INFECTION OF PROTOPLASTS DERIVED FROM LIQUID SUSPENSION CULTURE OF SOYBEAN WITH COWPEA MOSAIC AND SOUTHERN BEAN MOSAIC VIRUSES: A NEW SYSTEM FOR STUDYING VIRUS/HOST INTERACTIONS

By

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ABSTRACT

INFECTION OF PROTOPLASTS DERIVED FROM LIQUID SUSPENSION CULTURE OF SOYBEAN WITH COWPEA MOSAIC AND SOUTHERN BEAN MOSAIC VIRUSES: A NEW SYSTEM FOR STUDYING VIRUS/HOST INTERACTIONS

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A system has been developed for studying synchronous plant virus infection and replication that allows for axenic conditions to be maintained and yields a high degree of reproducibility between experiments. This was achieved by isolating and inoculating protoplasts from liquid suspension culture. Soybean cultures were initiated on agar and maintained in liquid medium. Protoplasts were enzymatically prepared from the culture tissue, washed in sorbitol and resuspended in a virus inoculation medium. After 20 minutes, protoplasts were washed with sorbitol plus 10 mM $CaCl_2$ and resuspended in culture medium.

Local lesion assays on Pinto bean leaves and fluorescent antibody assays showed infection of the inoculated soybean protoplasts by cowpea mosaic virus (CPMV) and southern bean mosaic virus (SBMV).

The contents of the inoculation medium were critical to obtaining a high percent of infected protoplasts. With CPMV, 70-80% of the protoplasts were infected with 0.5 µg CPMV/ml, 1.5 µg poly-L-ornithine/ml, 10 mM K phosphate buffer, pH 6.3, and 0.5 mM $CaCl_2$ at 23 C. Poly-L-ornithine was essential for infection. Phosphate buffer stimulated infection only in the range of pH 5.8 to 7.0, with infection peaking sharply at pH 6.3.

The stimulatory effect of calcium on protoplast infection was dramatic. Only 10-12% infection occurred when the inoculation medium was not amended with calcium, while 70-80% infection was obtained when 0.5 mM $CaCl_2$ was added to the inoculum. This seven-fold stimulation by Ca^{++} was considerably decreased by washing the protoplasts in sorbitol plus 10 mM $CaCl_2$ prior to inoculation.

Very little protoplast infection was obtained at an inoculation temperature of 0 C, but nearly maximal infection could be obtained at 12-15 C. After this, increasing the temperature of inoculation increased the percent of infection only slightly.

Infection of soybean protoplasts by southern bean mosaic virus was greatest at 2-2.5 µg SBMV/ml, 2 µg poly-L-ornithine/ml, 10 mM Tris-HCl buffer, pH 8.0, and 1 mM $MgSO_4$ plus 0.5 mM $CaCl_2$. A maximum of only 30-35% of the protoplasts were infected under these conditions. The infection was dependent on poly-L-ornithine. Infection in the presence of Tris-HCl buffer was nearly pH-independent over the range of pH 7.2 to 8.6. Several other relatively high pK buffers were also suitable for infection studies. The presence of a combination of calcium and magnesium salts increased the percent of infection 10-fold.

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Soybean tissue culture can provide a reproducible, homogeneous source for isolation of viable protoplasts. These protoplasts can be infected with outstanding reproducibility. This system, the first of its kind to be described, is suitable for use in plant virus/host interaction studies, especially those where synchrony of infection, axenic culture, defined growth conditions and reproducibility are of utmost importance.

This thesis is dedicated to my mother and father whose continuing love, support and enthusiasm have given me the confidence and courage to pursue my goals.

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LIST OF ABBREVIATIONS

BES	N,N-bis(2-Hydroxyethyl)-2-aminoethanesulfonic acid
bistrispropane	bis(2-Hydroxyethyl)imino-tris(hydroxymethyl)propane
CMV	cucumber mosaic virus
CPMV	cowpea mosaic virus
2'4'D	(2'4'-dichlorophenoxy)acetic acid
DIPSO	3- N-(bis-Hydroxyethyl) -amino -2-hydroxypropane-sulfonic acid
EDTA	ethylenediamine tetraacetic acid
FITC	fluorescein isothiocyanate
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HEPPS	N-Hydroxyethylpiperazine-N'-propanesulfonic acid
HEPPSO	N-Hydroxyethylpiperazine-N'-2-hydroxypropanesul-fonic acid
2ip	6(-Dimethylallylamino)-purine
LS salts	Linsmaier and Skoog salts
MES	2-(N-morpholino)ethanesulfonic acid
MOPS	3-(N-morpholino)propanesulfonic acid
MOPSO	3-(N-morpholino)-2-hydroxypropanesulfonic acid
PLO	poly-L-ornithine
SBMV	southern bean mosaic virus
TAPS	3- N-(tris-Hydroxymethyl)-methylamino -propane-sulfonic acid
TAPSO	3- N-(tris-Hydroxymethyl)-methylamino -2-hydroxy-propanesulfonic acid
TMV	tobacco mosaic virus

INTRODUCTION AND LITERATURE REVIEW

Basic mechanisms of virus infection and replication processes have been the focus for many molecular and microbiologists over the past 20 years. The literature on animal and bacterial viruses is voluminous - knowledge of replication processes is highly refined and virus-host interactions have been well characterized in many systems.

Plant virus studies, however, have lagged dismally far behind. Much of this has undoubtably been due to the lack of a suitable experimental system for the study of plant virus replication. Intact plants, though appropriate for host range and transmission studies, were found to be unsuitable for replication studies for three major reasons. First, the initial inoculation of whole plants results in the infection of an extremely small percentage of cells. This means that the first synchronously produced intermediates and products of replication are diluted greatly by the presence of healthy cells. Secondly, the subsequent spread of virus results in asynchronous virus replication, making it impossible to study sequential events in replication. Thirdly, uptake studies are extremely difficult and inefficient in whole plants.

Separated cells, enzymatically digested from infected leaves were an improvement in that they were able to take up labeled precursors, and sustained virus synthesis was successfully studied

(Jackson, et al. 1972). However, as in intact plants, replication was asynchronous and a relatively small population of cells was infected. Also cells degenerated rapidly.

The development of a system for cold-temperature synchronized replication in leaves (Dawson and Schlegel, 1973) and in tissue culture (White et al. 1977) improved percent of cells infected somewhat and synchronized the final stages in virus synthesis, but early infection of cells was not actually synchronized.

Therefore, it was a major breakthrough when techniques were developed by Cocking (1966) for the isolation and infection of protoplasts from leaf mesophyll tissue, and were subsequently refined by Otsuki and Takebe (1969), allowing an efficient and synchronous infection of a high precentage of cells.

Since these pioneering studies, over 25 protoplast-virus systems have been introduced and used successfully for studying virus replication as well as various other aspects of plant-virus interactions. These are well discussed in several recent review articles (Takebe, 1975; Takebe, 1978; Zaitlin and Beachy, 1974a; Zaitlin and Beachy, 1974b).

Protoplasts for such studies have been isolated almost exclusively from mesophyll cells, and although often over 90% of the cells can be synchronously infected (Takebe, 1978), the system has disadvantages. One problem has been microbial contamination. Although leaf sections can be surface sterilized and media autoclaved, antibiotics must still be added to mesophyll protoplast culture medium to avoid bacterial and fungal growth during short-term experiments.

A second problem has been in growing suitable plants for protoplast isolation. Plant age, lighting conditions, soil type, temperature, etc. are critical to the isolation of viable and uniformly infectible protoplasts (Kubo et al., 1975), and these parameters are often difficult to regulate due to seasonal variation throughout the year, and have been difficult to duplicate between laboratories.

These two problems have been circumvented in the case of tobacco (Murakishi, et al., 1971) and more recently with soybean (Wu and Murakishi, 1978) through the inoculation of agar- or liquid-grown cells. Although this system avoids problems of microbial and large scale cell variation and labeled precursor uptake studies pose less problems, the infection is 100 to 1000 times less efficient due to the presence of cell walls and cell clumps than in tobacco protoplast studies (Takebe and Otsuki, 1969). Also, only a relatively small percentage of cells are initially infected.

An examination of the advantages and disadvantages of systems using callus cultures and those using protoplasts from mesophyll leads to the obvious conclusion that a procedure using protoplasts derived from tissue culture would avoid some of the problems and would incorporate the primary advantages of each system.

Although Takebe has indicated the feasibility of this type of study using Vinca tissue culture (Takebe, unpublished results), no workable system has hitherto been developed for the inoculation of protoplasts from tissue culture. It was in an attempt to fulfill this need for a usable, efficient system that the present study was undertaken.

Soybean provides an excellent host for this type of study for several reasons. Cultures of soybean are readily initiated and maintained (Gamborg et al., 1968; Wu and Murakishi, 1978) and protoplasts have been isolated from liquid suspension by Kao et al., (1970) and others (Chu and Lark, 1976). Moreover, protoplasts have not as yet been successfully isolated from soybean mesophyll tissue, evidencing a need for the use of an alternate system. Another distinct advantage to using soybean is its potential for adaptation to the study of many different viruses. Soybean has been reported to be susceptible to over 50 viruses (Sinclair and Dhingra, 1975), including such well characterized viruses as alfalfa mosaic, cowpea chlorotic mottle, cowpea mosaic, southern bean mosaic, bean pod mottle, soybean mosaic and cucumber mosaic viruses.

Cowpea mosaic virus (CPMV), a multicomponent icosahedral virus (Breuning and Agrawal, 1967) was chosen for this study for several reasons. It is easily propagated and purified in large quantities and is stable in vitro (Van Kammen, 1967). Also, a precedent exists for protoplast infection by CPMV in that both cowpea (Hibi et al., 1975; Beier and Breuning, 1975) and tobacco mesophyll protoplasts (Huber et al., 1977) have been efficiently infected with the virus.

Southern bean mosaic virus (SBMV) was also used in this study. It is an icosahedral single component virus of 6.6×10^6 daltons (Miller and Price, 1946), and as with CPMV, it is easily purified and quite stable. Although there has been no precedent for the study of this virus in protoplasts, soybean tissue culture has been successfully inoculated with SBMV (Wu and Murakishi, 1978). This increased the probability that it would prove infectious in protoplasts

from the same tissue culture.

Preliminary work in this lab (Jarvis and Murakishi, 1978) indeed indicated the feasibility of using the soybean protoplast-SBMV system for virus studies. Between 40 and 50% infection of tissue culture-derived protoplasts was obtained.

This thesis describes the depth of that preliminary work and recounts the continued studies of the infection of soybean protoplasts by SBMV. In addition it includes the investigation of conditions necessary to obtain efficient infection by CPMV.

It has been shown for other protoplast systems that several factors are critical in obtaining efficient infection of protoplasts. These factors must be optimized in each protoplast-virus system to yield maximal infection.

1) Viability of cells: Viability and infectability of protoplasts is critically dependent on the growth conditions of source tissue and on the protoplast isolation procedures (Kubo et al., 1975). Unless these parameters have been optimized, protoplasts degenerate rapidly and little or no virus synthesis occurs.

2) Presence of a polycation: The infection in many protoplast-virus systems has been shown to be dependent on or stimulated by the presence of a polycation (most commonly poly-L-ornithine) in the inoculum (Takebe, 1978). Optimal poly-L-ornithine concentrations generally are between 0.5 µg/ml and 1.5 µg/ml. It has been hypothesized that the role of the polycation is to decrease electrostatic repulsion between the virus particle and the cell membrane, both

of which often bear a net negative charge.

3) Buffering conditions: All protoplast systems studied thus far have required a buffered inoculum for virus infection to occur. The most common buffer employed is K citrate at pH 5.0 to 5.5 (Takebe, 1975); phosphate buffer, pH 6.5 to 6.7 (Kubo et al., 1974) and Tris KCl buffer at pH 8.0 (Motoyoshi and Oshima, 1976) have been more recently introduced and have been found to be superior to K citrate in some systems.

4) Other parameters: The effect of the presence of various salts and the temperature of inoculation on percent of cells infected have been investigated in only a few systems. A calcium preinoculation wash of protoplasts has been found by Motoyoshi et al. (1974) to decrease infection of tobacco protoplasts by cowpea mosaic chlorotic mottle virus. Calcium or magnesium salts, when included in the inoculum at 10 mM, completely inhibited infection of several kinds of protoplasts by brome mosaic virus (Furusawa and Okuno, 1978).

Infection of tobacco protoplasts by cowpea chlorotic mottle virus has been shown to occur independently of inoculation temperature over the range of 4 C to 23 C (Motoyoshi et al., 1974). However, Alblas and Bol (1977) have shown that virus yield from infected protoplasts is much higher when inoculation is carried out at 4 C rather than at the standard 23 C.

Clearly, then, optimal infection is obtained under different inoculation conditions for each protoplast-virus system. We have found that infection of soybean protoplast by CPMV and SBMV is greatly influenced by all of these parameters. Therefore, this present study was conducted to examine each of these parameters and find the combination of inoculation conditions necessary to achieve a high percentage of infection by CPMV and SBMV. As a result of the investigation of these various parameters, several interesting phenomena surfaced, giving possible leads for future virus-membrane interaction research. Hopefully this system has reached the level of sufficient control and reproducibility to be easily adopted by other researchers for continuation of these studies.

