thus, the difference in specific attachment abilities of resistant and susceptible protoplasts would become apparent. There was no difference in attachment between the protoplasts in any of the inoculation media. Different inoculation media were able to inhibit or stimulate virus attachment. In general, the polycations stimulated CPMV attachment, by decreasing the electronegative charge of the plasmalemma and the proteins inhibited

attachment. The protoplasts' ability to attach virus after pretreatment with the inoculation medium plus additive was also compared. Again, no difference was seen between protoplast cultivars in their ability to attach CPMV. From these data, it was concluded that specific attachment of CPMV to the protoplast does not play a role in determining a protoplast's susceptibiltiy to infection.

LITERATURE CITED

Agrawal, H. 1964. Identification of cowpea mosaic virus isolates. Meded. Landbouwhogesch. Wageningen 64: 1-53.

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

Aoki, S., and I. Takebe. 1969. Infection of tobacco mesophyll protoplasts by tobacco mosaic virus ribonucleic acid. Virology 39: 439-448.

Ball, E. M. 1974. Serological tests for the identification of plant viruses. The American Phytopathology Society, St. Paul.

Beier, H., and G. Bruening, 1975. The use of an abrasive in the isolation of cowpea leaf proplasts which support the multiplication of cowpea mosaic virus. Virology 64: 272-276.

Beier, H., D. J. Siler, M. L. Russell,and G. Bruening. 1977. Survey of susceptibility to cowpea mosaic virus among protoplasts and intact plants from Vigna sinensis lines. Phytopathology 67: 917-921.

Beier, H., G. Bruening, M. L. Russell, and C. L. Tucker. 1979. Replication of cowpea mosaic virus in protoplasts isolated from immune lines of cowpeas. Virology 95: 165-175.

Bohol, B. B. and E. L. Schmidt. 1968. Nonspecific staining: Its control in immunofluorescent examination of soil. Science 162: 1012-1014.

Brants, D. H. 1964. The susceptibility of tobacco and bean leaves to tobacco mosaic virus infection in relation to the condition of ectodesmata. Virology 23: 588-594.

Burgess, J., F. Motoyoshi, and E. N. Fleming. 1973a. The mechanism of infection of plant protoplasts by viruses. Planta 112(4): 323-332.

Burgess, J., F. Motoyoshi, and E. N. Fleming. 1973b. Effect of poly-L-ornithire on isolated tobacco mesophyll protoplasts: Evidence against stimulated pinocytosis. Planta 111: 199-208.

Campbell, D. H., J. S. Garvey, N. E. Cremer, and D. H. Sussdorf. 1970. Ammonium sulfate precipitation. Pages 189-191 in Methods in Immunology. W. A. Benjamin Inc., Reading, Mass.

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

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

Chen, L. C. 1981. Studies of soybean mosaic virus strains by enzyme-linked immunosorbent assay. Master's thesis, Iowa State University.

Cocking, E. C. 1960. A method for isolation of plant protoplasts and vacuoles. Nature 187: 962-963.

Cocking, E. C. 1966. An electron microscopic study of the initial stages of infection of isolated tomato fruit protoplasts by tobacco mosaic virus. Planta 68: 206-14.

Cocking, E. C. 1970. Virus uptake, cell wall regeneration and virus multiplication in isolated plant protoplasts. Int. Rev. Cytol. 28: 89-124.

Cocking, E. C., and E. Pojnar. 1969. An electron microscopic study of the infection of isolated tomato fruit protoplasts by tobacco mosaic virus. J. Gen. Virol. 4: 305-32.

Curtain, C. C. 1961. The chromatographic purification of fluoroscein-antibody. J. Histochem. Cytochem. 9: 484-486.

Dales, S. 1965. Penetration of animal viruses into cells. Progr. Med. Virol. 7: 1-43.

Daubert, S. D., G. Bruening, and R. C. Najarian. 1978. Protein bound to the genome RNAs of cowpea mosaic virus. Eur. J. Biochem. 92: 45-51.

de Jager, C. P. 1976. Genetic analysis of cowpea mosaic virus mutants by supplementation and reassortment tests. Virology 70: 151-163.

de Zoeten, G. A. 1981. Early events in plant virus infection. Pages 221-239 in Plant diseases and vectors. Academic Press, New York.

de Zoeten, G. A., A. M. Assink, and A. Van Kammen. 1974. Association of cowpea mosaic virus induced double straneded RNA with cytopathological structure in infected cells. Virology 59: 341-355.

