STUDIES ON THE BIOSYNTHESIS OF SOUTHERN BEAN MOSAIC VIRUS IN SOYBEAN CALLUS CELLS

I. EFFECTS OF DIFFERENT CULTURAL CONDITIONS ON INFECTION

II. SYNTHESIS OF VIRUS AND VIRUS-INDUCED RNA

III. ELECTRON MICROSCOPY OF VIRUS DISTRIBUTION AND CYTOPATHIC EFFECTS

Dissertation for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY

FANG-SHENG WU

1977

This is to certify that the

thesis entitled

Studies on the biosynthesis of southern bean mosaic virus in soybean callus cells. I. Effects of different cultural conditions on the infection. II. Synthesis of virus and virus-induced RNA III. Electron microscopy of virus distribution and cytopath. effects. presented by

FANG-SHENG WU

has been accepted towards fulfillment
of the requirements for

Ph. D. degree in Botany and Plant Pathology

Harry H. Murakishi
Major professor

Date August 12, 1977

O-7639

ABSTRACT

STUDIES ON THE BIOSYNTHESIS OF SOUTHERN BEAN MOSAIC VIRUS
IN SOYBEAN CALLUS CELLS
I. EFFECTS OF DIFFERENT CULTURAL CONDITIONS ON INFECTION
II. SYNTHESIS OF VIRUS AND VIRUS-INDUCED RNA
III. ELECTRON MICROSCOPY OF VIRUS DISTRIBUTION AND CYTOPATHIC EFFECTS

By

Fang-Sheng Wu

Soybean (Glycine max 'Harosoy') callus cells grown in different media were inoculated with southern bean mosaic virus (SBMV) and then incubated in either liquid or agar media. The composition of the medium and whether the medium was liquid or agar significantly affected the growth curve of the virus. The liquid grown-liquid incubation conditions using Linsmaier and Skoog medium under constant shaking gave the fastest growth rate of virus. Vortexing of the cell suspension-virus inoculum was not required for infection.

To improve the synchronization of virus multiplication the inoculated cells were preincubated at 6°C for 4 days and then transferred to 25°C. The synthesis of ^{3}H-uridine labeled complete virus closely paralleled the increasing rate of infectivity and reached maximum during 37 to 45 hour incubation period after transfer to 25°C. The total amount of ^{3}H-uridine incorporated into cells dropped sharply while the synthesis of complete virus was in its exponential phase. An RNA with a molecular weight close to the supposed replicative form (RF) of SBMV-RNA was synthesized in significant amounts during 26 to 34 hour incubation period preceding the major period of virus RNA

synthesis which reached maximum during 40 to 48 hours. Pulse-chase experiments revealed the possible precursor role of RF and replicative intermediate (RI) in the synthesis of virus RNA. The RNase resistant analysis of LiCl-soluble RNA showed the presence of RF in infected cells. Accumulation of a heterodisperse high molecular weight RNA with its polyacrylamide gel electrophoresis profile of radioactivity corresponding to RF + RI of SBMV-RNA was detected in infected cells incubated at 6°C from 84 to 96 hours. Actinomycin D did not inhibit the synthesis of viral-related RNA and was employed through the biochemical studies.

Virus particles were observed in cytoplasm, vacuole, and nuclei of infected cells under the electron microscope. Crystalline arrangements of virus particles were seen in cytoplasm 10 days after inoculation. During later stages of infection (10 days and after), the virus particles were most often found in vacuoles as irregular clumps. Association of virus particles with a double membrane structure which was freely distributed in cytoplasm occurred in the early stage of infection (5 days). The cytoplasm of infected cells contained inclusions of various types. Amorphous bodies and dark irregular spots mixed with striated diffuse fibers were seen most frequently. Microfilaments were commonly present in cytoplasm, nuclei, and occasionally in mitochondria and chloroplasts. The cristae of mitochondria was firmly packed and electron-dense substances were present. Phytoferritin particles frequently accumulated in chloroplasts and many single membrane-bounded vesicles were observed adjacent to chloroplast membranes. Paramural bodies largely present between cell wall and plasmolemma and the plasmodesmata in cell wall occasionally contained virus particles.

STUDIES ON THE BIOSYNTHESIS OF SOUTHERN BEAN MOSAIC VIRUS IN SOYBEAN CALLUS CELLS

I. EFFECTS OF DIFFERENT CULTURAL CONDITIONS ON INFECTION

II. SYNTHESIS OF VIRUS AND VIRUS-INDUCED RNA

III. ELECTRON MICROSCOPY OF VIRUS DISTRIBUTION AND CYTOPATHIC EFFECTS

By

Fang-Sheng Wu

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

1977

This dissertation is dedicated to

my parents and wife

ACKNOWLEDGMENTS

I wish to express my deepest appreciation and gratitude to Professor Harry H. Murakishi for his guidance and enthusiasm during the course of this study. His ready help in the preparation of the manuscript is gratefully acknowledged. His continuous interest and dedication to the area of plant virology provided the atmosphere and incentive that made my graduate study a continuing adventure.

Special appreciation is expressed to Dr. Gary R. Hooper, whose breadth of knowledge, enthusiasm and assistance in the ultrastructure studies allowed the accomplishment of Part III of this dissertation. His generosity in providing the equipment, supplements, and help for the electron microscopic work is gratefully acknowledged. The capable technical assistance of Mrs. June Mack and Dr. Flegler Stanley is also deeply appreciated.

My gratitude is expressed to members of the guidance committee, Drs. Peter Carlson and Gene Safir, for their critical evaluation of the dissertation and their efforts on behalf of my doctoral program. The inspiration and assistance in tissue culture work by Dr. Carlson was extremely valuable.

I would like to thank Drs. Robert Bandurski, Norman Good, Philip Filner, Hans Kende, and Peter Wolk for the use of their instruments without which the biochemical studies of this dissertation would not have been possible.

Thanks are extended to the faculty members, graduate students, and secretaries of this department who contributed in many ways to the advancement of this study and who made my tenure as a graduate student here a memorable one. In this regard, special thanks are due to Dr. William Tai, for his encouragement and Miss Nancy Jarvis for her friendship and assistance in the preparation of this manuscript.

The continuous moral support of my father and mother from thousands of miles away across countries are above all beyond thanks.

My fondest appreciation is given to my wife, Meng-Ying, for her patience, understanding, and willingness to sacrifice her time and leisure that I might complete this work, and to my daughter, Minming, for her enlivening smiles which greatly inspired me.

I would finally like to thank the Department of Botany and Plant Pathology for selecting me as the 1977 Ernest A. Bessey Award winner in recognization of high scholarship and research. I feel greatly honored in receiving this prize.

Financial support which made this research possible was given by Michigan Agricultural Experiment Station and the State of Michigan.

TABLE OF CONTENTS

LIST OF FIGURES

GENERAL INTRODUCTION AND LITERATURE REVIEW

The study of the process in which viruses reproduce in cells is one of the most interesting and active areas of modern plant virology. Success of bacteriophage replication studies provided models for investigating the infection process of other types of viruses. It also inspired animal virologists as well as plant virologists to develop techniques for infecting cells in culture so that the study of virus replication would be free from the complicating factors of the whole organism. The progress of plant virus replication studies have lagged behind those of animal and plant bacterial viruses. This is largely due to the unavailability of a plant cell system that provides synchronized virus infection as with bacterial and many animal viruses. Obviously, to develop such a system is one of the most important requirements for progress in the understanding of plant virus replication.

To achieve this goal, tobacco tissue culture-tobacco mosaic virus (TMV) has been studied (Kassanis et al., 1958; Motoyoshi and Oshima, 1968; Hildebrandt, 1973). The efficiency of infection and the multiplication rate of TMV in their systems have been too low to allow biochemical study of virus replication. Murakishi et al. (1970, 1971) improved the system by vortexing friable, suspension cultures of tobacco callus with virus suspension and incubating the vortexed callus on agar medium. This system was subsequently proved to be successful in biochemical studies of TMV replication (Pelcher et al, 1972;

Beachy, 1973). Their success should encourage the study of other plant virus replication by using tissue culture as a starting material.

Part I of this dissertation concerns the investigation of parameters which affect the infection and multiplication of southern bean virus (SBMV) in soybean tissue culture cells. This was an effort to establish a cultured cell system that would provide high multiplication rate and improved synchronization of virus infection so that it could be used to study the biochemical process of SBMV replication.

SBMV is a spherical virus, having a single stranded RNA with molecular weight of 1.39×10^6 in its virion (Diener, 1965). The ease of obtaining SBMV in large quantities and the availability of a dependable host for the bioassay of virus should have facilitated biochemical investigations of its replication in vivo. Nevertheless, no report was found on how SBMV is replicated in the cells.

It is generally accepted that replication of most RNA viruses occurs by means of a double-stranded RNA replication intermediate (Bishop and Levintow, 1971). Evidence for the presence of virus-induced double-stranded RNA in leaves infected with TMV (Jackson et al., 1972), barley stripe mosaic virus (Pring, 1971), pea enation mosaic virus (German and de Zoeten, 1975), and many other plant viruses have been reported (Siegel and Hariharasubramanian, 1976). This double-stranded RNA is RNase resistant and consists of a complete viral RNA strand (plus strand) and a complete complementary strand (minus strand) (Bishop and Levintow, 1971). This type of RNA has been called the replicative form (RF). Another type of double-stranded RNA which is

characterized by single stranded tails of different lengths attached to base-paired duplex of constant size is called replicative intermediate (RI). It is also found in cells infected by many plant viruses (Siegel and Hariharasubramanian, 1976).

RF has been suggested to be a by-product in the synthesis of viral RNA and is produced as a consequence of the turnover of RI (Baltimore and Girard, 1966; Baltimore, 1968). It has also been shown that the formation of RF precedes that of RI during the in vitro synthesis of QB RNA by its specific replicase (Pace et al., 1968). Thus, there appeared to be two classes of RF molecules, one is a precursor to RI and another is an inactive endproduct produced during the replication.

In the replication of RNA phages, the minus strand RNA is the first progeny strand which can be detected in infected cells and its synthesis and accumulation continue until RNA synthesis ceases (Bishop and Levintow, 1971). The newly synthesized RI which contains newly formed minus strand turns over giving rise to increasing amounts of stable, inactive RF (Baltimore, 1968). It is therefore suggested that RI or its in vivo equivalent, is the immediate precursor to viral progeny RNA. In TMV, the amount of RI is greater than RF at early stages of TMV infection (Kielland-Brandt and Nilsson-Tilgren, 1973). This is consistent with the above conclusion. Further studies by Kielland-Brandt (1974) showed that minus strands are synthesized at the highest rate at vein-clearing or shortly after. Thereafter, the minus strands are synthesized at a twofold lower rate when the main

accumulation of plus strands occurs. The amount of minus strand remains at 0.1% of the total RNA when their breakdown is small. Most, if not all, minus strands are present in completely RF or partially double-stranded structures in RI after the extraction. RF and RI are probably interconvertible in the process of plus-strand synthesis and are the precursors of TMV RNA.

Part II of this dissertation concerns investigation of the process of SBMV replication in cells: the kinetics of virus particle formation in relation to virus RNA synthesis and the involvement of RF and RI in the replication of SBMV RNA. Whether the SBMV replication follows the general model stated above for the replication of other RNA viruses will be discussed.

The association of ultrastructural components with double stranded RNA formed during virus replication has been established in turnip yellow mosaic virus (TYMV) (Ralph et al., 1971) and cowpea mosaic virus (CPMV) (deZoeten et al., 1974). By high resolution autoradiography and RNase studies in TYMV infected cells, it has been shown that ^{3}H-uridine labelled viral specific RNA synthesis in the presence of actinomycin D was specifically associated with chloroplast vesicles and was RNase resistant. The biochemical studies revealed that virus specific RNA-polymerase was associated with chloroplast fractions which contain vesicles as judged by electron microscopy of pelleted fractions. It was therefore concluded that the vesicles of the chloroplast was associated with RF as well as viral RNA-polymerase activity and was most likely the site for virus RNA synthesis.

Recently, deZoeten et al. (1974) showed that the RF of CPMV was associated with a cytopathic structure in infected cells. Both high resolution autoradiography and pelleted density gradient fractions from infected cells suggested such association. Molecular hybridization with ^{3}H-uridine labelled virus RNA revealed that the fraction of cytopathic structures contained 90% of the hybridizable material. This is strong evidence that the replication of plant virus is in the cytoplasm.

It should be noted that only in the case of TMV, TYMV, CPMV, where cell fractionation methods were supplemented by electron microscopy, has localization of the site of viral genome replication been achieved. Other plant viruses seem to evoke membraneous structures in infected cells reminiscent of those observed in CPMV-infected cells, as is the case for polio virus (DeZoeten, 1976). Therefore, as a first step in associating the biochemical steps of SBMV replication with cytopathic structure at the ultrastructure level in infected cells, the localization of virus in infected cells and cytopathic effects caused by virus infection were investigated. Part III of this dissertation is undertaken for such studies.

PART I

EFFECTS OF DIFFERENT CULTURAL CONDITIONS ON INFECTION

Ine

ells or p

ology st

ketchy an

., 1970,

separate

35) hav

ation an

callus

with ra

., 1955

as attr

ell (ha

efficien

the p

ism.

ducate

ignifi

udied

Most e

INTRODUCTION

The prospect of using pipettable suspensions of virus infected cells or protoplasts has elicited considerable excitement among plant virologists according to Zaitlin and Beachy (1974). Callus cultures (Beachy and Murakishi, 1970; Motoyoshi and Oshima, 1968; Murakishi et al., 1970, 1971; Murakishi and Pelcher, 1974; Pelcher et al., 1972), separated cells (Zaitlin and Beachy, 1974), and protoplasts (Takebe, 1975) have been used by different investigators to study virus replication and virus-host interactions. Early studies of virus infection of callus cultures resulted in low efficiency of infection and slow growth rate of virus (Hildebrandt, 1973; Kassanis, 1967; Kassanis et al., 1958; Linsmaier and Skoog, 1965). The low efficiency of infection was attributed to the inability of virus to penetrate the cellulose wall (Hildebrandt, 1973). Murakishi et al. (1970) improved infection efficiency by vortexing friable, suspension cultures of tobacco callus in the presence of virus and incubating the vortexed callus on agar medium. When callus was not vortexed or when inoculated cells were incubated in liquid medium the infection efficiency was reduced significantly (Murakishi et al., 1971).

Plant virus-plant tissue culture relationships have been studied so far using tobacco tissue and tobacco mosaic virus (TMV) almost exclusively (Hildebrandt, 1973; Kassanis, 1967; Motoyoshi and

Oshima, 1968). Other viruses with different morphology, transmission characteristics and host range have not been studied because a suitable experimental system was not available. Strains of southern bean mosaic virus (SBMV) are world-wide in distribution affecting primarily bean (Phaseolus vulgaris L.), soybean (Glycine max L.) and cowpea (Vigna sinensis L.) (Shepherd, 1971). The SBMV is a small spherical virus with a diameter of 25 mm (Miller and Price, 1946). It may be seed-borne and is transmitted by the bean leaf beetle (Ceratoma trifurcata) as well as by leaf rubbing (Shepherd, 1971). The yield of SBMV from intact plant tissue is relatively high; the purirification procedure of SBMV is simple and a dependable bioassay host is also available (Diener, 1965). Callus from its host plants can be easily induced and maintained in chemically defined media. These favorable experimental conditions encouraged us to study SBMV-callus interactions. This report describes several parameters that affect the multiplication rate of SBMV in plant cell cultures. My objective was to determine the parameters of soybean tissue culture system that would provide a high multiplication rate and better synchronization of SBMV synthesis so that it could be used for the study of SBMV replication in cells at macromolecular levels.