MATERIALS AND METHODS

Propagation and purification of CPMV. The Sb strain of cowpea mosaic virus (CPMV), generously donated by Dr. G.B. Bruening, Department of Biochemistry and Biophysics, University of California, Davis, was propagated in Vigna sinensis cv. Blackeye by inoculating newly expanded primary leaves with virus diluted in 0.1 M K phosphate buffer, pH 7.0. Infected leaves were harvested 14 days after inoculation and stored at -25 C until use. Virus was isolated using a modification of the polyethylene glycol-NaCl method described by van Kammen (1967). Frozen leaves were triturated in 0.1 M K phosphate buffer, pH 7.0, 1.5 ml per gram of tissue, in a Waring blender at 4 C. The homogenate was expressed through 4 layers of cheesecloth and chloroform-butanol (1:1) was added to the filtrate to 8% of the final volume (Soong and Milbrath, 1975). The mixture was stirred for 60 minutes at 4 C and coagulated plant debris was removed by centrifugation for 10 minutes at 10,000 rpm (Sorvall type SS-34). NaCl was added with stirring to the supernatant to 0.2 M and 4% (w/v) polyethylene glycol (m.w. 5,700-6,700) (General Biochemicals, Chagrin Fall, OH) was dissolved into the mixture (Hebert, 1963). Precipitate was allowed to form at room temperature for 1 hour and was collected by centrifugation at 10,000 rpm for 15 minutes. This pellet was resuspended in 0.1 M K phosphate buffer, pH 7.0 at 1/5 the original volume, and the suspension was subjected to 2 rounds of differential centrifugation at 10,000 rpm for 15 minutes and 34,000 rpm for 90 minutes (Rotor no. 50, Spinco model L centrifuge) (van Kammen, 1967).

The final pellet was resuspended in 0.1 M K phosphate buffer, pH 7.0 and was freed of microorganisms by passage through a sterile ultrafine Corning sintered glass filter. The preparations had 260/280 nm absorbancy ratios of between 1.58 and 1.70. An extinction coefficient of 8 cm^2/mg at 260 nm was used to estimate virus concentration (van Kammen, 1967).

Propagation and purification of SBMV. The bean strain of southern bean mosaic virus (SBMV), was propagated in leaves of Phaseolus vulgaris cv. Bountiful. Infected leaves were harvested 14-18 days after inoculation and stored at -25 C until use. SBMV purification was accomplished according to the procedure used for CPMV with the following modifications. Leaf tissue was triturated in 0.2 M K phosphate buffer, pH 7.5. Virus was precipitated using 0.2 M NaCl and 6% w/v polyethylene glycol. The virus-PEG precipitate was collected by centrifugation at 10,000 rpm for 15 minutes and the pellet was resuspended in 0.02 M K phosphate buffer, pH 7.2 at 1/10 the original sap volume. The suspension was subjected to 2 rounds of differential centrifugation (10,000 rpm for 15 minutes and 22,500 rpm for 2.5 hours) and the final pellet was resuspended in 0.02 M K phosphate buffer, pH 7.2 (Shepherd and Fulton, 1962). The preparation was cleared of brown pigment by passage through a 2 cm X 7 cm column of DEAE cellulose and freed of microorganisms as was CPMV. These preparations had 260/280 nm absorbancy ratios of 1.60-1.65. Virus concentration was estimated by using an extinction coefficient of 5.8 (Sheperd, 1971).

Culture Initiation. Callus culture of soybean (Glycine max cv. Harosoy 63) were initiated from hypocotyl sections on R3 agar medium (Table 1) as described by Wu and Murakishi (1978), and maintained by transfer to fresh R3 every four weeks. To initiate liquid suspension culture, a section of callus was transferred to 30 or 50 ml of medium in 125 or 250 ml Erlenmeyer flasks and grown under continuous light of 861 lux (80 ft-c) from Gro-Lux fluorescent lamps at 23 C $\pm$ 2 C on a rotary shaker at 80 rpm (model 6140, Eberbach Corporation, Ann Arbor, MI). In these studies, R3A medium was used for liquid culture, a modified R3 medium containing 2X the concentration of 2'4' dichlorophenoxy acetic acid present in R3, and no indole-3-acetic acid. The liquid suspension culture was maintained on R3A for one year, transferring 1 ml culture per 5 ml fresh medium every 4 days, prior to use as a protoplast source.

Protoplast Isolation and Inoculation. All manipulations of culture cells and protoplasts, excluding assays, were performed under sterile conditions in a laminar flow hood (Contamination Control, Inc., Kulpsville, PA). Protoplast isolation was accomplished using a modification of the procedure outlined by Constabel (1975). Liquid suspension cultures of soybean, 36 to 48 hours from subculture, were transferred to 50 ml graduate conical tubes. Cells were washed once with R3A and allowed to resettle for 10 minutes. The supernatant fluid was carefully removed and digestion medium was added in an amount equal to the settled cell volume. Driselase at 2% (Kyowa Hakko Kogyo Co., N.Y., NY), 1% Macerase and 2% Cellulysin (both Cal-biochem) in 0.4 M sorbitol, pH 6.0 were used in the digestion

Table 1 Composition of R3 Medium[1]

Linsmaier and Skoog minerals[2]	amount/l	mM
NH_4NO_3	1.65 g	20
KNO_3	1.9 g	18.8
$CaCl_2 \cdot 2H_2O$	0.44 g	3.0
$MgSO_4 \cdot 7H_2O$	0.37 g	1.5
KH_2PO_4	0.17 g	1.2
H_3BO_3	6.2 mg	0.1
$MnSO_4 \cdot H_2O$	16.8 mg	0.1
$ZnSO_4 \cdot 7H_2O$	10.6 mg	0.04
KI	0.83 mg	0.005
$Na_2MoO_4 \cdot 2H_2O$	0.25 mg	0.001
$CuSO_4 \cdot 5H_2O$	0.025 mg	0.0001
$CoCL_2 \cdot 6H_2O$	0.025 mg	0.0001
Na_2EDTA	33.7 mg	0.1
$FeSO_4$	27.8 mg	0.1
Thiamine-HCl	1.0 mg	0.003
Pyridoxine-HCl	0.5 mg	0.002
Nicotinic Acid	0.5 mg	0.004
Inositol	100 mg	0.56
Sucrose	30 mg	88.0
(2'4'-dichlorophenoxy)acetic acid	0.5 mg	0.0025
Indole-3-acetic acid	5.0 mg	0.028
Kinetin	0.3 mg	0.0014
	pH to 6.0 with .2N KOH	

[1] Christiansen, in press
[2] Linsmaier and Skoog, 1965

medium (Nagata, I., personal communication) which were centrifuged at 10,000 rpm (Sorvall model SS-34) for 10 minutes and then sterilized by passing through a 0.45 μ Metricel GA-6 filter (Gelman Instrument Co., Ann Arbor, MI). The cell-enzyme mixture was incubated in the dark for 2.5 hours at 27 C in thin (2-3 mm) layers in flat sided prescription bottles. Gentle shaking at 30 excursions per minute (Thermo-shake bath, model 2562, Forma Scientific, Marietta, OH) aided protoplast liberation. Subsequently, cells were harvested by centrifugation at 100 g for 4 minutes (Damon/IEC model HN-SII, rotor #958, Needham Heights, MA), and cells were resuspended in 0.3M sorbitol. This process was repeated, and the second resuspension of cells was passed through a stainless steel screen, 74 u mesh (Cistron Corp., Lebanon, PA). Cell concentration was determined by hemocytometer count (Brightline hemocytometer, American Optical Co., Buffalo, NY) and cells were distributed to 15 ml tubes to give 2-5 x 10^6 protoplasts per tube. Inoculation was accomplished by a modification of the procedures of Takebe and Otsuki (1969) and Motoyoshi et al. (1973). The protoplasts were pelleted as before, the supernatant was carefully removed, and the pellet was quickly resuspended in 5 ml of inoculation medium (0.4 M sorbitol, buffer, virus, and usually poly-L-ornithine (PLO) mw 120,000 Pilot Chemical Company, Boston) and diluted with 5 ml of 0.4 M sorbitol. After a 20 minute inoculation period, cells were harvested by centrifugation for 4 minutes at 100 g and washed twice in 0.3 M sorbitol plus 10 mM $CaCl_2$. The third pelleting of protoplasts was resuspended in protoplast culture medium J (Table 2), modified from Constabel (1975) to give 2-5 x

Table 2. Composition of Protoplast Culture Medium J *

R3A, 1.5 X normal strength, no sucrose		
Casein Hydrolyzate	1.5 g/l	
$CaCl_2 \cdot 2H_2O$	0.7 g/l	4.8 mM
$CaH_4(PO_4)_2 \cdot H_2O$	0.2 g/l	0.8 mM
Glucose	25.0 g/l	0.14 mM
Sorbitol	54.6 g/l	0.3 mM
pH	6.0	

*modified from Constabel (1975)

10^5 protoplasts per ml. These protoplasts were incubated in sealed 15 x 100 mm Dispo plastic petri dishes (Scientific Products, McGraw Park, IL), 5 ml of suspension per plate under continual diffuse room light.

Protoplast Viability. Protoplast viability was assessed in the folowing ways:

(1) In light microscopy:

-presence of cytoplasmic strands: Percent of viable protoplasts was obtained by determining the percent of cells showing distinct and well distributed cytoplasmic strands when examined with the light microscope.

- Evans Blue dye: Percent of viable cells was also obtained by determining the percentage of cells that excluded Evans Blue dye.

(2) In fluorescence microscopy:

-Cells were considered to be viable if they exhibited a slight light green fluorescence, especially around the nucleus and cytoplasmic strands or if they fluoresced brightly with virus-specific stain. Dead cells exhibited an orange-brown cast.

Infectivity Assay. At specified times following inoculation, protoplasts from 5 ml aliquots of known protoplast concentration were harvested by centrifugation at 100 g for 4 minutes, washed once with culture medium J, and stored as a pellet at -25 C until assay. To determine infectivity of the preparation, each sample was thawed, diluted with 0.5 ml of 0.02 M K phosphate buffer pH 7.0 and ground in a Bellco glass homogenizer. Debris was removed by centrifugation

at 1600 g for 10 minutes (Damon/IEC model HN-SII, rotor #958, Needham Hts., MA), and the supernatant was diluted appropriately with buffer. Each sample was then assayed by determining the infectivity of the diluted supernatant on 6-8 primary leaves of 12-14 day old Phaseolus vulgaris cv.Pinto. Discrete necrotic SMBV lesions developed after 3-4 days on plants incubated at 23 C under continuous light of 780 lux from Gro-Lux fluorescent lamps. For CPMV infectivity determination, Pinto bean leaves were excised immediately following inoculation and stored at 100% humidity in the dark. Discrete chlorotic lesions developed after 4-5 days.

Mounting and staining of protoplasts. For determining percent of virus-infected cells, protoplasts were sampled after specified inoculation periods and stained using a modification of the procedure outlined by Otsuki and Takebe (1969). Protoplasts from approximately 0.5 ml of the cultured suspension were pelleted by centrifugation at 100 g for 2 minutes and the pellet was resuspended in a small volume of culture medium. Duplicate drops of this concentrated preparation were placed on a microscope slide previously coated with a thin film of Mayer's albumin, and allowed to dry. Cells were fixed for 5 minutes in 95% ethanol, then were washed for 5 minutes in phosphate buffered saline (PBS) (0.01 M K phosphate buffer, pH 7.0, containing 0.85% NaCl). Slides were carefully blotted and protoplast spots covered with the appropriate fluorescent antibody solution. These slides were incubated at saturated humidity at 37 C for 1 hour or 33 C for 2.5 hours, following which the unbound antibody was diluted by washing the slides in a large volume of the buffered saline.

The specimen was mounted with buffered saline:glycerol (9:1) and examined for fluorescence under a Zeiss GFL microscope (epi-luminescent) equipped with exciter filters KP-490 and LP-445, dichroic reflector 510 and barrier filter 520 (catalog no. 48-77-09). Percent of infected cells was determined by rating at least 300 viable protoplasts in the sample as fluorescent or non-fluorescent. The ratio of fluroescent to total viable protoplasts counted gave the percent fluorescent protoplasts.

Photography. High speed daylight Ektachrome, ASA 400 (Eastman Kodak Co., Rochester, NY) was used for all fluorescent photography. At maximum voltage, an exposure time of 15 minutes was necessary for observation of the background fluorescence of non-infected cells.

Other photomicrographs of living protoplasts and cultures were taken using a Wide M20 microscope using bright-field illumination. High speed Ektachrome (tungsten), ASA 200 (Eastman Kodak Co.) required exposures of 1/5 to 1/10 second.

Antibody Preparation and Conjugation. The production of CPMV-specific antibodies was elicited in a rabbit (New Zealand white, female) by subcutaneously injecting 1 mg of CPMV in 1 ml of 0.01 M K phosphate buffer, pH 7.0 emulsified with 1.4 ml of Freund's incomplete adjuvant (Difco Laboratories, Detroit, MI). Two more injections were administered at 2 week intervals, and 8 days after the last injection, the rabbit was bled. After clot formation, serum was decanted and frozen at -25 C until use. In serum thus prepared, CPMV antibody titre was determined to be 2048 by the tube precipitin test as described by Ball (1974) using 0.05 mg/ml virus and antiserum

concentrations from 1/8 to 1/2048. Rabbit γ-globulin was isolated from 17.5 ml of antiserum using the procedure of Otsuki and Takebe (1969), yielding 6 ml of γ-globulin solution. The protein content was estimated to be 17 mg/ml by using an extinction coefficient of 1.8 cm^2/mg at 280 nm (McGuigan and Eisen, 1968).

Conjugation of the γ-globulin with fluorescein isothiocyanate (FITC) was accomplished using the techniques developed by S. Kubo (personal communication) as follows:

To a 2 ml preparation of γ-globulin, 1.2 ml of FITC solution in 0.1 M Na_2HPO_4 was added dropwise so as to mix a ratio of 15 mg of FITC to 1 g of protein. The solution was diluted with 0.4 ml of 0.1 M Na_2HPO_4 and the pH was raised to 9.4-9.6 by the addition of 0.4 ml of 0.04 N NaOH. After stationary incubation for 30 minutes at 25 C, the mixture was charged onto a Sephadex G-25 column (1.5 x 16 cm) at 4 C, and eluted with PBS, separating the conjugated antibody from the uncoupled dye. The first band was collected and centrifuged for 30 minutes at 15,000 rpm to remove aggregated conjugates. The supernatant then contained a conjugated antibody titre of 512 and the FITC:protein ratio of the CPMV-specific fluorescent antibody was calculated to be 1.6*. The conjugated antibody was then stored in 0.8 ml aliquots at -80 C, and prior to use was thawed and diluted 1:20 with PBS for protoplast staining.

