

THESIS



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Studies on the Mechanisms of Plant Virus
Infection in Two Leguminous Protoplast
Systems: I. Bean Pod Mottle Virus Infection
of Suspension Culture Soybean Protoplasts
II. Infection of Bean Leaf Protoplasts
presented by

MARK STEVEN LESNEY

has been accepted towards fulfillment
of the requirements for

Ph. D . degree in Botany & Plant Pathology

A handwritten signature in cursive script, reading "H. H. Murakishi".

Major professor

15 May 1980

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STUDIES ON THE MECHANISMS OF PLANT VIRUS INFECTION
IN TWO LEGUMINOUS PROTOPLAST SYSTEMS
I. BEAN POD MOTTLE VIRUS INFECTION OF SUSPENSION
CULTURE DERIVED SOYBEAN PROTOPLASTS
II. INFECTION OF BEAN LEAF PROTOPLASTS WITH BEAN
POD MOTTLE AND COWPEA MOSAIC VIRUSES

By
Mark Steven Lesney

A DISSERTATION

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in partial fulfillment of the requirements
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ABSTRACT

STUDIES ON THE MECHANISMS OF PLANT VIRUS INFECTION IN TWO LEGUMINOUS PROTOPLAST SYSTEMS I. BEAN POD MOTTLE VIRUS INFECTION OF SUSPENSION CULTURE DERIVED SOYBEAN PROTOPLASTS II. INFECTION OF BEAN LEAF PROTOPLASTS WITH BEAN POD MOTTLE AND COWPEA MOSAIC VIRUSES

By

Mark Steven Lesney

Protoplasts derived from soybean (Glycine max L. cv. 'Harosoy 63') liquid suspension cultures were inoculated with Bean Pod Mottle Virus (BPMV) in order to ascertain the effects of various inoculum amendments on the virus infection system with the ultimate goal of elucidating possible infection mechanisms. Among the parameters examined were the effects of virus concentration, protoplast concentration, buffer (pH and concentration), poly-L-ornithine (PLO), calcium or magnesium chloride, and temperature on percentage infection obtained.

Virus concentration effects showed a sigmoidal increase in infection with concentration increase. Protoplast concentration showed an inverse relationship to infection. Potassium phosphate buffer showed a strong optimum for infection at pH 5.6. This was used as partial evidence for the involvement of histidine in the infection process. Effects of buffer concentration were pH dependent. PLO was not required for BPMV infection, but was found to be stimulatory. A synergistic effect on increasing virus infection was demonstrated between PLO and CaCl_2 at low virus concentrations. CaCl_2 proved more stimulatory to BPMV infection than did MgCl_2 . Pre-incubation experiments for both buffers and the divalent cations showed that pre-incubation with the virus was necessary for most of the stimulatory effect to be obtained, but that PLO-induced

infection was actually somewhat improved when pre-incubation was not allowed. BPMV was relatively temperature independent in the presence of inoculum amendments (buffer, PLO, CaCl_2), but temperature dependent in their absence; infection then was better at higher temperatures.

Protoplasts were isolated from bean (Phaseolus vulgaris L. cv. 'Pencil Pod Wax') leaves and infected with both BPMV and cowpea mosaic virus (CPMV) in order to compare infection for the two viruses in the two different systems.

The same PLO/calcium synergy was evident as in the soybean system. The optimum pH in the bean system for BPMV was 5.6. The primary difference observed between the two systems was the ability of pH 6.0 buffer to eliminate the PLO/calcium synergy when present in the bean system, but not in the soybean system for both viruses. The similarities seen between the leaf and suspension culture protoplasts were suggestive of the latter being equally 'natural' as a system, as well as one which is more reliable.

The kind and number of the complexities seen in the two virus/protoplast systems were advanced as circumstantial evidence for the possibility of receptor mediated endocytosis as the mode of infection by plant viruses in protoplasts - especially when considering such a theory as an alternative to the membrane 'wounding' hypothesis.

**This dissertation is dedicated to
my parents and my brother**

Journal of Interpersonal Violence 26(10)

$$P_{\text{eff}} = P_0 \left(1 - \frac{1}{2} \frac{P_0}{P_{\text{eff}}} \right) \quad (1)$$

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I would like to thank Dr. Harry Murakishi for providing the free environment in which I could grow and develop as a researcher, for providing the guidance and inspiration needed to work on this project, and for helping me to realize and experience the fact that science is nothing if it is not a joy and a fascination.

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GENERAL INTRODUCTION AND LITERATURE REVIEW

Introduction: Mechanisms of infection.

Some of the key questions involved in the study of physiological plant pathology are those dealing with infection mechanisms. The difficulties arising during such studies are enormous due to two major factors: a.) the microscale nature of the phenomena involved and b.) the subtlety and complexity of the interactions occurring. Studies of the infection mechanisms of plant viruses suffer from both of these drawbacks. Viruses are the smallest infectious units possible, and, despite a relatively simple structure, they are subject to many more subtle influences regarding infection effectors than might have been expected on the basis of their level of organization.

History of Infection Mechanism Studies.

With the increasing availability of sophisticated manipulatory techniques, the history of such studies of plant virus infection mechanisms has progressed from whole plant to plant part to tissue culture and finally to protoplast systems.

The earliest types of studies concentrated on such aspects as the number, kind and lifetime of infectible sites formed on intact leaves as the result of mechanical inoculation. The use of abrasives was seen to dramatically increase infection in intact plants. As discussed by Matthews (1970), this is thought to enable the production of wounds through the intact leaf surface (cuticle, pectin, cell wall) to expose the actual infectible sites. Evidence has been presented to implicate

ectodesmata in permitting virus entry into the cells, necessitating merely the breaking of the cuticle to allow access to the channels (Brant, 1966, Thomas and Fulton, 1968). Carborundum and bentonite abrasives were thought to serve as a mechanism for breaking through the cell wall.

The "phosphate effect" discovered by Yarwood (1952) demonstrates the sensitivity of the infection process even in whole plants to chemical additives. In this instance, the addition of dipotassium phosphate increased the infectivity of several viruses for bean leaves. Matthews (1970) suggests that this effect is unlikely to be directly on the virus or a nutritive one for the plant. For some viruses 10mM $MgCl_2$ greatly enhanced the phosphate effect (Kado, 1963). In another case it was seen that phosphate increased the adsorption of TMV to cell debris in vitro (Taniguchi, 1966). An interesting phenomenon described by Matthews, (1970) in relation to these others is the effect of water rinsing subsequent to inoculation on the number of local lesions obtained. Spraying, washing or dipping leaves in water within 2-4 h post-inoculation can substantially decrease lesion number (Yarwood, 1955). Yarwood suggests that this effect is due to the dilution of ions necessary for the attachment or penetration of the virus. Matthews and Proctor (1956) found that spraying Mg-nitrate and certain other metal salts onto the leaves within a few hours greatly increased infectivity. Air drying within 1 sec increased the number of local lesions seen on cowpea inoculated with cucumber mosaic virus more than a hundredfold, whereas the effect was much less dramatic with tobacco mosaic virus (TMV) on tobacco (Yarwood, 1963).

The lifetime of infectible sites has been shown to be fairly short,

falling off quickly after abrasion. Seventy percent of sites infectible by TMV in Nicotiana glutinosa leaves lost susceptibility within 90 sec with the other sites taking up to one hour to lose susceptibility (Furumoto and Wildman,1963). To show the extreme complexity of the phenomenon, however, and to point out the difficulty of postulating uniform, simplistic mechanisms of infection, Jedlinski (1956,1964) found that during the first ten minutes post-wounding, the number of infectible sites can either decrease, increase, or not change, depending on the virus-host system used.

The difficulties with all of these systems employing the use of whole plants is that more detailed and controlled experiments giving meaningful data cannot be done because of the extremely small number of cells in the population that become infected at even high inoculum concentrations and because of the lack of synchrony of infection involved. Only tantalizing clues can be discovered.

The ability to infect plant tissue cultures provided a new system for studying plant/virus interactions (Murakishi,et al,1971). In depth biochemical studies were possible (Pelcher et al,1972; White and Murakishi,1977), and recently the system was expanded to include soybeans (Wu and Murakishi,1978). Synchrony of replication was improved using cold temperature treatments (White et al,1977), but the system was not suited to extensive studies of the infection process itself. Efficiency in terms of numbers of virus particles needed was still relatively low, and synchronous virus replication by cold temperature treatments does not imply synchronization of infection. However some interesting observations were made.

Tobacco callus required vortexing into small aggregates for TMV

infection (Murakishi et al,1971). The soybean callus cells did not require such vortexing for successful infection with SBMV (Wu,1977). PLO was not required for infection in either virus system.

The development of high frequency infection of plant protoplasts occurred at a time of tremendous interest in the replication process (Takebe and Otsuki,1969) and it was immediately adopted by the great majority of workers interested in such studies. Parameters for plant virus adsorption and penetration were examined during this period more from the expediency of achieving synchronous, efficient infection of protoplasts for replication studies rather than from an interest in the infection process itself.

But these have still provided, if somewhat indirectly, an abundance of information on what might be happening during the infection process, at least in these in vitro systems.

Initially, Cocking (1966) reported apparent pinocytotic vesicles containing TMV particles in inoculated tomato fruit protoplasts. Apparent endocytotic vesicles containing virus were also seen using EM in tobacco mesophyll protoplasts (Otsuki et al,1972) leading to the theory that PLO induced endocytosis in plant cells as it did in animal cells (Takebe et al,1975). Pinocytosis was reported as the penetration mode of brome mosaic virus (BMV) into barley protoplasts (Okuno and Furusawa, 1978).

In contrast, Burgess et al (1973) offered evidence of a PLO-induced wounding leading to direct penetration of TMV into tobacco protoplasts. Similar evidence was reported for tobacco ringspot virus (TRSV) in tobacco protoplasts by Kubo et al (1976), and the wounding hypothesis was further discussed in the case of cowpea chlorotic mottle virus

(CCMV) infection of tobacco protoplasts by Motoyoshi et al (1974).

In both cases, that of pinocytosis and the wounding theory of the mode of virus entry, these conclusions were based solely on EM data with little or no kinetic or biochemical back-up information. Such studies are subject to artifact and misinterpretation, and the fact that EM data has been used to support both positions points to the weakness of such 'proofs'!

Perhaps the more sensible approach is the examination of the biochemical requirements for successful virus infection. Of necessity these will have been the result of and therefore point to various aspects of the processes involved. Binding studies have been done to provide one such indication.

Several studies of adsorption and binding of virus to protoplast membranes have been carried out recently. Zhuravleev et al (1975,1976) studied attachment of labelled TMV to tobacco protoplasts and some effects of various competitors on the attachment process. Infectious TMV, non-infectious TMV, and TMV protein enhanced labelled virus retention whereas serum albumin and casein hydrolysate did not. The addition of added infectious virus to the inoculation medium decreased final virus yield. The presence of TMV-RNA in the inoculation medium reduced both attachment/retention and yield. Attachment has been shown to be non-physiological in terms of being independent of temperature and the presence of metabolic inhibitors. Wyatt and Shaw (1975) and Zhuravleev et al (1973) showed that 10-15% and 6% of the inoculum virus respectively was retained by protoplasts even after inoculation.

Okuno et al. (1977) did studies on retention of labelled BMV on

isolated barley protoplasts in relating to PLO concentration and compared this to infection. It was found that above a certain concentration retention of label ceased to correlate with increased percent infection.

The biochemistry of infection has also been investigated for a wide range of protoplast/virus systems. Although modes of infection have not been the primary interest of most of these studies, much interesting data has been collected.

The consensus is that there are several requisites to successful infection of plant protoplasts. The requirements are, simply but inclusively:

1. viable protoplasts

2. infectious virus

3. osmoticum

4. buffer (different kinds and pHs)

5. PLO (generally required).

In each of the above listed cases concentration has been shown to be important in determining the ability to infect and the extent of infection obtained. It is worthwhile at this time to examine each of these parameters briefly.

Viable protoplasts are the first pre-requisite for studies of successful infection and the mode of obtaining them has remained something of an "art". According to Takebe(1977): "Since these conditions for obtaining stable protoplasts include factors such as temperature, humidity, day length, soil and method of watering, which usually differ according to where the plants are grown, it is difficult to standardize them, and each laboratory should ascertain its best conditions for plant growth". Thus, from the outset wide variations are introduced between laboratories and even within laboratories where new plants must be used

for each experiment and where exact standardization of environment is not at all times practical.

The leaf mesophyll protoplast systems available for use in virus research include the following host species: tobacco, tomato, cowpea, Chinese cabbage, turnip, barley, corn, wheat and oats. The viruses used have been even more numerous, and these virus/protoplast systems have been reviewed (Takebe,1978).

Specific infectivity of the virus preparations used is of course important to the results obtained. Storage of some viruses can lead to decreased infectivity with time(Hollings and Stone,1970). Methods of purification can change infectivity as well, including the presence of or absence of neutral salts or buffers (Gibbs and Harrison,1976). In the case of both CPMV and BPMV the stage of infection at which the plant material is harvested can have a strong effect on the ability of virus preparations to produce local lesions in plants (Bancroft,1962;Niblett and Semancik,1969). These phenomena have not been extensively studied in protoplasts. The tendency has naturally been to use only the best possible virus preparations.

Osmoticums used are generally sorbitol and mannitol. In one instance osmotic shock (increasing concentration of osmoticum sharply during inoculation)has been shown to increase infection of monocot protoplasts with BMV significantly (Okuno and Furusawa,1978).

In almost all cases previously reported, buffers have been used for obtaining infection. The most commonly used are: citrate, phosphate and Tris-HCl, with optimal concentrations ranging from 0.2mM to 10mM and pH ranging from 4.8 to 8.0.

Polycations, especially PL0, have been shown to be either essential

or stimulatory to virus infection. In theory the polycation allows for a balancing of electrostatic charge allowing the negatively charged virus to attach to the negatively charged membrane. Those few viruses which do not require PLO (pea enation mosaic virus (PEMV) in tobacco, CPMV in cowpea, and BMV in monocots) seem to have higher isoelectric points in their host systems than those which require it. Thus PLO would be less necessary for charge-balancing.

Recently, the first full-scale, workable system for the study of plant/virus interactions using protoplasts derived from liquid suspension cultures was developed. Soybean protoplasts from suspension cultures of variety Harosoy 63 have been infected with cowpea mosaic and southern bean mosaic viruses (Jarvis and Murakishi, 1979; Jarvis, 1979).

Although there were no firm indications of what mechanisms for adsorption and penetration were involved in the case of the two viruses, certain highly suggestive results pertaining to key parameters involved were obtained. These include:

1. A divalent cation effect: The presence of low levels of calcium and/or magnesium during inoculation (the period of early interaction) provided a 3 to 10-fold increase over untreated controls in protoplast infection with CPMV and BPMV in the soybean protoplast system. The viruses differed in their cation requirements and the extent of stimulation obtained. Pretreatment of the protoplasts with excess Ca^{2+} almost completely inhibited the stimulatory effects; post-inoculation treatment of the protoplasts had no apparent effect. Similar enhancement effects of these divalent cations have been seen during inoculation in the poliovirus system (Lonberg-holm and Philipson, 1974).

2. A temperature effect: Evidence pointed to a strong correlation between the effects of temperature on CPMV infection and known temperature effects on microviscosity (the fluid state of a membrane) (Jarvis,1979). Viscosity studies of plasmalemma from rose-petal protoplasts (Borochoy et al,1978) and of membrane phase changes in mung bean (Raison and Chapman,1976) have indicated that the membrane systems of plants in general undergo a phase change from a predominately gel phase below 11-15C, to a mixture of fluid and gel above this critical temperature range. The soybean/CPMV protoplast system demonstrates an abrupt change in rate of infection increase with temperature at about the 12C mark. From 0-12C the linear rate of increase is 4.4% increase in fluorescing protoplasts/degree C. From 12-37 C the linear rate of increase drops abruptly to 0.45% increase/degree C - a nearly ten-fold change in rate which occurs suddenly at the approximate phase transition point (as observed by fluorescent probes and electron spin resonance) common to most plant membrane systems studied. This apparently was the first reported instance of a correlation between protoplast infectibility and membrane viscosity. In modern membrane theory temperature effects on fluidity are considered crucial to the functioning of protein receptor sites and the occurrence of pinocytosis (Singer,1975).
3. Novel effects were obtained from the use of the "Good" sulfonic acid buffers. Using these buffers such as HEPES for inoculating soybean tissue culture derived protoplasts, Jarvis(1979) found complete elimination of the need for PLO or a similar polycation to be used for successful infection for both CPMV and SBMV. This

was an effect never seen before. Previously PL0 or the presence of a similar polycation has been shown to be either absolutely necessary for a particular virus/protoplast system, or it has proved to be merely stimulatory (Takebe,1977). It has never been previously reported as being necessary under one set of conditions for a particular system and then unnecessary for the same system under a set of different conditions.