MATERIALS AND METHODS

Tissue culture and media. Soybean (Glycine max 'H63') or bean (Phaseolus vulgaris L. 'Prince') seeds were surface-sterilized with 70% ethanol for 3 min and 20% chlorox for 20 min, rinsed in 0.01 N HCl, and washed several times with sterilized distilled water. The seeds were then transferred to sterile 50 ml test tubes in which filter paper was folded and placed at the bottom of the tubes to support the seeds. The seeds were kept moist by the addition of 9 ml medium diluted 1:4 with water. The seeds were allowed to germinate under the same light and temperature conditions used for growing tissue cultures. The hypocotyl was cut into 1 cm segments and placed on designated media solidified with 1% agar. The induced calli were then maintained either on liquid media (liquid-grown) or on media solidified with 1% agar (agar-grown). Gro-lux fluorescent lamps (80 foot candles) and a temperature of 24°C ± 1°C were used under all growth conditions. Unless otherwise specified, calli grown in liquid media were incubated in Erlenmeyer flasks and placed on a rotary shaker at 120 rpm.

R3 medium is composed of Linsmaier and Skoog minerals (1965), and 30 g sucrose supplemented with 0.5 mg pyridoxine, 0.5 mg nicotinic acid, 0.5 mg thiamine-HCl, 5.0 mg indole-3-acetic acid, 0.3 mg kinetin, and 0.5 mg 2,4-dichloroacetic acid (2,4-D) per liter of medium. The pH was adjusted to 5.8 with 0.2 N NaOH solution.

E152 medium was from Eriksson (1965) which had been found suitable for growing SBMV-infected bean callus (Murakishi and Pelcher, 1974). This medium contained 0.10 mg 2,4-D, 0.5 mg Naphthalene acetic acid (NAA) and 0.02 mg kinetin per liter of medium. E115 medium was Eriksson's medium containing 0.10 mg 2,4-D, 0.10 mg NAA and 0.02 mg kinetin per liter of medium. E152 provided better callus growth on agar medium than in liquid medium, while E115 produced the opposite effect.

Virus preparations. Bean strain of SBMV was propagated in leaves of Prince bean. Plants grown in greenhouse were harvested 3 to 4 weeks after inoculation and stored at -25°C until used. The virus was purified by homogenizing infected leaves in a 0.5 M potassium phosphate buffer, pH 7.5. The resultant slurry was passed through 4 layers of cheesecloth and N-butanol was then added to the sap to a final concentration of 10%. After stirring for 30 minutes, the mixture was centrifuged at 10,000 rpm for 10 minutes in a Sorvall SS-34 rotor. $(NH_4)_2SO_4$ was then added to the supernatant to a final concentration of 60% with constant stirring for 20 min and stored at 4°C for 1 hr. The precipitates were collected by centrifugation and resuspended in distilled water. The suspension was subjected to 4 or 5 cycles of differential centrifugation (12,000 x g for 15 min and 97,000 x g for 1 hr). The final pellet was resuspended in 0.01 M phosphate buffer, pH 7.2, and was freed of microorganisms by filtering through a 0.45 μ millipore filter.

Inoculation of virus. Callus grown either on agar or in liquid medium was collected in 1 g batches and placed in 50 ml test tubes containing 3 ml of liquid medium and SBMV at a concentration of 180 µg/ml. Unless specified, the mixture of callus and virus was immediately vortexed with a Model S8220 vortex mixer (Scientific Products, Evanston, Illinois) for 20 seconds at approximately 800 rpm. In experiments in which vortexing was omitted the callus-virus mixture was agitated on a rotary shaker at 120 rpm as is normally done when growing callus cells. After vortexing or shaking, the cells were collected on Miracloth-lined funnels and washed 3 times with fresh medium. To randomize the cell clumps and enhance the removal of unabsorbed viruses, the cells were stirred gently with a spatula during the washing. After washing, cells were transferred to liquid or agar medium. Incubation was under the same light and temperature conditions as those used for maintaining callus cell growth. The liquid medium was periodically renewed by adding fresh medium and the callus grown on agar medium was periodically transferred to fresh medium. This process of regular subculture maintained actively growing cells. At various periods after incubation, the cells were collected, weighed and stored at -25°C until used for infectivity assays.

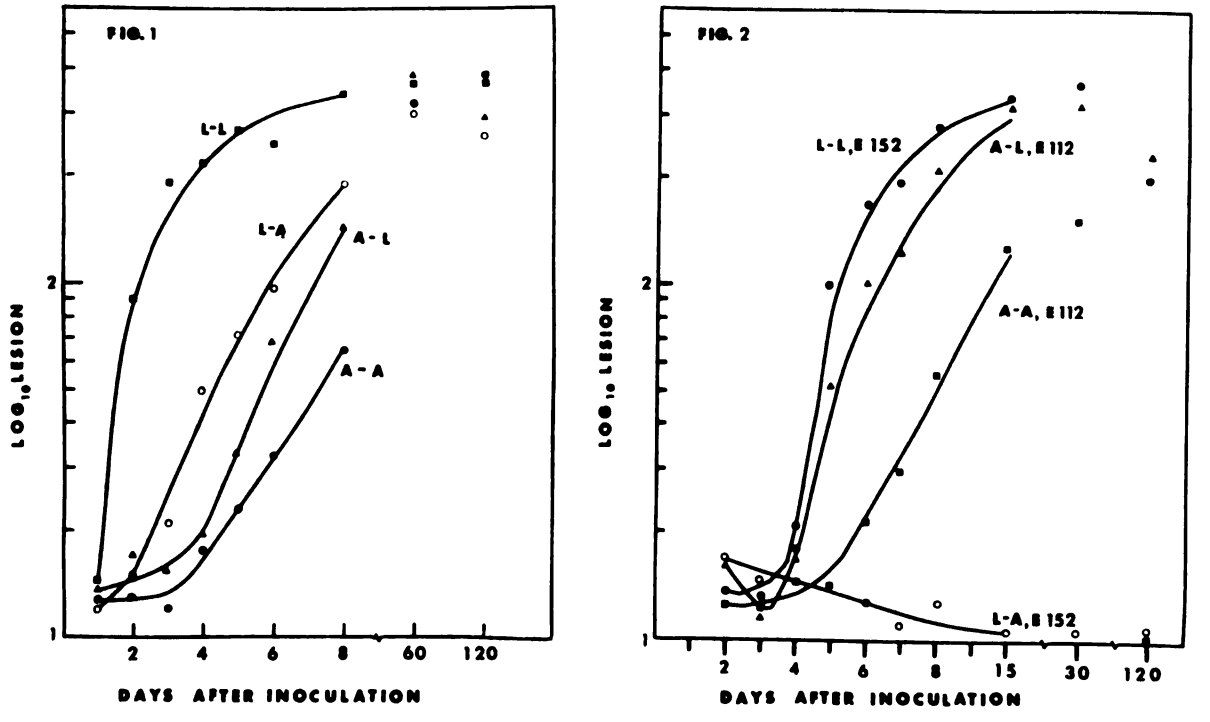
Bioassay. Frozen callus cells were homogenized with 3 ml of cold 0.5 M phosphate buffer, pH 7.5 per gram of callus in a Tenbroeck tissue grinder. The homogenized sap was diluted to 10^{-1} with 0.01 M phosphate buffer pH 7.1 for bioassay. Fully expanded-primary leaves of Pinto bean were used fro assay by the half-leaf method with 8-10

replications for each determination. The plants were preincubated in the dark for 4 days before the bioassay. This procedure increased the lesion number and size and gave more consistent results than non-predarkened leaves.

RESULTS AND DISCUSSION

Effects of liquid or agar medium. Callus cells grown either in liquid or on agar were inoculated with virus and then incubated in either liquid or agar. The four combinations of liquid and agar media resulted in different multiplication rates of virus in cells (Figures 1 and 2). When the R3 medium was used (Figure 1), the liquid grown-liquid incubation condition gave the fastest synthesis rate of virus in cells. Logarithmic increase of virus titer occurred at 2 days after inoculation and reached a maximum at 5 days. The agar grown-agar incubation condition had a 5 to 6 days lag period with a slow increase in virus multiplication rate occurring during the first 8 days after inoculation. The liquid grown-agar incubation condition did not show significant increase in virus titer until 4 days after inoculation. The virus titer increased slowly thereafter until it reached almost a maximum 8 days after inoculation. The agar grown-liquid incubation condition had a virus synthesis rate similar to liquid grown-agar incubation conditions but there was a 1 day delay in the initial increase of infectivity.

In all four conditions, the total amount of infectivity was approximately equal at 60 days after inoculation and the high amount of infectivity was maintained at 120 days. Lesion number near or above 250 at 10^{-1} dilution was considered maximum, although further dilution



Figures 1 and 2. Growth curve of SBMV in soybean callus cells grown in R3 (Figure 1) or Eriksson medium (Figure 2). The composition of Eriksson medium was either E112 or E152 as in materials and methods. Callus cells grown in liquid medium were inoculated with SBMV and then incubated either in liquid medium (L-L) or on medium solidified with 1% agar (L-A). Callus cells grown on agar-solidified medium were same inoculated with SBMV and then incubated either in liquid medium (A-L) or on agar-solidified medium (A-A). Infectivity was determined at intervals after inoculation.

to 10^{-2} of 60 day and 120 day samples often gave similar numbers of lesions. Not until they were diluted to 10^{-3}, did they show significant differences in lesion numbers which varied greatly among replications. The differences probably resulted from variation in spreading rate of virus in cell clumps rather than from an increase of virus in cells infected at the time of inoculation. Although spreading of virus could contribute to the total infectivity during earlier stages of infection, its amount is insignificant since the rate of virus spread in tissue culture was very slow (Kassanis et al., 1958). The infectivity increase during the first several days after inoculation was considered due to virus synthesis in cells infected at the time of inoculation.

In order to see if the effect of liquid or agar medium on virus multiplication rate was independent of different media used, Eriksson's medium was compared with R3 medium (Figure 2). The liquid grown-liquid incubation sequence in Eriksson's medium showed the most rapid increase in virus synthesis rate. The agar grown-liquid incubation condition gave a similar synthesis rate but had a less steeper curve during its exponential phase. The virus synthesis rate increased very slowly under the agar grown-agar incubation and the total amount of infectivity up to 30 days was less than that of liquid grown-liquid incubation and agar grown-liquid incubation conditions.

After prolonged incubation to 120 days, no infectivity was detected in any of the cultures. It seems possible that the virus was able to initiate multiplication in infected cells but failed to

continue the synthesis in newly divided cells. The virus synthesized in infected cells was then probably diluted by the cell divisions.

The liquid grown-agar incubation condition in Eriksson's medium did not show a detectable level of infectivity increase in the cells. Evidently, not only the composition of the medium but also the forms of the same medium can significantly affect the synthesis of virus in callus cells. The reason why the liquid grown-liquid incubation condition in both R3 and Eriksson's media gave the best results for virus multiplication are not known. Possibly the liquid media provided better growth condition for soybean callus cells and the actively growing cells were suitable for viral replication. When cultures were shifted from agar to liquid medium or vice versa there was a prolonged lag period before virus synthesis resumed and furthermore, the rate of synthesis declined. Interestingly the R3 liquid or agar medium was always suitable for supporting virus synthesis in cells for a long period of time, but when Eriksson's medium was used, only the liquid medium was capable of maintaining virus synthesis. Soybean callus cells grew better in R3 agar medium than in Eriksson's agar medium. From this experiment it may be suggested that actively growing cells in liquid medium are more susceptible to virus infection and that rapid proliferation of cells is essential to maintaining virus synthesis in cells. This is in contrast to TMV in tobacco tissue culture which required liquid grown-agar incubation conditions for high virus yield (Murakishi et al., 1971). Under most of the conditions used, SBMV was able to maintain viability in infected soybean callus for 120 days, the

longest duration so far tested. This is also different from TMV infected tobacco callus which lost TMV infectivity after several transfers of callus cells (Murakishi, unpublished data).

Effect of vortexing. Vortexing dissociates tobacco callus into small aggregates and single cells and probably causes minor wounds which help TMV to enter the cells (Murakishi et al., 1971). If minor wounds which expose the protoplasts to virus were needed for entry of the SBMV into cells, the vortexing of callus or use of abrasives would be required for the infection. As shown in Figure 3, the vortexing procedure was not necessary for SBMV infection of soybean callus cells. The vortexed callus had a steeper exponential increase in the virus growth curve than the unvortexed callus. It is possible the dispersion of cells from callus increased the number of cells coming in contact with virus, and therefore more cells became infected at the time of inoculation. Vortexed callus also produced broken cells and cell debris.

If an affinity between cell walls and virus exists, the cells and cell debris could absorb considerable amounts of virus from the inoculation medium and reduce the virus available for infection. In order to see if this was true, callus cells were vortexed with virus and filtered through Miracloth. The filtrates from vortexed mixture of the callus and virus which contain viruses and small cell debris served as inoculum and were added to healthy callus without further vortexing. This procedure had no effect on the virus synthesis rate in the cells (Figure 3).

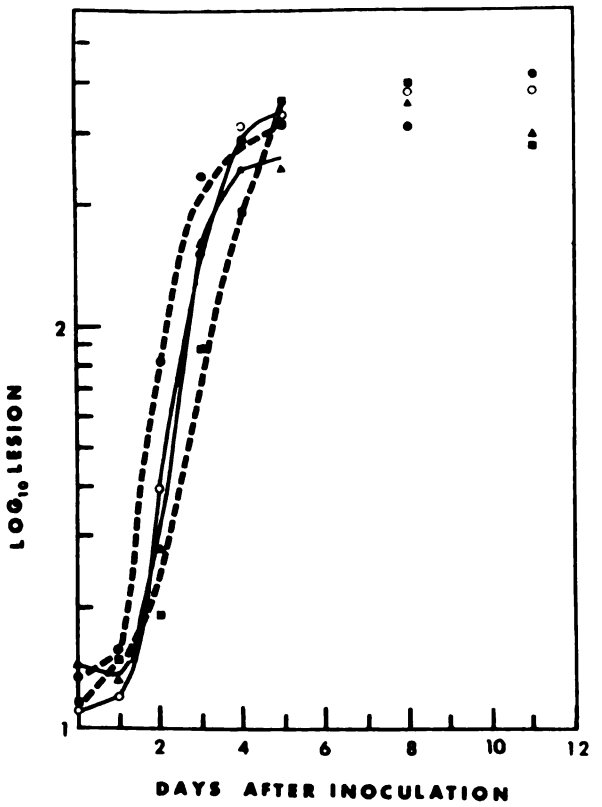


Figure 3. Growth curve of SBMV in soybean callus cells which were either vortexed or non-vortexed during the inoculation of virus. (●---●): vortexed as routine procedures; (o——o): non-vortexed; (▲——▲): non-vortexed with filtrate from vortexed mixture of virus and callus cells as inoculum; (■---■): non-vortexed with filtrate from vortexed healthy callus added into virus inoculum.