The same procedure was followed for the isolation and conjugation of SBMV γ-globulin. Yield was 5 ml of protein at 15.3 mg/ml from 36 ml of antiserum. The conjugated -globulin titre was 512, and the F/P ratio 1.15. A preparation diluted 1:16 with PBS was used for staining SBMV-infected protoplasts.

*The FITC to protein ration (F/P ratio) can be estimated by determining absorbance of the preparation at 495 nm (FITC) and at 280 nm (protein) and calculating the ratio as follows:

$$X = \frac{OD\ 495}{0.175}$$

$$Y = 0.75\ (OD\ 280 - 0.053\ X)$$

$$F/P = \frac{X}{389.4} \Big/ \frac{Y}{160}$$

RESULTS

Tissue Culture. Several media were tested for their suitability for soybean callus growth. Results are given in Appendix A. R3 agar medium was chosen for callus initiation because it supports compact and rapid growth of Harosoy 63 cells. At 6-8 weeks after initiation on R3 agar, callus was sufficient for transfer to liquid medium, although longer culturing periods on agar (transferring to new plates every 4 weeks) allowed the development of a viable callus that more readily adapted to liquid medium. R3 hormones (see Table 1) were modified to support growth of liquid suspension culture suitable for protoplast isolation. IAA has been reported to inhibit protoplast release (Reidand Galston, 1975), and therefore was omitted. This decrease of auxin concentration was compensated for by doubling the 2'4'D levels in R3A.

Two weeks after transfer of callus to liquid, spent medium was decanted and replaced with fresh, then at one week intervals, cells were transferred to new medium in decreasing amounts for 2 months. After one month, most cultures had large populations of single, elongated cells, and small clumps of round cells were sparsely distributed in the cultures. After 2-3 months, cultured cells began to be more uniform, and the growth rate increased. After 6 months, viable protoplasts could be isolated from the cultures, however the growth rate was faster and the isolation of viable protoplasts was more reliable after 9-10 months in liquid medium. At this time,

cultures appeared uniform, having small (10-15 cell) clumps of rounded cells, and little floating debris was observed.

A growth curve of a typical culture used in these experiments is shown in Figure 1. Doubling time of these cells is approximately 40 hours, and cells in early log growth (36-48 hours from transfer) have been found to be suitable for protoplast isolation. Under a four day transfer regime, the settled cell volume at this stage was characteristically 8-15% of the culture volume. Using these cells, nearly 100% digestion could be achieved, although some cell loss was observed due to breakage of weakened cells. Yield was recorded as protoplasts per ml settled cell volume; 3-5 X 10^6 protoplasts/ml were regularly obtained. Viability of untreated protoplasts after 48 hours was 65-75% as determined by the presence of cytoplasmic strands and 75-85% as determined by Evans Blue exclusion dye. Cells began to form irregular shapes within 24 hours, indicating cell wall formation, and division began to occur in 1-2% of the protoplasts.

Protoplast Inoculations. Protoplasts isolated from liquid suspension cultures of soybean have been successfully inoculated with CPMV and SBMV, obtaining 60-70% and 30-35% infection, respectively. General characteristics of infection and parameters which affect infection will be discussed. These parameters - buffer, pH, polycation concentration, presence of divalent cations and temperature - are mutually dependent. Therefore, although they are discussed in separate sections for convenience, they must be considered as a unit. An attempt has been made to optimize each parameter under conditions where all other factors have been optimized for high percentage of infection.

Figure 1. Growth curve of liquid suspension culture of soybean in R3 medium. Three mls of cells (settled volume at 20 minutes) were introduced into 50 ml of R3A culture medium at zero time. At various times, the culture was steriley transferred to a 50 ml conical graduated tube. After 20 minutes, settled cell volume was recorded and the culture was returned to its flask.

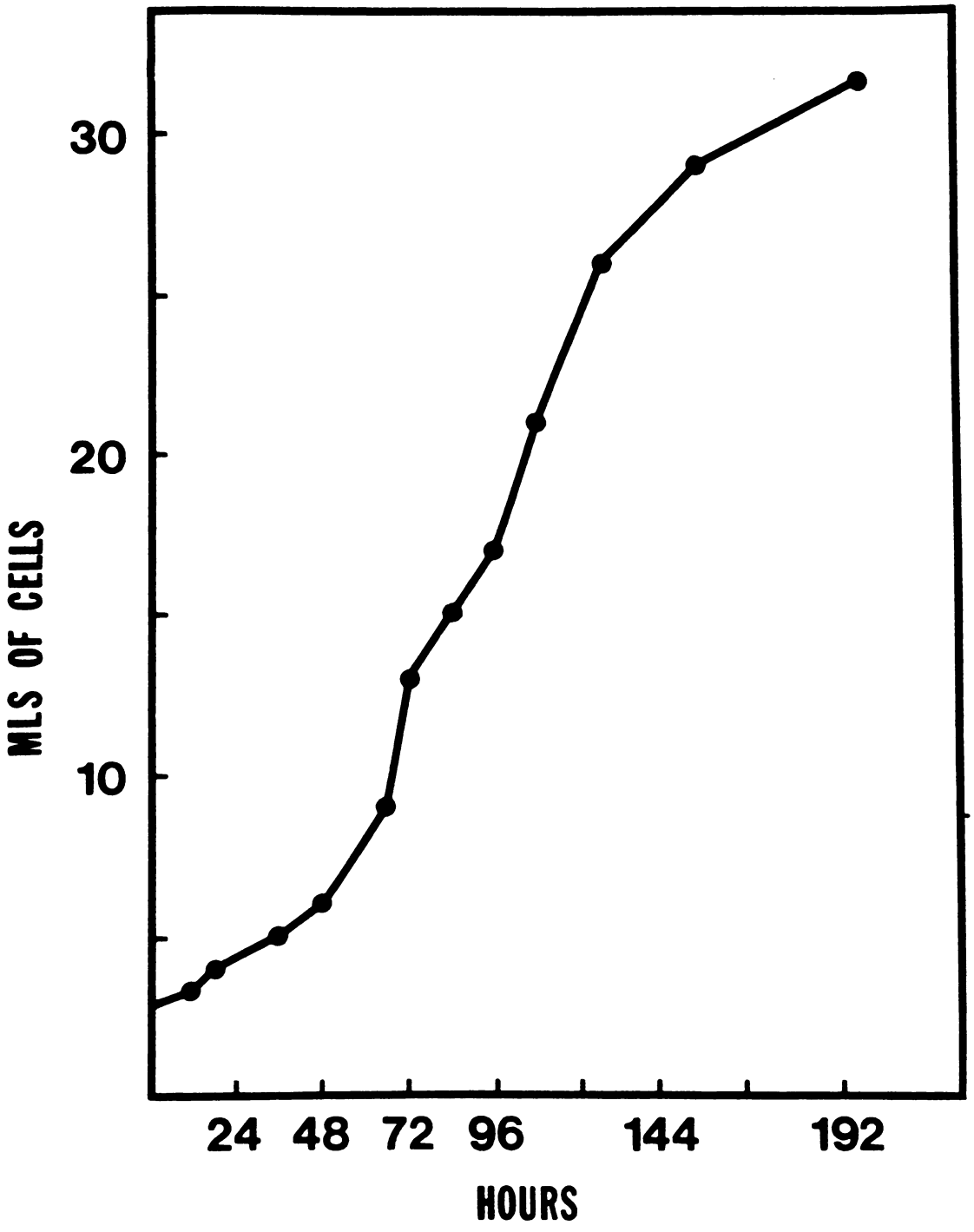


Figure 1

(1) Observations of fluorescent protoplasts. Figures 2a and b show a preparation of protoplasts harvested 48 hours after inoculation with SBMV. Fluorescence generally first appears in the nuclear area and later is observable in the cytoplasm as bright, discrete points of applegreen fluorescence. This fluorescence does not occur in inoculated cells at 0 hours after inoculation, in non-inoculated 48 hour controls, or in cells stained with a fluorescent antibody to a heterologous antigen (anti-CPMV antibody). Cowpea mosaic virus infection (Figure 3) shows a brighter, more diffuse fluorescence than does SBMV infection. Fluorescence often first appears near the nucleus, as observed in SBMV-infected protoplasts. Controls analagous to those described above in the case of SBMV infection show no such fluorescence.

(2) Development of Infectivity Over Time. Protoplasts begin to show visible fluorescence 24 hours after inoculation with SBMV, and the percent of cells fluorescing increases sporadically to 28% at 48 hours (Figure 4). Virus infectivity first was detected in SBMV-inoculated protoplasts by local lesion assay at 15 hours, rapidly increased to 24 hours, and leveled off (Figure 4). Fluorescence was observed at an earlier hour in CPMV-infected protoplast preparations (Figure 5). At 17 hours, 6% of the viable protoplasts fluoresced; this increased to 63% at 48 hours. The infectivity curve was similar to that of SBMV; local lesions first appeared from the 15 hour sample and by 21 hours the amount of infectious virus was already nearly maximal.

Figure 2a and 2b. SBMV-infected protoplasts stained with fluorescent antibody. I, infected protoplasts; N, non-infected protoplast.

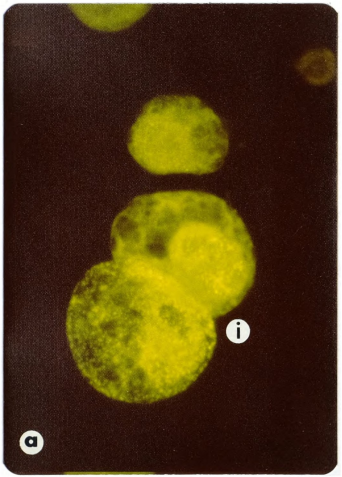


Figure 2a

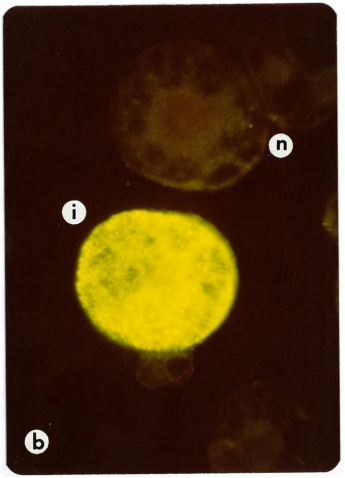


Figure 2b

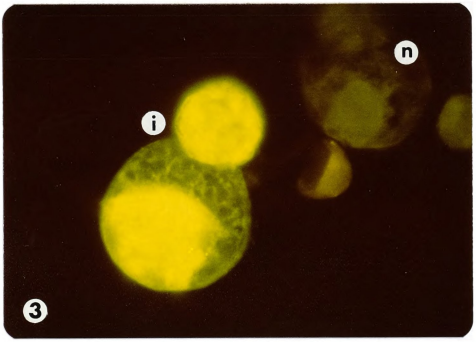


Figure 3. CPMV-infected protoplasts stained with fluorescent antibody. I, infected protoplasts; N, non-infected protoplasts.

Figure 4. Time course of SBMV synthesis in protoplasts. Protoplasts were inoculated with 5 µg SBMV/ml in the presence of 2 µg PLO/ml, 10 mM Tris-HCl buffer, pH 8.0 and half media strength Linsmaier and Skoog salts. Protoplasts were harvested at various times after inoculation and were stained with fluorescent antibody to determine percentage infection, or were assayed on half leaves of Pinto bean to determine infectivity of the virus. Percent fluorescent protoplasts with time (•———) and the number of local lesions with time (•- - - -→) from SBMV-infected protoplasts.

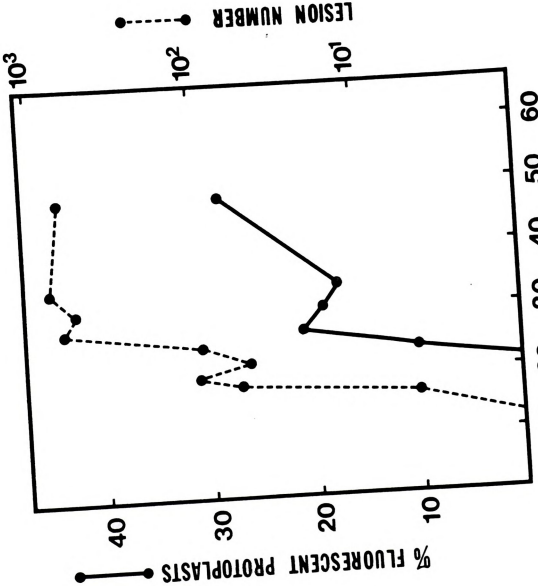

LESION NUMBER
10^3
10^2
10^1
% FLUORESCENT PROTOPLASTS
40
30
20
10
10
20
30
40
50
60
TIME OF INCUBATION, hours

Figure 4

Figure 5. Time course of CPMV synthesis in protoplasts. Protoplasts were inoculated with 5 µg CPMV/ml in the presence of 2 µg PLO/ml, 10 mM K phosphate buffer, pH 6.3 and half media strength Linsmaier and Skoog salts. Protoplasts were harvested at various times after inoculation and were stained with fluorescent antibody to determine percentage infection, or were assayed on half leaves of Pinto bean to determine infectivity of the virus. Percent fluorescent protoplasts with time (•———•) and the number of local lesions with time (•– – –•) from CPMV-infected protoplasts.