An added unique effect of the sulfonic acid buffers is that using them in the inoculation medium completely eliminated the temperature sensitivity of the system for CPMV. Equally good levels of infection were obtained at both high and low temperatures with no obvious changes at the probable membrane shift point.

This then was the general background of infection mechanism studies prior to the beginning of this investigation. The purpose of the presently reported research was threefold:

1. To expand and develop the soybean protoplast system by introducing a new virus into it,
2. To validate some of the observations in this system by comparing the behavior of viruses in it to that of the more traditional leaf mesophyll type system, (to which end the bean leaf mesophyll protoplast system was developed), and
3. To examine virus infection parameters in both systems in comparison to those other systems already known in order to attempt to come up with some indication of possible modes of infection.

This last, indeed, was the primary and most interesting purpose for conducting this entire investigation.

PART I

**BEAN POD MOTTLE VIRUS INFECTION
OF SUSPENSION CULTURE DERIVED
SOYBEAN PROTOPLASTS**

INTRODUCTION

Bean pod mottle virus was chosen for infection in the soybean protoplast system for several reasons:

1. It is an icosohedral virus, well-characterized and easy to purify (Semancik,1972).

2. It is a comovirus as is cowpea mosaic virus, and it was thought that this would make for interesting comparisons of infection requirements. In fact, these two viruses are the two closest related (non-strain) viruses capable of being put into one protoplast system. They have many similar properties (Bruening,1978).

3. The virus is an actual economic problem in soybeans causing 10 to 15% losses of yield by itself and up to 60% when associated with soybean mosaic virus (Compendium of Soybean Diseases,1975).

4. The virus has not been introduced into any other protoplast system previously, probably due to its host range being limited to legumes.

Thus the virus seemed the perfect candidate for incorporation into the soybean protoplast system.

MATERIALS AND METHODS

BPMV Purification. BPMV was inoculated to 8-10 day old(unifoliate leaf stage) Phaseolus vulgaris cv. Pencil Pod Wax plants in flats and harvested 14-21 days later when maximum symptoms were observed. Virus was purified using a modification of the method reported by Semancik (1972). Tissue was triturated in 0.2M pH 7.0 K-phosphate buffer (1.5 - 2.0ml/g tissue) and clarified by the addition of 10% v/v n-butanol + chloroform (1:1). After stirring for 60 min at 4C the denatured plant material was removed by low speed centrifugation and the virus was then pelleted for 3h in a #30 rotor at 22.5K. The pellet was resuspended in 0.1M pH 7.0 K-phosphate buffer. The virus was then precipitated by adjusting the pH to 5.0 with acetic acid as described (Semancik,1972), or alternatively precipitated with polyethylene glycol (PEG) MW 6000 (4%) and made 0.2M with NaCl. Resuspension was in 0.1M pH 7.0 K-phosphate buffer. Following two cycles of differential centrifugation the virus was finally resuspended in the phosphate buffer at a virus concentration of from 1-2mg/ml. At lower concentrations of buffer (10mM) and in distilled water the virus would precipitate out of solution.

Soybean Culture Initiation and Maintenance. Liquid suspension cultures of Glycine max cv. Harosoy 63 were initiated and maintained as described (Jarvis and Murakishi,1980). At present the cultures have been successfully maintained for over 2 1/2 years from initiation.

Protoplast Isolation. Protoplasts were isolated as described by

Jarvis and Murakishi (1980) with an added final filtration step through a 25 um mesh stainless steel screen to remove added cellular debris.

Protoplast Inoculation and Incubation. The inoculation procedure was similar to that outlined (Jarvis and Murakishi,1980) with the following variations: Pre-incubation of the inoculation medium was 15 min at 22 C. Between $0.5 - 1.0 \times 10^6$ protoplasts were pelleted and resuspended in 5 ml of 0.4M sorbitol followed by the immediate addition of 5 ml of inoculation medium containing virus and the various amendments in concentrations determined by the experiment being performed. Rapid mixing was accomplished by twice pouring the virus and protoplast suspension from one tube to another. The suspension was then incubated at 22 C for 15 min, whereupon the protoplasts were washed via centrifugation and incubated as described (Jarvis and Murakishi,1980).

Protoplast viability was determined after 48 h by the use of Evans Blue dye exclusion. Protoplast viability varied according to treatment conditions, but in general the least damaging treatments averaged 70% viability.

Fluorescent Antibody Preparation and Staining. Protoplasts were assayed for infection by staining with fluorescein isothiocyanate conjugated antibody following the methodology previously described (Jarvis and Murakishi,1980). The antiserum was titered by using the tube precipitin test (Ball, 1974).

Infectivity Assay. Protoplasts from 0.5 and 48 h following inoculation were harvested, pelleted, and stored at -20 C. A total of 1.2×10^6 protoplasts were resuspended in 0.5 ml of 0.2M K-phosphate buffer pH 7.0 and disrupted in a ground glass tissue grinder after thawing. Local lesion assay was performed by inoculating unifoliate leaves of

Phaseolus vulgaris cv. Pinto using matched opposite leaves for each of the two post-inoculation times being tested. After inoculation, leaves were detached and incubated on moistened filter paper in parafilm sealed petri-plates for 72 h in the dark at 25 C.

Variation in the System. In order to examine the inherent variation involved in studying the parameters of a biological process, three sources of variation were looked at in the soybean-BPMV system:

1. Within experiment variation in determining viability and fluorescence using slides.
2. Experimental variation due to the techniques and random differences between "identical" replications.
3. Variation introduced due to the use of different virus preparations.

Photography. High speed Daylight Ektachrome ASA 400 (Eastman Kodak Co., Rochester N.Y.) was used for all fluorescent photography as described by Jarvis (1979).

RESULTS AND DISCUSSION

Fluorescent Antiserum. The FITC-conjugated antiserum to BPMV had a titer of 4096 as determined by tube precipitin test using 0.05mg/ml BPMV. A dilution of 1:70 for actual assay test on protoplast slides gave good results.

Demonstration of Virus Infection.

Fluorescent Time Course. As seen in Figure 1, virus specific fluorescence was first seen to appear between 6 to 18 h post-inoculation. By 18 h over half the developing fluorescence had already appeared. Around 24 h the rate of increase in fluorescence began to level off markedly until little significant increase was seen between 36-60 h. The fluorescence seen was a very bright, apple green color throughout the whole cell (Figure 2) rather than the crystalline pinpoints as has been reported for TMV in tobacco protoplasts (Otsuki and Takebe, 1969). Non-inoculated control protoplasts showed no such development of fluorescence over time.

Infectivity Assay. Inoculation conditions for the protoplasts used in the local lesion assay were as follows: 0.6×10^5 protoplasts per ml final concentration were combined with an inoculation mixture containing 2.0 ug/ml BPMV, 1.0 ug/ml PLO, 10mM K-phosphate buffer pH 6.3 and 0.4M sorbitol. Infection determined after 48 h by fluorescent antibody assay was 31%. Protoplasts harvested after 0.5h post-inoculation resulted in an average of 3 lesions per unifoliate leaf inoculated. Protoplasts harvested at 48 h post-inoculation gave an average of 101 lesions per

Figure 1. Time course of BPMV synthesis in protoplasts. Protoplasts were inoculated with 4.5 ug/ml BPMV in the presence of 5mM K-phosphate pH 6.3 and 0.5mM CaCl_2 in the absence of PLO. Samples were removed and slides made from incubating protoplasts at the times indicated. Each of the data points represents a single replication only.

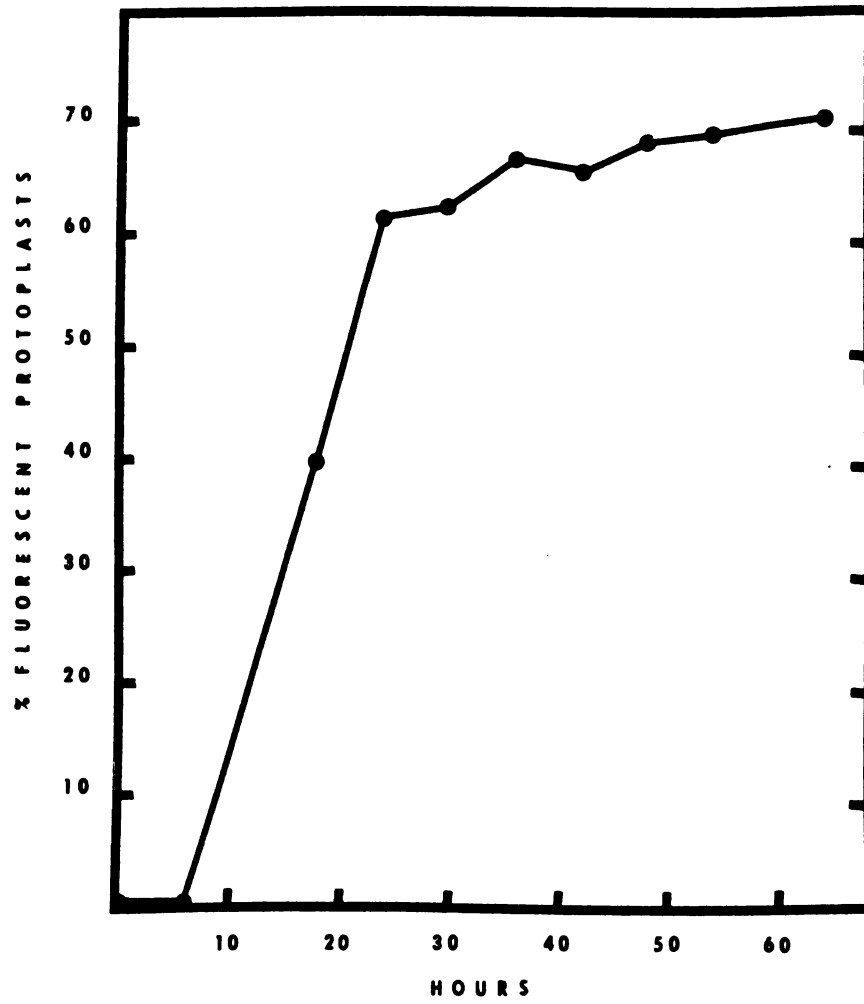
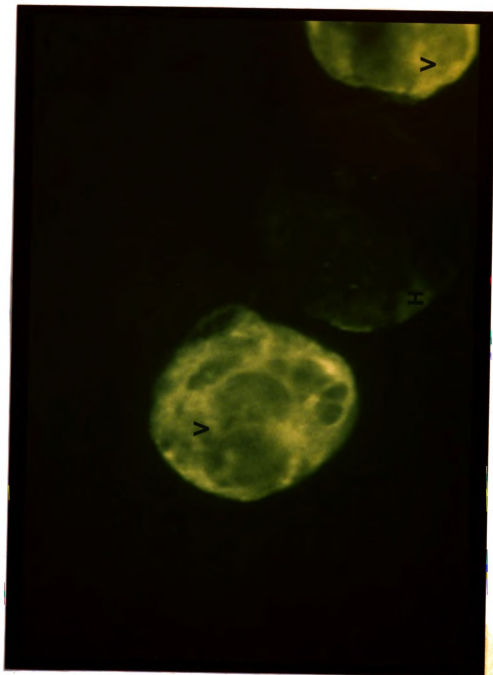


Figure 2. BPMV - infected protoplasts stained with fluorescent antibody.
V = virus infected protoplast; H = healthy protoplast.



unifoliate leaf inoculated. Seven plants were used and the two times were paired, each on an opposite unifoliate leaf to provide 7 matched pairs.

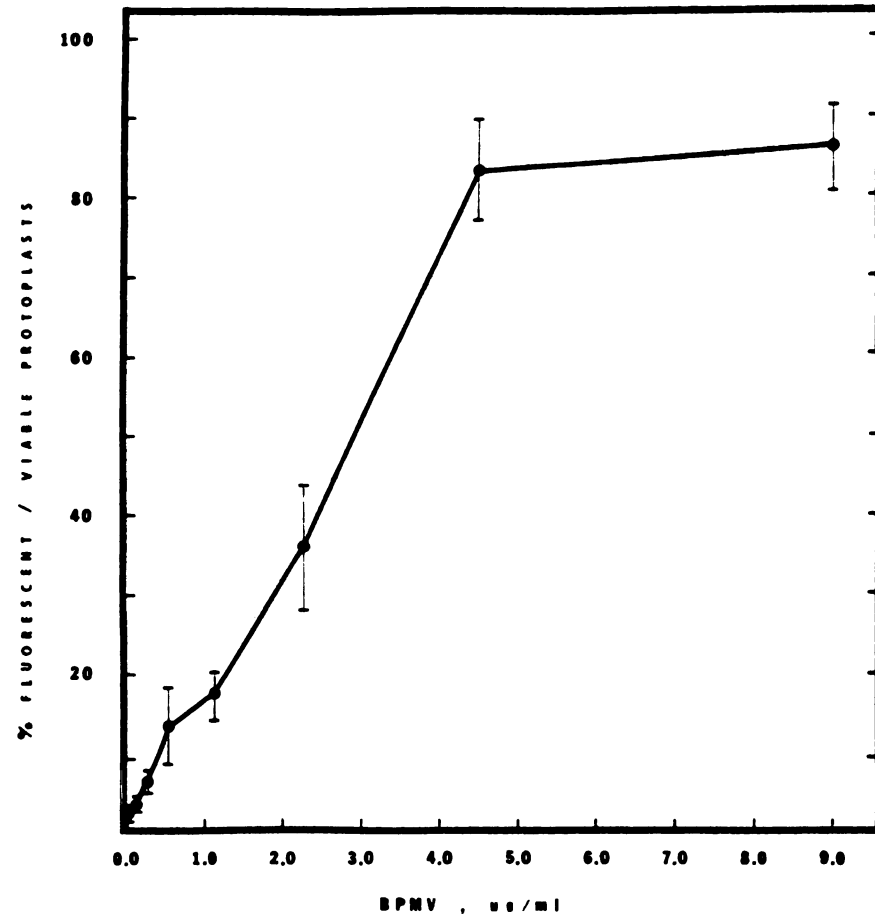
Variation in the System.

Variation Due to Assay Technique. Although an in-depth study was not performed, several early experiments indicated that, in order to insure minimal variability (generally within $\pm 10\%$) in counting percent viable protoplasts using Evans blue it was necessary to count at least 200 protoplasts total, using randomized scanning fields. In assaying fluorescent protoplasts, between 300-400 were routinely counted, which gave similar or better variability range.

Experimental Variation. To examine the sum total of variability involved routinely when using the above counting methods, an experiment was done to assay the effect of virus concentration on BPMV infection using three replicate tubes per concentration. Figure 3 shows the range of variability involved. As in all biological assays and measurements, the larger the number of samples taken, and the larger the number of replicates used, the greater the reliability that can be placed in the results.

Using the described counting regime, at least 2 replicates per treatment within most experiments and repeating experiments at least once seems to give dependable, reproducible results in this system. Error does inevitably creep in, however, such as will be seen in a few cases where greater than 100% infection is obtained (due to separate viability and infection counts). This demonstrates the absolute need to evaluate such experiments only within their range of acceptability (as indicative of trends and of numbers only within their error range). In

Figure 3. The effect of BPMV concentration on percentage of protoplasts infected and on experimental variability in the absence of amendments. Protoplasts were infected with BPMV concentrations as indicated. Each data point is an average of three replications, and the "I" bars indicate the + and - standard deviation.



order to do mathematically sound analysis, more replications would be advisable. It is this inattention to variability that makes much of the protoplast literature suspect. The beauty of the soybean protoplast system is that replications for routine work are easily performed, and, where more complex analysis is required, this too could be accomplished.