As a control to see if cell debris and cell contents released from broken cells might interfere with the susceptibility of callus cells to virus infection, healthy callus cultures were vortexed without virus and then were added together with virus inoculum into healthy callus cultures. The resulting growth curve was similar to other unvortexed samples (Figure 3). It seems therefore that the cell debris did not absorb any significant amount of virus. From our results, it appears that the virus attachment sites are maximally exposed to virus under these inoculation conditions without vortexing.

Effect of shaking medium. Liquid grown-liquid incubation condition in R3 medium provided the best environment for virus synthesis as shown in Figure 1. The medium was continuously shaken as described in the materials and methods section. When the liquid grown cells under shaking conditions were inoculated with the virus and transferred to a petri dish for incubation without shaking, the synthesis rate of virus slowed down and the lag period was prolonged (Figure 4). It was shown from this experiment that even with the same medium, a small change in the incubation condition can significantly affect the virus synthesis rate in tissue culture.

The shaking probably provided better contact between callus cells and the medium than stationary cultures. The initial stage of virus synthesis in cells was probably sensitive to the environmental change but once the cells adjusted to it, virus synthesis resumed its normal rate. This may be the reason for the prolonged lag period in stationary cells.

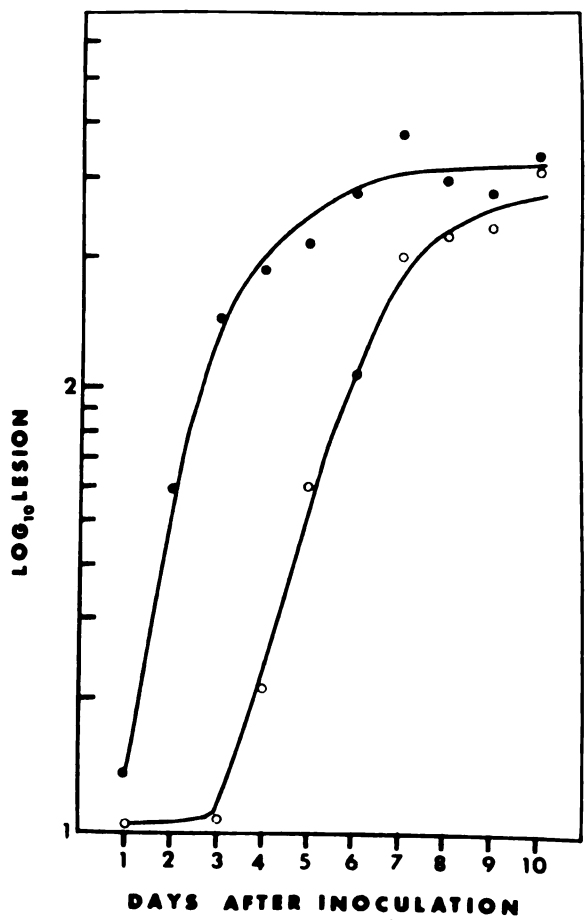


Figure 4. Growth curve of SBMV in soybean callus cells incubated with shaking at 120 rpm (●——●) or without shaking (o——o). Infectivity was determined at intervals after inoculation.

Sources of calli as the host for SBMV. Prince bean was used as propagation host for SBMV throughout the study. The yield of purified virus was about 300 µg per gram of fresh leaf and proved to be a good host for bean strain of SBMV. The callus derived from the plant was thought to be a good host for SBMV as well. Consequently, callus cells induced from different media were inoculated with SBMV and virus synthesis was monitored by bioassay. No evidence of infectivity increase in inoculated cells could be detected under all conditions tested except when Eriksson's medium was used (Table 1). A slight increase in infectivity was found in liquid grown-agar incubation cells and agar grown-agar incubation cells, but infectivity could not be maintained after long incubation periods. The calli from Prince bean were either not susceptible to SBMV infection or if they became infected, did not support a rapid rate of virus synthesis. Ultimately virus infectivity was eliminated from the cells.

Soybean tissue culture was a good host for SBMV as shown here but the soybean plant was a poor host for the same virus. The soybean plants were inoculated at the trifoliate stage and incubated in the greenhouse. After 8 days and 60 days all leaves except inoculated leaves were ground in 0.1 M phosphate buffer, pH 7.0 (1/3:w/v). The crude juice without dilution was then assayed on local lesion host as before. No lesions could be detected for plants inoculated after 8 days and only 15 lesions (average of 12 half-leaves with 4 replications) were shown in the 60 day-old plants.

Table 1. The infectivity of SBMV-inoculated Prince bean tissue culture in Eriksson's medium

Days after inoculation	Local lesion numbers[a]			
	L-L[b]	L-A	A-L	A-A
2	3	3	11	4
4	9	2	2	8
6	2	13	9	14
8	0	52	1	18
30	0	25	0	31
120	0	0	0	0
180	-	0	-	0

[a]Local lesions on Pinto beans without diluting the homogenate.

[b]Abbreviations are given in legend under Figures 1 and 2.

It is obvious that the soybean callus is more susceptible to SBMV infection and is a much better host for the multiplication of SBMV than the intact soybean plants. In contrast, tobacco callus derived from different species and cultures responded to TMV infection in a manner similar to that of intact tobacco plants (Beachy and Murakishi, 1970).

SUMMARY

Soybean (Glycine max 'Harosoy 63') callus cells grown in either Eriksson's or Linsmaier and Skoog's media were inoculated with southern bean mosaic virus (SBMV) and then incubated in either liquid or agar media. The composition and type (liquid or agar) of the medium significantly affected the growth curve of the virus. Assays on Phaseolus vulgaris 'Pinto' leaves indicated that liquid grown-liquid incubation conditions using Linsmaier and Skoog medium gave the fastest virus growth rate. Vortexing of the cell suspension-virus inoculum as is done for tobacco mosaic virus-tobacco callus infection was not necessary with the soybean callus-SBMV system. Infection was achieved by adding virus inoculum to a suspension culture of callus cells, washing the cells with fresh medium and incubating the cells in liquid media on a rotary shaker at 120 rpm.

REFERENCES

BALTIMORE, D. (1968). Structure of the poliovirus replicative intermediate RNA. J. Molec. Biol. 32: 359-368.

BALTIMORE, D., and GIRARD, M. (1966). An intermediate in the synthesis of poliovirus RNA. Proc. Natl. Acad. Sci. U.S. 56: 741-748.

BEACHY, R. N., and MURAKISHI, H. H. (1970). Local lesion and viral crystal formation in tissue culture cells of different species and cultivars of Nicotiana. Phytopathology 60: 1283. (Abstr.)

BEACHY, R. N., and MURAKISHI, H. H. (1973). Effect of cycloheximide on tobacco mosaic virus synthesis in callus from hypersensitive tobacco. Virology 55: 320-328.

BISHOP, J. M., and LEVINTOW, L. (1971). Replicative forms of viral RNA: Structure and function. Progr. Med. Virol. 13: 1-82.

DeZOETEN, G. A., ASSINK, A. M., and A. VAN KAMMEN. (1974). Association of cowpea mosaic virus-induced double-stranded RNA with a cytopathological structure in infected cells. Virology 59: 341-355.

DeZOETEN, G. A. (1976). Cytology of virus infection and virus transport in "Physiological Plant Pathology" (R. Heitefuss and P. H. Williams, eds.) Springer-Verlas, New York.

DIENER, T. O. (1965). Isolation of infectious ribonucleic acid from southern bean mosaic virus. Virology 27: 425-429.

ERIKSSON, T. (1965). Studies on the growth requirements and growth measurements of cell cultures of Haplopappus gracilis. Physiologia plantarum 18: 976-993.

HILDEBRANDT, A. C. (1973). Tobacco mosaic virus in plant tissue culture. In "Tissue Culture" (D. F. Kruse and M. K. Patterson, eds.), pp. 549-558. Academic Press, New York and London.

JACKSON, A. O., ZAITLIN, M., SIEGEL, A., and FRANCKI, R. I. B. (1972). Replication of tobacco mosaic virus I. Viral RNA metabolism in separated leaf cells. Virology 48, 655-665.

KASSANIS, B. (1967). Plant Tissue Culture. In "Methods in Virology" (K. Maramorosch and H. Koprowski, eds.), Vol. I, pp. 537-566. Academic Press, New York.

KASSANIS, B., TINSLEY, T. W., and QUAK, F. (1958). The inoculation of tobacco callus tissue culture with tobacco mosaic virus. Ann. Appl. Biol. 46: 11-19.

KIELLAND-BRANDT, M. C. (1974). Studies on the biosynthesis of tobacco mosaic virus. VII. Radioactivity of plus and minus strands in different forms of viral RNA after labelling of infected tobacco leaves. J. Mol. Biol. 87: 480-503.

KIELLAND-BRANDT, M. C., and NILSSON-TILLGREN, T. (1973). Studies on the biosynthesis of TMV. V. Determination of TMV RNA and its complementary RNA at different times after infection. Molec. gen. Genet. 121: 229-238.

LINSMAIER, E. M., and F. SKOOG. (1965). Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. 18: 100-127.

MILLER, G. L., and PRICE, W. C. (1946). Physical and chemical studies on southern bean mosaic virus. I. Size, shape, hydration and elementary composition. Archiv. Biochem. 10: 467-477.

MOTOYOSHI, F., and OHIMA, N. (1968). Multiplication of tobacco mosaic virus in suspension culture of tobacco cells. Japan J. Microbiol. 12: 317-320.

MURAKISHI, H. H., HARTMANN, J. X., PELCHER, L. E., and BEACHY, R. N. (1970). Improved inoculation of cultured plant cells resulting in high virus titer and crystal formation. Virology 41: 365-367.

MURAKISHI, H. H., HARTMANN, J. X., BEACHY, R. N., and PELCHER, L. N. (1971). Growth curve and yield of tobacco mosaic virus in tobacco callus cells. Virology 43: 62-68.

MURAKISHI, H. H., and PELCHER, L. E. (1974). Synthesis of southern bean mosaic virus in bean callus cells. Abstract No. 37. 3rd Internat. Congr. Plant Tissue and Cell Culture. Leicester, England.

PACE, N. R., BISHOP, D. H. L., and SPIEGELMAN, S. (1968). The immediate precursor of viral RNA in the Q β-replicase reaction. Proc. Natl. Acad. Sci. U.S. 59: 139-144.

PELCHER, L. E., MURAKISHI, H. H., and HARTMANN, J. X. (1972). Kinetics of TMV-RNA synthesis and its correlation with virus accumulation and crystalline viral inclusion formation in tobacco tissue culture. Virology 47: 787-796.

RALPH, R. U., BULLIVANT, S., and WOJCIK, S. J. (1971). Evidence for the intracellular site of double-stranded turnip yellow mosaic virus RNA. Virology 44: 473-479.

SIEGEL, A., and HARIHARASUBRAMANIAN. (1976). Reproduction of small plant RNA viruses. In "Comprehensive Virology" (H. Fraenkel-Conrat and R. R. Wager, eds.), Vol. 2, pp. 61-108. Academic Press, New York.

SHEPHERD, R. J. (1971). Southern bean mosaic virus. Descriptions of plant viruses. Commonw. Mycol. Inst. Assoc. Appl. Biol. Kew, Surrey, England, 4 p.

TAKEBE, I. (1975). The use of protoplasts in plant virology. Annu. Rev. Phytopathology 13: 105-125.

ZAITLIN, M., and BEACHY, R. N. (1974). The use of protoplasts and separated cells in plant virus research. Adv. in Virus Res. 19: 1-35.

PART II

SYNTHESIS OF VIRUS AND VIRUS-INDUCED RNA

INTRODUCTION

Several systems have been employed to study the replication of plant viruses at the macromolecular level. These include separated cells from infected leaf (Jackson et al., 1972); inoculated leaf at the vein-clearing stage (Nelsson-Tillgren et al., 1969); differential temperature treated leaves (Dawson et al., 1973); protoplasts (Takebe and Otsuki, 1969); and tissue culture (Pelcher et al., 1972). Among these, tissue culture has several advantages. The host cells are aseptic and homogeneous. They can be maintained in a metabolically active state indefinitely so the uptake of radioactive precursors and metabolic inhibitors occurs readily (Beachy and Murakishi, 1973). Conditions for growing cells can be strictly controlled and synchronization of virus synthesis may be approached (White et al., 1977). With the advantages that tissue culture possesses, no plant virus other than tobacco mosaic virus (TMV) has been used to study biochemical aspects of replication using this system. The reason may be attributed to the difficulty in developing a tissue culture system that allows sufficient synchrony of virus synthesis to make it feasible for biochemical studies. Before the tissue culture system was well developed, intensive experiments had been conducted on TMV replication in intact plant systems. The use of tissue culture and protoplasts then added only very little information on the knowledge of TMV replication. It

is from this point of view, Part II of this dissertation is to study the replication of southern bean mosaic virus (SBMV) using tissue culture system as a starting material.

SBMV is a spherical virus, having a single stranded RNA with a molecular weight of 1.39×10^6 in its virion (Diener, 1965). The ease of obtaining SBMV in large quantities and the availability of a dependable host for virus bioassay facilitate the biochemical investigation of its replication in vivo. This study employs a soybean suspension culture system which provides a high multiplication rate of SBMV and an improved synchronization of SBMV synthesis by preincubating infected cells at nonpermissive temperatures (White et al., 1977). This system was used to study biochemical events following infection. The medium was chemically defined and contained no coconut water which is needed for TMV infection in tobacco tissue culture. The growth and incubation medium was liquid rather than agar which enabled better uptake of labeled compounds.

The objective of this study was to investigate the synthesis of SBMV particles and SBMV-induced RNA at different times after infection and to characterize the RNA and their kinetics of synthesis using soybean cells in suspension culture.

MATERIALS AND METHODS

Virus preparation. Southern bean mosaic virus was propagated in Phaseolus vulgaris L. 'Prince' and purified as previously described in Part I.

Selection of uniform-sized cell aggregates for inoculation. Soybean (Glycine max. L. Harosoy 63) callus culture was maintained on R3 medium (Part I). The callus formed on agar-solidified R3 medium was transferred to liquid medium and periodically renewed with fresh medium until the growth rate of callus reached a steady actively growing phase. This was estimated by sampling a given volume of cell suspension and noting the steady increase of pelleted callus volume after standing for 5 minutes in a 15 ml conical centrifuge tube. The callus culture at this stage contained aggregates ranging from single cells to clumps up to 3 mm in diameter. The callus cells were filtered through a filtering pan with 864 μm screen openings (Cistron Corp., Lebanon, Pa.). Callus clumps which passed through the screen were collected and again filtered through another filtering pan with 406 μm screen openings. The callus clumps retained on the second filtering screen were collected and put into fresh R3 medium at a density of approximately one gram per 3 ml of medium. After incubation for 20 hours, uniformed sized-clumps were inoculated with virus.

Inoculation of virus and low temperature pre-incubation of inoculated callus. Callus clumps were inoculated with SBMV as described in Part I. The inoculated cells were washed with fresh medium, incubated at 25°C for 4 hours and then transferred to 6°C for incubation on a rotary shaker under 968 lux of light. After 4 days at 6°C, the inoculated callus cells were transferred to 25°C and the medium renewed 1 hour later. The time when the culture was transferred to 25°C represented the zero time of the incubation. This low temperature pre-treatment of inoculated callus enhanced the synchronization of virus synthesis in callus and was used throughout the experiment.