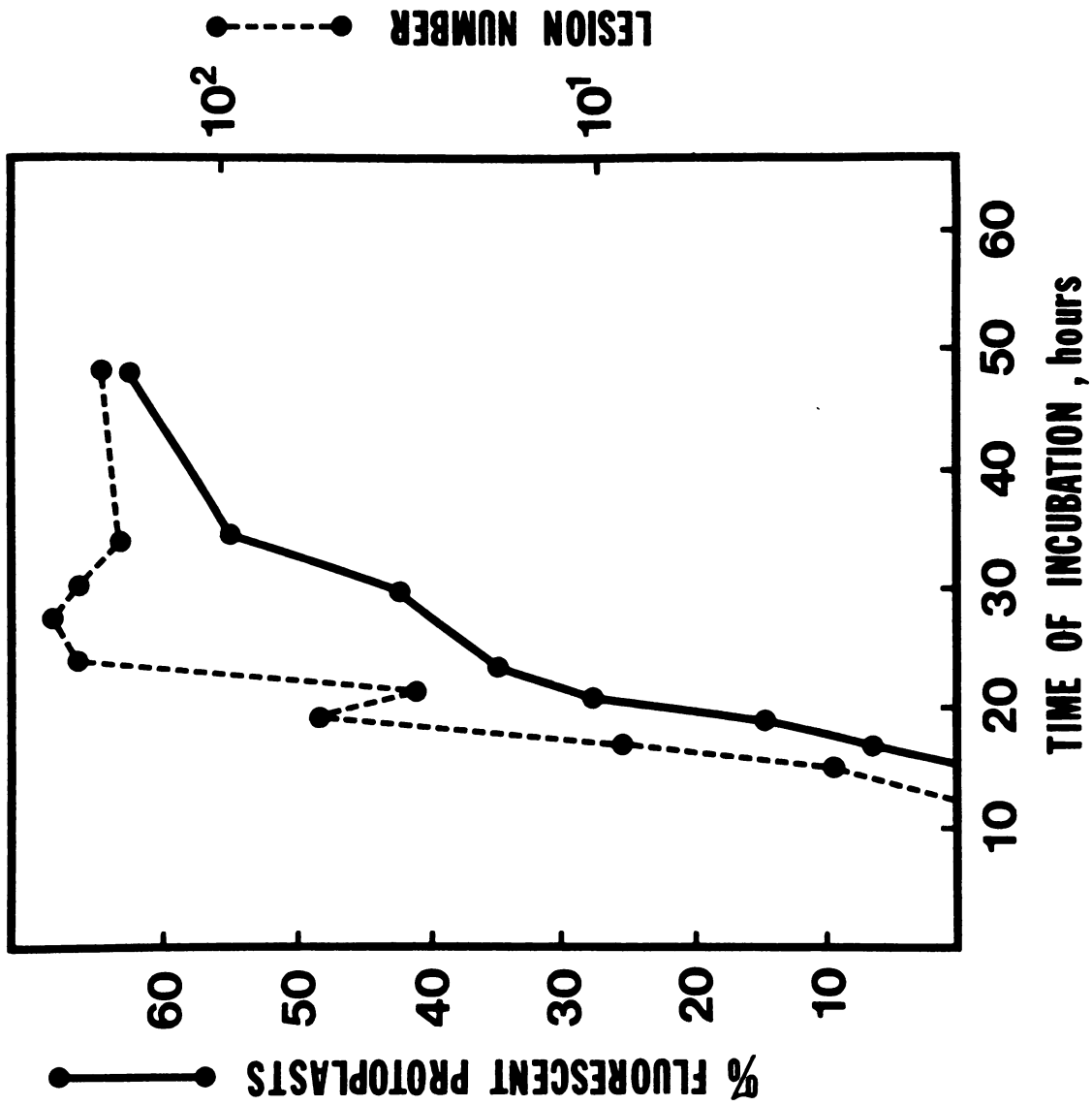

LESION NUMBER
10^2
10^1
% FLUORESCENT PROTOPLASTS
60
50
40
30
20
10
10
20
30
40
50
60
TIME OF INCUBATION, hours

Figure 5

Conditions Affecting Infection

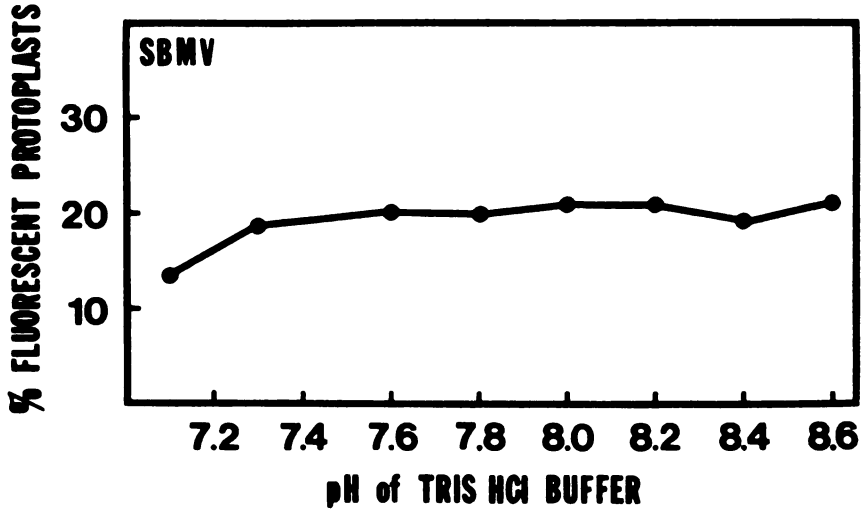
(1) Buffer. Buffering of the soybean protoplast preparation during inoculation was found to be necessary to obtain infection with both CPMV and SBMV. Table 3 shows the effect of four of the buffers tested in CPMV infection. Citrate, the buffer most widely used in virus-protoplast studies (Takebe, 1978), allowed ~1% infection at pH 5.5 and was found to be somewhat toxic to the protoplasts. A similar low infection was observed with Tris-HCl buffer, pH 8.0, although the viability was greatly improved at this pH. With 10 mM phosphate buffer at pH 6.3, however, a large percentage of the cells were infected and viability was high. The pH optimum for phosphate buffer infection is shown in Figure 7. Peak infection of 60% occurs at pH 6.3; infection drops off rapidly at higher and lower pH's. In the above case the pH study was done using inoculum medium containing Linsmaier and Skoog (LS) salts (Linsmaier and Skoog, 1965) which were found to greatly increase infection in a wide range of conditions (an effect which will be discussed later in this section). Interestingly, MES at pH 6.3 did not allow infection (Table 2). Other buffers were investigated for their use in protoplast infection. These are discussed in Appendix B.

Although it allowed very little infection with CPMV, 10 mM Tris-HCl at pH 8.0 was found most effective in stimulating infection of soybean protoplasts by SBMV (Table 3). Less than 5% infection was obtained using K phosphate buffer at pH 6.3 or citrate buffer at pH 5.5, while 36% occurred with Tris-HCl, pH 8.0. Table 3 also shows that higher pK buffers in general were the most effective of those

Figure 6. Effect of pH on percent of SBMV-infected protoplasts. Protoplasts were inoculated with 5 µg SBMV/ml in the presence of 2 µg PLO/ml, 10 mM Tris-HCl buffer at various pHs, plus 0.5 mM $CaCl_2$. Protoplasts were harvested at 48 hours and stained with fluorescent antibody to determine percent infection.

Figure 7. Effect of pH on percent of CPMV-infected protoplasts. Protoplasts were inoculated with 5 µg CPMV/ml in the presence of 2 µg PLO/ml, 10 mM K phosphate buffer at various pHs and with (•———•) or without (•— — — —•) half strength Linsmaier and Skoog salts. Protoplasts were harvested at 48 hours and stained with fluorescent antibody to determine percent infection.

Figure 6



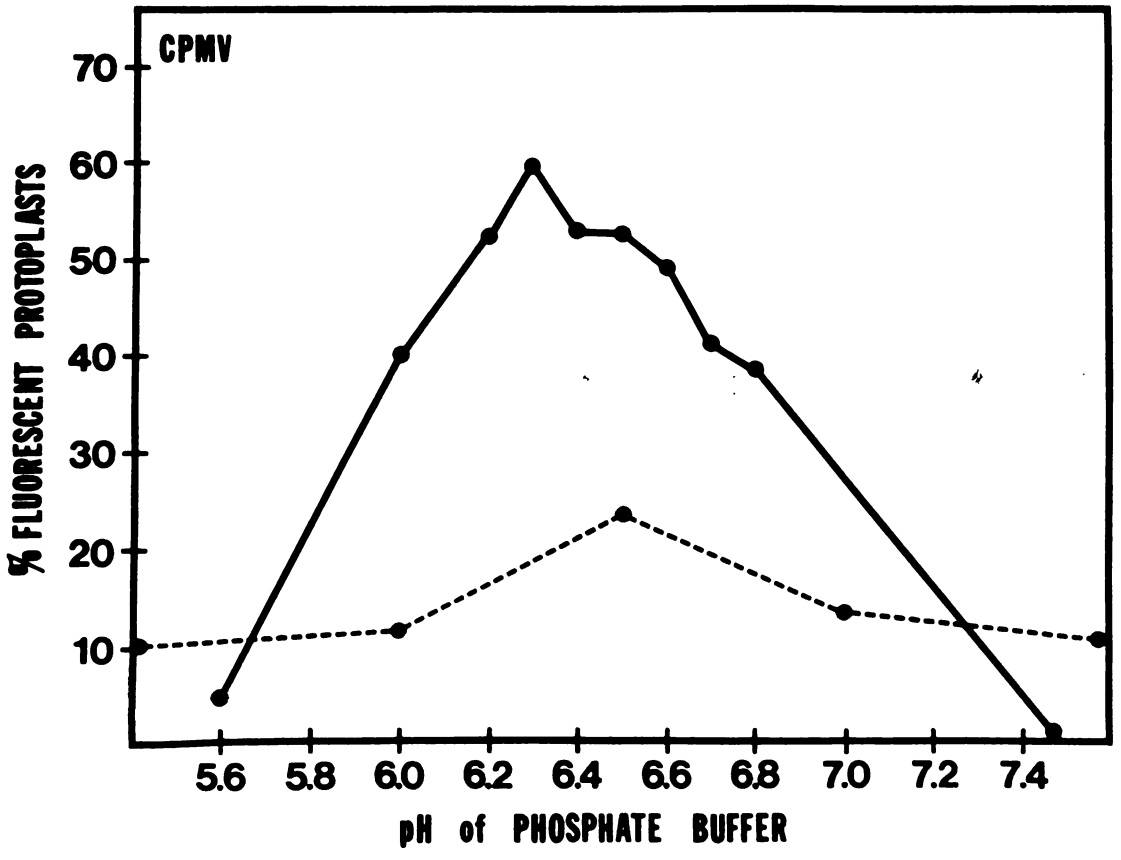


Figure 7

Table 3. Effect of the Addition of Various Buffers to the Inoculation Medium on Percent of Protoplast Infected with CPMV and with SBMV. Protoplasts were inoculated in the presence of 2 µg PLO/ml, a buffer as indicated, and either 5 µg CPMV/ml plus 0.5 mM $CaCl_2$ in experiment 1 or 5 µg SBMV/ml Linsmaier and Skoog salts, half media strength in experiment 2. Protoplasts were harvested 48 hours after inoculation and stained with fluorescent antibody to determine percent of CPMV- or SBMV-infected protoplasts.

Table 3

Experiment 1. CPMV

Buffer		Percent Fluorescent Protoplasts
10 mM K citrate,	pH 5.5	1%
10 mM Tris-HCl,	pH 8.0	4%
10 mM K phosphate,	pH 6.3	66%
10 mM MES,	pH 6.3	2%

Experiment 2. SBMV

Buffer		Percent Fluorescent Protoplasts
10 mM K phosphate	pH 6.5	0
10 mM Tricine,	pH 8.0	21%
10 mM Bistrispropane,	pH 7.0	32%
10 mM Tris-HCl,	pH 8.0	36%
10mM TAPS,	pH 8.0	29%
10 mM TAPSO,	pH 7.5	20%
10 mM BES,	pH 7.0	15%
10 mM DIPSO,	pH 7.5	31%
10 mM HEPES,	pH 5.0	8%

tested; tricine, pH 8.0, Bistrispropane at pH 7.5, BES at pH 8.0 and others were able to allow some infection, although the percentage was not as high and the viability was lower than with Tris-HCl. It is apparent from Figure 6 that the Tris-stimulated infection is nearly independent of pH over the range of 7.1 to 8.6.

(2) Poly-L-ornithine. Poly-L-ornithine (PLO) has been found to have a profound effect on infection of protoplasts by CPMV and SBMV. Figure 8 shows that under these conditions, no infection occurred in the absence of PLO. As increasing concentrations of PLO were added to the inoculum, an increasing percentage of infected cells resulted. At a PLO concentration of 1.5 µg/ml, maximal infection of 74% occurred. Higher PLO concentration proved to be supra-optimal - the percent of infected cells dropped slightly at 2 µg/ml and 2.5 µg/ml. Infection of soybean protoplasts by CPMV was possible in the absence of PLO only when a sulfonic acid buffer was used in the inoculum at low pH. This is discussed in Appendix B.

SBMV infection also is dependent on the presence of PLO. Table 4 shows that no infection occurred without PLO, 19% of the protoplasts were infected at 1 µg PLO/ml, and when the PLO concentration was raised to 2 µg/ml, a substantial increase to 30% infection occurred. PLO levels above 2 µg/ml proved to be toxic to protoplasts under these conditions.

Figure 8. Effect of various concentrations of poly-L-ornithine on percent of protoplasts infected with CPMV. Protoplasts were inoculated with 5 μg CPMV/ml in the presence of 10 mM K phosphate buffer, pH 6.3, 0.5 mM $CaCl_2$, and various concentrations of Poly-L-ornithine (PLO). Protoplasts were harvested 48 hours after inoculation and stained with fluroescent antibody to determine percent of CPMV-infected cells.