Variation Due to Different Virus Preparations. After a long series of experiments with BPMV it was discovered that under certain conditions a considerable amount of between experiment variation seemed to be introduced when different virus lots were used as inoculum. This is beyond the relatively low level of variability inherent in comparing different experiments to one another. The indications are that the virus behaves differently depending on which purification lot was used. Table 1 shows this variation between three different purified virus lots. Indications are that, in the absence of amendments, virus lots II and IV are quite similar, whereas virus lot III seems to have an extremely low infectivity on its own. The intriguing point is that, with amendment (Table 2), infectivity of preparation III rises to equivalent levels of lot II in a series of similar experiments. These differences seen held consistent over a number of experiments. The interesting phenomenon is that it is the unamended virus that differs from lot to lot and that calcium amendments can mitigate what is an apparently dramatic difference in initial specific activity. This particular type of phenomenon will be seen repeatedly - that no one component of the in vitro infection process can be regarded as exclusively dominant over infection percentages obtained, not even the apparent specific activity of the virus prep. In this case, calcium chloride seems capable of counteracting some lack in the virus itself in preparation III, raising it to levels similar to

Table 1. Infection variation among different BPMV preparations (unamended). Protoplasts were inoculated with virus lots purified from different batches of host plants at different times. No other amendments were present in the osmoticum. The %I/V represents single data points, not averages.

Table 1.

<u>Virus Lot</u>	<u>Experiment #</u>	<u>Virus Concentration</u> (ug/ml)	<u>%I/V</u>
II	25	2.3	29
		2.3	44
		2.3	35
	28	2.3	19
	29	2.3	55
	31	2.3	23
	32	2.3	27
III	40	2.0	4
		2.0	0
	44	2.0	3
		2.0	1
IV	45	2.1	53
		2.1	37

Table 2. Infectivity Variation Among Different BPMV Preparations (Amended).

<u>Virus Lot</u>	<u>Experiment #</u>	<u>Virus Concentration</u>	<u>CaCl₂</u>	<u>%I/V</u>
II	26	2.3 ug/ml	0.5mM	48
	28	2.3 ug/ml	"	47
III	40	2.0 ug/ml	"	27
		2.0 ug/ml	"	51
	43	2.0 ug/ml	"	66
		2.0 ug/ml	"	63

Protoplasts were inoculated with different virus lots (same nomenclature as Table 1) in the presence of CaCl₂ at 0.5mM. The %I/V represents single data points, not averages.

those obtained with preparation II. Of equal interest is the fact that there is no equivalent increase of effectiveness of preparation II upon the addition of calcium. It is as if preparation II had already reached some maximal infectivity level which the addition of CaCl_2 could not dramatically change.

One source of this specific infectivity difference between virus preparations can be speculated upon. It has been seen by other researchers that BPMV is processed by host enzymes to a less infectious form over time (Niblett and Semancik, 1970). Therefore, depending on the physiological age of the disease at the time of leaf harvest, the purified BPMV from one preparation could be either more or less infectious than from another. No attempt was made in this study to assay the percentage of the less infectious form in each individual virus preparation used since this phenomenon was not recognized until late in the investigation. This infectivity phenomenon will be discussed more in the section on buffer effects.

Effect of Various Inoculation Parameters.

Virus Concentration Effects. BPMV, unlike most other viruses used in plant protoplast systems, has extremely minimal requirements for efficient, successful infection. Poly-L-ornithine, significant quantities of buffer, and other amendments are not required. Figure 3 shows the effect of changing virus concentration on percent protoplasts infected. No more than 0 to 0.1mM K-phosphate buffer pH 7.0 is present from the virus stock solution - an amount not capable of exerting a significant buffering effect in the system, and less concentration than seems necessary to keep milligram quantities of purified virus in solution. No other amendments are present. As can be seen from Figure 3 percent

infection increases rapidly between 0 to 4.5 ug/ml virus, after which a strong levelling off occurs with little infection increase even when BPMV concentration was increased up to 9 ug/ml.

The general trend of the curve shows sigmoidicity at the lower virus concentration ranges. This would seem to signify some sort of concentration cooperativity effect which in this case, since we are primarily dealing with the presence of virus alone, is probably due to the threshold effect caused by BPMV being a multi-component virus requiring two genomically distinct particles to infect. The leveling off of infection, even at increasing virus concentrations, at less than 100% infection seems to indicate some sort of saturation effect. Since there are no other significant amendments it would seem that this saturation is due to some sort of interaction between virus and protoplasts.

Protoplast Concentration Effects. As has been reported elsewhere (Mayo, 1978), protoplast concentration can have a marked effect on infection. In early experiments using fully amended inoculum containing virus, PLO, CaCl_2 and buffer, increasing protoplast concentration above the 1×10^5 protoplasts/ml level led to significantly decreased infection (Figure 4). Generally the best infection was obtained when $0.6 - 0.9 \times 10^5$ protoplasts/ml were used. The former is the lowest volume of protoplasts that can be used in the centrifugation process without great difficulties.

Buffer Effects. Although added buffer was not needed for infection, it was shown to be stimulatory (Table 3). Potassium phosphate buffer showed an optimum at pH 5.6 in the absence of PLO and CaCl_2 (Figure 5). In the presence of these two amendments the pH optimum appeared to shift slightly towards 5.4, but more significantly, infection leveled off

Figure 4. The effect of protoplast concentration on percentage of BPMV infection. Protoplasts were inoculated with 1.0 ug/ml BPMV in the presence of 10mM K-phosphate buffer pH 6.3. 1.5 ug/ml PLO and 0.5mM CaCl_2 . Protoplast concentration varied as indicated. Data points represent single replicates only.

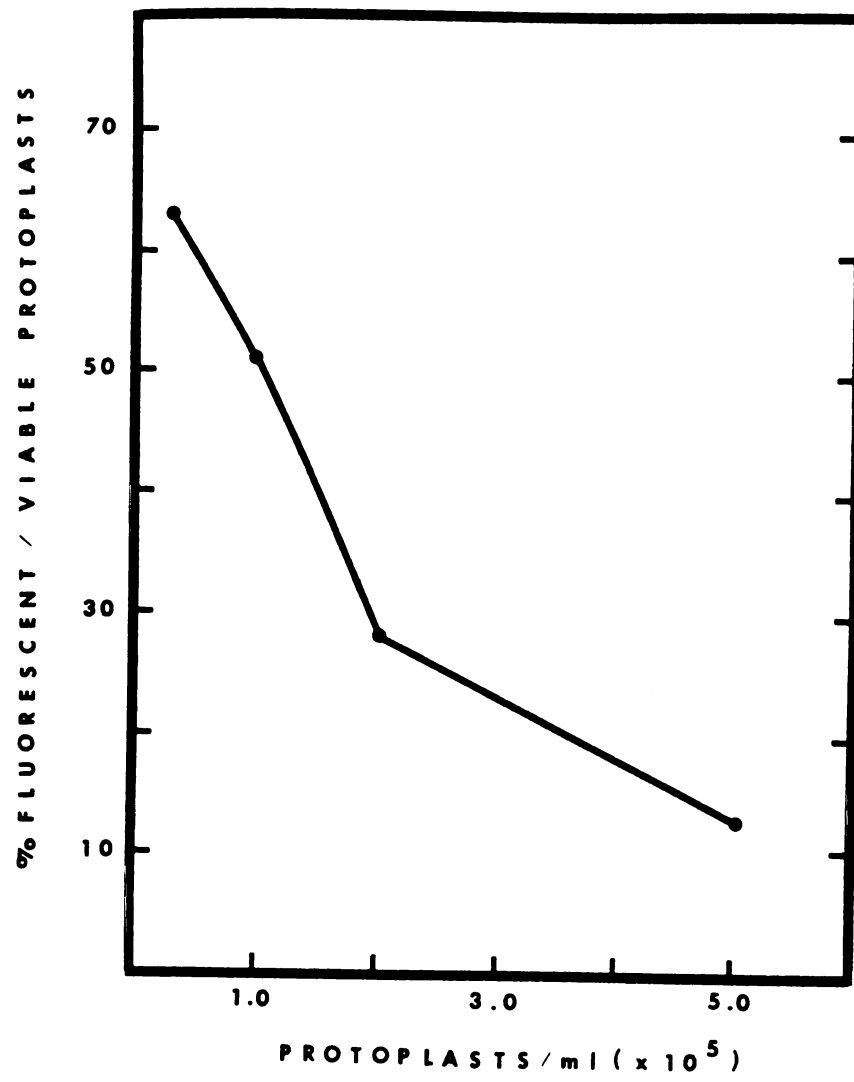
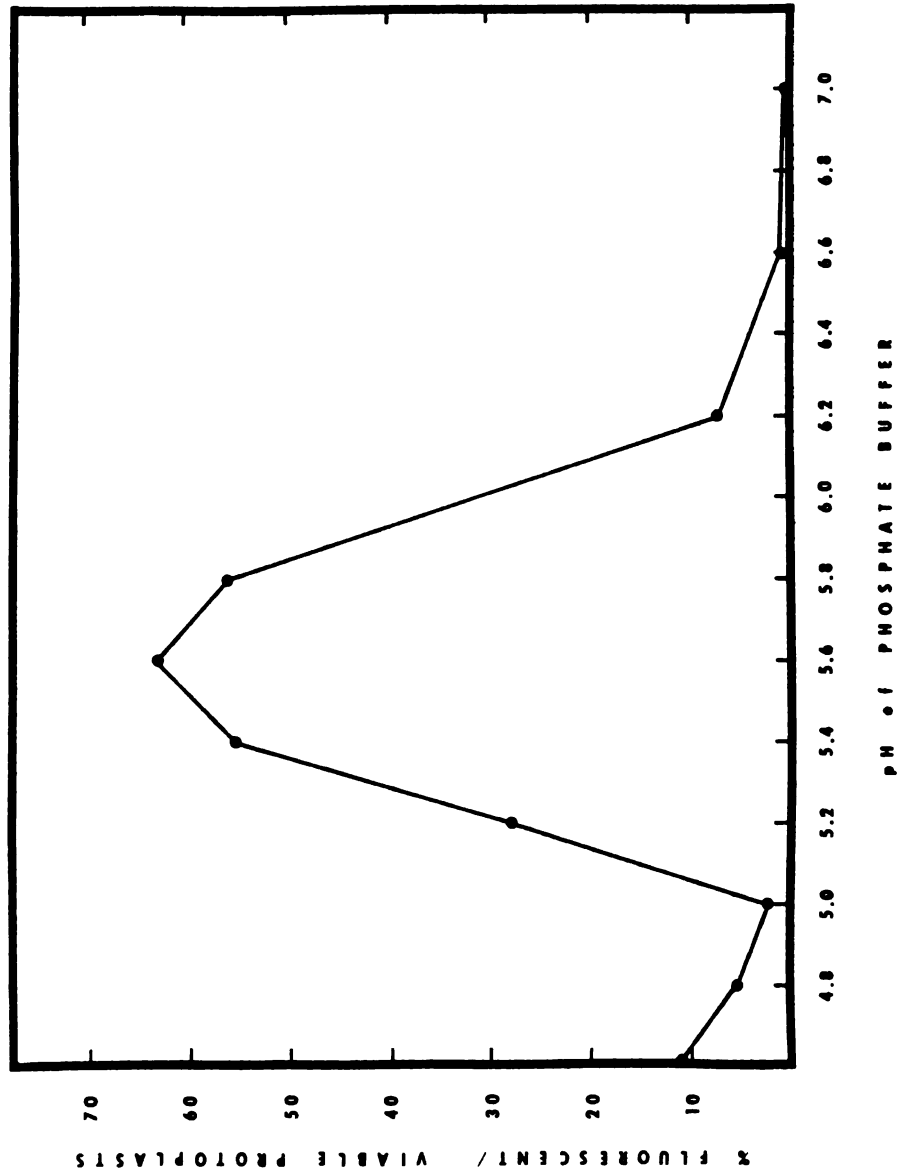


Table 3. Effect of pH 5.6 K-phosphate Buffer Concentration on Percentage of BPMV Infection of Protoplasts.

<u>Experiment #</u>	<u>Virus Concentration</u>	<u>K-phosphate</u>	<u>pH</u>	<u>%I/V</u>
36	2.0 ug/ml	5mM	5.6	7
	2.0 ug/ml	10mM	5.6	29
	2.0 ug/ml	20mM	5.6	20
37	4.0 ug/ml	0mM	**	21
	4.0 ug/ml	10mM	5.6	66

Protoplasts were inoculated with virus and pH 5.6 buffer at various concentrations. The pH of the protoplast mix without buffer (**) was 5.5. The %I/V represents the average of two replications per experiment.

Figure 5. The effect of pH of K-phosphate buffer on percentage BPMV infection of protoplasts (unamended). Protoplasts were inoculated with 4.0 ug/ml BPMV in the presence of 10mM k-phosphate buffer at the pHs indicated with no other added amendments. Each data point represents the average of two replicates.



rather than continued to decrease between pH 6.2-7.0 (Figure 6).

Of three buffer concentrations tested at pH 5.6, 10mM seemed to be optimal. The lower concentration resulted in less infection (Table 3) and the higher concentration lead to no improvement or an actual decrease in infection when only virus, buffer and osmoticum were present in the inoculation mix.

In contrast, at a suboptimal pH of 6.3 (chosen initially because it is the optimal pH for the closely related CPMV in the soybean protoplast system as reported by Jarvis and Murakishi (1980)) increasing concentrations of K-phosphate proved ineffective or inhibitory at all virus concentrations tested (Figure 7) when only virus, buffer and osmoticum were present. The addition of CaCl_2 to the inoculum made this decrease even more dramatic (Figure 8).

The use of Na-phosphate buffer at pH 6.3 showed a similar concentration dependent decrease in infection. However, in this case, a lower (2.5mM) buffer concentration proved stimulatory to infection (Figure 9).

It may be speculated that all of this demonstrates a separation of pH effects from buffer concentration effects: That particular buffer ions (K^+ , $\text{PO}_4^{=}$, Na^+) are desirable for infection at certain concentrations, but that an optimal pH is also desirable. A very low concentration of the wrong pH buffer could perhaps provide enough of the desirable ion but be insufficient to pull the system to an inhibitory pH. Increasing the buffer concentration at the wrong pH pulls the system more and more to the inhibitory pH values causing more of an inhibition.

This would explain why adding pH 5.6 buffer to the unbuffered system (normal pH being 5.4-5.5 as determined experimentally) is so tremendously stimulatory : The buffer compound itself is stimulatory.

Figure 6. The effect of K-phosphate buffer on percentage BPMV infection of protoplasts (amended). Protoplasts were inoculated with 1.0 ug/ml BPMV in the presence of 10mM K-phosphate buffer at the pHs indicated with PLO (1.5 ug/ml) and CaCl_2 (0.5mM) added as amendments. Each data point represents the average of two replicates.