Dunn, A. and J. Arditti. 1968. Preparation of media and reagents. Page 265 in Experimental physiology. Holt, Rinehart and Winston Inc., New York.

Ehra, Y., and G. I. Mink. 1980. Cucumber mosaic virus and peanut stunt virus differ in an initial event that occurs during the infection of cowpea leaf epidermal cells. Virology 104: 258-261.

Fisher, D. J. 1979. Studies of plant membrane components using protoplasts. Plant Sci. Letters 15: 127-133.

Fowke, L. C., and O. L. Gamborg. 1980. Applications of protoplasts to the study of plant cells. Intern. Rev. Cytol. 68: 9-51.

Gaard, G., and G. A. de Zoeten. 1979. Plant virus uncoating as a result of virus-cell wall interactions. Virology 96: 21-31.

Geelen, J. L. M. C., A. Van Kammen, and B. J. M. Verduin. 1972. Structure of the capsid of cowpea mosaic virus. The chemical subunit: Molecular weight and number of subunits per particle. Virology 49: 205-213.

Gibbs, A., and B. Harrison. 1976. Plant virology: The principles. John Wiley and Sons, New York.

Gornell, A. C., C. Barvdaivell and M. David. 1949. Determination of serum proteins by means of the biurett reaction. J. Biol. Chem. 177: 751-766.

Herridge, E. A., and D. E. Schlegel. 1962. Autoradiographic studies of tobacco mosaic virus inoculations on host and non-host species. Virology 18: 217-523.

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

Karonen, S. L., P. Mörsky, M. Siren, and U. Seuderling. 1975. An enzymatic solid-phase method for trace iodination of proteins and peptides with ^{125}Iodine. Anal. Biochem. 67: 1-10.

Kassanis, B., R. F. White, R. H. Turner, and R. D. Woods. 1977. The mechanism of virus entry during infection of tobacco protoplasts with tobacco mosaic virus. Phytopathol. Z. 88: 215-228.

Kiho, Yukio. 1974. Model system for the adsorption of tobacco mosaic virus. Japan J. Microbiol. 18(4): 313-320.

Koike, M., T. Hibi, and K. Yora. 1977. Infection of cowpea mesophyll protoplasts with cucumber mosaic virus. Virology 83: 413-416.

Kontaxis, D. G., and D. E. Schlegel. 1962. Basal septa of broken trichomes in Nicotiana as possible infection sites for tobacco mosaic virus. Virology 16: 244-247.

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

Lee, R. F., L. B. Johnson, and C. L. Niblett. 1975. Effect of host enzyme extracts on the electrophoretic forms and specific infectivity of cowpea mosaic virus. Physiol. Plant Pathol. 7: 59-69.

Lewis, V. J., W. L. Jones, J. B. Brooks, and W. B. Cherry. 1964. Technical considerations in the preparation of fluorescent-antibody conjugates. J. Appl. Microbiol. 4: 343-348.

Luria, S. E., and J. E. Darnell. 1967. General Virology. John Wiley and Sons, New York.

Mattheus, R. E. F. 1970. Turnip yellow mosaic virus. In Descriptions of plant viruses. No. 2 Commonwealth Mycological Institute and Association of Applied Biologists, Surrey, England.

Maule, A. J., M. I. Boulton, and K. R. Wood. 1980. Resistance of cucumber protoplasts to cucumber mosaic virus: A comparative study. J. Gen. Virol. 51: 271-279.

McLaughin, M. R., G. R. Bryant, J. H. Hill, H. I. Benner, and D. P. Durand. 1980. Isolation of specific antibody to plant viruses by acid sucrose density gradient centrifugation. Phytopathology 70: 831-834.

Merkens, W. S. W., G. A. de Zoeten and G. Gaard. 1972. Observations on ectodesmata and the virus infection process. J. Ultrastruct. Res. 41: 397-405.