Actinomycin D (AMD) treatment and ^{3}H-uridine labeling. At designated intervals after the transfer to 25°C, callus cells weighing approximately 5 g were transferred to a flask containing 40 μg/ml of AMD in fresh medium and incubated under dark for 2 hours. ^{3}H-uridine was then added to a final concentration of 200 μCi/ml and the incubation was continued under dark conditions for 8 hours. Callus cells were then washed five times with 10 ml fresh medium on a funnel lined with miracloth and weighed. Nucleic acids were extracted immediately.

Extraction of nucleic acids from callus cells. Extraction buffer was glycine buffer (0.1 M glycine, 0.1 M NaCl, and 0.02 M EDTA, pH 9.4) containing 0.4% bentonite, and 1.5% sodium lauryl sulfate (SDS). Extraction phenol was water-saturated phenol containing 10% m-cresol (v/v) and 0.1% 8-hydroxyquinoline (w/v). Callus cells were mixed with extraction buffer and extraction phenol at a ratio of 1 g:4 ml:8 ml, respectively. The mixture was homogenized with an Omnimixer (Sorvall)

in an ice bath for 3 min at a speed setting of 6. The homogenate was centrifuged at 8,000 g for 10 min and the aqueous phase collected and stored in an ice bath. The interface and phenol phase was reextracted with fresh buffer and phenol at 50°C for 5 min, and the aqueous phase after centrifugation, was combined with the aqueous phase collected before.

The combined aqueous phases were again extracted twice with 2 volumes of extraction phenol. The final aqueous phase was centrifuged at 15,000 g for 50 min to remove the bentonite. After adding several drops of 3 M sodium acetate to the supernatant, the nucleic acids were precipitated with 2 volumes of 95% ethanol and stored at -20°C overnight. The nucleic acids were collected by centrifugation at 20,000 g for 30 min, dried on N_2 gas briefly, suspended in SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) and digested with DNase (15 μg/ml) for 20 min at 35°C. They were then reextracted with phenol to remove the DNase. The nucleic acids collected after alcohol precipitation were resuspended in SSC. The absorption spectra were determined in a Cary 15 spectrophotometer. The absorbance ratio of nucleic acids at 240: 260:280 nm were 1:2:1, respectively. The concentration of nucleic acid was determined assuming an $E\ \frac{0.1\%}{260\ nm} = 26$. Extreme precautions were taken to avoid RNase contamination on glasswares and solutions (Sreevalsan, 1973).

Sucrose density gradient centrifugation. Nucleic acid preparations were layered on 5-20% linear-log sucrose gradients made in SSC. Centrifugation was performed in a Beckman Model L

ultracentrifuge at 4°C. The SW 25.1 rotor was run at 22,000 rpm for 16 hrs, and the SW 50.1 rotor at 40,000 rpm for 3 hrs. The fractions were analyzed at 254 nm by an ISCO Model D density gradient fractionator coupled with a Model UA4 UV analyzer. An adapter was made to allow the use of SW 50.1 tubes. The fractions from the gradient were collected by an LKB 7000 Ultrorac fraction collector.

For further purification of SBMV particles, the virus purified from 4 cycles of differential centrifugation was resuspended in 0.01 M phosphate buffer, pH 7.1 and layered on 10-40% linear-log sucrose density gradients made in the same buffer. Centrifugation was at 23,000 rpm for 3 1/2 hrs with SW 25.1 rotor. The band corresponding to SBMV was collected and dialysed against the same buffer.

Preparation of SBMV-RNA. The procedure for extracting RNA from SBMV-virions was based on the reports that EDTA at high pH solutions disrupted the structure of SBMV-virions (Sehgal, 1973; Wells, 1969). SBMV purified from the sucrose density gradient centrifugation was adjusted to a concentration of 2.20 mg/ml and was mixed with an equal volume of glycine buffer containing 20 mg/ml purified bentonite and 1.5% SDS. The mixture was shaken gently for 20 min at 25°C and stored at 4°C for 1 hr. The SDS precipitate and the bentonite were removed by centrifugation at 15,000 g for 50 min at 0°C. The supernatant was subjected to 5-20% sucrose gradient centrifugation in SW 25.1 rotor and run at 23,000 rpm for 16 hrs. The RNA band was collected, dialysed against SSC overnight and precipitated with 2 volumes of 95% alcohol. The precipitates were then dried briefly with N_2 gas and resuspended in SSC.

Polyacrylamide-agarose gel electrophoresis (PAGE). The basic methods described by Loening (1967) and Bourque and Naylor (1971) were modified for the separation of high molecular weight RNA on 2.25% polyacrylamide gels containing 0.5% agarose. Agarose (7.2% w/v) was refluxed for 20 min and stored in 50°C water bath until used. Acrylamide stock solution (20%) was made by dissolving 10 g acrylamide and 0.5 g bisacrylamide. Tenfold electrophoresis buffer (EP buffer) consisted of 0.4 M tris, 0.2 M Na-acetate, 0.01 M Na-EDTA; pH was adjusted to 7.8 with glacial acetic acid. NNN'N'-Tetramethylethylenediamine (TEMED) was made 3.2% and ammonium persulfate was made 0.8% just before use. The gels were prepared by mixing the stock solution sequentially as follows: Agarose 44.4 ml, 10 x EP buffer 6.4 ml, acrylamide 7.2 ml, TEMED 4.0 ml. The mixture was degassed under vacuum for 2 min and 2.0 ml of ammonium persulfate was added to the mixture. The mixture was poured into Plexiglass tubes (6.5 mm internal diameter x 10.2 mm long). The agarose solidified in a few minutes, but the polymerization of acrylamide gels required 20-30 min at room temperature. The gels were stored at 4°C overnight before use and were pushed out of the tubes for about 2-5 mm and each gel was cut with a previously flamed razor blade to make the surface flat and each gel the same length. Electrophoresis was carried out at a constant current of 5 mA per gel. Heavy rRNA (25S) and light rRNA (18S) extracted from unlabeled healthy cells were used as markers. Gels were pre-electrophoresed for 1 hr before layering the samples. RNA preparations which were adjusted to 1 mg/ml and 40-80 µl containing 0.8% sucrose, were layered on each

gel. Durations of electrophoresis runs are given under the figure legends. After electrophoresis the gels were scanned at 260 nm by a Gilford Model 2000 spectrophotometer equipped with gel scanner. The baseline was adjusted by substrating the background absorbance with the digital absorbance control of the equipment. After the scan, the gels were frozen with dry ice and sliced on a gel slicer (Mickel Lab. Eng. Co., England). Each slice (1 mm) was placed into a scintillation vial and 0.8 ml of a NWT mixture consisting of NCS solubilizer: water:toluene = 9:1:10 was added. The vials were capped tightly and incubated at 55°C for 2 hours. After the vials were cooled, 10 ml of scintillation fluid (6 g PPO and 75 mg POPOP per liter of toluene) was added to each vial. Vials were kept in the dark overnight and the radioactivity was determined in a Beckman cpm-100 liquid scintillation system.

Determination of acid-insoluble radioactivity. Aliquots from sucrose gradients were precipitated with trichloroacetic acid (TCA) at a final concentration of 8%. Crude, commercial RNA was added as a coprecipitator at 200 µg/ml. After 30 minutes at 0°C, the precipitates were filtered through Whatman GF/C glass fibre filter papers (prewetted briefly with 10% TCA), washed twice with 10 ml 95% ethanol. Each filter paper was dried at 60°C for 2 hours in a scintillation vial, digested with 0.8 ml NWT mixture at 55°C for 4 hours and the radioactivity determined as described above.

Total amounts of ^{3}H-uridine incorporated into whole cells was determined by adding 1 ml of 20% TCA into 0.5 ml of homogenate from

the mixture of callus, extraction buffer and extraction phenol during the extraction of RNA. The acid-insoluble radioactivity was then determined.

RNase, DNase, and NaOH treatments of RNA. One ml fractions from SW 25.1 sucrose density gradients were divided into five 0.2 ml aliquots. Each aliquot was diluted by adding 10 x SSC and H_2O to make the final salt concentration 1 x SSC for treatment of control, DNase and NaOH; 2 x SSC and 0.1 x SSC for treatment of RNase at high salt and low salt, respectively. DNase was at a final concentration of 5 μg/ml. RNase digestion included RNase A 1.0 μg/ml and RNase T_1 50 units/ml. NaOH concentration was 0.2 N. Incubation of enzyme digestion was at 35°C for 30 min. Alkali degradation was prolonged to 15 hrs. After the incubation periods, the acid-insoluble radioactivity was determined as described above.

Infectivity assays. Primary leaves of Phaseolus vulgaris L. Pinto were used for bioassay by a half-leaf method as described in Part I of this dissertation.

Reagents. Sucrose: Density gradient grade (RNase-free), Schwarz/Mann; [5,6-^{3}H]-uridine: New England Nuclear Corp.; Agarose: Bausch and Lomb; RNase A, RNase T_1, and DNase: Sigma; Acrylamide: Biorad, electrophoresis purity grade; NNN'N'-tetramethylethylene diamine (TEMED): Eastman; NCS solubilizer: Amersham/Searle; Actinomycin D: Calibiochem.

Purification of Reagents. Bentonite, 325 Mesh (E. H. Sargent and Co.) was purified as described by Frankel-Conrat (1966). SDS was purified by dissolving in boiling 80% ethanol and filtering through Whatman No. 1 filter paper on a prewarmed Buchner funnel. The filtrate was brought to a 95% ethanol concentration and chilled to precipitate SDS. The precipitates were collected on a Buchner funnel, washed several times with cold ethanol and acetone and dried at 80°C. Bisacrylamide was purified as described by Loening (1967). Phenol was purified by redistillation and stored at -25°C for not more than 5 months before use.

RESULTS

Synthesis of complete virus in relation to total amount of ^{3}H-uridine incorporation of cells. The amount of ^{3}H-uridine incorporated into complete virus was estimated by purifying SBMV from infected callus cells at intervals after transfer to 25°C and counting the radioactivity present in purified virus. Because the amount of virus synthesized during the initial periods was too small to be recovered by the routine purification procedure, 1 mg of purified virus was homogenized with each gram of callus cells to serve as a carrier for pelleting the extracted ^{3}H-uridine labeled virus during differential centrifugation. The final pellet was resuspended in 0.5 ml distilled H_2O and radioactivity was determined after adding 10 ml of Bray's scintillation fluid.

The results show that the incorporation rate of ^{3}H-uridine into complete virus closely paralleled the increased rate of infectivity during the initial period of virus synthesis (Figure 1). The synthesis of complete virus rose exponentially during the first 34 hrs and kept increasing throughout the 80 hrs tested, although not as dramatically as in the initial period.

The total amount of ^{3}H-uridine incorporated into whole cells increased slightly during the 7 to 15 hr period, but decreased rapidly thereafter until it reached the minimum amount during the 37 to 45 hr

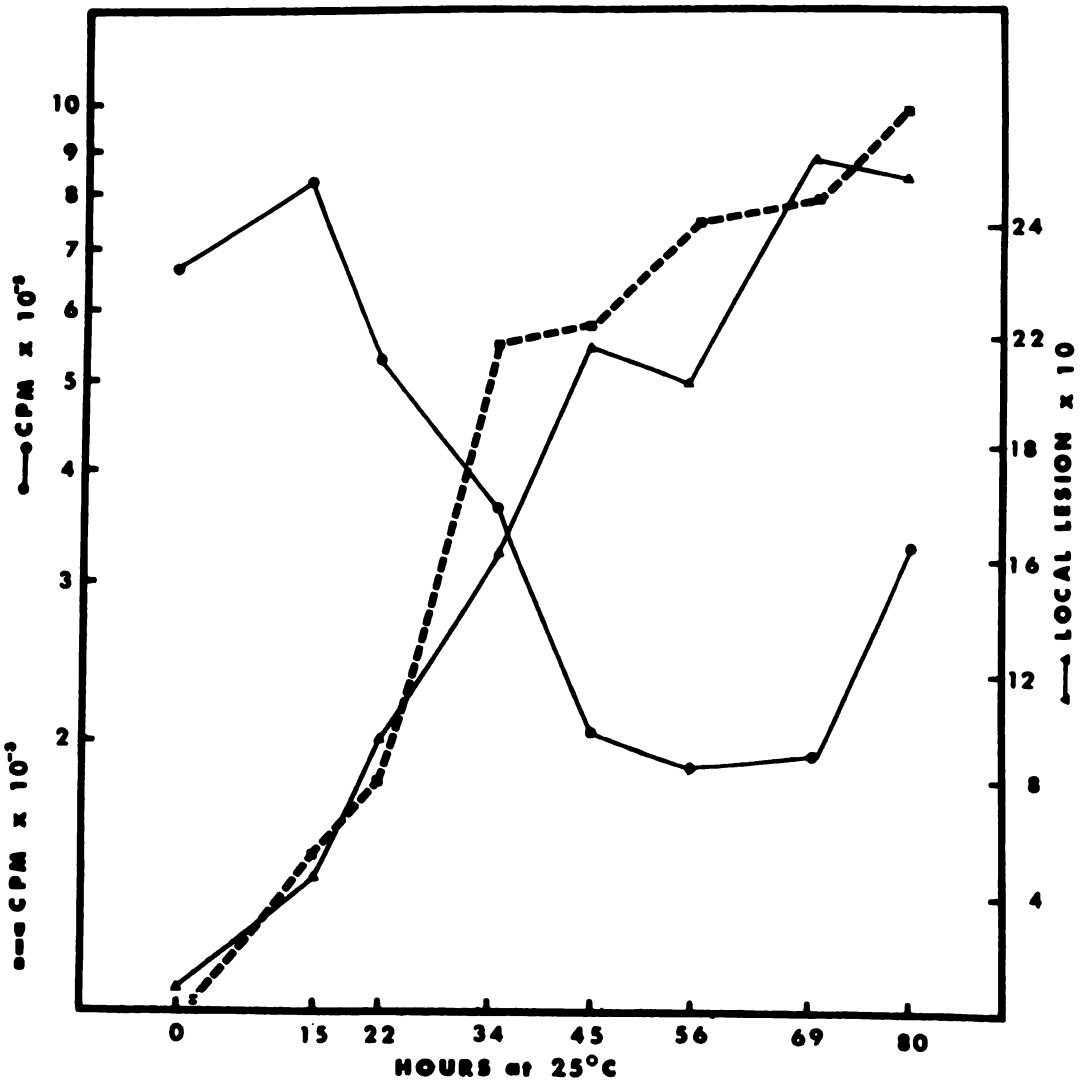


Figure 1. Time course of the total ^{3}H-uridine incorporation into one gram of infected callus cells (●—●), and into complete virus purified from one gram of callus cells (■---■), and infectivity (▲—▲) of callus cells infected with SBMV. The callus cells were preincubated at 6°C for 4 days before they were transferred to 25°C for incubation. The time at the transfer represented zero time of incubation. All callus cells were treated with AMD for 2 hours prior to ^{3}H-uridine labeling. Callus cells were allowed to incorporate ^{3}H-uridine for 8 hours at a concentration of 200 μci/ml. The hours indicated in the group were at the termination of 8 hour-radioactive labeling periods.

incubation period. Incorporation then remained at this minimum until it started to increase again during the 73 to 80 hr incubation period. While the synthesis of complete virus was at its exponential growth period, the total amount of ^{3}H-uridine incorporated into cells dropped sharply. During the 7 to 15 hr incubation period, only 0.18% of the total radioactivity incorporated into cells was present in complete virus particles, but during the 48 to 56 hr period, it increased to 4.4%. Thus, the percentage of radioactivity incorporated into complete virus increased 25 times during the rapid synthesis period of virus.