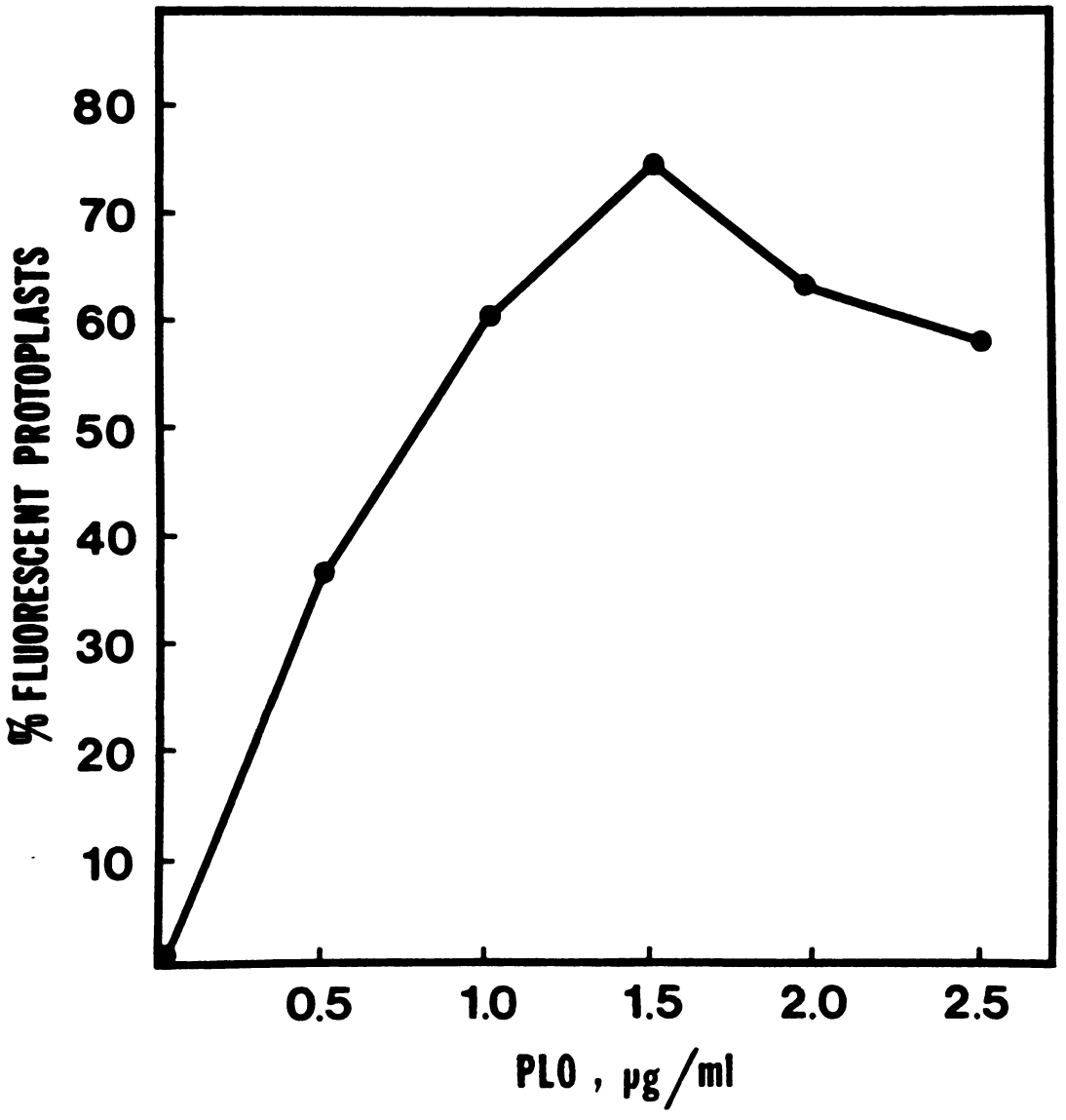

% FLUORESCENT PROTOPLASTS
80
70
60
50
40
30
20
10
0.5
1.0
1.5
2.0
2.5
PLO , µg/ml

Figure 8

Table 4. Effect of Various Concentrations of Poly-L-ornithine on Percent of Protoplasts Infection with SBMV.

Poly-L-ornithine concentration, μg/ml	Percent Fluorescent Protoplasts
2	30%
1	19%
0	0%

Protoplasts were inoculated with 5 μg SBMV/ml in the presence of 10 mM Tris-HCl buffer, pH 8.0 and Linsmaier and Skoog salts at half media strength plus various concentrations of poly-L-ornithine. Protoplasts were harvested 48 hours after inoculation and stained with fluorescent antibody to determine percent of SBMV-infected protoplasts.

(3) Salt Environment. Various salts were added to the inoculation medium in order to determine their effect on percentage of cells infected by CPMV and SBMV. Table 5 shows the results of the addition of several salts to CPMV-inoculation medium. Clearly experiment 1 shows that the cationic moiety of the salt largely determines the effect on infection; the type of anion appears to be of little or no consequence. In both experiment 1 and 2 all magnesium salts tested increased CPMV infection by about 40%. This increase may be obtained with as little as 1 mM $MgSO_4$ (see Appendix C). Addition of a magnesium chelator (EDTA) dramatically decreased infection,

Table 5. The Effect of the Addition of Various Salts to the Inoculation Medium on Percent of CPMV-Infected Protoplasts. Protoplasts were inoculated with 5 µg CPMV/ml in the presence of 2 µg PLO/ml, 10 mM K phosphate buffer, pH 6.3 and ammendments as listed. Protoplasts were harvested 48 hours after inoculation and stained with fluorescent antibody to determine percent of CPMV-infected protoplasts.

Table 5

Experiment 1.

Inoculum Amendments	Percent Fluorescent Protoplasts
none	18%
5 mM $Mg(NO_3)_2$	24%
5 mM $MgCl_2$	26%
5 mM $MgSO_4$	22%
2.5 mM $MgSO_4$ + 2.5 mM MgEDTA	8%
5 mM MgEDTA	6%
5 mM $NiCl_2$	0%
5 mM $CaSO_4$	65%
5 mM $CaH_4(PO_4)_2$, H_2O	55%
5 mM $NaNO_2$	17%
5 mM KNO_3	5%
5 mM LiCl	5%

Experiment 2.

Inoculum Amendments	Percent Fluorescent Protoplasts
none	11%
1 mM $MgSO_4$	15%
0.5 mM $CaCl_2$	62%
1 mM $CaCl_2$	57%
1 mM $CaSO_4$	64%
0.25 mM $CaCl_2$ + 1 mM $MgSO_4$	45%
0.5 mM $CaCl_2$ + 1 mM $MgSO_4$	64%
1 mM $CaCl_2$ + 1 mM $MgSO_4$	67%
1.5 mM $CaCl_2$ + 1 mM $MgSO_4$	66%
LS salts, half strength media concentration	60%

even when extra Mg^{2+} ions were added to the inoculum. A second divalent cation-containing salt, $NiCl_2$, completely inhibited infection by CPMV. All monovalent cations tested were also not effective at stimulating infection. Na^+, K^+ and Li^+ ions either did not affect percentage of infection or were quite inhibitiory. Ca^{2+} ions, however, caused a dramatic increase in infection. Both experiments showed that various concentrations of calcium salts stimulated between 57 and 65% infection. In the presence of 0.5 mM $CaCl_2$, 62% infection occurred, nearly a 6-fold increase over the non-amended control.

To investigate the possibility that a combination of stimulatory salts might allow an even higher percent of infected cells, magnesium and calcium salts were mixed in various proportions in the inoculation medium. Experiment 2 in Table 5 shows the results. Infection stimulated by $CaCl_2$ was not significantly further increased by the addition of 1 mM $MgSO_4$. Also, the combination of salts present in soybean culture medium was tested for its stimulatory ability. Linsmaier and Skoog (LS) salts (Linsmaier and Skoog, 1965) at 0.5 media strength contains 10 mM NH_4NO_3, 9 mM KNO_3, 1.5 mM $CaCl_2$, 0.75 mM $MgSO_4$ and 0.6 mM KH_2PO_4. This mixture stimulated 60% infection, which was not an increase in percentage over that obtained with CaC_2 alone. In fact, no combination of salts tested was significantly more effective at stimulating CPMV infection than 0.5 mM $CaCl_2$.

However, a disadvantage of using only $CaCL_2$ in the inoculum is the tendency for debris to accumulate with the protoplasts. When Ca^{2+} ions are added to the inoculum, cell membranes from broken protoplasts tend to clump and pellet with the protoplasts. Although

this phenomenon does not appear to affect viability of protoplasts or virus proliferation, this type of preparation may not be suitable for some studies unless a purification step is added. This problem may also be alleviated by inoculating in the presence of a combination of salts, such as those in the LS medium. The membrane-aggregation phenomenon was considerably lessened when several salts were combined.

Infection by SBMV is also dramatically affected by the addition of divalent cations (Table 6). However, the stimulatory effects of magnesium and calcium appear to be additive, having 6% infection with $MgSO_4$ alone, 19% with $CaCl_2$ alone and 30% when they are in combination. These two salts appear to account for the stimulatory effect of LS salts on percent of infection.

Table 6. The Effect of the Addition of Various Salts to the Inoculation Medium on Percent of SBMV-Infected Protoplasts.*

Inoculum Amendments	Percent Fluorescent Protoplasts
None	3%
1 mM $MgSO_4$	6%
1 mM $Mg(NO_3)_2$	6%
1 mM $CaCl_2$	19%
1 mM $MgSO_4$ + 0.5 mM $CaCl_2$	30%
LS salts, one tenth media concentration	30%
LS salts, half media concentration	32%

*Protoplasts were inoculated with 5 ug SBMV/ml in the presence of 2 ug PLO/ml, 10 mM Tris-HCl buffer, pH 8.0 and amendments, as listed.

(4) Virus Concentration. A study was undertaken to determine the effect of virus concentration on the percent of protoplasts infected in the presence of various salts. Figure 9 shows curves of CPMV concentration vs. percent fluorescence without additional salts and in the presence of 0.75 mM $MgSO_4$, 0.5 mM $CaCl_2$ or LS salts, 0.5 media strength. Without salts, infection reached a maximum at 1 ug CPMV/ml and never rose above 15%. $MgSO_4$ generally increased percent of infection, reaching a maximum of 40% around 1 ug CPMV/ml. Addition of 0.5 mM $CaCl_2$, however, increased infection to over 70%. At 0.1 ug CPMV/ml, infection was already 52% and maximum infection occurred at 0.5 ug CPMV/ml. The addition of LS salts at 0.5 media strength generally had the same effect as did $CaCl_2$ alone.

SBMV infection was much less efficient than CPMV infection (Figure 10). Percent of infection rose slowly and did not level off until 2 to 2.5 ug SBMV/ml. Infection was actually maximal at much higher concentrations, although no attempt was made to increase the virus concentration above 10 ug/ml.

(5) Additional Calcium Effects. Calcium has been shown to be highly stimulatory to virus infection, however this effect is definitely dependent on when the protoplast and/or virus is exposed to the calcium ions. Table 7 shows the results of an experiment where $CaCl_2$ was included in the preinoculation sorbitol wash, the inoculation medium, the post inoculation sorbitol wash, or a combination of the three. When the standard prewash sorbitol-post wash

Figure 9. The effect of CPMV concentration on percentage of protoplasts infected in the presence of various salts. Protoplasts were inoculated in the presence of 2 ug PLO/ml, 10 mM K phosphate buffer, pH 6.3, and various concentrations of CPMV. The following amendments were added to the inoculation medium: (a) no additional salts; (b) 0.75 mM $MgSO_4$; (c) half media strength Linsmaier and Skoog salts (see text) and (d) 0.5 mM $CaCl_2$. Protoplasts were harvested 48 hours after inoculation and stained with fluorescent antibodies to determine percent of CPMV infection.

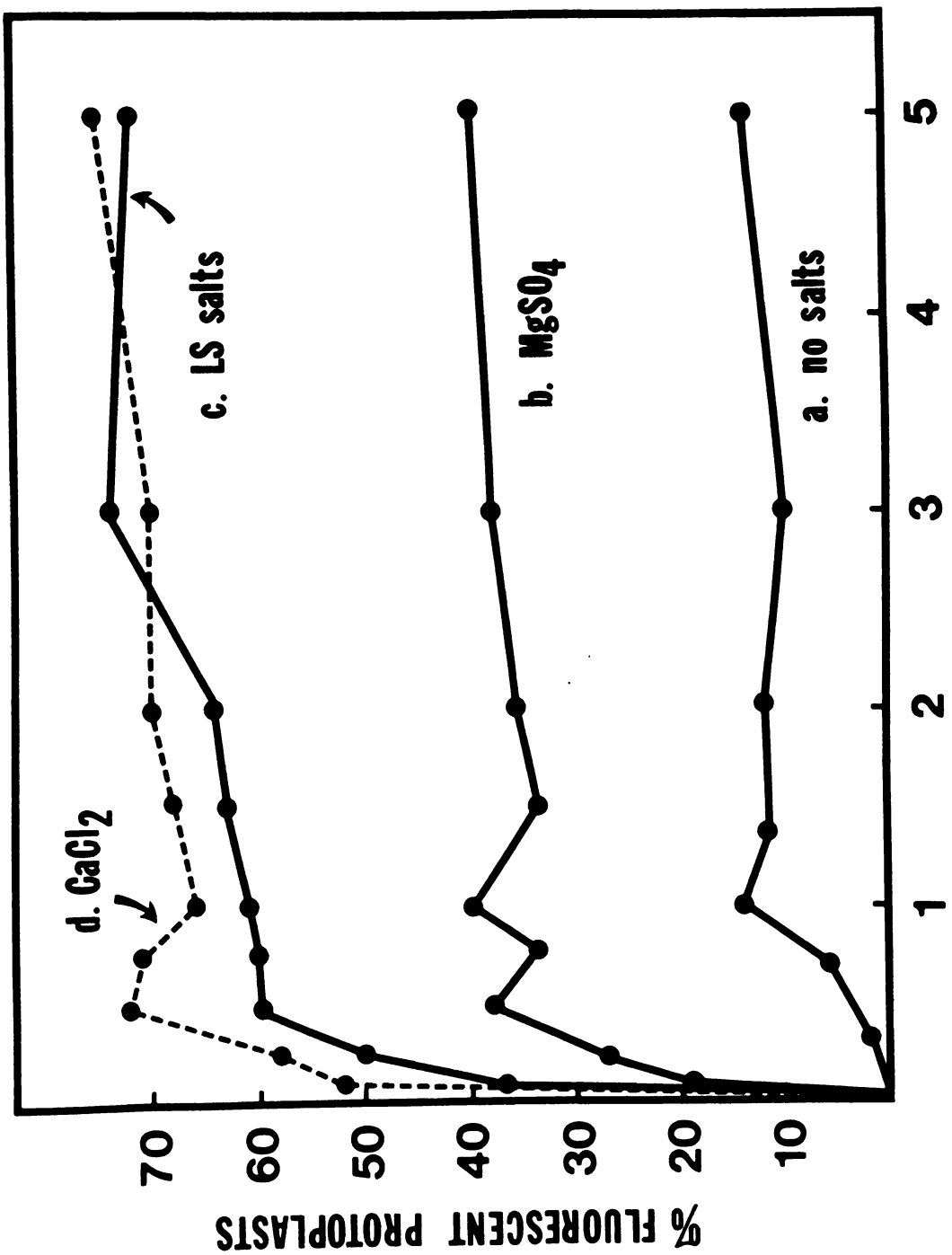

% FLUORESCENT PROTOPLASTS
70
60
50
40
30
20
10
1
2
3
4
5
CPMV CONCENTRATION, µg/ml
a. no salts
b. $MgSO_4$
c. LS salts
d. $CaCl_2$

Figure 9

Figure 10. The effect of SBMV concentration on percentage of protoplasts infected. Protoplasts were inoculated in the presence of 2 ug PLO/ml, 10 mM Tris-HCl buffer, pH 8.0, half strength Linsmaier and Skoog salts (see text) and various concentrations of SBMV. Protoplasts were harvested 48 hours after inoculation and stained with fluorescent antibody to determine the percentage of SBMV-infected protoplasts.