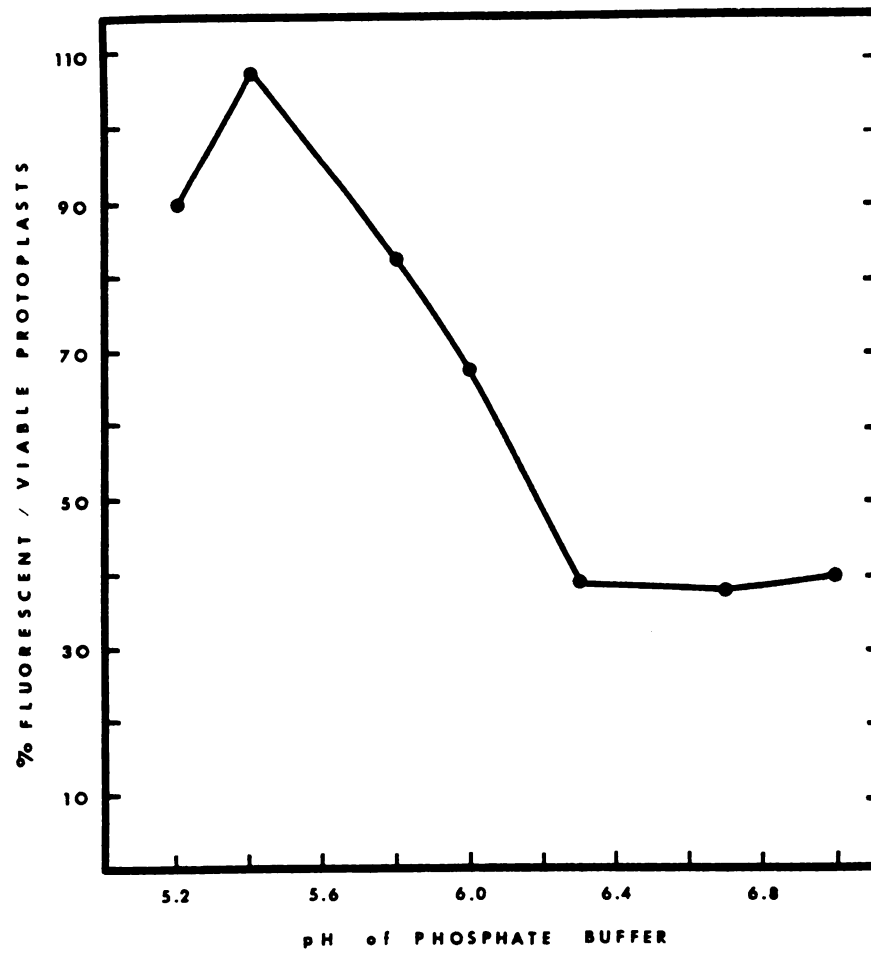


Figure 7. Effect of pH 6.3 K-phosphate buffer concentration on percent BPMV infection of protoplasts. Protoplasts were inoculated at various BPMV concentrations in the presence of K-phosphate pH 6.3 at 0mM (● — ●); 5mM (○ — ○); 10mM (▲ — ▲); and 20mM (▼ — ▼) buffer concentrations in the absence of other amendments. Each data point represents only one replicate.

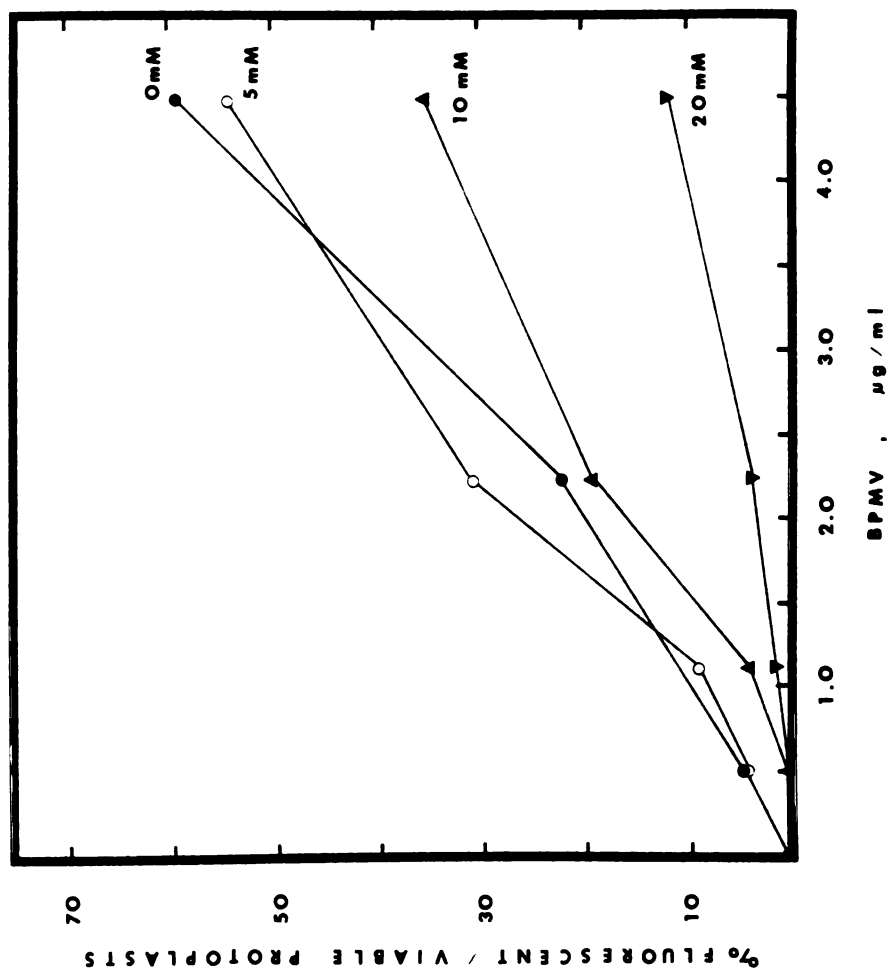


Figure 8. Effect of CaCl_2 on pH 6.3 K-phosphate buffer induced inhibition of BPMV infection of protoplasts. Protoplasts were inoculated at the virus concentrations indicated. Treatments included were: no amendments ($\circ$ — $\circ$); 10mM K-phosphate buffer, pH 6.3 ($\blacktriangledown$ — $\blacktriangledown$); 10mM K-phosphate buffer, pH 6.3 + 0.5mM CaCl_2 ($\blacktriangle$ — $\blacktriangle$); and 0.5mM CaCl_2 ($\bullet$ — $\bullet$). Each data point represents only one replication.

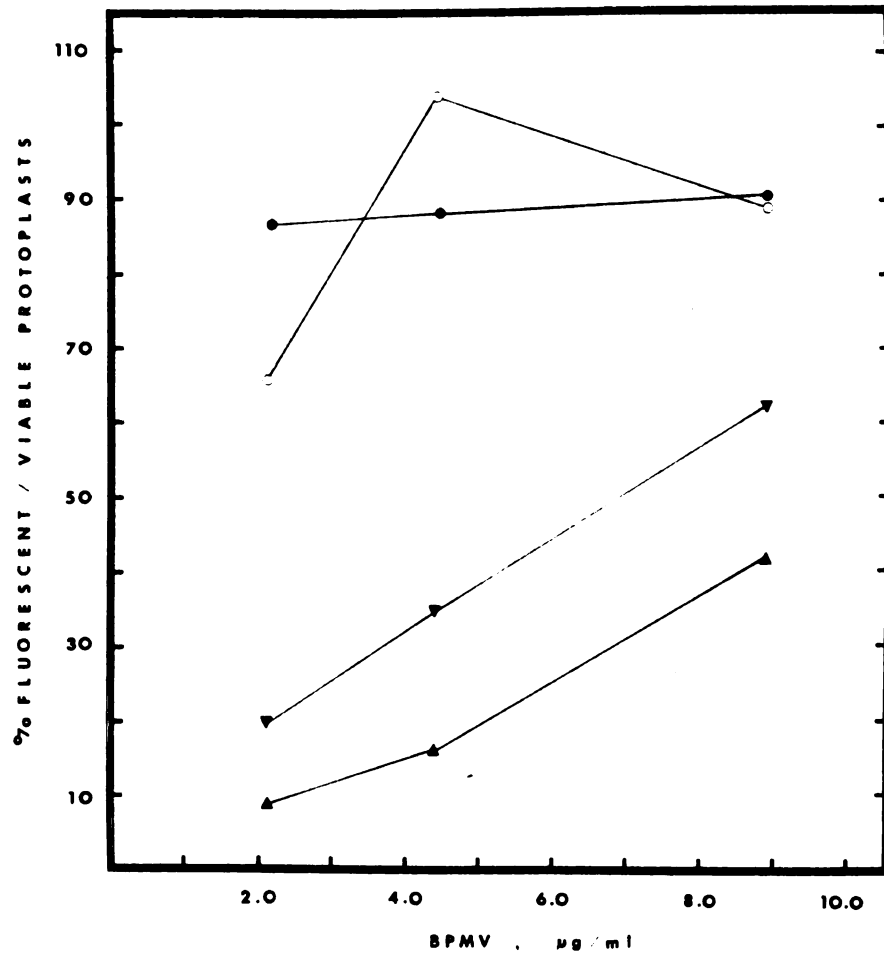
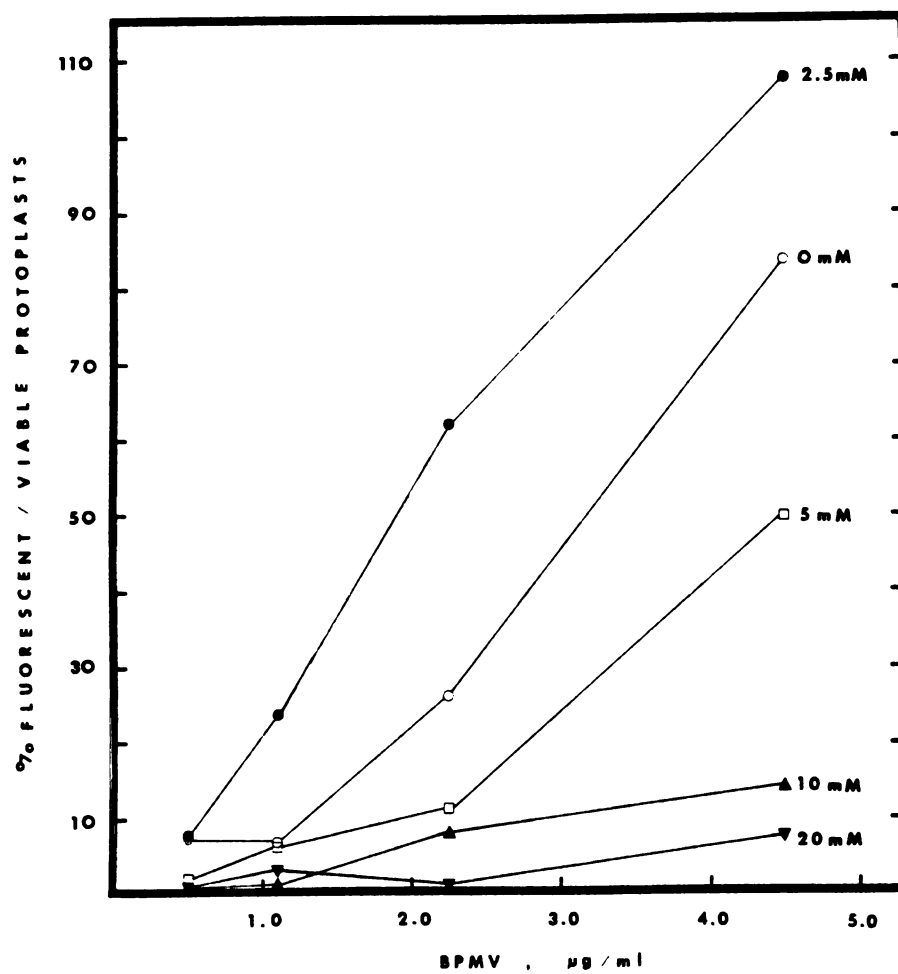


Figure 9. Effect of pH 6.3 Na-phosphate buffer concentration on percent BPMV infection of protoplasts. Protoplasts were inoculated at various BPMV concentrations in the presence of Na- phosphate, pH 6.3 at 0mM ( — ); 2.5mM ( — ); 5mM ( — ); 10mM ( — ); and 20mM ( — ) buffer concnetrations in the absence of other amendments. Each data point represents only one replication.



The wrong pH buffer can only be stimulatory (or show no great inhibitory effect) when it is in quite low concentrations.

Such observations are in line with the fact that it is not only buffer pH, but the kind of buffer used as well that seems to be important in other virus/protoplast systems (Takebe,1978). The evidence thus seems to be for a separable effect.

Experiments were done to localize the stimulatory effect of buffer on BPMV infection. Stimulation of infection over the basal level occurred only when the virus was pre-incubated with the buffer, (Table 4). This would indicate that the primary effect of the buffer is somehow involved with the virus particle itself.

Speculation on the molecular site of operation of this effect can be attempted (since it is acting on the virus particle itself). The pH effect of buffer alone on infection shows a bell-shaped peak at 5.6 with lows on either side, below 5.0 and above 6.3 (Figure 6). This indicates that infection efficiency is predicated upon the protonization of a participatory group with a pK_a of 5.2 and the deprotonization of a participatory group with a pK_a of 6.1 (this analysis being standard for interpreting pH dependence in biological reactions (Engel,1977)). A quite similar, but pH shifted effect was seen by Jarvis (1979) for the same buffer and the closely related CPMV in the soybean protoplast system. This dual protonization/deprotonization requirement at this particular pH range is highly indicative of the participation of histidine in the buffer-effect on the virus, histidine being the only amino acid with an ionization pattern that falls into the observed range (in particular the imidazole group of the histidine molecule), (Barnard and Stein,1958). The indication that an individual amino acid can be involved in virus

Table 4. Effect of Pre-incubation with Virus of K-phosphate Buffer on Percentage of BPMV Infection.

<u>Experiment #</u>	<u>Treatment</u>	<u>%I/V</u>
41	Buffer pre-incubated with virus	35
	Buffer not pre-incubated with virus	18
51	Virus alone	18
	Buffer not pre-incubated with virus	16
	Buffer pre-incubated with virus	42

Protoplasts were inoculated with either 2.0 (Experiment #41) or 0.53 ug/ml BPMV (Experiment #51) with or without pre-incubation of virus with 10mM K-phosphate pH 5.6 buffer in the inoculation medium for 15 minutes. No other amendments were present. The %I/V represents the average of two replications per treatment.

infectivity, particularly for the comoviruses, is not unique. Changes in coat protein residues have been coupled to the observed phenomenon of the differences in infectivity of virus electrophoretic forms for this taxonomic grouping, including BPMV and CPMV (Niblett and Semancik, 1970).

It has been demonstrated for BPMV that enzymatic cleavage of specific acidic amino acids from the virus coat by host enzymes leads to a conversion of electrophoretic form from Fast (F) to Slow (S) with a concomitant decrease in specific infectivity (Niblett and Semacik, 1970). This demonstrates the importance of coat protein to infectivity, the specific role of individual amino acids, and lends credence to the possibility that an individual amino acid, in this case histidine as the pH data seems to indicate, may indeed be highly important.

Divalent Cation Effects. When used as an amendment to the virus alone, CaCl_2 improved infection markedly with increasing concentrations until its effects leveled off and showed indications of causing a decrease (Figure 10).

As previously indicated, calcium caused a decrease in infection at several virus concentrations when used in the presence of buffer at the wrong pH (pH 6.3) (Figure 8). In the presence of buffer at the 'right' pH (pH 5.6) calcium alone had, in contrast, a stimulatory effect (Table 5).

Magnesium chloride also showed a stimulatory effect on virus infection when used as the sole inoculum amendment. In an experiment done to compare MgCl_2 to CaCl_2 and to localize these effects it was found that:

1. CaCl_2 was better at stimulating infection than MgCl_2 , and
2. Both compounds had stimulatory effects over the basal level of infection only when allowed to pre-incubate with the virus

Figure 10. Effect of CaCl_2 concentration on percentage BPMV infection at several virus concentrations. Protoplasts were inoculated in the presence of various CaCl_2 concentrations in 3 separate experiments, each at a different virus concentration: 1.06 ug/ml BPMV ($\square$ — $\square$); 2.0 ug/ml BPMV ($\bullet$ — $\bullet$) and 4.5 ug/ml BPMV ($\blacktriangle$ — $\blacktriangle$). Each data point represents only one replication except for BPMV = 2.0 ug/ml where each data point represents the average of two replications.