Effects of actinomycin D on RNA metabolism. AMD at a concentration of 40 μg/ml inhibited rRNA synthesis completely, but slightly stimulated the synthesis of low molecular weight RNA in the healthy cells (Figure 2E). The synthesis of SBMV RNA was not inhibited by AMD, and AMD stimulated the synthesis of low molecular weight RNA in infected cells (Figure 2F). The SBMV-RNA had the same sedimentation value as heavy cellular rRNA, therefore, they were indistinguishable from each other in the sucrose density gradient centrifugation. Without the AMD treatment, the RNA profile of healthy and infected cells were essentially the same except that the amount of radioactivity present at the 25 S region was greater in RNA extracted from infected cells (Figure 2C, D). The additional amount of radioactivity in the 25S region of infected cells was presumably contributed by SBMV-RNA. In order to investigate the SBMV-RNA synthesis rate at different times after infection, it became necessary to suppress the host rRNA synthesis by employing AMD.

Figure 2. Photometric scanning (————) and acid-insoluble radioactivity (·····) patterns of nucleic acids subjected to 5-20% linear-log sucrose density gradient centrifugation in Beckman SW 25.1 rotor for 16 hours at 22,000 rpm. Nucleic acids were extracted from: (A) healthy callus incubated 14-24 hours; (B) SBMV-infected callus incubated 14-24 hours; (C) health cells incubated from 38-48 hours; (D) SBMV-infected cells incubated from 38-48 hours; (E) healthy cells incubated from 38-48 hours with AMD; (F) SBMV-infected cells incubated from 38-48 hours with AMD.

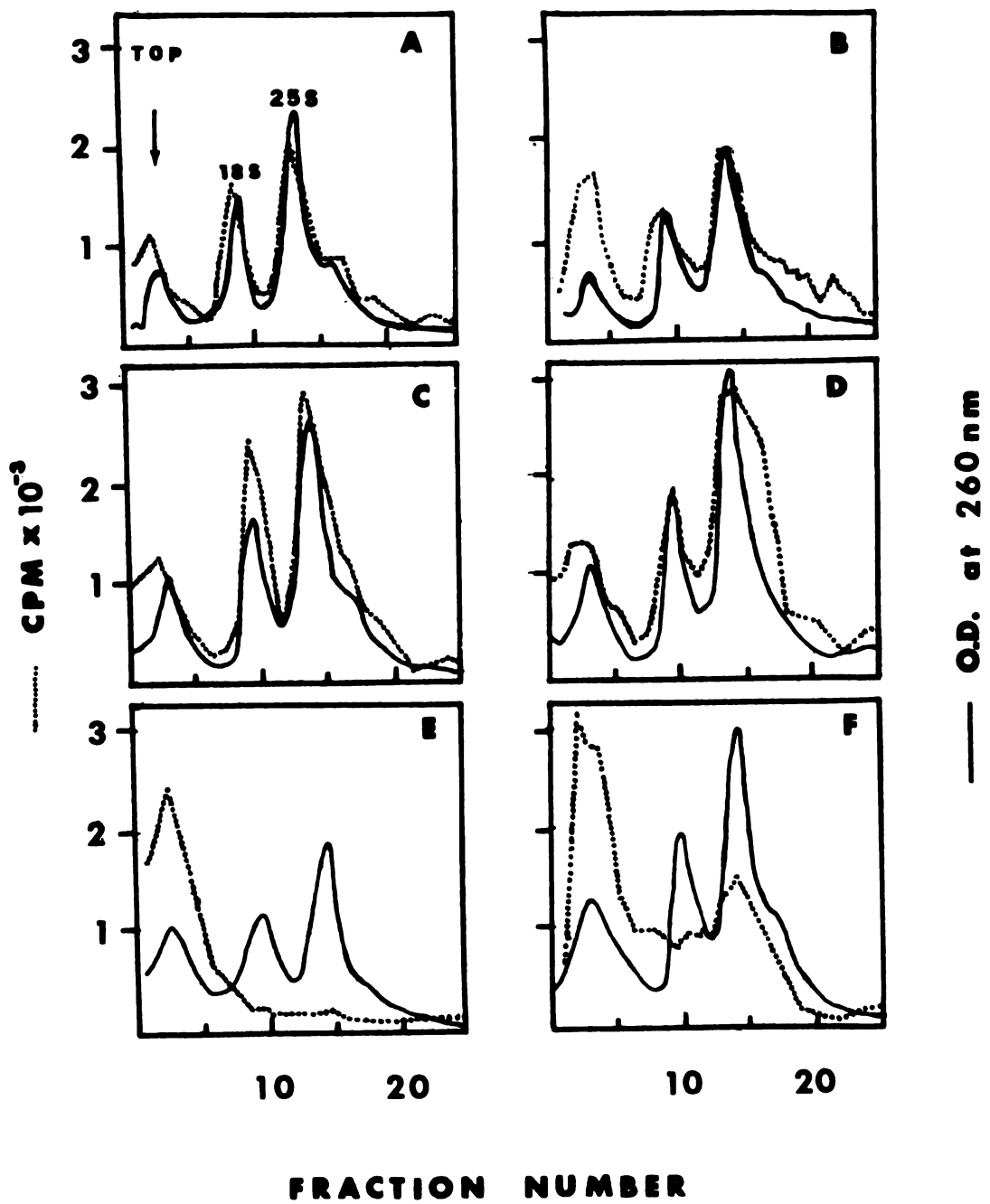
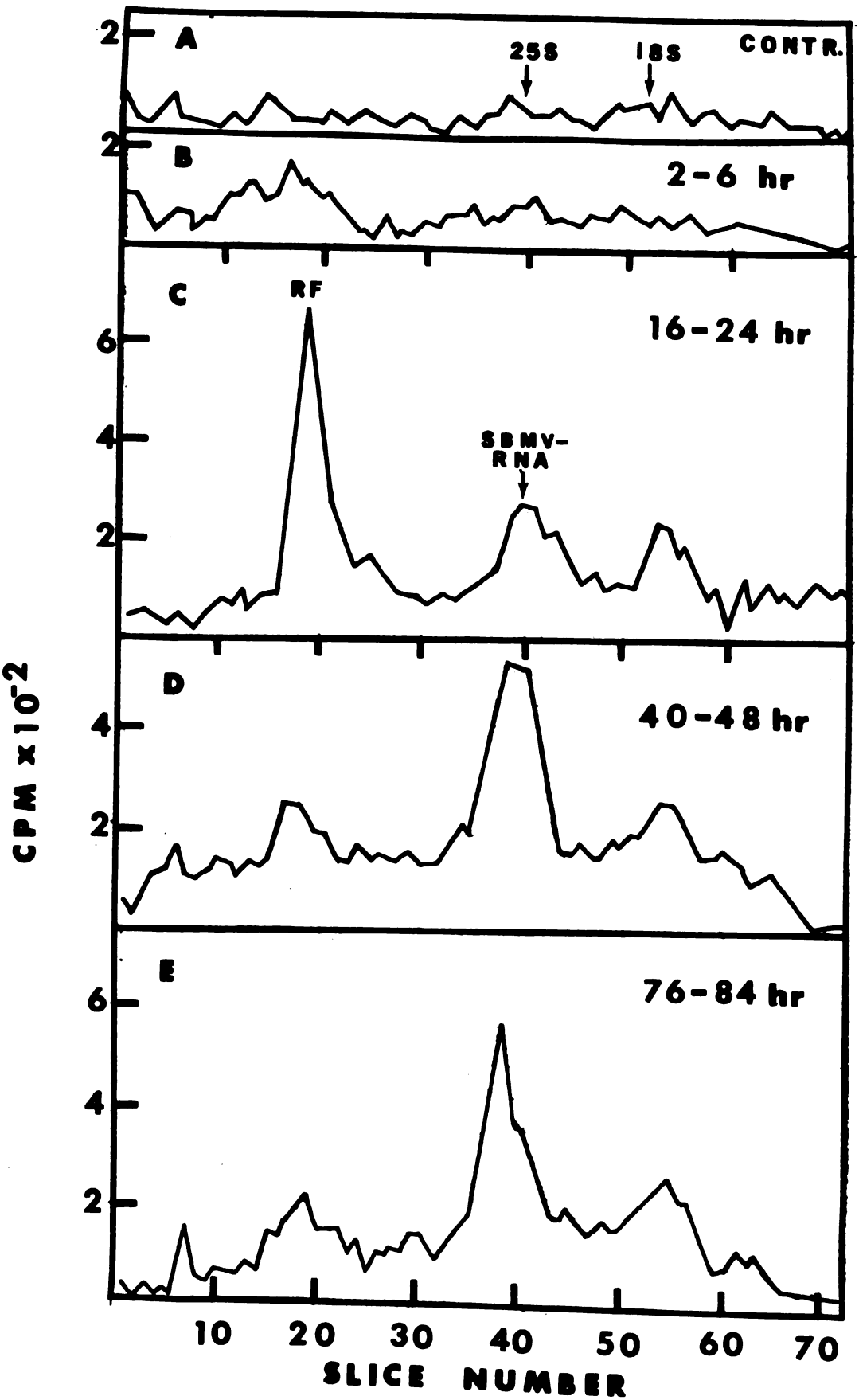

TOP
A
B
C
D
E
F
18S
25S
3
2
1
10
20
CPM x 10⁻³
O.D. at 260 nm
FRACTION NUMBER

Figure 2

Synthesis of virus-induced RNA. PAGE profiles from infected callus cells (Figure 3) revealed that during the first 6 hrs after the transfer to 25°C, a small amount of heterogenous high molecular weight RNA was synthesized. No evidence of SBMV-RNA synthesis was shown at this time. During 16 to 24 hrs a major peak of high molecular weight RNA was apparent. When the molecular weight of this RNA was extrapolated from its mobility in relation to those of marker RNA (Figure 5), it was estimated to be 2.78×10^6 daltons which is in agreement with the expected value of replicative form (RF) of SBMV-RNA. It is not possible to determine accurately the molecular weight of double stranded RNA by using single stranded RNA markers since their conformations are different, and even with DNA or double stranded RNA as markers, relative molecular radii are revealed rather than molecular weight (Fischer and Dingman, 1971). Nevertheless, the absence of this RNA in healthy cells and the time course kinetics of its appearance in relation to SBMV-RNA synthesis suggested that it is virus-induced RNA. From our present knowledge of viral RNA synthesis, RF would be the most likely candidate for this high molecular weight RNA. The SBMV-RNA isolated from purified virus migrated to the same distance as the heavy rRNA from healthy cells in PAGE (Figure 4). In AMD treated healthy cells, the RNA profile did not show any detectable level of ^{3}H- counts in this region. In AMD-treated infected cells, the RNA profile from the 16 to 24 hr incubation period showed considerable amount of radioactivity at 25 S and 18 S, and a polydisperse radioactivity between these two regions. The amount of radioactivity in

Figure 3. Distribution of radioactivity in 2.25% polyacrylamide-agarose gel electrophoresis of nucleic acids extracted from AMD treated healthy and SBMV-infected soybean callus cells at various periods after incubation at 25°C. The RNA samples were DNase-treated prior to electrophoresis. Electrophoresis was at a constant current of 5 mA/gel for 3 hours at 4°C. Gels were sliced 1 mm per slice, digested with NCS solubilizer and radioactivity of ^{3}H-uridine determined as described in Materials and Methods. Heavy rRNA (25 S) and light rRNA (18 S) extracted from unlabeled healthy cells were used as markers. (A) Nucleic acids extracted from control healthy callus incubated the same as infected cells from 16-24 hours after transfer to 25°C; (B-E) Nucleic acids extracted from SBMV-infected callus at intervals during the incubation at 25°C. Before the incubation, both healthy and infected cells were pre-incubated at 4°C for 4 days. The hours indicated in the graph represent the incubation periods of infected callus in fresh medium containing 200 µci/ml ^{3}H-uridine starting at the time of transfer to 25°C as a zero time. All callus cells were pretreated with 40 µg/m actinomycin D before the radioactive labeling.


A
25S
18S
CONTR.
B
2-6 hr
C
RF
16-24 hr
SBMV-RNA
D
40-48 hr
E
76-84 hr
CPM x10^-2
SLICE NUMBER
10
20
30
40
50
60
70

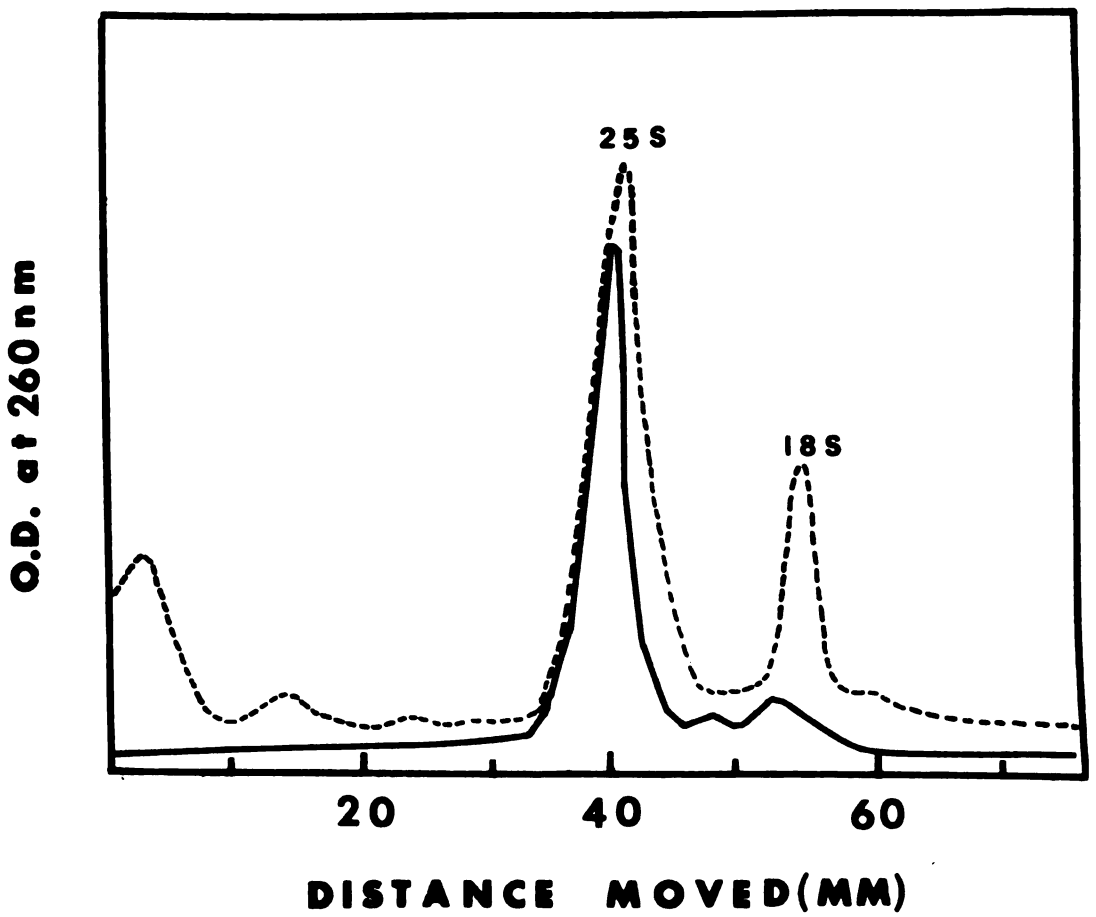


Figure 4. Electrophoresis of SBMV-RNA isolated from purified virions (———). The nucleic acids extracted from healthy soybean callus cells were used as markers (- - - -). Electrophoresis was carried out in 2.25% polyacrylamide-agarose gels at 5 mA/gel for 3 hours. Gels were scanned at 260 nm.