Mink, G. I. 1976. Use of inactivating agents to detect and partially characterize very early events in the infection of cowpea epidermal cells by peanut stunt virus. Virology 72: 291-298.

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

Nagata, T., and G. Melchers. 1978. Surface charge of protoplasts and their significance in cell-cell interaction. Planta 142: 235-238.

Niblett, C. L. 1975. Attachment of plant viruses to host and nonhost plants. Proc. Am. Phytopathol. Soc. 2: 87.

Niblett, C. L., and J. S. Semancik. 1969. Conversion of the electrophoretic forms of cowpea mosaic virus *in vivo* and *in vitro*. Virology 38: 685-693.

Niblett, C. L., and J. S. Semancik. 1970. The significance of the coat protein in infection by electrophoretic forms of cowpea mosaic virus. Virology 40: 350-355.

Novikov, V. K., and J. G. Atabekov. 1970. A study of the mechanism controlling the host range of plant viruses. I. Virus-specific receptors of Chenopodium amaranticolor. Virology 41: 101-7.

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

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

Pelham, H. R. B. 1979. Synthesis and proteolytic processing of cowpea mosaic virus proteins in reticulocyte lysates. Virology 96: 463-477.

Quail, P. H. 1979. Plant cell fractionation. Ann. Rev. Plant. Physiol. 30: 425-484.

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

Reijnders, L., A. M. J. Albers, A. Van Kammen, and R. W. J. Thuring. 1974. Molecular weights of plant viral RNAs determined by gel electrophoresis under denaturing conditions. Virology 60: 515-521.

Rezelman, G., R. Goldbach, and A. Van Kammen. 1980. Expression of bottom component RNA of cowpea mosaic virus in cowpea protoplasts. J. Virol. 36: 366-373.

Rottier, P. 1980. Viral protein synthesis in cowpea mosaic virus infected protoplasts. Meded. Landbouwhogesch. Wageningen 80: 1-96.

Rottier, P. J. M., G. Rezelman, and A. Van Kammers. 1979. The Inhibition of cowpea mosaic virus replication by actinomycin. Virology 92: 299-309.

Semancik, J. S. 1966. Studies on electrophoretic heterogeneity in isometric plant viruses. Virology 30: 698-704.

Shaw, J. G. 1969. In vivo removal of protein from tobacco mosaic virus after inoculation of tobacco leaves. II. Some characteristics of the reaction. Virology 37: 109-116.

Shaw, J. G. 1972a. Effect of surface charge of virus particles on their attachment to leaves during mechanical inoculation. Phytopathology 62(7): 789.

Shaw, J. G. 1972b. Effect of poly-L-ornithine on the attachment of tobacco mosaic virus to tobacco leaves and on the uncoating of viral RNA. Virology 48: 380-385.

Stanley, J., P. Rottier, I. W. Zabel, and A. Van Kammen. 1978. A protein linked to the 5'-termini of both RNA components of the cowpea mosaic virus genome. Nucleic Acids Res. 5: 4505-4522.

Stanley, J., R. Goldbook, and A. Van Kammen. 1980. The genome-linked protein of cowpea mosaic virus is coded by RNA from the bottom component. Virology 106: 180-182.

Takagi, Y., and M. Takahushi. 1972. Attachment of tobacco mosaic virus to host and non-host plants. Bull. Univ. Osaka Pref. 24: 40-42.

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

Takebe, I., and Y. Otsuki. 1969. Infection of tobacco mesophyll protoplasts by tobacco mosaic virus. P.N.A.S. USA 64: 843-848.

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

Thomas, P. E., and R. W. Fulton. 1968. Correlation of ectodesmata number with nonspecific resistance to initial virus infection. Virology 34: 459-469.

Thomason, B. M., and J. G. Wells. 1971. Preparation and testing of polyvalent conjugates for fluorescent-antibody detection of Salmonellae. Appl. Microbiol. 22: 876-884.

Thongmeearkom, P., and R. M. Goodman. 1978. Complementation and pseudorecombination between ribonucleic acids from two natural isolates of cowpea mosaic virus. Virology 85: 75-83.

Tower, B. B., B. R. Clark, and R. T. Rubin. 1977. Preparation of ^{125}I polypeptide hormones for radioimmunoassay using glucose oxidase with lactoperoxidase. Life Sci. 21: 959-966.