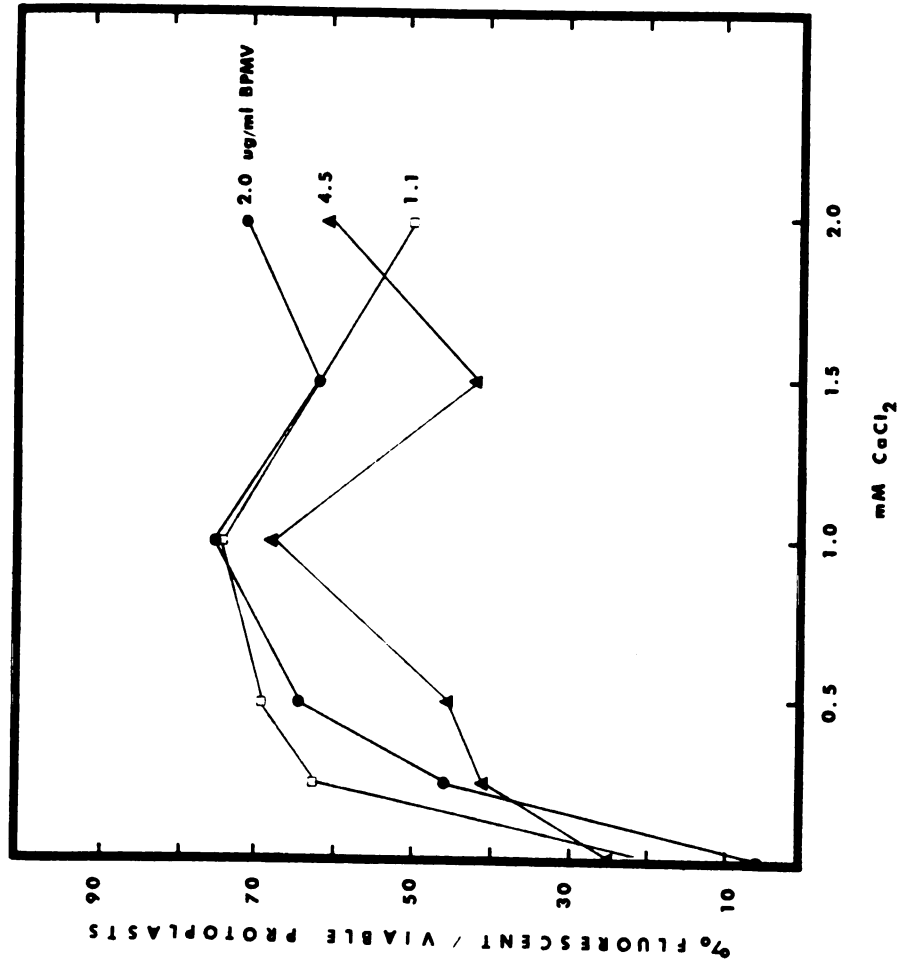


Table 5. Effect of Calcium Presence on Infection with pH 5.6 K-phosphate Buffer in the Inoculum.

<u>Experiment #</u>	<u>Virus Concentration</u>	%I/V	
		<u>+ CaCl₂</u>	<u>- CaCl₂</u>
45	2.1 ug/ml	110	69
46	0.53 ug/ml	110	51
	0.27 ug/ml	55	1
49	4.2 ug/ml	90	77
	2.1 ug/ml	90	84

Protoplasts were inoculated at various virus concentrations in the presence or absence of CaCl₂. All treatments were amended with 10mM K-phosphate buffer, pH 5.6. The %I/V represent single data points.

(Table 6).

Thus, the two divalent cations seem to act upon the virus particle first, just as the buffer was indicated to do. To some extent there is an antagonistic or competitive effect between the divalent cations and the buffer apparently, because the presence of buffer can weaken the extent to which the calcium induces a stimulation and, in the case of the wrong pH, can even turn it to a noticeably inhibitory effect.

That calcium is a better stimulus than magnesium seems to indicate some sort of selective specificity and not just an effect of positive charges.

As will be discussed in the next section, calcium chloride shows a strong, almost synergistic effect with the presence of PLO at the lower virus concentrations in terms of infection stimulation.

The effect of CaCl_2 on the virus particle would seem to obviate the role of calcium in terms of membrane fluidity as discussed by Jarvis (1979) although such effects might indeed be involved in the case of the infection decrease induced by a high concentration of CaCl_2 observed by that author.

Poly-L-ornithine Effects. Like the other viruses that do not require PLO for infection in protoplast systems (BMV, (Okuno et al, 1977); PEMV, (Motoyoshi and Hull, 1974)), BPMV has a fairly high isoelectric point as compared to those viruses which do require a polycation. This seems to reinforce the electrostatic view of early virus/protoplast interaction as proposed by various researchers (Takebe, 1978).

Although PLO is not required for successful infection, it is generally stimulatory. However, at concentrations above 1.5 $\mu\text{g/ml}$ inhibition often occurs. Without the presence of CaCl_2 or greater than minimal

Table 6. Effect of Divalent Cations on BPMV Infection of Protoplasts.

Experiment #	Treatment	%I/V
40	Virus alone	2
	Virus + calcium (no pre-incubation)	32
	Virus + calcium (pre-incubation)	39
51	Virus alone	18
	Virus + calcium (no pre-incubation)	14
	Virus + calcium (pre-incubation)	48
	Virus + magnesium (no pre-incubation)	14
	Virus + magnesium (pre-incubation)	32

Protoplasts were inoculated with 2.0 (Experiment #40) or 0.53 ug/ml BPMV (Experiment #51) amended where indicated with 0.5mM CaCl_2 or MgCl_2 . No other amendments were present. Salt pre-incubation with virus was for 15 minutes. The %I/V represents the average of two replicates per treatment.

buffer concentrations, such high levels of PLO can prove tremendously damaging to the protoplasts, especially at virus concentrations of 2 ug per ml and lower (Figure 11).

Experiments done to determine if pre-incubation of PLO with virus was necessary for maximal effect showed that, on the contrary, infection was enhanced if no pre-incubation was permitted. The PLO provided maximal stimulation when it and the virus were not mixed together until the very moment of inoculation (Table 7) although, unlike the buffer and divalent cation effects, PLO showed still greater than basal levels of stimulation even when added in the "wrong" order (in this case when pre-incubation was allowed). The presence of buffer seemed to have no effect on this phenomenon.

Since PLO is most efficient in its stimulatory effect when it was not pre-incubated with the virus, but rather was best when added only during the actual inoculation itself, it is unlikely that the mode of action for the polycation is to produce virus aggregates of BPMV as has been proposed by researchers for tobacco rattle virus (Mayo and Roberts, 1978). Nor is it likely that charge-balancing is the real mode of action such as PLO acting as a charge bridge for TMV (Takebe, 1978). Pre-incubation of PLO with BMV was seen to be slightly inhibitory by Okuno and Furasawa (1978) as well. They suggested that the PLO must be acting on the protoplast membrane primarily. It is interesting to note that BMV is one of those few viruses that, like BPMV, does not require PLO for successful infection. All of this points to the fact that PLO, as they suggest, has the possibility for at least two distinct modes of action, because pre-incubation was necessary for those viruses which require the polycation.

Figure 11. Effect of PLO concentration (with and without CaCl_2) on the percentage BPMV infection of protoplasts. Protoplasts were inoculated with 2.0 $\mu\text{g}/\text{ml}$ BPMV in the presence of several PLO concentrations with 0.5mM CaCl_2 (● ————— ●) and without the calcium amendment (○ ————— ○). No other amendments were included. Without CaCl_2 all protoplasts died at PLO concentrations of 1.5 $\mu\text{g}/\text{ml}$ and above (*). Each data point is the average of two replicates.

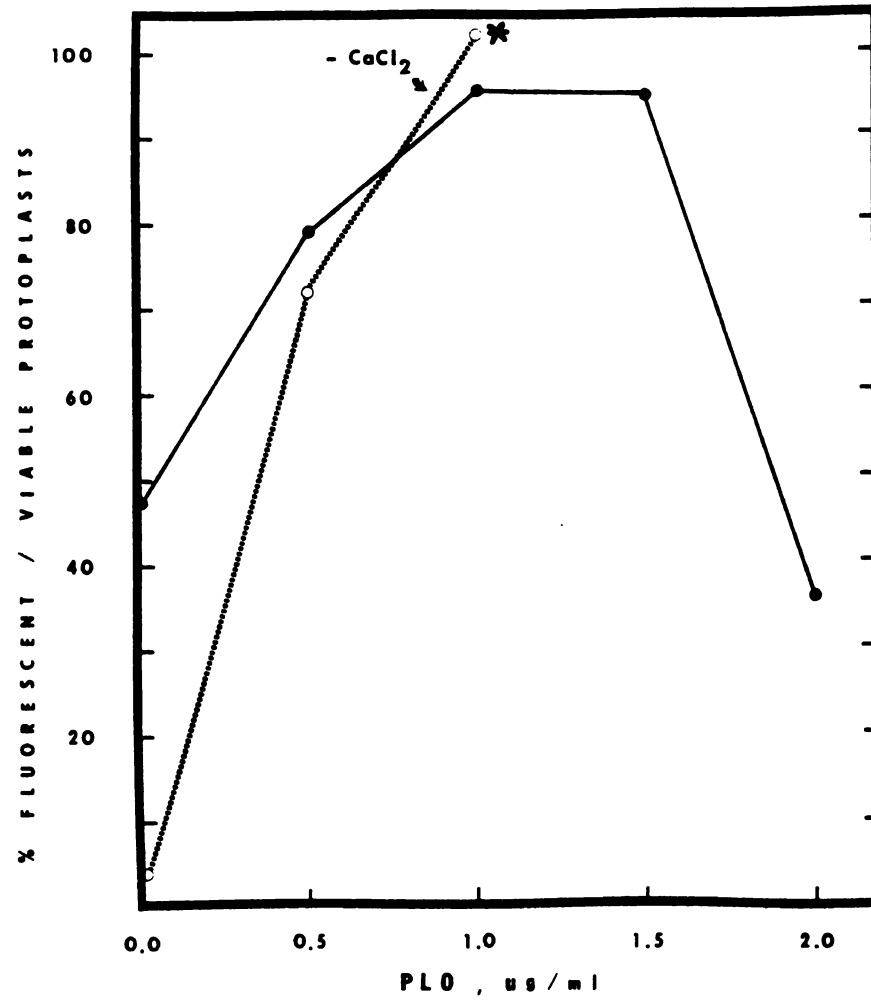


Table 7. Effect of Pre-incubation of PLO with Virus on Percentage BPMV Infection of Protoplasts.

<u>Experiment #</u>	<u>Treatment</u>	<u>%I/V</u>
40	Virus alone	2
	Virus + PLO (pre-incubation)	62
	Virus + PLO (no pre-incubation)	110
42	Virus + PLO (no pre-incubation)	92
	Virus + PLO (pre-incubation)	78
51	Virus alone	18
	Virus + PLO (pre-incubation)	79
	Virus + PLO (no pre-incubation)	83
41	Virus + buffer	35
	Virus + buffer + PLO (pre-incubation)	77
	Virus + buffer + PLO(no pre-incubation)	97

Protoplasts were inoculated with 2.0 (Experiments #40, 41 and 42) or 0.53 ug/ml BPMV (Experiment #51) and amended with 1.0 ug/ml PLO and/or 10mM K-phosphate buffer pH 5.6 as indicated. PLO pre-incubation with virus was 15 minutes. The %I/V represents the average of two replications per treatment.

At lower levels of virus concentration, PLO showed an apparently synergistic effect with CaCl_2 , increasing infection much more than the sum of these two compounds separately when used with K-phosphate buffer, pH 6.3. At higher virus concentrations a particular amount of PLO + CaCl_2 was no better than PLO alone (Figure 12).

PLO can be seen to raise the maximal level of infection as compared to the presence of calcium alone (increase in infection "Vmax" if we may borrow a term from enzymology). This shows quite a different phenomenon from that of the addition of CaCl_2 along with PLO where, instead of a change of "Vmax" we have a remarkable shift of the virus "Km" (virus concentration that gives half maximal infection) to the left. This would indicate a probable cooperative interaction between all of the various components since it is virus concentration dependent as well. It is tempting to speculate that, since, in this system CaCl_2 acts on the virus, PLO acts on the protoplast, and there is some sort of cooperative effect occurring with virus concentration; that there is some sort of virus-membrane complex involved with PLO and CaCl_2 as active, participatory priming agents.

Temperature Effects. Unlike CPMV under similar infection conditions (Jarvis, 1979), BPMV shows a relative temperature independence, infection being the same or somewhat better at lower rather than higher temperatures (Figure 13). It is interesting, however, that this situation reverses itself in the absence of inoculum amendments and shows infection proportional to temperature and a leveling off at higher temperatures. This sort of peculiar dependence of temperature upon inoculum amendments was also seen by Jarvis (though in reverse) for the sulfonic acid buffers and CPMV. In this case not only did these buffers eliminate the need for

Figure 12. Synergistic effects of PLO and CaCl_2 on percentage BPMV infection of protoplasts. Protoplasts were inoculated with various virus concentrations in the presence of 10mM K-phosphate buffer at pH 6.3 and the following amendmerits: 0.5mM CaCl_2 ($\circ$ — $\circ$); 1.5 ug/ml PLO ($\blacktriangle$ — $\blacktriangle$); 0.5mM CaCl_2 + 1.5 ug/ml PLO ($\bullet$ — $\bullet$). Each data point represents only one replicate.

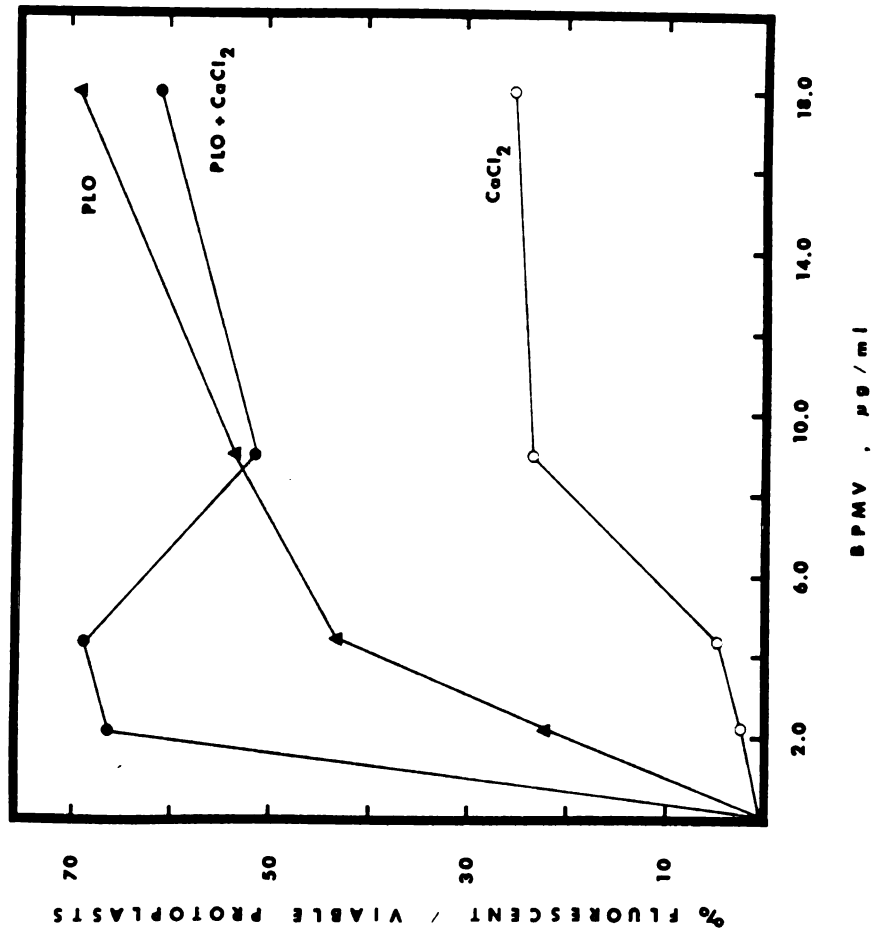
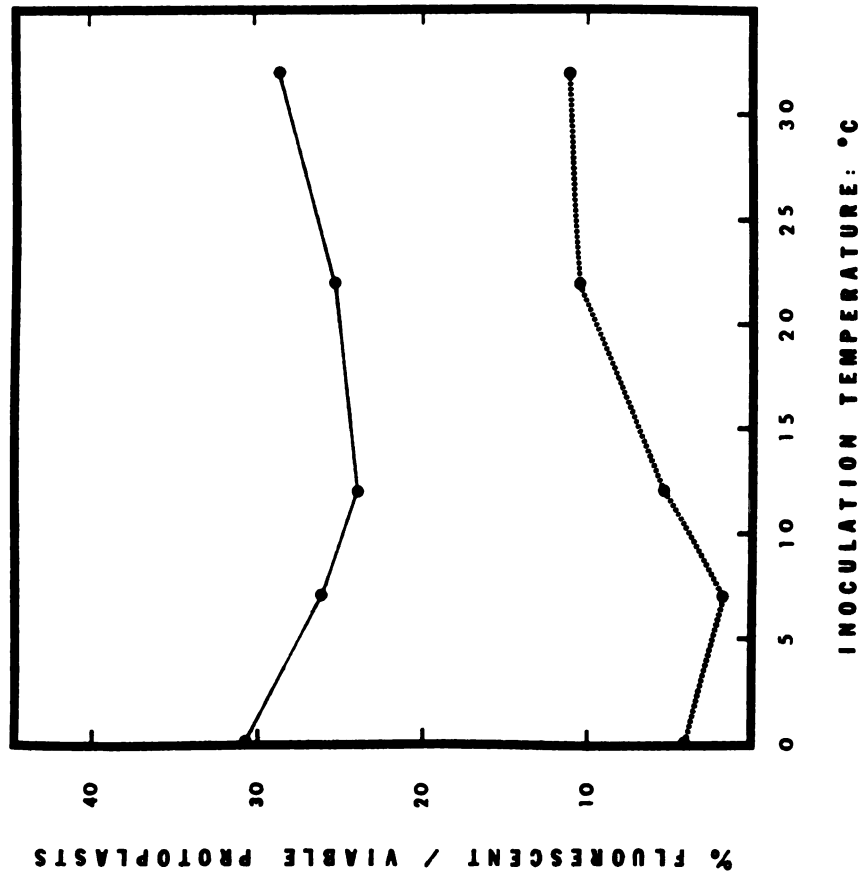


Figure 13. Effect of temperature on percentage BPMV infection of protoplasts. Protoplasts were inoculated with 2.25 ug/ml BPMV alone (●-----●); and in the presence of 10mM K-phosphate pH 6.3 + 1.5 ug/ml + 0.5mM CaCl_2 (●—————●) at a range of five different temperatures. Each data point for the virus alone curve represents one replicate; each data point for the amended virus curve represents the average of three.