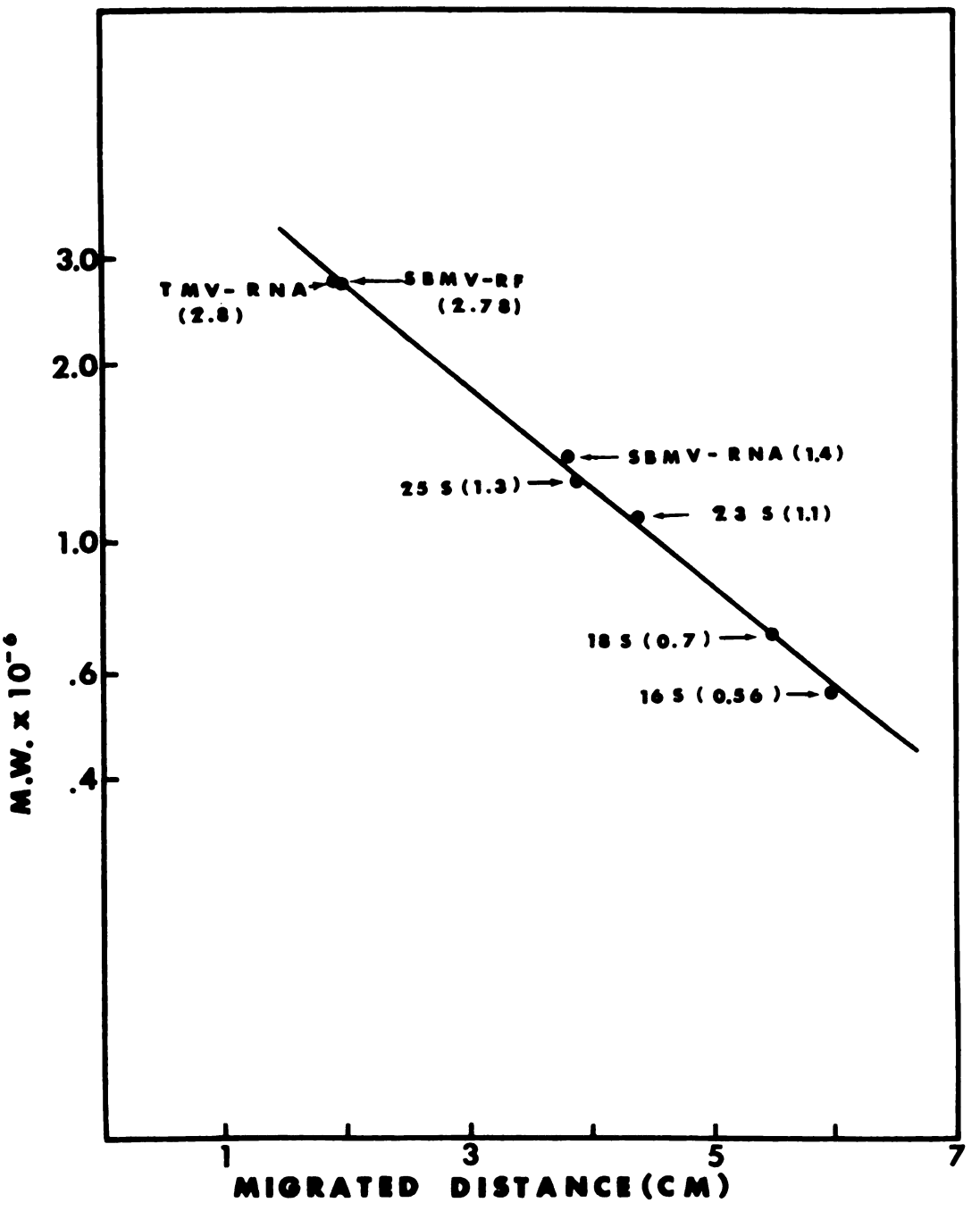


Figure 5. Plot of log molecular weight (M.W) versus distance of migrations in polyacrylamide-agarose gel electrophoresis. Conditions for PAGE were the same as in Figure 3. The ribosomal RNA extracted from healthy Prince bean leaves were used as markers. Numbers in parenthesis represent approximate molecular weight in x 10^6 daltons.

the 25 S peak followed the time course of virus synthesis closely, but the 18 S peak did not. It is assumed that the 25 S RNA peak present in infected cells is SBMV-RNA and the 18 S together with the polydisperse region below 25 S resulted from degradation of SBMV-RNA during the extraction. When phenol was added to the extraction buffer during the isolation of SBMV-RNA from purified virus, a similar heterodisperse feature was shown. Phenol seems to have had a deleterious effect on the SBMV-RNA during the extraction. This hindered the accurate estimation of the amount of SBMV-RNA in the PAGE, and a precise kinetic model of SBMV-RNA synthesis in relation to complete virus synthesis was not attempted. It is still possible to follow the time course of approximate amounts of SBMV-RNA synthesized during the incubation periods. The synthesis rate of SBMV-RNA reached a maximum between 40 and 48 hrs, at the time when the synthesis of complete virus was at its exponential phase. The amount of RF was greatly reduced at this time. During the 74-82 hrs, the synthesis rate of virus RNA decreased but was still the major RNA synthesized. The amount of RF increased slightly at this time, possibly resulting from a second cycle of virus replication which is thought to occur through the spreading of newly synthesized virus into neighboring uninfected cells (Pelcher et al., 1972). This is consistent with the fact that the synthesis rate of virus again increased rapidly at this time (Figure 1). The appearance of SBMV-RNA seemed not to precede the appearance of complete virus. The reason may be due to the extraction procedure which differentially recovers encapsidated

viral RNA better than free viral-RNA which when newly synthesized is very labile under the phenol extraction procedures.

Pulse-chase experiment. In order to determine the possible precursor role of the high molecular weight RNA found in PAGE, a pulse-chase experiment was conducted. Twenty-six hours after incubation, the cells were treated with AMD (40 μg/ml) for 2 hrs and then pulse-labeled for 1 hr in fresh medium containing 200 μci/ml of ^{3}H-uridine. The cells were washed on a miracloth-lined funnel with washing solution containing fresh medium and 1 mg/ml of cold uridine and then divided into 3 equal parts. One was frozen with dry ice immediately (pulse); the others (chases) were further incubated in the washing solution for 3 hrs and 18 hrs, respectively. The nucleic acids were then extracted and analyzed on PAGE (Figure 6 A-D). During the 1 hr pulse, a polydisperse high molecular weight RNA with its major peak migrating into the expected position of SBMV-RF was found (Figure 6A). Because of its heterogeneity, it was assumed to include RF and RI of SBMV-RNA which were not separated by the method used. The amount of its radioactivity was reduced to about one-half during the 3 hrs chase (Figure 6B) and no significant decrease of the radioactivity was shown after chasing for 18 hrs (Figure 6C). The synthesis of virus RNA was not obvious during the 1 hr pulse, but was in a distinguishable peak after chasing for 3 hrs and was further increased after chasing for 18 hrs. The amount reduced from the supposed RF + RI peak was much less than the amount increased in the virus RNA peak.

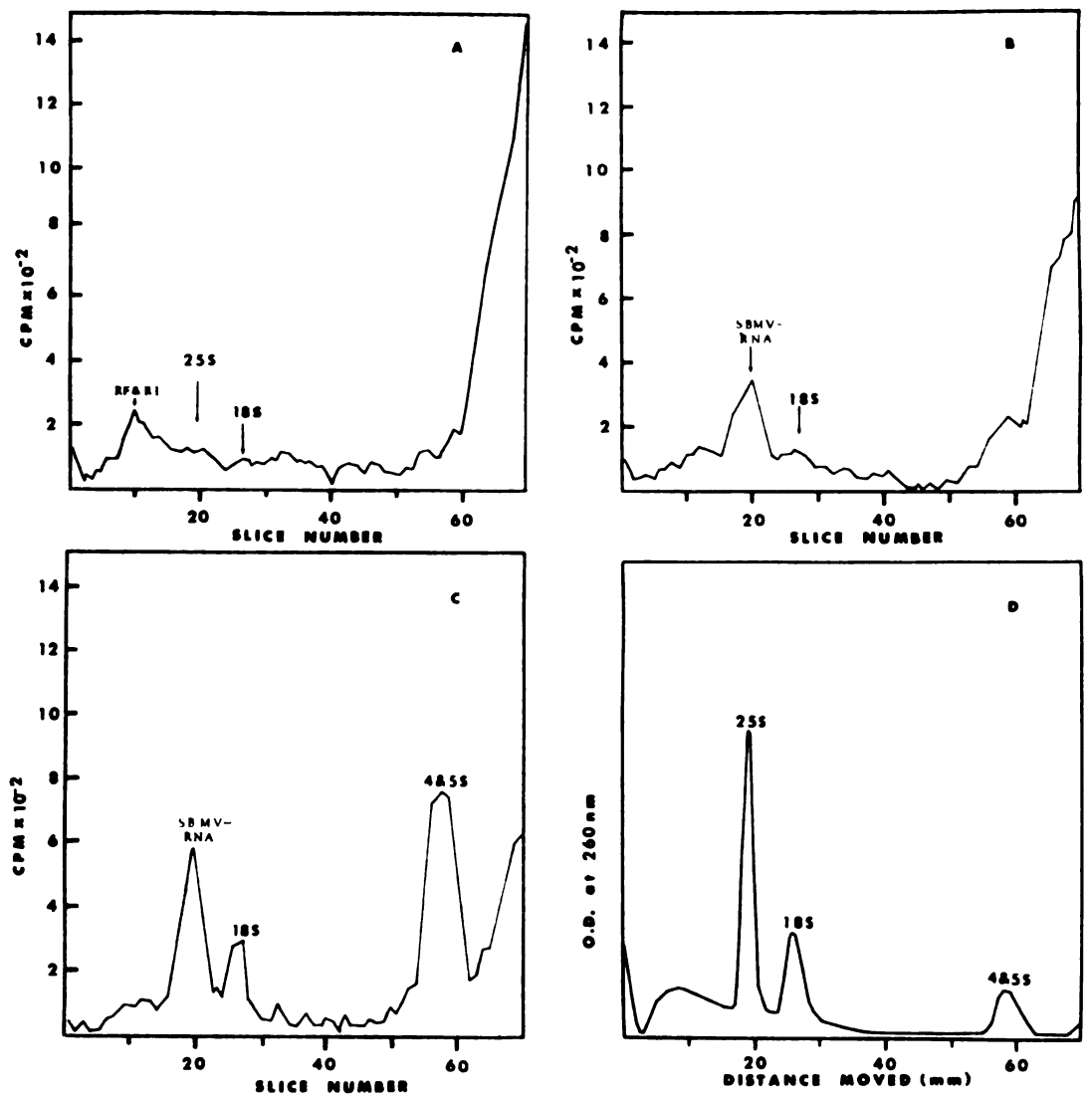


Figure 6. Electrophoresis profiles of RNA isolated in pulse-chase experiment. SBMV-infected soybean callus cells were pulse-labeled for 1 hour (A) in fresh medium containing 200 μCi/ml ^{3}H-uridine and 40 μg/ml AMD, followed by washing away the ^{3}H-uridine and chased with fresh medium containing 40 μg/ml AMD and excess unlabeled uridine for 3 hours (B) or for 18 hours (C). RNA was extracted and electrophoresed on 2.25% polyacrylamide-agarose gels at 5 mA/gel for 100 minutes at 4°C. Gels were scanned at 260 nm and sliced 1 mm each slice. Radioactivity was determined as described in Materials and Methods. Optical absorbance profiles of gels at 260 nm 1 hour-pulse (D) and pulse-chased samples were essentially the same.

This raises a question that the radioactivity which appeared in the virus RNA peak was not all from the RF + RI peak but originated elsewhere. It is possible that ^{3}H-uridine was incorporated into low molecular weight RNA or soluble nucleotide pools in the cell and was then used for construction of viral RNA. This is based on the fact that most of the radioactivity was present in the less-than 4 S region of PAGE profiles during the 1 hr chase and the radioactivity amount in this region greatly reduced following each of the 3 hr and 18 hr chases. During the 18 hr chase the major RNA synthesized was 4-5 S RNA which agreed with the previous results (Figure 2E) that AMD does not inhibit synthesis of low molecular weight RNA in host cells. The only possible source of radioactive precursor for this 4-5 S RNA was the small molecular weight RNA present at the end of PAGE.

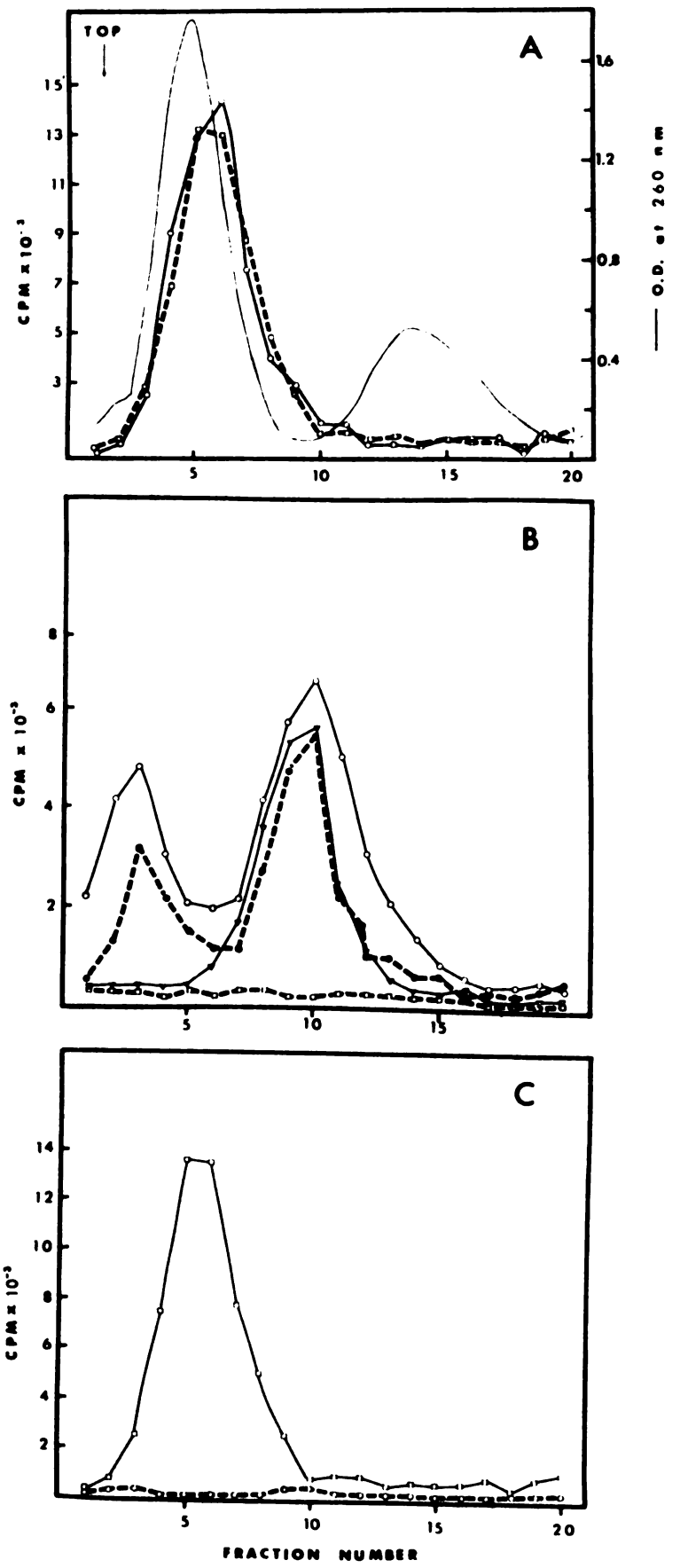
Possibly these small molecular weight RNAs were degraded and joined the cellular nucleotide pool which may be reutilized to build higher molecular weight RNA. Scholtissek (1972) reported that AMD not only inhibits the synthesis of RNA, but can also produce an artificial breakdown of RNA.