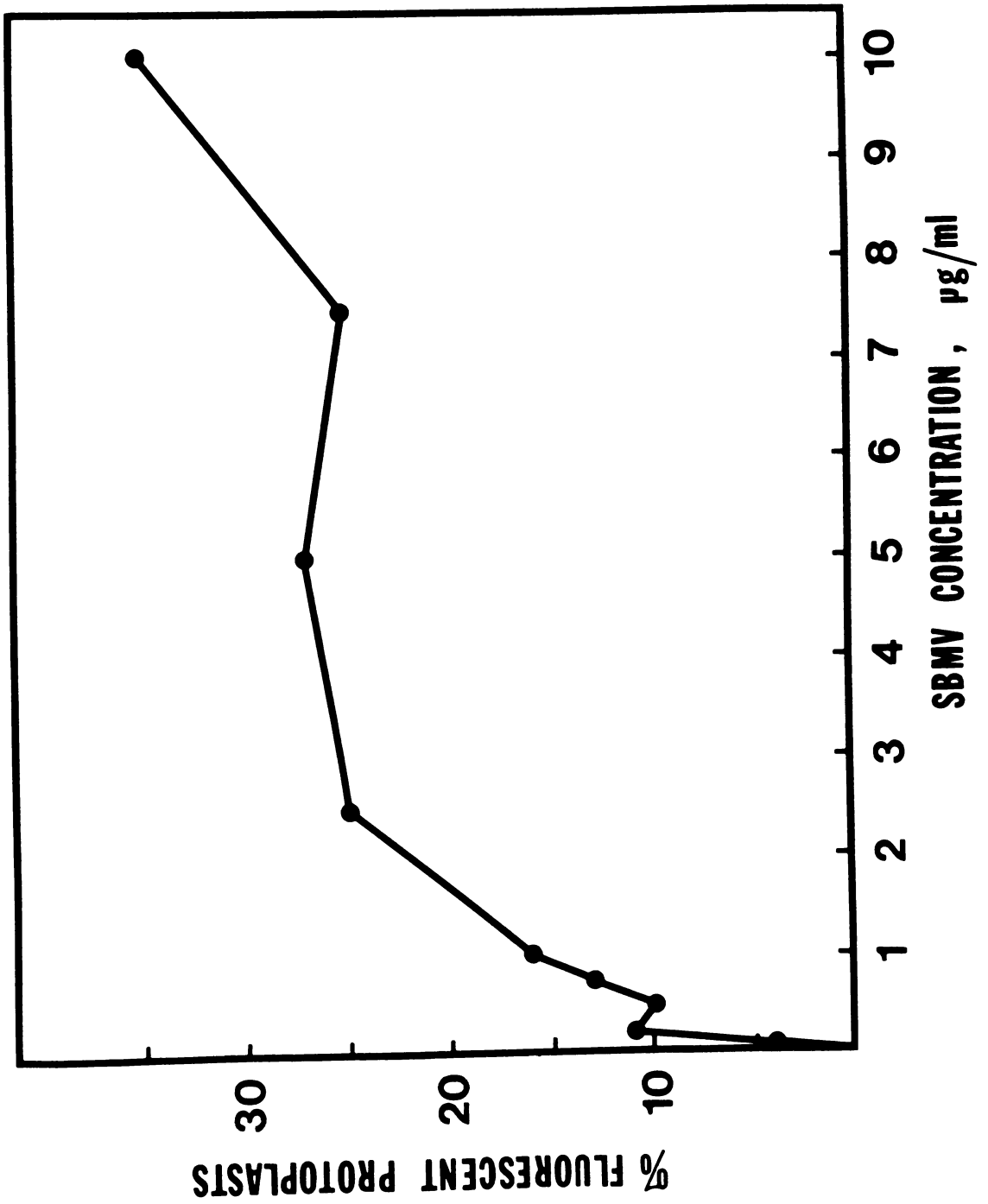

% FLUORESCENT PROTOPLASTS
10
20
30
SBMV CONCENTRATION, µg/ml
1
2
3
4
5
6
7
8
9
10

Figure 10

sorbitol plus $CaCl_2$ was used, familiar results were obtained; with Ca^{2+} in the inoculum, 63% infection was obtained, and 12% when no Ca^{2+} was included. However, when 10 mM $CaCl_2$ was included in the pre-inoculation wash medium, only 31% infection was obtained from the calcium-containing inoculum, representing only 1/2 of that obtained with no pre-wash. When no $CaCl_2$ was in the inoculum, infection actually increased slightly when cells were washed with $CaCl_2$ prior to inoculation.

The post-wash affected neither the percent of cells infected when calcium was present nor that when calcium was absent. However under these conditions, calcium was definitely affecting the protoplasts. When $CaCl_2$ was included in the wash medium, protoplasts clung tenaciously together in a slime-like mass, and became extremely hard to resuspend.

No calcium pre-wash experiments were done with SBMV.

Table 7. Effect of $CaCl_2$ on Infection of Protoplasts by CPMV when present in the Pre-Inoculation Wash, Inoculation Medium or Post-Inoculation Wash.

Pre-wash Amendment	Inoculum Amendment	Post-wash Amendment	Percent Fluorescent Protoplasts
None	None	10 mM $CaCl_2$	12%
None	0.5 mM $CaCl_2$	10 mM $CaCl_2$	63%
10 mM $CaCl_2$	None	10 mM $CaCl_2$	20%
10 mM $CaCl_2$	0.5 mM $CaCl_2$	10 mM $CaCl_2$	31%
None	0.5 mM $CaCl_2$	None	65%
None	None	None	10%

Protoplasts were washed with either 0.3 M sorbitol or 0.3 M sorbitol plus 10 mM $CaCl_2$ and inoculated with 5 ug CPMV/ml in the presence of 2 ug PLO/ml, 10 mM K phosphate buffer, pH 6.3. The inoculum medium was amended with 0.5 mM $CaCl_2$ or not, as indicated. After inoculation, protoplasts were washed with 0.3 M sorbitol or 0.3 M sorbitol plus 10 mM $CaCl_2$. Protoplasts were harvested 48 hours after inoculation and stained with fluorescent antibody to determine percent of CPMV-infected protoplasts.

(6) Temperature of Inoculation. A study was made to determine percent of infected protoplasts vs. temperature of inoculation. Tubes of inoculation medium were equilibrated in water baths to temperatures between 0 C and 35 C after the 10 minute PLO-virus incubation period. Protoplast pellets were briefly chilled or warmed

in the water baths prior to inoculation. Results are given in Figure 11. Percent infection was lowest at 0 C and rose sharply to about 12-13 C. After this temperature, increase in percent infection occurred only slowly with further increase in temperature. Two distinct lines may be drawn intersecting at 12-13 C. Between 0 C and 12-13 C, percent of infected protoplasts increased 4.4 per degree; after 12-13 C, an increase of only 0.45 percent infection per degree was observed.

Figure 11. Effect of temperature of inoculation on percent of protoplasts infected with CPMV. Protoplasts were inoculated with 5 μg CPMV/ml in the presence of 1.5 μg poly-L-ornithine/ml, 10 mM K phosphate buffer and 0.5 mM $CaCl_2$ for 20 minutes at various temperatures. Protoplasts were harvested 48 hours after inoculation and percent fluorescence was determined by staining with CPMV-specific antibody.

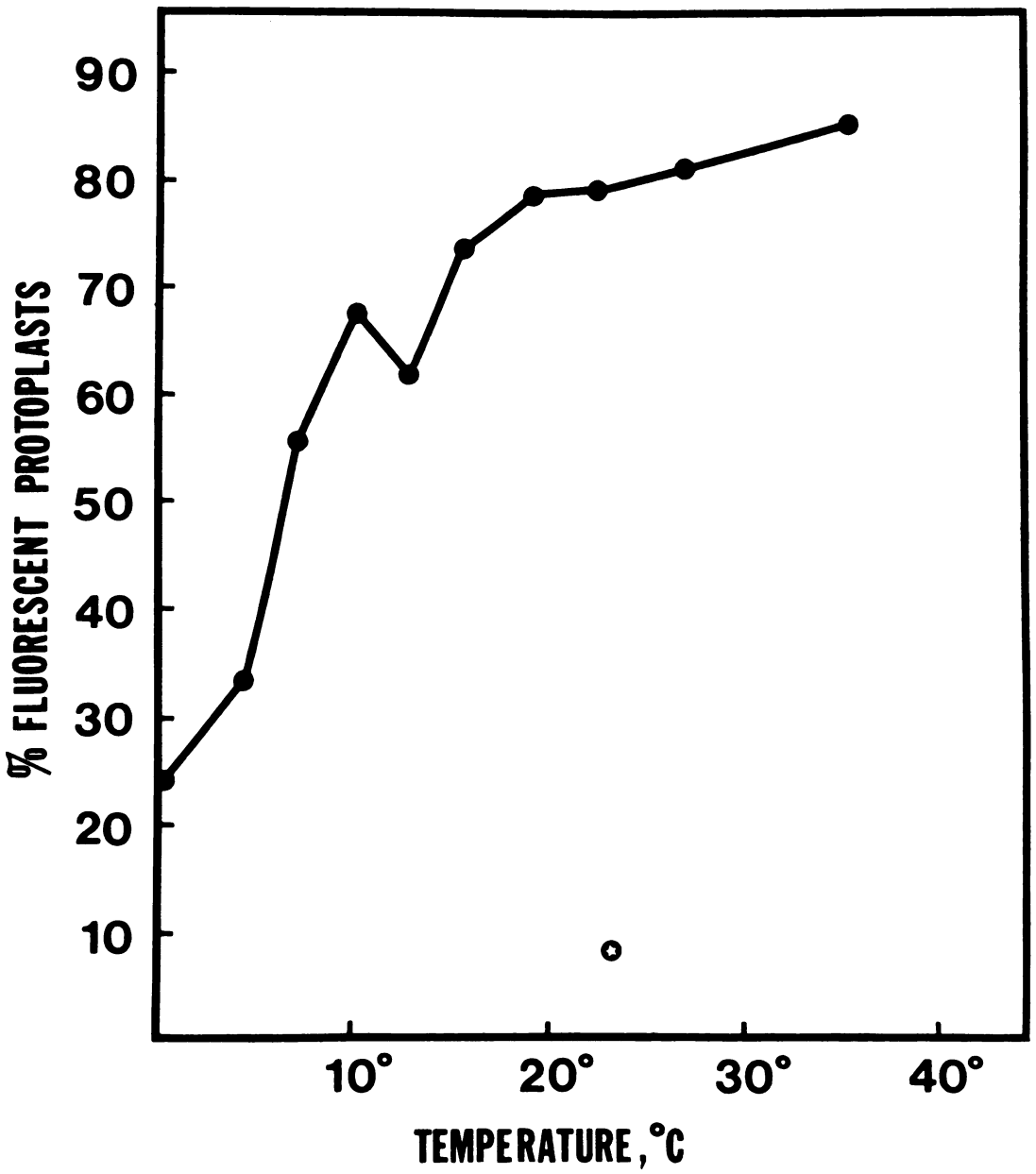

90
80
70
60
50
40
30
20
10
% FLUORESCENT PROTOPLASTS
10°
20°
30°
40°
TEMPERATURE, °C

Figure 11

DISCUSSION

Protoplasts have been used in plant virus research since the mid 60's. A procedure for the efficient inoculation of tobacco mesophyll protoplasts with tobacco mosaic virus was introduced by Takebe in 1969, and since then many other protoplast-virus systems have been developed. In these studies, protoplasts have been isolated almost exclusively from mesophyll tissue. Although development of this system was a great advance over the use of whole plants for virus study, difficulties in achieving sterility, uniformity and reproducibility still plague plant virologists.

In an attempt to overcome these problems, we have developed the first workable system, to our knowledge, for the efficient inoculation of protoplasts from a new source - tissue culture. This system has many advantages over those using mesophyll protoplasts. First, tissue culture and tissue culture protoplasts may be manipulated under sterile conditions, obviating the necessity for antibiotics which are universally used in mesophyll protoplast culture. This absolute freedom from microbial growth and potentially inhibitory chemicals available through the use of tissue culture-derived protoplasts is of critical importance to any biochemical study. Secondly, callus protoplasts often regenerate cell walls more readily than protoplasts from leaf (Constabel, 1975). This property, combined with the ability to maintain sterile cultures, allows long-term experiments to be undertaken.