PL0, they changed the temperature requirements of the system such that low temperatures were no longer inhibitory, but were in fact better than higher temperatures. Thus even temperature cannot be deemed as having a constant effect on the in vitro infection of plant viruses.

In all , the complexity of the results seen in this investigation of parameters of the BPMV/soybean protoplast system demonstrates that highly complicated interactions between all of the various inoculum components seem to be involved in the process of virus infection. In this light, certain summary statements can be made:

1.) All of the various functional components of the system (except temperature) involve charged moieties.

2.) The system (particularly the virus) can differentiate between calcium and magnesium ions, which would seem to imply some sort of biological, almost enzyme-like specificity rather than mere charge effects alone.

3.) The system can differentiate between various pHs.

4.) The system is relatively temperature insensitive when amendments are present, and temperature sensitive when they are not.

5.) The system can differentiate between high and low buffer concentrations and does this differently at different pHs.

6.) The system can achieve similar levels of infection through the manipulation of a number of various parameters.

7.) The system can be either inhibited or stimulated by the presence of the same effector when used under different conditions (CaCl_2 , PL0, K-phosphate, and even temperature).

8.) The system has very definite ordering requirements as demonstrated by the PL0, CaCl_2 , MgCl_2 and buffer pre-incubation experiments.

9.) In the presence of certain effectors the system can be saturated at levels of infection lower than those maximally possible, as is shown in Figure 12 for calcium in the presence of the "wrong" pH buffer.

10.) There are synergistic, cooperative effects possible between amendments, as seen in the case of PLO + CaCl_2 .

11.) Based on the use of Evans Blue testing for viability throughout these and other experiments, damage to the protoplast population (at least as determined by comparative viability at 48 h) showed no overall correlation to increased infection rates (data not shown).

12.) The soybean protoplast system as a whole can differentiate between BPMV and CPMV (two relatively closely related viruses in the same taxonomic group) on the basis of pH, temperature and PLO requirements.

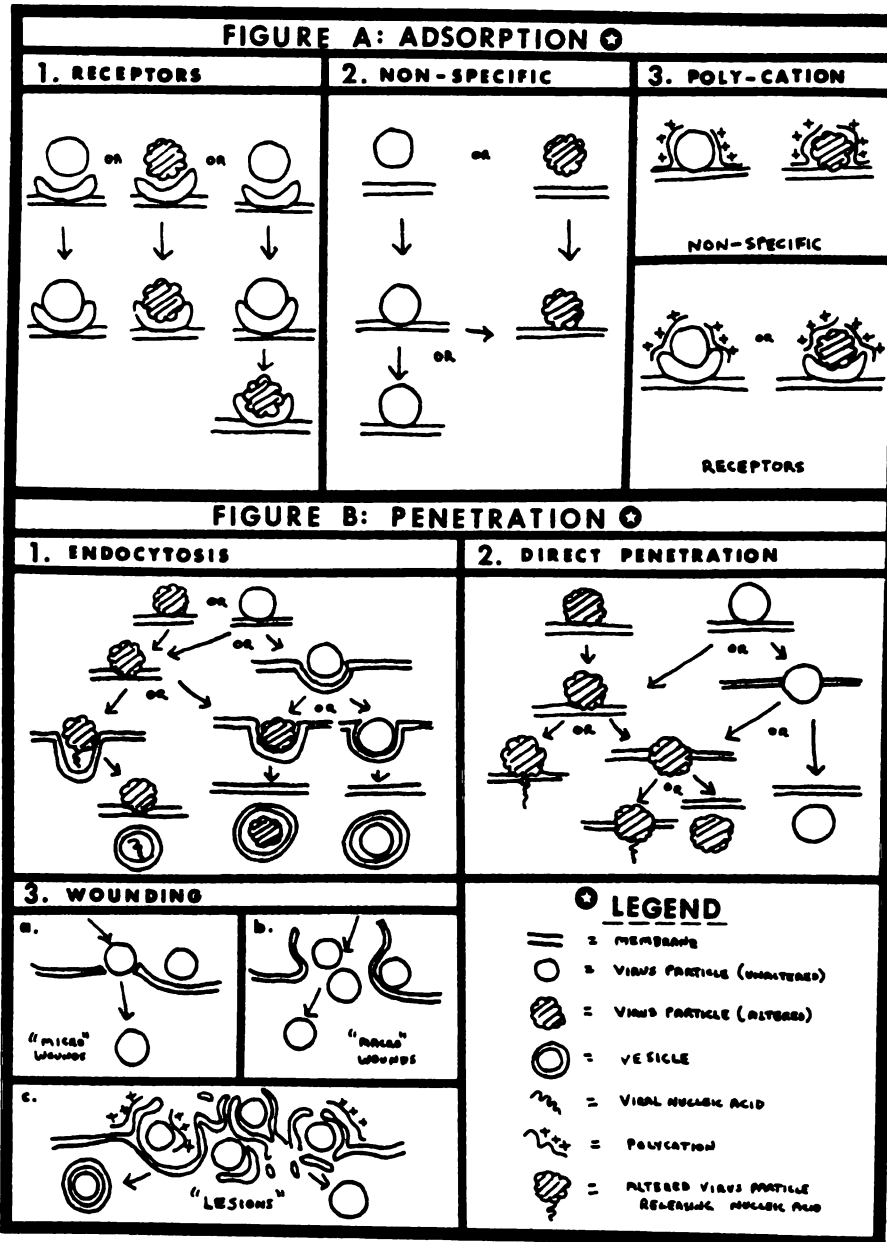
Many, if not most of these various types of phenomena have already been observed scattered throughout other virus/protoplast systems, with especially similar results having been seen by Okuno and Furusawa (1978) in the BMV/barley protoplast system. But these others have not been examined to the extent of demonstrating all of these complexities in one system, and none have reported such dramatic differences in infection requirements in the same protoplast system for such closely related viruses as BPMV and CPMV.

In order to discuss what might be happening, the current hypotheses for the mechanisms of adsorption and penetration of viruses into protoplasts must be examined (Figure 14a and 14b).

Which of these possibilities does the BPMV/soybean system (and other virus-protoplast systems) most seem to support?

Figure 14a. Illustration of the primary hypotheses for adsorption of an individual plant virus particle to a protoplast membrane. In each case the virus can either approach the membrane in its normal in vitro state, or one altered by the specific environment provided during inoculation.

Figure 14b. Illustration of the primary hypotheses for penetration of non-enveloped plant viruses into the host protoplast. In each case adsorption is assumed to have occurred and receptors, whether present or not, are not shown. Figure 14b (a) depicts the endocytotic theory of virus entry. Figure 14b (b) shows some of the variations possible in the direct penetration hypothesis. In Figure 14b (c) the wounding hypothesis is shown.



First and foremost, the action of buffer and the divalent cations upon the virus particles themselves seems to indicate that alteration in the state of the virus is necessary for highly efficient infection. Because calcium is better than magnesium at fulfilling this function, non-specific alteration of charge by positive ions is not likely, and this is borne out for the buffer as well, due to the narrowness of the activating pH. Such selectivity might indicate the occurrence of conformational changes in the virus structure.

The main question then becomes - what is the function of this sort of alteration for the virus infection process? One possibility is that the virus structure is stabilized, leading to protection of the nucleic acid. But this is unlikely for the following reasons:

- 1.) The virus particles are stable at the pH range in use (and over a much wider range as indicated by other researchers (Bancroft,1962) in terms of infectivity.

- 2.) The longevity in vitro in the presence of plant enzymes is several orders of magnitude greater than the 15 min period during which adsorption and/or penetration must occur (Semancik,1972). Therefore, no greater sensitivity to RNase degradation is indicated for BPMV virions as compared to other viruses used in protoplast systems.

- 3.) Successful virus purification and storage requires neither the addition of divalent cations nor the optimum infection pH to maintain virus infectivity for long periods of time (Semancik,1972; Bancroft, 1962).

If we tentatively dismiss virus stabilization as the probable site of action for the alteration's beneficial effects, we are left with three major possibilities. Namely, the alteration permits:

- 1.) Better attachment to the protoplast membrane,
- 2.) Better penetration through the membrane, or
- 3.) Ease of virus uncoating.

The last of these possibilities is unlikely because of the pre-incubation requirement of the buffer and the divalent cations. It appears that the virus must be primed immediately prior to its exposure to the protoplasts and that the 15 min exposure to the amendments during the inoculation has no real effect. This would imply that the amendment-induced stimulation is required immediately for infection to be initiated, otherwise it seems logical that the virus could pick up the required alterations while waiting to "go in" if attachment and penetration were independent of the amendments' effects on the virus. Also, it seems likely that divalent cations at least could be picked up without much trouble within the cell and that unless uncoating is part of the process of adsorption and penetration it is not the real site of the amendment induced stimulation seen.

If it were only a charge phenomenon involved, (ie. the more positive the virus particle, the greater the affinity to the negatively charged membrane) why is there a pH-indicated requirement for a deprotonization reaction, which would remove a "plus" charge from the virus? Also, why the preference for calcium over magnesium? Furthermore, why does PL0 act more efficiently if not pre-incubated with the virus? This is especially surprising if PL0, as suggested (Takebe, 1978) acts to make the virus charge more positive as one of its functions. All of these facts seem to indicate that simple charge effects alone are not the primary arbiters of infection.

Such complexity of interaction as seen in this and other protoplast

systems would seem to imply either a subtlety in or a multiplicity of phenomena effected. This would seem to cast doubts upon such relatively gross, non-biological phenomena as wounding as the sole or primary mechanism of infection.

The evidence seems to imply the specific interaction of a complex battery of charged components affecting both the virus and the protoplast in a manner which permits much subtlety and variation of effect over fairly narrow concentrations and pH ranges.

A mechanism that could most simulate such phenomena would be one involving a complex balance of virus and host cell plasma membrane interactions leading to competent binding (probably at specific virus receptive sites) followed by subsequent virus uptake by some form of endocytosis as suggested by various workers (Takebe et al., 1975; Okuno and Furusawa, 1978).

A schema for such an interaction using the BPMV-soybean system for a model can be envisioned. In this schema, unamended BPMV is capable of weakly and reversibly binding to the virus receptive site. The addition of buffer at appropriate pH and ion concentrations and the presence of divalent cations cause an alteration of virus charge and/or conformation which allows for tighter binding to the proposed receptive site. Penetration would be the result of endocytosis stimulated by a threshold concentration of bound virus; i.e. as postulated for a number of endocytotic systems (Stossel, 1977), a certain number of particles per unit area must be bound before uptake is initiated. At any particular virus concentration, weak, reversible binding would lead to less bound virus per unit area per time than would tight binding, at least until saturation was achieved. Anything that would alter the virus (buffer or

calcium?) to a state permitting tighter attachment would allow a lowering of the effective virus concentration and an increase in infection would be the result. If PLO, acting on the membrane, provides a generalized stimulation of endocytosis, as well as some sort of synergistic effect with the CaCl_2 -primed virus (Figure 12), the picture is complete in outline and the apparent increase in virus "Km" for the combination of amendments can be explained.

Such a theory of infection is consistent with the results obtained for BPMV in the soybean system and for BPMV as seen in the bean leaf protoplast system (Part II). The theory can easily be expanded to include other viruses, specifically those which require PLO.

In these cases, PLO would serve a dual function. It would provide for aggregation and charge-balancing of the more electronegative viruses (Takebe, 1978; Mayo and Roberts, 1978). Aggregation would help to mitigate the threshold effect (aggregated particles having been shown to be more effective in inducing endocytosis than non-aggregated particles (Stossel, 1977)). PLO could also help to maintain the tighter binding to negatively charged membrane sites. A generalized stimulation of endocytosis by PLO might also occur as suggested (Takebe, 1978).

The postulated existence of virus receptive sites does not imply that these are necessarily specific for the viruses involved or that these are the primary source of host range specificity. The concept of "receptors" simply implies that for a virus to penetrate biologically into a cell, it must attach to a non-random, endocytosis-triggering site. Successful attachment would depend upon proper virus and receptor coordination in terms of charge and conformation. A detailed discussion of this type of phenomenon in animal virus systems has been presented

(Lonberg-Holm and Philipson, 1974). The concept of receptive sites as seen in animal virus systems could easily account for the kind and complexity of phenomena observed in the soybean and other protoplast systems.

Obviously there is no concrete proof for the existence of such receptor sites as yet, though indications that some such system is involved seems strong. It is therefore necessary to examine the primary alternative hypothesis: that of the relatively non-biological process - "wounding" of the membrane, as being the source of virus entry. Does this theory have equal or greater weight of evidence behind it than the receptor-mediated endocytosis theory described above? Can it account for the data?

The wounding hypothesis is the result, primarily, of the following observations:

1.) One must wound plants during mechanical inoculation to get infection.

2.) PL0-induced damage seems to correlate in a number of virus/protoplast systems with increased infection.

3.) PL0 causes damage to protoplasts, including complex "lesions" visible under the electron microscope.

4.) Repeated agitation (via centrifugation - pelleting and resuspension) increases virus infection.

5.) The evidence for endocytosis in plants is not at all as well developed as in animal systems.

6.) Cold temperature inoculation does not substantially interfere with virus infection (the implication being that endocytosis, an energy-requiring process could therefore not be occurring).

It seems difficult to reconcile the idea of damaged membranes as being the port of virus entry with the complexity of the infection process as has been seen in the soybean and other protoplast systems. The number of parameters involved and their effects (changing for every virus in every system) argues, perhaps, for a more biological, less simplified mechanism.

It is important to examine, therefore, each of the above points used to develop the wounding hypothesis, to determine their strengths and weaknesses and whether other interpretations of the observations are plausible. Taking the points listed in order then:

1.) The fact that the plant cuticle and cell wall must be ruptured during mechanical inoculation is well documented. The idea that this wounding involves the membrane itself is a view with no such research support. Researchers such as Matthews and others (Matthews, 1970) have made no suggestion of this being probable or necessary from their observations.

2.) PLO causes damage to protoplasts under certain conditions: This is not inevitable. Other researchers have shown that PLO stabilizes nucleic acids and allows their uptake by plant protoplasts without causing membrane damage, at least as assayed by the ability of the protoplasts to regenerate cell walls and undergo subsequent division to the same percentage as those untreated with PLO (Hughes et al, 1979). In the soybean/BPMV system, increasing PLO concentrations in the absence of CaCl_2 can cause extensive damage. Adding CaCl_2 increases viability (less damage) greatly, without decreasing infection in the slightest. In fact, adding more PLO, which should in theory cause even more damage, decreased infection without any dramatic change in viability.