Van Kammen, A. 1967. Purification and properties of the components of cowpea mosaic virus. Virology 31: 633-642.

Van Kammen, A. 1971. Cowpea mosaic virus. in Descriptions of plant viruses. No. 47. Commonwealth Mycological Institute and Association of Applied Biologists, Surrey, England.

Van Kammen, A., and L. J. D. Van Griensven. 1970. I. Further characterization of the nucleoprotein components of cowpea mosaic virus. Virology 41: 274-280.

Wood, H. A. 1972. Genetic complementation and pseudo-recombination between the two nucleoprotein components of cowpea mosaic virus. Virology 49: 592-598.

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

Zaitlin, M., and R. N. Beachy. 1974. The use of protoplasts and separated cells in plant virus research. Advances in Virus Research 19: 1-35.

Zhuravlev, Y. N., Y. G. Reifman, L. A. Shumilova, Z. S. Yudakova, and N. F. Pisteskaya. 1975. The absorption of phosphorus-32 labeled tobacco mosaic virus in isolated tobacco protoplasts and the deproteinization of the virus in them. Fiziol. Rust. 22(5): 1075-1077.

Zhuravlev, Y. N., Z. S. Yudakova, and N. F. Pisetskaya. 1976. Effects of viral protein viral RNA and some other poly electrolytes on infection of tobacco protoplasts by tobacco mosaic virus. Virology 73: 454-460.

ACKNOWLEDGMENTS

I thank Dr. D. P. Durand and Dr. J. H. Hill for their advice during this project and helpful criticism while preparing this manuscript. I would also like to thank my fellow graduate students for their assistance during my sojourn at Iowa State University.

APPENDIX

Table 7. Optimization of coating antibody concentration for detection of purified CPMV by ELISA

Coating antibody concentration (µg/ml)	Absorbance
0.00[a]	0.15[b]
0.01	0.60
0.05	1.21
0.10	2.17
0.15	2.54
0.50	5.54
1.00	5.88
5.00	5.51
10.00	5.88

[a]Purified CPMV at a concentration of 5.0 µg/ml and a conjugate dilution of 1:1,000 were used.

[b]Average A_{405} of duplicate samples.

Table 8. Optimization of enzyme-conjucted antisera dilution for detection of purified CPMV by ELISA

Antigen	Dilution of enzyme-conjugate					
	1×10^{-4}	2×10^{-4}	1×10^{-3}	2×10^{-3}	1×10^{-2}	2×10^{-2}
CPMV	0.33[a]	1.29	2.56	4.92	14.97	17.71
PBS-Tween	0	0	0	0.01	0.21	0.54
v/c Ratio[b]	--	--	--	351.40	72.30	32.74

[a]Average A_{405} values of duplicate samples: coating antibody concentration was 0.5 µg/ml; CPMV antigen was used at 5 µg/ml; PBS-Tween was used as the control.

[b]Calculation of v/c ratio is described in the text.

Table 9. Sensitivity of ELISA for detection of purified CPMV

CPMV (g/ml)	A_{405}
0.00 (PBS-Tween)	0.03[a]
0.75	0.38
1.50	0.68
3.00	1.54
6.00	2.47
12.00	2.94
24.00	3.73
48.00	4.91
95.00	9.36

[a]Average A405 values of duplicate samples: coating antibody concentration at 0.5 µg/ml; conjugated-enzyme antisera used at 1:1,000 dilution; PBS-Tween used as the control.

Table 10. Relative infectivities of protoplast extracts at various times post inoculation

Cultivar Extracts (P.I. number)	Relative infectivities					
	Hours post inoculation					
	0	12	24	36	48	60
293453	103[a]	92	72	33	31	20
293467	97	157	258	314	291	266
293514	100	144	172	238	308	268
364495	98	151	225	296	330	302
Calif. Bl.	104	171	221	249	284	334
Calif. Bl. Control[b]	5	6	4	0	0	0

[a]Average of duplicate cultures calculated from total lesions on 8 half leaves as described in text.

[b]Control represents Calif. Bl. protoplasts mock inoculated without CPMV.