Perhaps the most important advantages of using tissue culture protoplasts, however, are the ease of maintenance and, particularly, uniformity of tissue. Laboratories using mesophyll protoplasts must cope with many problems inherent in using whole plants as source material. Seasonal, even diurnal fluctuations, variation between plants and parts of plants, variations of exact conditions between labs, etc. can cause considerable non-uniformity of material, and therefore some degree of irreproducibility. Although growth and isolation conditions for the tissue culture protoplast system are not less critical than those for the mesophyll protoplasts, they are less of a source for irreproducibility because growth conditions for callus - light, temperature, growth media, etc. - may be more easily defined and regulated. With regular subculturing, a source of homogeneous cells is continuously available for protoplast isolation. We hope that these defined conditions will allow this system to be easily adapted by other laboratories for use in specific virus-protoplast studies.

Those conditions which are critical to the success of the soybean protoplast-SBMV and -CPMV systems have been examined in this thesis and can be divided into two categories - culture growth and inoculation conditions.

Culture Growth. Clearly optimal culture growth is important in the isolation of viable and infectible protoplasts. Three factors are of utmost importance in this regard.

(1) Growth rate. A rapid growth rate is desirable for the following reasons. Fast growing cultures have a high proportion of recently divided "young" cells which have more easily hydrolyzed walls than do older cells. This allows protoplasts to be released more rapidly, avoiding prolonged exposure to enzymes which may have deleterious effects on cells, and possibly on virus-receptor sites.

(2) Clump Size. Obtaining fine cultures with no more than 20 cell per clump is necessary for the isolation of a high yield of viable cells. Intuitively, smaller clumps with all cells exposed to the enzymes digest faster than do large clumps. Transferring from the top layers of the culture and frequent transfer help to select for fine cultures.

(3) Culture Age. The optimal age for isolating protoplasts from culture is 36-48 hours from last subculture, or approximately at the first doubling time when the probability is high that a large population of the cells have recently divided. Culture of more than 48 hours tend to yield less viable protoplasts, and of those that are viable, fewer become infected with virus. This may correspond to the tendency toward increasing resistance to virus infection with age in many whole plants (Matthews, 1970).

Inoculation Conditions. Once suitable protoplasts can be isolated, other factors become critical to obtaining a high percentage of infection. Four important factors to regulate are, buffer used, presence of a polycation, virus concentration, and salt environment. These factors are all intimately involved in defining the inoculation environment during which infection takes place.

(1) Buffer Used. The kind of buffer used, its concentration and pH have been shown, in the case of other protoplast systems, to be of critical importance for optimal infection. The most effective buffering conditions depend on the virus and the type of protoplasts used. Most of these same observations held true for obtaining a high level of infection of soybean protoplasts by CPMV and SBMV. Optimal buffering conditions for these two viruses are very different and perhaps reveal some interesting aspects of infection.

CPMV infection: Out of the non-sulfonic acid buffers tried, only phospate was successful at stimulating infection of soybean protoplasts by CPMV. Interestingly enough, citrate was ineffective at stimulating any infection, although CPMV infection of tobacco (Huber, et al., 1977) and cowpea (Hibi et al., 1975) meosphyll protoplasts reached 75% and 95% respectively when K citrate buffer was used; yet cowpea mesophyll protoplasts were also infectible with CPMV using 0.1 M K phosphate buffer, pH 6.5, buffering conditions similar to those used successfully for soybean-CPMV infection with the soybean system, optimal CPMV infection occurred at pH 6.3 for phosphate buffer. However, MES buffer at pH 6.3 has no stimulatory effect on infection even though this is well within its optimal buffering range. Clearly then, stimulation is not simply a buffering or pH phenomenon. It is possible that K phosphate itself is causing a stimulation of infection as well as functioning as a buffer, or that MES is simultaneously buffering yet inhibiting infection. This phenomenon could be investigated through several approaches: examining the effects of MES-phosphate combination buffers; adding

potassium ions to MES buffer; using sodium instead of potassium phosphate buffer, or testing other non-sulfonic acid buffers with pK_a's around 6.3-6.7.

SBMV infection: All buffers tested having high pK values successfully stimulated SBMV infection of soybean protoplasts at relatively high pH values. Buffers used at lower pH values (phosphate, pH 6.5 or citrate pH 5.5) were unsuccessful. Tris-HCl gave the best infection among those high pK buffers tested, and its effectiveness was relatively independent of pH over the range of 7.3 to 8.6. No data was obtained with phosphate at pH 7.2 to 8.6, although it would be interesting to note whether SBMV's inability to infect soybean protoplasts with phosphate buffer is due to the compound or simply the low pH. A higher concentration of Tris-HCl (50 mM) has been used successfully in other systems, (Motoyoshi and Oshima, 1976) and should be tested here.

It can be seen, therefore, that infection of soybean protoplasts by CPMV and SBMV differ greatly in buffering requirements between the two viruses. SBMV infection appears to be stimulated at relatively high pH values; no infection is obtained with CPMV above pH 7 using Tris or phosphate buffers. CPMV's infection is stimulated by K phosphate buffer and is quite pH critical, while SBMV does not infect soybean protoplasts in the presence of K phosphate at pH 6.3 and its infection in the presence of Tris-HCl buffer is pH independent at the higher pH values tested. These differences may be paritally due to differences in electrophoretic mobility and ion and charge requirements of the two viruses, which will be discussed in the next sections.

(2) Presence of Polycation. Long chain polycations, when present in inoculation medium, have generally been stimulatory or essential to virus infection of protoplasts (Takebe, 1978). It has been suggested that addition of poly-L-ornithine (PLO) to inoculation medium enhances binding of the virus to the protoplasts (Takebe, 1978) by forming a complex with the virus. This complex is then much more likely to become associated with the membrane, possibly at specific sites, and result in infection. Clearly the effectiveness of this interaction and the necessity for the cationic liaison depends on the net negative charge of the virus and on the charge of the membrane, or of specific sites on the membrane. It has been found that cowpea mesophyll protoplasts have been able to be infected with CPMV (Hibi et al., 1975) and cucumber mosiac virus (CMV) (Koike et al., 1977) to a very high percent without PLO; also some tobacco mosaic virus (TMV) infection occurs in the absence of PLO in cowpea protoplasts (Koike et al., 1976). However, CPMV (Huber et al., 1977), CMV (Otsuki and Takebe, 1973) and TMV (Otsuki et al., 1972) infectivity in tobacco cells is completely dependent on PLO. Perhaps cowpea mesophyll protoplast membranes, or appropriate portions of the membranes are less negatively charged than corresponding areas of tobacco membranes.

Virus charge also apparently plays a role in the interactions. Brome mosaic virus, strain V5 and pea enation mosaic virus have relatively high isoelectric points (Okuno et al., 1977; Motoyoshi and Hull, 1974) and their infection of tobacco mesophyll protoplasts (Motoyyoshi et al., 1974d; Motoyoshi and Hull, 1974) does not require

PLO. Thus these relatively positively charged particles can overcome the relative negative charge of the membrane or the membrane sites of tobacco protoplasts.

With soybean tissue culture protoplasts, infection by both CPMV and SBMV was absolutely dependent on the presence of poly-L-ornithine under the conditions used in this study.

CPMV infection: The requirement for PLO in the CPMV-soybean system perhaps could be considered as analogous to the situation found for the tobacco mesophyll-CPMV system. According to the PLO-charge neutralization theory, it could be that both tobacco and soybean protoplasts have membranes or membrane sites that are negatively charged, and CPMV, whose isoelectric point is between 3.7 and 4.5, would tend to be discouraged from binding to the highly negative sites unless PLO was present. A less negatively charged membrane, such as is hypothesized with cowpea mesophyll protoplasts, could allow infection in the absence of PLO. It would certainly be interesting to determine whether a virus (such as pea enation mosaic virus) with a relatively high isolecetric point would require PLO for infection of soybean protoplasts.

In the soybean-CPMV system the levels of 1.5 to 2 ug PLO/ml found optimal for infection are somewhat higher than normal. CPMV infection of tobacco and cowpea mesophyll protoplasts was optimal at only 0.5 ug PLO/ml.

SBMV infection: Since SBMV infection of soybean absolutely requires PLO, we may predict, according to the aforementioned theory, that the isoelectric point of SBMV bean strain is relatively low.

Electrophoretic data has been presented for several other stains of SBMV and isoelectric points range from 4.5-6.0 (Magdoff-Fairchild, 1967). However, data regarding the bean strain used in this study is not yet available, although we probably may guess that the isoelectric point does not fall much above 6.0. Appropriate isoelectric potential data could help understand this system and play a role in supporting or revising the present theory.

(3) Virus Concentration. For virus-protoplast interactions requiring PLO, percent of infected protoplasts generally increases in proportion to the log of the inoculum concentration over a certain range (Otsuki and Takebe, 1973; Otsuki et al., 1974). After the concentration giving the maximum percentage of infection is reached (usually 0.5 to 5.0 ug virus/ml), increasing the amount of virus in the inoculum does not generally increase percent of infection, and usually some protoplasts will not become infected even if excess virus is used. This type of curve, characteristic for mesophyll protoplasts infection, is also observed in CPMV and SBMV infection of soybean tissue culture protoplasts.

CPMV infection: Percent of CPMV-infected protoplasts was dependent on virus concentration within the range of 0 to 0.5 ug CPMV/ml. Under optimal conditions, maximum infection occurred at 0.5 ug CPMV/ml and higher levels of infection could not be obtained by raising the virus concentration. Based on the average molecular weight for an infectious CPMV particle of 5.5×10^6 daltons, this represents approximately 10^{11} virus particles/ml or 5×10^5 particles/protoplast when $2.5\text{-}3 \times 10^5$ protoplasts/ml were inoculated. This optimum is substantially lower than that found for CPMV-infection of mesophyll

protoplasts. In cowpea and tobacco mesophyll protoplasts, 3-4 ug CPMV/ml was needed to obtain maximum infection (Hibi, et al., 1975) and Huber et al., 1977).

SMBV infection: Infection of soybean tissue culture protoplasts was somewhat less efficient with SBMV than with CPMV, and maximum infection required a high concentration of SBMV. At 2-2.5 ug SBMV/ml, maximum percent of infection occurred. Although this percentage was relatively low, the shape of the curve is comparable to infection of viruses in many mesophyll protoplast systems. It is likely, however, that when conditions are optimized that it will be possible to obtain 100% infection of viable cells.

(4) Salt Environment. Aside from the information that can be garnered from buffer studies, extremely little is known about the effect of the ionic environment on viral infection on plant protoplasts. However, studies in several other systems make this aspect of protoplast-virus interactions potentially interesting. In several animal virus studies, ionic strength is of critical importance in early cell-virus interactions (Lonberg-Holm and Philipson, 1974). Monovalent cations in the medium promote maximum attachment of several viruses (Holland and McLaren, 1959; Neurath et al., 1970). Divalent cations Mg^{2+} and Ca^{2+} have also been shown to increase attachment rate of Coxsackie A9 and human rhinovirus type 2 to HeLa cells. This stimulation can be inhibited by EDTA (Lonberg-Holm and Karant, 1972).

In whole plant studies there have been some indications that the presence of certain metallic ions can greatly affect the extent of infection. In 1956, Matthews and Proctor showed that when

$Mg(NO_3)_2$ at 10 mM was included in tobacco necrosis virus inoculum, number of lesions on Phaselus vulgaris cv. 'Black Prince', approximately doubled over the no $Mg(NO_3)_2$ control. $Ca(NO_3)_2$ at 10 mM had no effect on lesion numbers when applied alone, although it did partially annul the inhibitory (hypothesized to be chelating) effects of 0.01 N succinic acid. Potassium and ammonium nitrates had no effect on the production of local lesions. Matthews remorses that "With bacterial viruses it is possible to control the concentration of compounds in the medium in which virus establishment occurs. No such control is possible with plant leaves." Certainly the addition of various metal ions in various ionic strengths is a simple matter in protoplast-virus studies.

One group has reported having studied effects of Ca^{2+} and Mg^{2+} on protoplast infection. Furusawa and Okuno (1978) have observed that $MgCl_2$ and $CaCl_2$ at 10 mM inhibit infection of wheat, barley, maize and Japanese radish mesophyll protoplasts with brome mosaic virus. No data or methods were given.

However, in the tissue culture system studied here, ionic strength and composition proved to be exceedingly important with SBMV and CPMV. Calcium salts in particular greatly stimulated infection.

CPMV infection: Infection of soybean tissue culture protoplasts by CPMV was stimulated to different degrees by Mg^{2+} and Ca^{2+}. When 1 mM $Mg(NO_3)_2$, $MgSO_4$ or $MgCl_2$ were included in the inoculation medium, infection increased approximately 40% over the control where no divalent cation was added. This stimulation was strongly inhibited by the addition of ethylenediamine tetraacetic acid (EDTA), indicating chelation of the stimulatory cation(s).

A more marked, seven-fold increase was obtained when 0.5 mM $CaCl_2$ was included in the inoculum. This is quite a dramatic increase in infection, particularly since the level of Ca^{2+} ions required is quite low.

These salts could be having several effects on the system.

(a) These divalent cations (especially Mg^{2+}) could be having an effect by stabilizing the virion or the viral RNA, the net effect being an increased specific infectivity of the population.

(b) The effect may be largely at the membrane. Calcium in particular is well known to have manifold effects on fluidity and permeability of the membrane (Hauser et al., 1976), on binding sites and a whole host of other phenomena. It is almost inconceivable that Ca^{2+} would not have membrane effects in this system. It is highly possible that Ca^{2+} alters the membrane, perhaps at quite specific sites, allowing it to be more readily accessible to the virus.