The 18 S RNA did not appear in the 3 hr chase but was present in the 18 hour chase. It was presumed to be light rRNA rather than degraded virus RNA because of its sharp peak. The effectiveness of AMD was probably reduced during the long incubation period and a slight synthesis of cellular rRNA probably occurred. In such a situation, the 25 S rRNA should also be synthesized. The amount of radioactivity which appeared in the 25 S peak would then be the sum of 25 S rRNA and SBMV-RNA. After substracting the amount of

radioactivity present in 3 hr chase, the remaining radioactivity was approximately the amount of 25 S rRNA synthesized. It is then reasonable to assume that there was no more radioactivity incorporated into SBMV-RNA during the 18 hr chase period. If the low molecular weight RNA was reutilized to build SBMV-RNA, the amount of radioactivity in SBMV-RNA would be continuously increased during the 18 hr chase as long as the ^{3}H-uridine-labelled low molecular weight RNA was being degraded. This was not the case in this experiment; therefore, the radioactivity present in SBMV-RNA peak could only have originated from the supposed RF and RI. The reason for the less than expected amount of radioactivity transferred from RF + RI to viral RNA is probably due to the large RNA precursor pool in eukaryotic cells which interfered quantitatively the transfer as discussed by Aoki and Takebe (1975).

RNase-resistant analysis of LiCl-soluble RNA. To enrich the RF, 4M LiCl was mixed with equal volume of RNA preparation and stored at 4°C overnight. After centrifugation, the supernatant was added to 2 volumes of 95% alcohol, and the precipitates were collected by centrifugation and resuspended in SSC. The LiCl-soluble RNA was subjected to 5-20% sucrose density gradient centrifugation in a SW 25.1 rotor as described before. The absorbance of 254 nm of the gradient showed two peaks: a major one from fractions 3 to 10 and a minor one from fractions 11 to 17. When each fraction was treated with TCA and acid-insoluble radioactivity determined, it showed only a large, wide peak from fractions 3 to 10 (Figure 7A). No difference was shown between healthy and infected samples. In order to see if RF was present in

Figure 7. Analysis of LiCl-soluble RNA on 5-20% sucrose gradient rate zonal centrifugation. Centrifugation was performed with Beckman SW 50.1 rotor at 50,000 rpm for 3 hours at 4°C. (A) total acid-insoluble radioactivity from RNA extracted from healthy (□--□) and infected (○---○) callus cells was determined by TCA-filter paper techniques as described in Materials and Methods. Optical absorbance profiles from healthy and infected cells (———) were virtually the same. (B) RNase resistant radioactivity present in RNA extracted from SBMV-infected callus cells. Each fraction from gradients was treated with RNase A 1.5 μg/ml and RNase T1 40 units/ml for 30 min at 35°C in 2 x SSC (○——○), and 0.1 X SSC (●---●), or treated with tenfold RNase (15 μg/ml and 40 units ml for RNase A and T1, respectively) in 2 x SSC (△——△), and 0.1 x SSC (□--□). TCA-precipitable radioactivity was then determined. (C) Acid precipitable radioactivity after treatment with 0.2 N NaOH for 16 hours at 35°C (○---○), and after digestion with pancreatic DNase at a concentration of 10 μg/ml for 30 min at 35°C (□——□).


TOP
A
B
C
CPM x 10⁻³
O.D. at 260 nm
FRACTION NUMBER

the LiCl-soluble RNA fraction, each fraction from the gradients was treated with RNase at high salt (2 x SSC) and low salt (0.1 x SSC) solution concentrations. RNase treatment at high salt solution revealed two RNase-resistant RNAs with their highest radioactivity peaks at fractions 3 and 10. Increasing RNase concentration to tenfold revealed the same peaks with less acid-insoluble radioactivity present. Fraction 10 always served the major peak and was less influenced by the higher RNase concentration. With RNase treatment at low salt solution, the fraction 10 peak was still present, although in lesser amounts than when it was at high salt. When the RNase concentration was increased to tenfold, this peak was eliminated and no acid-insoluble RNA could be detected in the gradient. Edy et al. (1976) reported that dsRNA was very resistant to RNase T_1 even in low-salt media but its resistance was dependent on the concentration of both salts and enzyme. This could explain the presence of dsRNA in LiCl-soluble RNA and the RNase resistant radioactivity present in fractions 7 to 12 at low salt and its degradation at tenfold RNase. It is believed that this RNA, having its highest RNase resistant radioactivity at fraction 10, is a complete double stranded RNA, and is the RF of SBMV-RNA. This is because of its high RNase resistance, its low sedimentation rate in the sucrose gradient, and its presence only in infected cells. The RNase resistant RNA having its acid-insoluble radioactivity peak at fraction 3 may represent the hairpin-like structures in soluble cellular RNA, or it may represent dsRNA normally synthesized by cells, since fraction 3 peak was also

found in healthy cells although in less quantity. The existence of dsRNA in normal cells has been reported (Stern and Friedman, 1971; Kronenberg and Humphreys, 1972). However, the nature of this RNase-resistant RNA present in both healthy and SBMV-infected callus cells is not yet identified. Because of its extremely slow sedimentation property in a sucrose density gradient, it is unlikely to be the RF of SBMV-RNA.

No infectivity associated with cells incubated under low temperature. During the 4 days preincubation period at 6°C, no complete virus synthesis could be detected either by infectivity assay or by the purification procedures described before. Up to 15 days incubation period at 6°C of the inoculated cells were tested in separate experiments and no infectivity could be recovered at any intervals tested. The possibility that improved synchronization of virus synthesis by preincubation at 6°C could be due to virus spreading among callus cells is unlikely since there was no infective virus available at this time.

High molecular weight RNA synthesized at 6°C in infected cells. A heterodisperse high molecular weight RNA (major radioactive peak at 2.8×10^6 daltons in PAGE) accumulated in infected cells incubated at 6°C from 84 to 96 hrs. Healthy cells under the same conditions showed a much smaller RNA peak (Figure 8). Scanning the gels at 260 nm did not reveal any difference between healthy and infected cells, but the difference in radioactivity between them is very prominent.

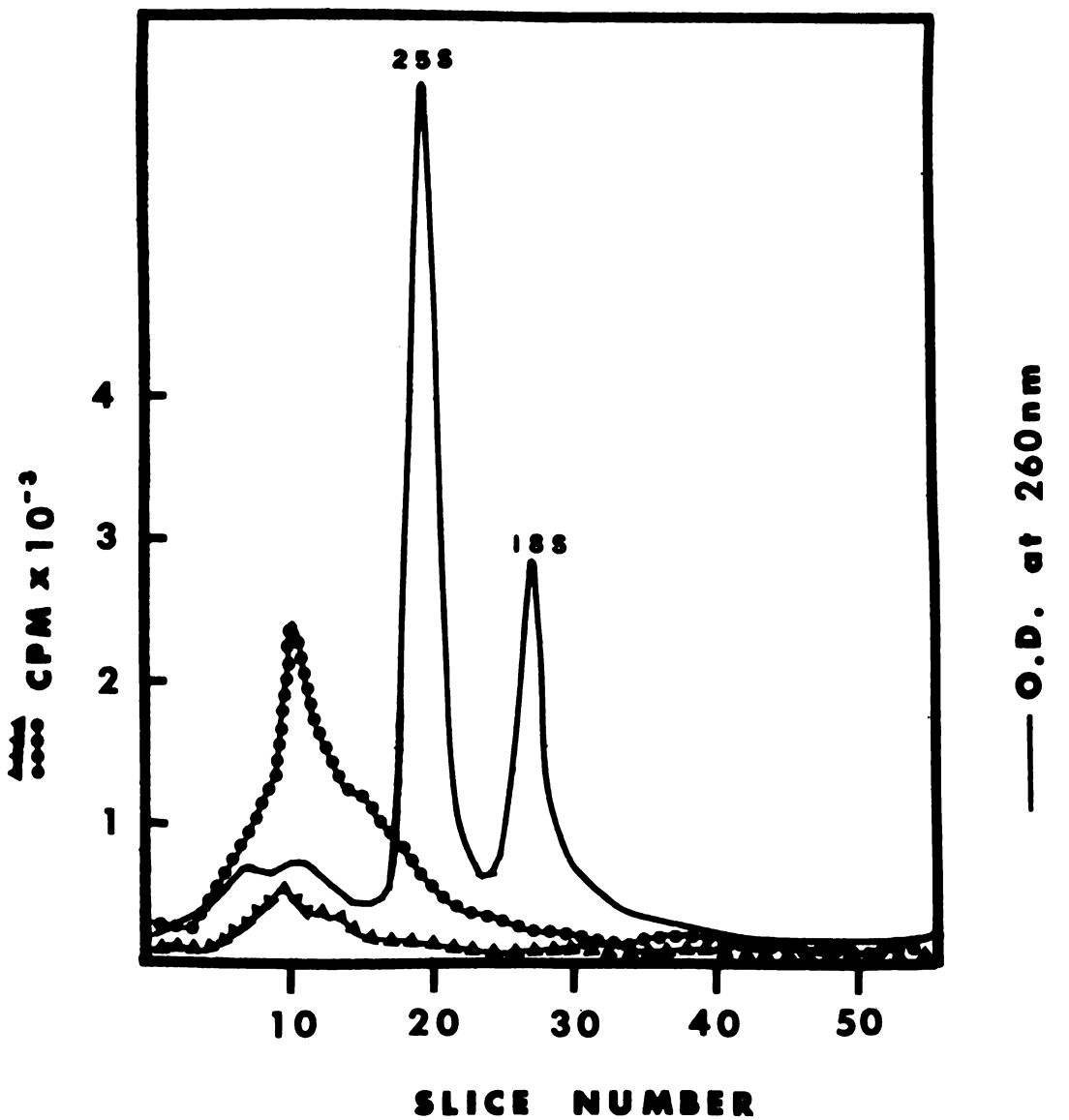


Figure 8. Electrophoresis profiles of RNA extracted from healthy and SBMV-inoculated soybean callus incubated at low temperature from 84-96 hours. Callus cells were inoculated with SBMV, incubated at 25°C for 4 hours and were then incubated at 6°C for 82 hours. AMD was added into incubation medium to a final concentration of 40 µg/ml, and callus cells were further incubated for 2 hours before ^{3}H-uridine was added into medium. The cells were allowed to incorporate ^{3}H-uridine at a concentration of 200 µci/ml for 12 hours. Nucleic acids were extracted and analyzed on 2.25% polyacrylamide-agarose gel electrophoresis as in Figure 6. (●●●●●) Infected callus; (▲▲▲▲▲) Healthy callus; (———) optical density of both infected and healthy callus.

The migration distance of this heterodisperse RNA in PAGE was similar to the high molecular weight RNA synthesized in 1 hr pulse-labeled RNA (Figure 6A) and the RF synthesized during the 16 to 24 hr incubation period after transfer to 25°C (Figure 3). This similarity suggested the possibility that they are of the same RNA. The small amount of radioactivity present in healthy cells is not understood but it may be the precursor of rRNA which was not completely suppressed by AMD under low temperature.

DISCUSSION

It has been shown in this study that the suspension cultured soybean callus cells is a suitable system for the study of virus replication. ^{3}H-uridine was readily taken up by callus cells and was incorporated into virion RNA as well as other virus-induced RNAs. It was surprising that while the amount of ^{3}H-uridine incorporated into complete virus increased sharply with time, the total amount of radioactivity taken up by whole cells decreased rapidly (Figure 1). This is in contrast to the TMV system in which both the incorporation rates and radioactivity into cells and virus increased linearly in infected cells (Jackson et al., 1972). Pring (1971) reported that the host RNA synthesis decreased during or after a period of rapid RNA accumulation in barley stripe mosaic virus infected cells. Inhibition of host RNA synthesis is a common phenomenon in virus-infected animal cells. Although the mechanism of inhibition of host RNA synthesis upon virus infection is not clear, it has been reported that in Newcastle disease virus-infected cells, the cellular RNA synthesis was turned off by a protein which was presumably virus-specific (Wilson, 1968). Wertz and Youngner (1970) reported that in vesicular stomatitis virus-infected cells, the inhibition of macromolecular synthesis was the critical factor in induction of interferon synthesis. Stephenson and Dimmok (1974) observed a decrease in the labeling of cytoplasmic

RNA species and a degradation of the smaller rRNA precursors of nucleus of Influenza virus-infected cells. In SBMV-infected soybean callus cells treated with AMD, the total incorporation rate of ^{3}H-uridine decreased during the initial virus synthesis period but the synthesis of cellular cytoplasmic RNA was not affected when the cells were not treated with AMD. SBMV infection alone inhibited neither the total ^{3}H-uridine incorporation nor cellular RNA synthesis. The reason why total ^{3}H-uridine incorporation was suppressed through the combination of SBMV and AMD treatments and suppression only occurred during the exponential phase of virus synthesis is not known.

AMD was reported to selectively inhibit chain elongation but not chain initiation of RNA which leads to a marked decrease in average chain length of RNA formed (Maitra et al., 1967). This may account for the large amount of low molecular weight RNA present in both healthy and SBMV-infected cells treated with AMD (Figure 2). For viruses which do not depend on DNA for their replication, AMD has been widely used in the studies of virus-mediated RNA synthesis without interference from cellular RNA synthesis. Although the synthesis of influenza virus was reported to be inhibited by AMD, the production of virus RNA was not affected if AMD was added more than 2 to 3 hrs after infection (Duesberg and Robinson, 1967). A time-dependent inhibition of cowpea mosaic virus and cowpea chlorotic mottle virus synthesis is also caused by AMD (Lockhart and Semancik, 1968; 1969). A discrepancy exists among reports on the action of AMD on TMV-infected cells (Sinha, 1972). In SBMV-infected callus cells reported here, the synthesis of virus

and virus-mediated RNA was obviously not inhibited by AMD at a concentration which inhibits all the cellular rRNA synthesis. The possibility that AMD had a time-dependent effect on the uptake of ^{3}H-uridine by infected cells cannot be ruled out.