In other experiments there was no evidence that increasing membrane damage, (as assessed by lowered viability) was accompanied by a concomitant increase in infection. In many cases, the presence of added buffer was seen to increase both viability and infection greatly. Thus the correlation between membrane damage and infection does not hold for PL0 or the other amendments in this system as a universal phenomenon.

3.) PL0-induced "lesions" have been one of the strongest pieces of evidence used by the wounding hypothesis supporters. But even in their key paper (Burgess et al. 1973) it was stated that "in particular it is impossible to judge whether all the membrane systems comprising the lesion derive from the protoplast to which it is attached. Conceivably at least some of the closed membraneous units could derive from a population of protoplasts which became extensively fragmented during treatment." This statement weakens greatly the entire argument - that these lesions are actually the evidence of wounds. In the soybean system PL0 induces protoplast clumping under a large number of conditions and often clumping and sticking of debris to the protoplasts was evident even at a light microscope level. It seems highly probable that such sticking of membrane fragments would be seen under electron microscopy after PL0 treatment and that this could easily be the origin of the "lesions" observed as suggested. In any event, in such cases as has been discussed, EM data is suspect in that it can be used to support both sides of the argument as when endocytosis was claimed to have been seen by several researchers (Hibi and Yora, 1972; Otsuki et al, 1972).

4.) At first glance the case for wounding is much strengthened by the fact that repeated pelleting and resuspension increase infection of protoplasts. This has been ascribed to "added wounding," (Motoyoshi et al,

1974). However, in biochemical and biophysical cell studies, such treatments have been shown to have numerous other effects which could be equally, if not more, suspect as having an important role in virus infection. For example:

a.) forcing cells into close proximity with one another and then rapid separation leads to the production of dramatic changes in the membrane electropotential (Oshima, 1977).

b.) agitation of cultured plant cells leads to a period of "shock" which greatly alters membrane transport functions (Thoiron et al, 1974). With such demonstrated alternative effects of added agitation such as would occur during centrifugation, the claim that increased damage is the real effect is somewhat weakened.

5.) The evidence as to whether endocytosis really does or does not occur in plants is indeed not perfect. At present it is almost a matter of belief as to whether what a number of researchers have detected is real or artifact. To this researcher, however, the evidence is at least as strong as that presented for wounding, if not stronger. However, more stringent research, not so dependent on EM is definitely needed.

6.) The idea that, because low temperature inoculation can lead to infection, an energy requiring process is not involved, need not be the case. Attachment may be the non-energy requiring process which would have to occur before endocytosis is likely to occur; attachment having been seen to occur at ice-bath temperatures in endocytotic conditions (Stossel, 1977). The temperature independence of the phenomenon during inoculation is not an important argument because the protoplasts are subsequently raised to incubation temperatures and it is at that point that endocytosis can be suggested to occur.

In conclusion, the entire infection process in protoplasts remains something of a mystery. The evidence presented in this study hints at certain types of mechanisms - those involving some sort of biological specificity. The possibility of receptors seems to be an extremely important one to investigate, especially since the probability of "wounding" being a viable alternative seems to diminish under close consideration. However, in neither case can what is essentially only circumstantial evidence be enough. In depth biochemical studies and the search for receptors is the only logical next step with any hope of answering the multitudinous questions involved.

It is in this light that BPMV in the soybean protoplast system seems uniquely useful for studying the infection process due to its quite minimal requirements for successful infection and due to its sensitivity to the addition of inoculum amendments in various concentrations and combinations. It is particularly valuable in that it provides the soybean protoplast system with a virus with such different infection requirements, but one that is fairly closely related to the previously introduced CPMV.

The following section will detail some of the parameters of both BPMV and CPMV in another protoplast system. It will be seen that many of the above observations, where investigated, hold equally well for beans as well as soybeans. As will be discussed, this may indicate that the postulated infection mechanisms may be capable of explaining observations in yet another study system.

PART II
INFECTION OF BEAN LEAF PROTOPLASTS
WITH BEAN POD MOTTLE AND
COWPEA MOSAIC VIRUSES

INTRODUCTION

In order to determine the universality of any proposed mechanism of virus infection of protoplasts, it is necessary to examine the infection behavior of the same viruses in different protoplast systems.

BPMV and CPMV have been introduced into the soybean suspension culture-derived protoplast system. Initially it was thought ideal to try and develop a soybean leaf mesophyll protoplast system to directly compare the suspension culture and leaf derived protoplasts as to their infection requirements. This was to enable examination to determine if there were any peculiarities due solely to the use of protoplasts from culture as opposed to leaves. Such an approach did not prove feasible, however, because soybean leaf protoplasts could not be obtained using commercial enzyme preparations. Therefore, in compromise, a bean leaf protoplast system was developed in which the two viruses could be examined in the same leaf protoplast system. (The only other legume protoplast system available - that of cowpea - was not a good option for this kind of comparison because BPMV does not infect cowpea as a host).

MATERIALS AND METHODS

Plant Source Material. Phaseolus vulgaris L. cv Pencil Pod Wax were planted and grown under fluorescent lights under greenhouse conditions. Protoplasts could be isolated from both unifoliate and trifoliate leaves, although trifoliate leaves gave more reproducible results in isolation. The key age requirement was that the leaves had become just fully expanded. Younger leaves were too easily damaged; older leaves did not digest with as great efficiency and many single cells remained. As with all leaf protoplast systems, occasional failures at isolation occurred, but these were usually due to using plants that had become too old, or had become too spindly due to lack of light. After 3-4 weeks, even the newest trifoliate leaves ceased to yield good protoplasts.

Protoplast Isolation. Abaxial surfaces of unifoliate or trifoliate leaves were sprayed with carborundum and then rubbed with a small piece of carborundum dusted sponge until a watersoaked appearance of the leaves was observed. Cut leaf pieces (2-4cm sections) were floated, rubbed surface down, on an enzyme solution containing 0.5M mannitol, 2% Cellulysin and 0.2% Macerase (both from Cal-Biochem, Co). The leaf pieces were incubated with gentle shaking at 30 C for 3-5 h.

Protoplasts were harvested by filtration through Miracloth, and washed from enzyme solution 3 times with 0.5M mannitol using centrifugation for 4-6 min at 100g prior to inoculation.

Virus Purification. BPMV was purified as described in Part I. Cowpea mosaic virus was purified as described by Jarvis, (1979).

Protoplast Inoculation. Techniques for protoplast inoculation were virtually identical to those listed for the soybean suspension culture protoplasts, except for the use of mannitol in place of sorbitol.

Infection Assay, Viability Determination and Photography. Infection assay using fluorescent antiserum was identical for BPMV as reported in Part I. Similar techniques, but using fluorescent antiserum prepared to CPMV were used for that virus as described by Jarvis,(1978). Viability determination and photography were as described in Part I.

Protoplast Incubation. After inoculation, protoplasts were incubated for 48 h in 0.5M mannitol containing 10mM CaCl_2 and antibiotics (0.3mg/ml Pyopen (Beecham Laboratories, Tenn.) and Nystatin at 0.03mg/ml).

RESULTS

Protoplast Isolation. Good protoplasts were isolated using the techniques described (Figure 15). There was generally very little problem with debris.

Infection Assay and Viability Determination. Using fluorescent antibody assay, zero-time slides and non-inoculated controls showed only weak background fluorescence for either virus. At 48 h infection was clearly discernable by the development of the bright apple-green fluorescence as described in Part I. Over 80% viability as determined by Evans Blue dye exclusion was routinely obtained after the 48 h incubation period.

Poly-l-Ornithine Effects. As in the soybean system, BPMV does not require PLO for infection in the bean protoplast system, although it is highly stimulatory, increasing its effects with increasing concentration up to 0.75 ug/ml after which a decrease is seen, similar to that apparent in the soybean system (Figure 16).

CPMV, on the other hand, does require PLO for infection as in the soybean system. It reaches a leveling off at 0.75 ug/ml of PLO as seen for BPMV. Infection levels were lower however than for BPMV, perhaps due to a lower specific infectivity for this CPMV preparation (Figure 16).

Calcium Effects. Both BPMV (Figure 17) and CPMV (Figure 18) showed the same synergistic reaction of PLO with the presence of CaCl_2 , identical to that seen in the soybean system in this study for BPMV, thereby demonstrating that the behavior of these amendments on the viruses is nearly

Figure 15. Bean leaf protoplasts after harvesting from enzymes and washing in 0.5M mannitol.

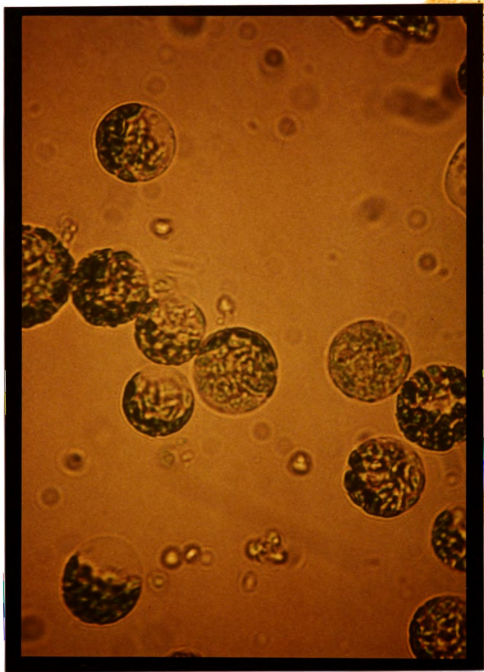


Figure 16. Effect of PLO concentration on BPMV and CPMV infection of bean protoplasts. Protoplasts were inoculated either with 2.6 ug/ml BPMV (● ——— ●) or 2.1 ug/ml CPMV (▲ ——— ▲) with 0.25mM CaCl_2 in the presence of various concentrations of PLO. No buffer was added. Each data point represents only one replicate.

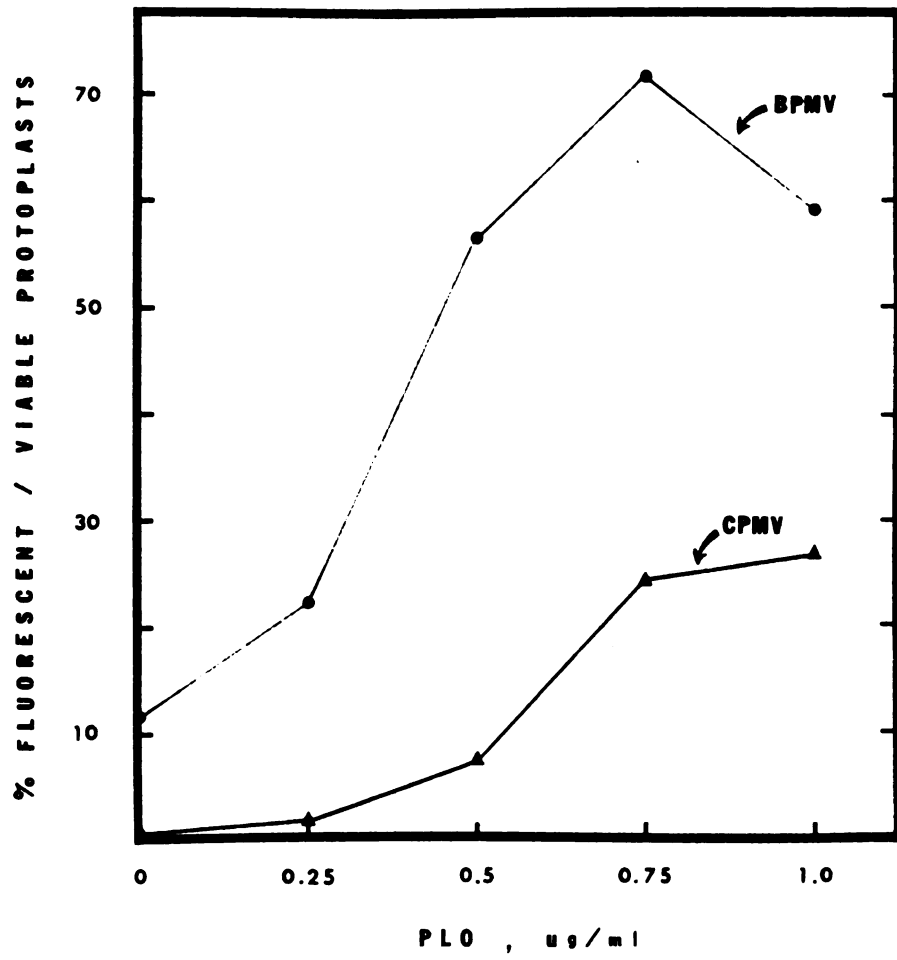


Figure 17. Effect of PLO and CaCl_2 on infection of bean protoplasts with BPMV. Protoplasts were inoculated with BPMV at various concentrations in the presence of less than 0.05mM buffer with the following amendments: 0.5mM CaCl_2 (▲ ————— ▲); 1.0 ug/ml PLO (▼ ————— ▼); and 0.5mM CaCl_2 + 1.0 ug/ml PLO (● ————— ●). Each data point represents only one replicate.

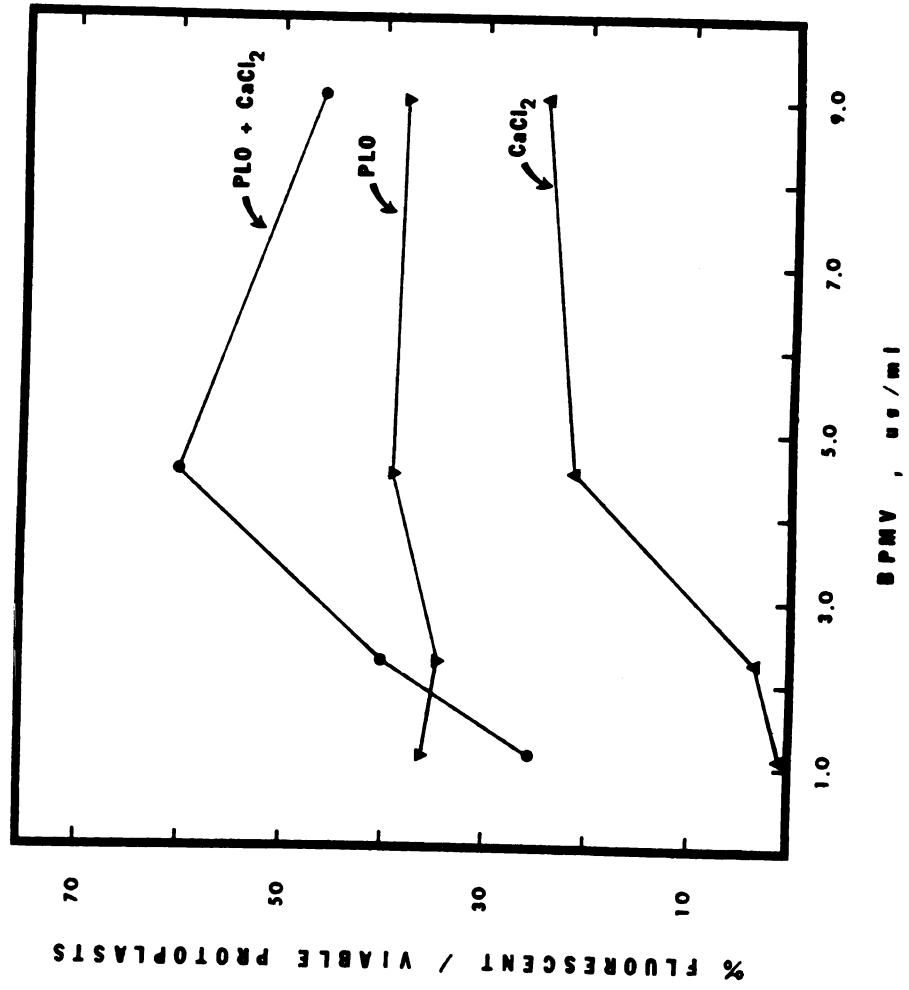
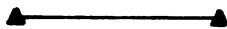
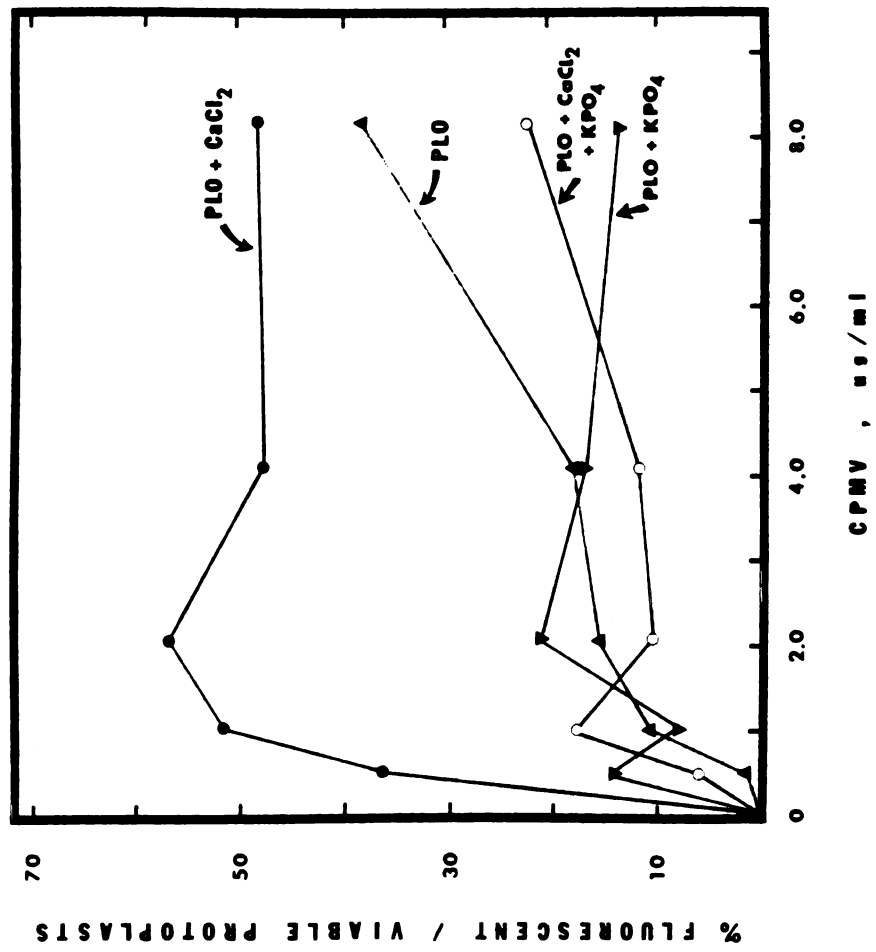