(c) A third ion effect may be involved here either alone or, likely, in combination with one or both of the above. This is that an effect on the virus-membrane interaction is occurring. It is possible that calcium ions can form a bridge between virus and cell. There is certainly precedence for Ca^{2+}-induced binding between membranes (Kretsinger, 1976), and there is some indication that this type of interaction can occur between cells and macromolecules (Lonberg-Holm and Philipson, 1974).

SBMV infection: Similar phenomena could well be occurring during the SBMV infection of protoplasts. However the observation that this infection appears to be stimulated by a combination of magnesium ions and calcium ions suggests that perhaps virus stabilization is a substantial factor (Magnesium effect) as well as calcium-mediated membrane or virus-membrane effects.

The observation that a pre-inoculation $CaCl_2$ wash greatly decreases the calcium-stimulated CPMV infection leads to further speculation. In this case, calcium appears to be having a direct effect on the protoplasts. This could be occurring in several ways:

1) At 10 mM, $CaCl_2$ could be altering the net charge or configuration of the hypothesized binding site, making conditions unfavorable for virus adsorption and/or uptake.

2) $CaCl_2$ at 10 mM may be having a profound effect on the fluidity of the membrane. Its addition may have an inhibitory effect on the lateral or vertical movement in the membrane of any receptor, a function which might be necessary for binding (see next section on temperature).

3) A third hypothesis is that it might be essential that the membrane be exposed simultaneously to the virus and to calcium, and calcium pre-treatment inhibits the virus-calcium-membrane interaction necessary for infection.

The decrease in infection at low inoculation temperatures is interesting for several reasons. First, it has not been reported for any other protoplast-virus system. Indeed, the reverse has been reported for one system (Alblas and Bol, 1977) and no temperature

effect has been found for others (Motoyoshi et al., 1974b). Secondly, temperature-sensitivity provides a handle for the study of virus-membrane interactions. In these initial experiments, the transition appears to be rather distinct between the great increase in infection with temperature at low temperatures and a very small increase with temperatures above about 13-15 C. This observation, if it is substantiated by further experimental data, would be particularly valuable for understanding virus-membrane interaction.

Speculations can be made regarding the basis for this temperature-sensitivity. It has been hypothesized in the case of temperature-sensitive animal-cell interactions (Holland and McLaren, 1959; Lonberg-Holm and Philipson, 1974) that a multi-protein receptor complex is necessary for virus adsorption and penetration. Thus the virus binds weakly to an initial receptor, and a second (or more) receptor must migrate to that binding site and engage in the interaction, forming a complex. This latter migration would of course be temperature-sensitive.

Penetration of the virus into the membrane, if it is an active process, would also be inhibited at lower temperatures. Takebe (1978) has hypothesized that virus entry occurs by a pinocytotic mechanism which would be sensitive to low temperatures.

SUMMARY

A procedure has been described for the efficient infection of soybean protoplasts isolated from tissue culture with two plant viruses.

Infection by southern bean mosaic virus requires the presence of poly-L-ornithine, and is stimulated by a combination of magnesium and calcium salts. The infection occurs in the presence of Tris-HCl buffer at pH 8.0 and several other buffers having relatively high pK values.

Infection by cowpea mosaic virus also requires poly-L-ornithine. This infection is stimulated seven-fold by the addition of 0.5 mM $CaCl_2$. Of the buffers tested, only K phosphate was effective at stimulating infection. Infection occurred in a narrow pH range around pH 6.3. The infection could be inhibited by washing the protoplasts with $CaCl_2$ prior to inoculation. Infection was also considerably decreased at temperatures below 10 C.

This system is highly suitable for many plant virus-host studies for the following reasons:

1) Sterility may be maintained.

2) Protoplast source tissue is uniform and reproducible.

3) A constant tissue culture source is easily maintained.

We hope that these advantages will make this new system valuable for the study of many aspects of plant virology. The calcium- and temperature-dependency of the interaction should provide a probe for further virus-membrane interaction studies.

APPENDICES

APPENDIX A

Soybean callus growth medium study

Table A1. Growth of Soybean Callus on Agar Medium Supplemented with Various Hormones*

Hormonal Composition (μg/ml)				Increase in weight (g)	Callus characteristics
2'4'D	Kinetin	2ip	1AA		
-	-	-	-	-5	no growth
-	-	0.3	-	-6	no growth
1.0	-	-	-	352	very friable, soft callus
1.0	0.3	-	-	1365	friable, dry callus
1.0	0.3	-	0.5	1135	same
1.0	-	0.3	-	1401	friable, moist callus
1.0	-	0.3	0.5	1355	same
0.5	0.3	-	5.0	1352	very compact, hard crumbly callus
0.5	-	0.3	5.0	1277	same

*Sections of soybean hypocotyl were grown on R3 minerals, vitamins and sugars (Table 1) containing 0.8% agar, and supplemented with various combinations of hormones. After 2 months on agar medium, calluses were harvested and wet weights were recorded.

2'4'D alone stimulated some growth, but increased proliferation could be achieved by the addition of a cytokinin. While the addition of 1AA does not appear to further stimulate growth, its presence alters the callus characteristics. R3 medium, which contains 2'4'D, kinetin and 1AA, produced a very compact callus, having small and densely packed cells. R3A medium, containing twice as much 2'4'D and no 1AA, however, stimulated a friable and loosely-growing callus of large (highly vacuolated) cells. The compact callus grown on R3 proved to be most readily adapted to liquid suspension culture.

APPENDIX B

Further inoculation buffer investigations

Several sulfonic acid buffers were tested for their effectiveness in allowing or stimulating infection of soybean protoplasts by CPMV and SBMV. As shown in Table A3, CPMV infection was obtained with several of these compounds. These experiments demonstrate the following points: 1) Infection occurred in the presence of phosphate buffer only if poly-L-ornithine was included in the inoculum, 2) All the sulfonic acid buffers with the exception of TAPS allow some infection with or without poly-L-ornithine. 3) Tricine, a non-sulfonic acid buffer, does not cause CPMV infection under any conditions tested. 4) Percentage infection by phosphate is fairly constant between experiments, but there is considerable variation between percentage of infection in those experiments using sulfonic acid buffers. 5) The pK's of all the compounds used are much higher than 5, so that none of these compounds (with the possible exception of MES) are buffering substantially at the pH used in these experiments. Therefore their stimulatory effect is one other than buffering.

Viability (data not shown) was far better for phosphate and tricine than any of the sulfonic acid buffers, especially MES. Therefore, due to buffer toxicity and variation in experimental results, under these conditions the sulfonic acid buffers are not suitable for use for protoplast inoculations. However, variation of pH, buffer concentration, etc. may alleviate these problems and may make their use more desirable.

SBMV infection could be stimulated by the sulfonic acid buffer HEPES. Results are shown in Tables A3-A5. Poly-L-ornithine was not essential for infection nor in most cases was significantly stimulatory (Tables A3 and A4). SBMV infection occurred equally well at inoculation temperatures of 4C and 23C (Table A3). From Table A4 it can be seen that lower HEPES pH favored a high percent of infection. Protoplast stability decreased greatly at a pH less than 5 (data not shown).

In the presence of 10 mM HEPES, pH 5.0, percent of protoplasts infected increased as the concentration of SBMV in the inoculum was increased (Table A5). This was also found to be the case for HEPES-stimulated CPMV infection (Table A6). No poly-L-ornithine was included in the inoculation medium in these two experiments.

Therefore, it can be concluded from these experiments that the characteristics of sulfonic acid-stimulated virus infection of soybean protoplasts differ dramatically from those observed for phosphate- or Tris-HCl-stimulated infections described in the body of this thesis. The major differences are (1) No poly-L-ornithine is required for sulfonic acid-stimulated infections while a polycation is absolutely essential in obtaining infection with other buffers. (2) Sulfonic acid-stimulated infection is equally effective at 4C and 23C; CPMV infection with PLO and phosphate is temperature sensitive (no data for SBMV). (3) Using phosphate and PLO or Tris-HCl and PLO, saturation is reached at a very low virus concentration, while with HEPES, even at 40-50 µg/ml, increasing virus concentration still causes an increase in percent of cells infected. (4) Viability is much

poorer with any of the sulfonic acid buffers at pH 5.0 than with phosphate or Tris-HCl at higher pH's.

It appears that different mechanisms are involved in those infections occurring with sulfonic acid buffers and those obtained with non-sulfonic acid buffers. It is possible that the sulfonic acid buffers may be allowing infection simply by injuring the membrane and permitting entry of the virus particles, while the function of other less toxic buffers is to provide an environment which allows a more complex virus-cell interaction. Much more work must be done, however, to reveal the respective roles these buffers play in the infection process.

Table A2. CPMV Infection of Protoplasts With or Without Poly-L-ornithine in the Presence of Various Inoculation Buffers[a]

		Percent Viable Protoplasts Infected[b]										
		Experiment										
Buffer	pH	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11
Phosphate	6.5	-	-	-	-	-	16(0)	27(0)	19(0)	14(0)	17(0)	16(0)
Tricine	5.0	0(0)	-	-	-	-	0(0)	-	-	-	-	0(0)
HEPES	5.0	6(2)	17(2)	69(18)	18(0)	0(0)	21(12)	10(2)	8(2)	14(0)	3(0)	-
TAPS	5.0	-	0(0)	-	-	-	-	-	-	-	-	-
TAPSO	5.0	-	28(7)	-	-	-	-	-	-	-	-	35(13)
MOPS	5.0	-	-	75(36)	-	-	-	6(23)	-	-	22(2)	-
MOPSO	5.0	-	83(64)	-	-	-	-	71(55)	-	-	53(15)	-
BES	5.0	-	-	-	-	-	-	45(31)	-	-	-	30(29)
MES	5.0	-	-	83(83)	-	-	-	-	-	-	36(31)	-
HEPPS	5.0	40(40)	-	-	-	-	43(21)	-	-	55(34)	-	-
HEPPSO	5.0	53(50)	-	-	-	-	76(41)	-	-	37(31)	-	-
DIPSO	5.0	-	54(20)	-	65(60)	33(5)	-	-	-	-	-	53(20)

[a]Protoplasts were inoculated with 15 µg CPMV/ml with or without 2 µg poly-L-ornithine/ml in the presence of various buffers at 10 mM. Percent infection was determined by harvesting cells at 48 hours, staining with CPMV-specific fluorescent antibody and examining for percent fluorescence.

[b]Number in parentheses represents percent infection in the absence of poly-L-ornithine.

Table A3. SBMV Infection of Protoplasts under Various Inoculation Conditions in the Presence of HEPES Buffer*

SBMV concentration (µg/ml)	PLO concentration (µg/ml)	temperature of inoculation	percent viable protoplasts infected
8.3	0	4	30
8.3	1.0	4	47
8.3	0	23	33
8.3	1.0	23	35
16.6	0	4	45
16.6	1.0	4	32
16.6	0	23	39
16.6	1.0	23	31

*Protoplasts were inoculated with 8.3 or 16.6 µg SBMV/ml with or without 1.0 µg poly-L-ornithine/ml (PLO) in the presences of HEPES buffer. Percent of protoplasts infected was determined by harvesting protoplasts at 48 hours, staining with SBMV-specific antibody and examining for percent fluorescence.

Table A4. SBMV Infection of Protoplasts in the Presence of HEPES at Various pH's*

	Percent Viable Protoplasts Infected	
pH	(-)PLO	(+)PLO
4.8	36	44
5.2	21	32
5.8	10	39
6.0	3	0.5
6.4	0.5	0.5
7.2	0.5	0.5
7.6	0.5	0.5

*Protoplasts were inoculated with 10 μg SBMV/ml with (+) or without (-) 2 μg poly-L-ornithine (PLO)/ml in the presence of 10 mM HEPES buffer at various pH's. Percent of protoplasts infected was determined by harvesting protoplasts 48 hours after inoculation, staining with SBMV-specific antibody and examining for percent fluorescence.

Table A5. SBMV Infection of Protoplasts in the Presence of HEPES Buffer Using Various Virus Concentrations*

SBMV concentration (μg/ml)	percent viable protoplasts infected
0	0
1	5
2	13
5	21
10	28
15	28
20	45
30	52
40	56
50	61

*Protoplasts were inoculated at 4°C with various concentrations of SBMV in the presence of 10 mM HEPES buffer, pH 4.8. Percent infected protoplasts was determined by harvesting protoplasts 48 hours after inoculation, staining with SBMV-specific fluorescent antibody, and examining for percent fluorescence.

Table A6. CPMV Infection of Protoplasts in the Presence of HEPES Buffer using Various Virus Concentrations*

CPMV concentration (µg/ml)	percent viable protoplasts infected
0	0
1	1
5	2
10	9
15	26
20	28
30	40

*Protoplasts were inoculated at 23°C with various concentrations of CPMV in the presence of 10 mM HEPES buffer, pH 5.0. Percent infected protoplasts was determined by harvesting protoplasts 48 hours after inoculation, staining with CPMV-specific antibody, and examining for percent fluorescence.

APPENDIX C

Magnesium concentration in the inoculum and its effect on CPMV infection of protoplasts

Table A7. Effect of $Mg(NO_3)_2$ on Infection of Protoplasts when Present in the Inoculation Medium at Various concentrations*

$Mg(NO_3)_2$ concentration	% fluorescent protoplasts
0	8
0.5	17
1.0	30
2.0	30
3.0	30
5.0	36
10.0	33

*Protoplasts were inoculated with 5 µg CPMV/ml in the presence of 2 µg poly-L-ornithine/ml, 10 mM K phosphate buffer, pH 6.3 and various concentrations of $Mg(NO_3)_2$. Protoplasts were harvested at 48 hours and percent fluorescence was determined by staining with fluorescent antibody to CPMV.

It can be seen from Table A7 that infection is maximally stimulated at 1 mM $Mg(NO_3)_2$. Raising the concentration past this level does not further stimulate infection.

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LITERATURE CITED

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