Figure 18. Effect of PLO, CaCl_2 and buffer presence on infection of bean protoplasts with CPMV. Protoplasts were inoculated with CPMV at various concentrations with the following amendments: 1.0 ug/ml PLO (); 1.0 ug/ml PLO + 0.5mM CaCl_2 (); and 1.0 ug/ml PLO + 0.5mM CaCl_2 + 10mM K-phosphate, pH 6.0 (); and 1.0 ug/ml PLO + 10mM K-phosphate, pH 6.0 ().



the same both in the legume system from mesophyll and in that from suspension culture protoplasts.

Buffer Effects. Limited pH studies were done for the effect of 10mM K-phosphate buffer on both BPMV and CPMV in the bean protoplast system. BPMV infectivity showed a strong peak at pH 5.6 (although lower pHs were not tested) dropping strongly by pH 6.0. Cowpea mosaic virus, however, did not show any great pH stimulation at all and gave fairly low infection at all pHs tested, although these did drop off to nearly zero on either side of a very weak "plateau" (Figure 19).

An interesting effect of the presence of 10mM K-phosphate buffer was seen in the case of CPMV (Figure 19). Infection was significantly lowered by the presence of buffer in inoculum amended with both PLO and CaCl_2 . This was somewhat different from the effect of buffer on the PLO alone amended inoculum where a decrease in infection occurred only at the higher virus concentrations. As can be seen, the presence of the buffer generally inhibited the effectiveness of the other amendments, especially the calcium-PLO synergy.

The presence of pH 6.0 buffer had similar effects on BPMV infection. In separate experiments comparing the effect of buffer presence it was seen that neither calcium alone, PLO alone, nor calcium + PLO had any differing stimulatory effects on infection in the presence of buffer (Figure 20). In the absence of the buffer, however, strong differences between the three sets of amendments were seen (Figure 17).

Figure 19. Effect of pH of K-phosphate buffer on BPMV and CPMV infection of bean protoplasts. Protoplasts were inoculated with 4.2 ug/ml BPMV (● ——— ●) or 4.1 ug/ml CPMV (▲ ——— ▲) with 0.5 ug/ml PLO + 0.5mM CaCl_2 and 5mM K-phosphate buffer at the pHs indicated. Each data point represents only one replicate.

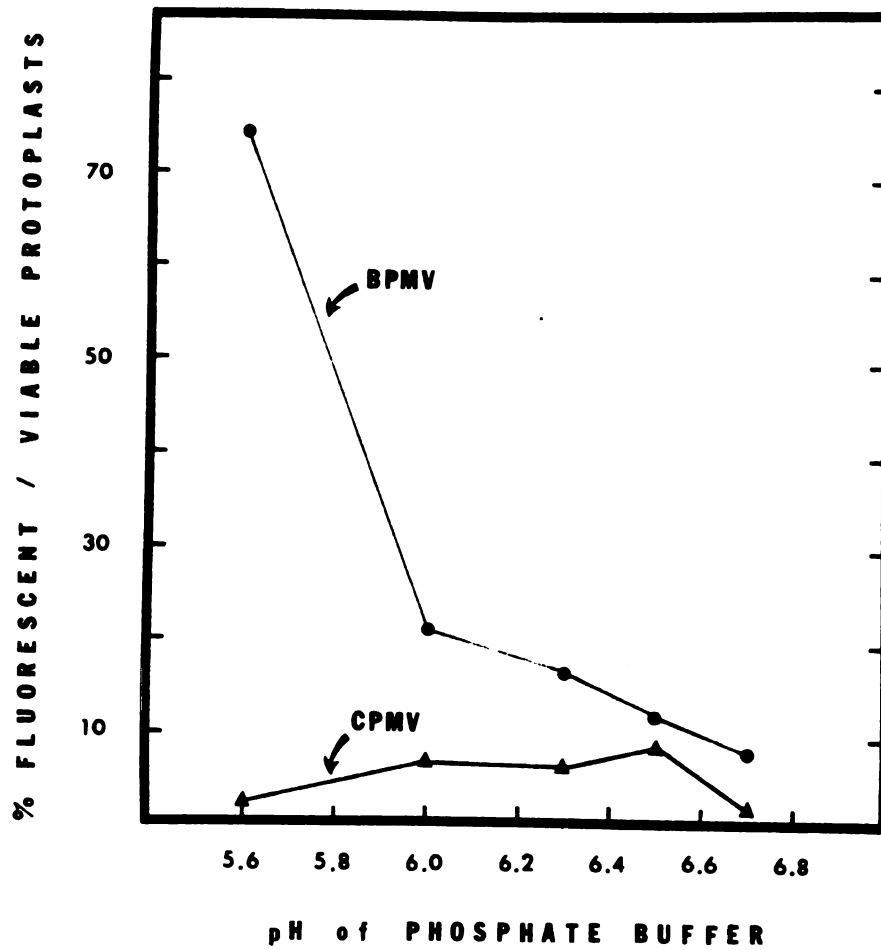
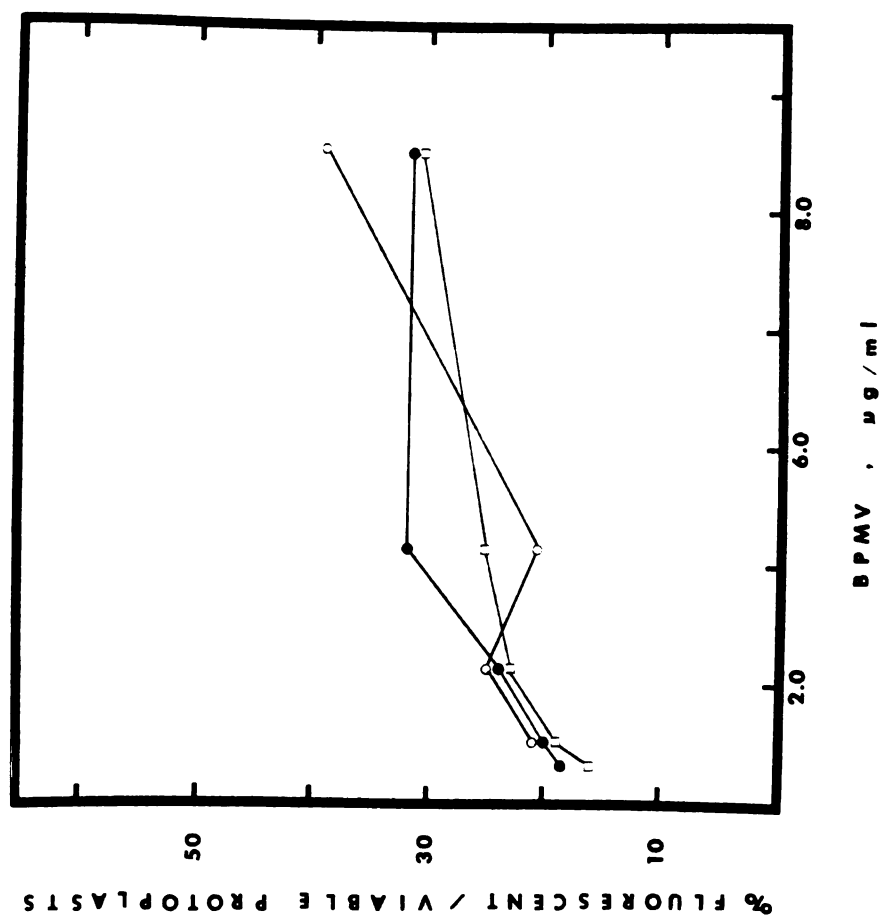


Figure 20. Effect of the presence of pH 6.0 K-phosphate buffer on PLO and CaCl_2 effects on BPMV infection of bean protoplasts. Protoplasts were inoculated with BPMV at various concentrations in the presence of 10mM K-phosphate buffer, pH 6.0 with the following amendments: 0.5mM CaCl_2 ( — ); 1.0 ug/ml PLO ( — ); and 0.5mM CaCl_2 + 1.0 ug/ml PLO ( — ). Each data point represents only one replicate.



DISCUSSION

What do these preliminary results in the bean protoplast system tell us firstly about the similarities for the two viruses in this as compared to the soybean system? Secondly, what do these results indicate as to in vitro infection mechanisms of plant viruses?

Comparison of the Bean and Soybean Systems. It has been shown that the PL0 requirements for both viruses remain constant for each of the two viruses for both systems. The PL0/calcium synergy is shown to exist in both systems and there is limited indication that the pH preferences of BPMV hold true in both protoplast systems. Similar too in both systems is the fact of a "wrong" pH inhibition of the effect of other amendments.

However, there are noticeable differences between the two systems, even in this limited comparison. CPMV in the bean system is not as dramatically effected by pH as demonstrated by Jarvis (1978) in the soybean system. More significantly, perhaps, the calcium-PL0 synergy for both viruses can be inhibited by the presence of buffer in the bean system, whereas this does not seem to be the case in the soybean system.

Mechanisms of Virus Infection. The comparison provides and interesting quandry. The same two viruses, the same set of parameters, but in different protoplast systems behave in some cases similarly and in others differently. If infection were a simple process involving only charge balancing and membrane wounding, it would be expected that the parameters maintain some sort of consistency between the two protoplast

systems.

In the soybean system the effect of buffer was seen to be initially on the virus (ie. pre-incubation was required). No interference with PLO + calcium synergy was ever observed. If buffer was simply balancing charge on the virus, or enhancing its stability against enzyme attack, there should, it would seem likely, be no differences here between the two systems. Since this is not the case, we are left again with an apparently inexplicable complexity - inexplicable if we look for crude infection mechanisms to be involved.

The theory of virus receptive sites could be invoked to, if not explain, at least allow for some of these peculiarities. If receptive sites differed subtly in the bean protoplast system from the soybean protoplast system, then it might be possible that the presence of buffer could interfere with calcium/PLO synergy in the one system (bean), but not in the other because the membrane-virus-amendments complex would be slightly different in each.

In any event, something fairly complex must be invoked to explain the fact that all of the system components influence each other and do so differently, though not consistently so, in the two different systems. Whatever infection mechanism(s) is finally seen to be involved in the protoplast system (and hopefully by extension in whole plant infection) it will have to explain many, many such complexities as presented for these two legume systems and for the many other protoplast systems currently under study. Proof does not exist for any of the prominent theories advanced, and until such is obtained, ideas on mechanisms must remain merest speculation. Of the current theories available, however, it would seem that the weight of circumstantial evidence must rest on

the side of the more biological theory of receptor-mediated endocytosis as compared to the more simplified "physical" theory of membrane wounding.

Validation of the Soybean Protoplast System for Infection Studies.

One last, and fairly important point must be discussed. Whatever the infection mechanism involved, it would seem that none of the observations made on such things as the divalent cation stimulation and the PL0/calcium synergy are unique to protoplasts derived from liquid suspension culture, having been seen in a leaf mesophyll system for the same two viruses as well. Thus any doubts as to the validity of using suspension culture derived protoplasts as compared to leaf protoplast systems for studying virus infection can be reasonably discounted. One system is apparently no more "unnatural" than the other. This assurance, coupled with the numerous benefits involved in using suspension culture derived protoplasts as opposed to those from leaves (discussed in some depth by Jarvis,(1978)) should make development of such systems the method of choice for virus/protoplast interaction studies in the future.

Recommendations for Early Interactions Studies in Protoplast Systems.

In the light of all that has been seen in this study on infection parameters it would seem necessary to raise a strong cautionary note upon interpreting the protoplast literature already extant on this subject and a word of warning to those who would proceed further in this field.

There are relatively few hard and fast rules in this system. Changing one parameter can be seen to change the effects of some or all of the other parameters in the system. To make blanket statements

about a buffer or pH being optimum, or a particular concentration of this or that amendment being best is to risk confusion and contradiction unless all other parameters are taken into account. Until an inclusive theory about the systems is worked out for protoplast infection mechanisms in general, all observations must be looked on as isolated data bits and not used to make sweeping generalizations.

Very detailed studies must be carried out before systems can be properly understood, much less compared to one another. No such study yet has come even close to being sufficiently in depth.

If a crude analogy might be raised, it is as if protoplast infection studies were now at that state of the art that enzymologists were many decades ago - where what was at first seen as simple catalysis had suddenly become a system replete with inhibitors, activators, substrate competition, pH effects and allosteric controls. Complex studies, mathematically precise studies of the biochemistry involved were the only hope then, and would seem to be the only hope now for understanding systems that have become too complex for mere qualitative analysis to make sense of them.

GENERAL SUMMARY

Infection parameters have been described for BPMV in protoplasts derived from soybean liquid suspension culture.

The virus does not require PLO but is stimulated by it under most conditions. A synergistic effect is seen between PLO and calcium in improving infection at low virus concentrations. The pH requirements of the virus show a narrow peak at pH 5.6 for K-phosphate buffer. Pre-incubation studies have shown that the primary effect of divalent cations and the buffer are on the virus ant first, and that the primary effect of PLO in this system is probably on the membrane. An extreme complexity among the relationships of the various amendments to one another in the system has been demonstrated. Infection at low temperatures was equal to or better than that at higher temperatures in the presence of full amendments.

The theory of receptor-mediated endocytosis has been invoked to account for the possibility of, if not to explain, the presence of such complex interdependencies in a somewhat more convincing manner, it is hoped, than is possible with the alternative hypothesis - that of membrane wounding.

A procedure for the easy isolation and infection of bean leaf protoplasts with two plant viruses has been described and preliminary results reported on comparing this system to the soybean system.

Similar results were obtained, except for noticeable differences that the presence of buffer can have on the elimination of the PLO/calcium

synergy in the bean, but not in the soybean system.

The similarities between the bean leaf and soybean systems in most other respects was shown to validate the use of suspension culture derived protoplasts in that the same two viruses behave similarly on most counts in both systems. This would seem to indicate that there should be no reservations to studying infection processes in laboratory grown suspension culture derived protoplasts in that they behave just as naturally (or unnaturally) as do those protoplasts directly isolated from whole plants grown in the greenhouse.

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