The high molecular weight RNA synthesized during the 16 to 24 hr incubation period has an estimated molecular weight of 2.78×10^{8} daltons from the distance migrated in PAGE (Figure 5). This is in agreement with the expected RF value of SBMV-RNA. The presence of RF and RI in cells infected with bacteriophages, animal viruses and plant viruses is well known, but the active role of RF and RI is not well understood. In SBMV-infected callus cells, the presence of RF and RI and its involvement in SBMV replication was indicated by: (1) the presence of highly RNase-resistant RNA at 2 x SSC in LiCl-soluble RNA fraction of nucleic acids extracted from infected cells but not from healthy cells; (2) its susceptibility to RNase at 0.1 x SSC; (3) its resistance to DNase treatment and sensitivity to degradation by alkaline treatment; (4) the rapid synthesis of this high molecular weight RNA preceded the rapid synthesis of SBMV-RNA and the fast decrease in its synthesis when the amount of virus-RNA was at maximum (Figure 3); (5) within limitations, the pulse-chase experiment revealed a precursor-product relationship between this RNA and virus RNA (Figure 6); (6) its synthesis is AMD-insensitive.

MacNaughton et al. (1976) reported that the presence of RF occurred in infected cells only when they were treated with AMD, whereas the synthesis of RI was not affected by the drug. It was

suggested that RF is an artifactual structure derived from RI as a result of AMD treatment. Whether the RF in SBMV-infected callus cells is AMD-dependent has not yet been investigated, however, the kinetics of its synthesis and virus synthesis indicated that RF is more likely actively related to SBMV replication.

The pulse-chase experiment did not definitely show the precursor role of the polydisperse high molecular weight RNA, which may include RF and RI, in the synthesis of SBMV-RNA. It met the same situation as in separated leaf cells (Jackson et al., 1972) as well as in protoplasts (Aoki and Takebe, 1975) in that the RNA precursor pool in plant cells is too large to completely permit the chased label moving from precursor to product. However, the distinct radioactivity peak of SBMV-RNA during the 3 hr chase, together with the conspicuous decrease in radioactivity of this high molecular weight RNA, suggested its precursor role in the formation of SBMV-RNA similar to those of RF and RI found in other plant virus and animal virus replications (Bishop and Levintow, 1971). The results here have shown that the replication of SBMV-RNA is associated with a polydispersed high molecular weight RNA having a molecular weight of 2.78×10^6 in its major peak. The nature of this high molecular weight RNA remains to be elucidated, but it is reasonable to suggest that it included RF and RI with RF in a larger amount.

The accumulation of a high molecular weight RNA similar to that found in pulse-chase experiments was observed in infected cells incubated at 6°C for 4 days (Figure 8). It is possible that they are

the same kind of RNA which included mainly RF and also RI. If this is true, it would mean that the improvement of synchronization of virus synthesis by preincubating inoculated cells at low temperature was caused by a blocked virus replication at the RF and RI stage. Thus, when the cells were transferred to 25°C, the RF and RI accumulated in the low temperature were then ready to synthesize virus RNA all at the same time and the synthesis of virus was then synchronized. This may explain the much shorter lag period before the increase of infectivity was detected in cold temperature preincubated cells. The dsRNA blocked at low temperature may even spread through plasmodesmata into adjacent cells which were not infected at the time of inoculation, such that more cells became infected at the time of transfer to 25°C. This would not only contribute to the synchronization of virus synthesis, but also increase the total virus infectivity recovered from callus clumps. During the first 8 hrs of incubation after low temperature treatment, the synthesis of SBMV-RNA was not obvious, and a heterodisperse high molecular weight RNA was synthesized (Figure 3). This could represent a period for the cells to recover from low temperature to its normal growth temperature. After this period, the cells were recovered and the active replication of virus RNA started. The significant amount of SBMV-RNA and the massive RF produced during 16 to 24 hr incubation period could represent the stage of virus replication in which the virus RNA, newly synthesized from RF and RI accumulated at 6°C, was reutilized to synthesize RF, so that the RF became the dominant RNA during this time.

It is still too early to predict the RNA replication model of SBMV although RF and RI are thought to be associated with virus infection. Purification and characterization of RF and RI would be necessary to verify their roles in the replication of SBMV in callus cells.

This study established a tissue culture system that shows promise for the investigation of SBMV replication. The use of liquid suspension culture in a chemically defined medium together with the improved synchronization of virus replication should encourage more experiments in "problem solving-attempts to answer some of the challenging questions of how plant virus replicate and how they affect their host" as stated by Zaitlin and Beachy (1974). In fact, this study has already attempted to answer the question of whether RF and RI are involved in SBMV replication, and has indeed, although only partially, answered the question.

SUMMARY

The synthesis of ^{3}H-uridine labeled complete virus, viral RNA, replicative form (RF), and replicative intermediate (RI) in the replication of southern bean mosaic virus in soybean tissue culture were studied. To improve the synchronization of virus multiplication, the inoculated cells were preincubated at 6°C for 4 days and then transferred to 25°C. Actionomycin D did not inhibit the synthesis of viral-related RNA and was employed throughout the study.

The synthesis of complete virus closely paralleled the increasing rate of infectivity and reached maximum during 37 to 45 hr incubation period after the transfer to 25°C. The total amount of ^{3}H-uridine incorporated into cells dropped sharply while the synthesis of complete virus was in its exponential phase. An RNA with a molecular weight close to the supposed replicative form (RF) of SBMV-RNA was synthesized in significant amount during 26 to 34 hr incubation period preceding the major period of virus RNA synthesis which reached maximum during 40 to 48 hr. Pulse-chase experiment revealed the possible precursor role of RF and RI in the synthesis of virus RNA. The RNase resistant analysis of LiCl-soluble RNA by sucrose density gradient centrifugation showed the presence of RF in RNA extracted from infected cells. Accumulation of a heterodisperse high molecular weight RNA with its polyacryamide gel electrophoresis profile of radioactivity corresponding to RF + RI of SBMV-RNA was detected in infected cells incubated at 6°C from 84 to 96 hr.

REFERENCES

AOKI, S., and TAKEBE, I. (1975). Replication of tobacco mosaic virus RNA in tobacco mesophyll protoplasts inoculated in vitro. Virology 65: 343-354.

BEACHY, R. N., and MURAKISHI, H. H. (1973). Effect of cycloheximide on tobacco mosaic virus synthesis in callus from hypersensitive tobacco. Virology 55: 320-328.

BISHOP, J. M., and LEVINTOW, L. (1971). Replicative forms of viral RNA: Structure and function. Progr. Med. Virol. 13: 1-82.

BOURQUE, D. P., and NAYLOR, A. W. (1971). A simple electrophoretic procedure for separation of RNA on mixed agarose acrylamide gel columns. J. of Chromatography 56: 79-86.

DAWSON, W. O., and SCHLEGEL, D. E. (1973). Differential temperature treatment of plants greatly enhances multiplication rates. Virology 53: 476-478.

DIENER, T. O. (1965). Isolation of infectious ribonucleic acid from southern bean mosaic virus. Virology 27, 425-429.

DUESBERG, P. H., and ROBINSON, W. S. (1967). On the structure and replication of influenza virus. J. Mol. Biol. 25: 383-405.

EDY, V. G., SZEKELY, M., LOVING, T., and DREYER, C. (1976). Action of nucleuses on double-stranded RNA. Eur. J. Biochem. 61: 563-572.

FISHER, M. P., and DINGMAN, C. W. (1971). Role of molecular conformation in determining the electrophoresis properties of polynucleotides in agarose-acrylamide composite gels. Biochem. 10: 1895-1899.

FRAENKEL-CONRAT, H. (1966). Preparation and testing of tobacco mosaic virus-RNA. In "Procedures in Nucleic Acid Research" (G. L. Canton and D. R. Davies, eds.), pp. 480-687. Harper and Row, New York.

JACKSON, A. O., ZAITLIN, M., SIEGEL, A., and FRANCKI, R. I. B. (1972). Replication of tobacco mosaic virus I. Viral RNA metabolism in separated leaf cells. Virology 48: 655-665.

KRONENBERG, L. H., and HUMPHREY, T. (1972). Double-stranded ribonucleic acid in sea urchin embryos. Biochem. 11: 2020-2026.

LOCHART, B. E. L., and SEMANCIK, J. S. (1968). Inhibition of the multiplication of a plant virus by actinomycin D. Virology 36: 504-506.

LOCKHART, B. E. L., AND SEMANCIK, J. S. (1969). Differential effect of actinomycin D on plant-virus multiplication. Virology 39: 362-365.

LOENING, U. E. (1967). The fractionation of high-molecular-weight ribonucleic acid by polyacrylamide-gel electrophoresis. Biochem. J. 102: 251-257.

MACNAUGHTON, M. R., COOPER, J. A., and DIMMOCK, N. J. (1976). Rhinovirus multistranded RNA: Dependence of replicative form on the presence of actinomycin D. Virology 18: 926-932.

MAITRA, U., NAKATA, Y., and HURWITZ, J. (1967). The role of deoxyribonucleic acid in ribonucleic acid synthesis. XIV. A study of the initiation of ribonucleic acid synthesis. J. Biol. Chem. 242: 4908-4918.

NILSON-TILLGREN, T., KOLEHMAINEN-SEVEUS, D., and WETTSTEIN, D. VAN. (1969). Studies on the biosynthesis of TMV. I. A system approaching a synchronized virus synthesis in tobacco leaf. Mol. Gen. Genetics 104: 124-141.

PELCHER, L. E., MURAKISHI, H. H., and HARTMANN, J. X. (1972). Kinetics of TMV-RNA synthesis and its correlation with virus accumulation and crystalline viral inclusion formation in tobacco tissue culture. Virology 47: 787-796.

PRING, D. R. (1971). Viral and host RNA synthesis in BSMV-infected barley. Virology 44: 54-66.

SEHGAL, O. P. (1973). Factors affecting dissociation of southern bean mosaic virus. Phytopath. 63: 629-633.

SCHOLTISSEK, C. (1972). Unphysiological breakdown of fast-labeled RNA by actinomycin D in primary chick fibroblasts. Europ. J. Biochem. 28: 70-73.

SINHA, R. C. (1972). Inhibitors of plant viruses and mycoplasma. In "Metabolic Inhibitors" (R. M. Hochster et al., eds.), pp. 277-304. Acad. Press, New York and London.

STEPHENSON, J. R., and DIMMOCK, N. J. (1974). Interaction of influenza virus with the host cell: Inhibition of ribosome biosynthesis. In "Negative Strand Viruses" (R. D. Barry and B. W. J. Machy, eds.), pp. 485-490. Acad. Press, New York.

SREEVALSAN, T. (1973). Isolation of single-stranded viral ribonucleic acids. In "Nucleic Acid Biosynthesis" (A. I. Laskin and J. A. Last, eds.), pp. 63-92. Marcel Dekker, Inc., New York.

STERN, R., and FRIEDMAN, R. M. (1971). Ribonucleic acid synthesis in animal cells in the presence of actinomycin. Biochem 10: 3634-3645.

TAKEBE, I., and OTSUKI, Y. (1969). Infection of tobacco mesophyll protoplasts by tobacco mosaic virus. Proc. Nat. Acad. Sci. U.S. 64: 843-848.

WELLS, J. M., and SISLER, H. D. (1969). The effect of EDTA and Mg^{2+} on the infectivity and structure of southern bean mosaic virus. Virology 37: 227-236.

WERTZ, G. W., and YOUNGNER, J. S. (1970). Interferon production and inhibition of host synthesis in cells infected with vesicular stomatitis virus. J. Virol. 6: 476-491.

WHITE, J. L., WU, FANG-SHENG, and MURAKISHI, H. H. (1977). The effect of low temperature pre-incubation treatment of tobacco and soybean callus cultures on rates of tobacco- and southern bean mosaic virus synthesis. Phytopath. 67: 60-63.

WILSON, D. E. (1968). Inhibition of host-cell protein and ribonucleic acid synthesis in Newcastle disease virus-infected cells. J. Virol. 2: 1-6.

PART III

ELECTRON MICROSCOPY OF VIRUS DISTRIBUTION AND CYTOPATHIC EFFECTS

INTRODUCTION

Southern bean mosaic virus (SBMV) has a host range restricted to only the Leguminosae (Shepherd, 1971). Two of its major strains, cowpea and bean, have different host ranges in that the bean strain of SBMV does not infect the hosts of cowpea strain and vice versa. Prominent virus crystals occurred in nuclei and cytoplasm of cowpea plants but were lacking in mesophyll cells of bean plants (Weintraub and Ragetli, 1970a). This noncrystallization of the bean strain SBMV was also reported by DeZoeten and Gaard (1969). It has not been possible to study whether the crystallization is virus strain-dependent or host plant-dependent because of the unusual restricted host range of SBMV. Other cytopathic effects of SBMV-infection also raise the same question.

The soybean suspension callus cell system was used since it provided a high multiplication rate for the bean strain of SBMV in a previous study (Part I). Since previous studies have used cells from intact plants, it would be of interest to compare the response of suspension cultured cells to the virus.

Electron microscopic study of virus-infected cells is an indispensable way to investigate the distribution of virus in cells and to find out cytopathic structures that could be related to the biochemical events of virus replication. The association of ultrastructural components with double stranded RNA formed during virus

replication has been established in turnip yellow mosaic virus (TYMV) (Ralph et al., 1971) and cowpea mosaic virus (CPMV) (DeZoeten et al., 1974). The ultrastructural study obviously plays an important role in the understanding of virus replication. The present study was undertaken to determine the distribution of the virus in callus cells and to investigate the cytopathic effects caused by virus infection.

MATERIALS AND METHODS

Soybean (Glycine max 'Harosoy-63') callus derived from hypocotyl of young seedlings were maintained in Linsmaier and Skoog's liquid medium and inoculated with the bean strain of SBMV as described in Part I. Callus cells were sampled 5, 10, 15, and 20 days after inoculation. Callus clumps having diameters of approximately 0.8 mm were taken from incubation medium, resuspended in fresh medium, and mixed with equal volume of 5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.1, containing 4% sucrose. The calli were fixed in glutaraldehyde at room temperature for 30 min and then transferred to 4°C to continue the fixation. After 1 hr the fixation solution was pipetted off and replaced with cold 5% glutaraldehyde made in the same buffer. Fixation was continued 2 hrs further at 4°C. After fixation, the calli were washed with the above buffer for 30 min with several changes of the buffer and then post-fixed in 1% osmium tetroxide in the same buffer for 30 min at 25°C and then 3 hrs at 4°C. The calli were washed with the same buffer for 30 min with 4 changes, followed by 5 min in distilled water with 2 changes. Dehydration was performed at 10% increments in alcohol solutions starting from 10% to 80%, and then 95%, and 100%. The dehydration time in each increment was 20 min at 25°C. After the first 100% change, the calli were dehydrated overnight with several more changes. The calli were embedded in Spurr's embedding medium (Spurr, 1969). Unless specified,

thin sections cut on glass or diamond knives were stained with uranyl acetate saturated in a mixture of 95% ethanol and absolute methanol (1:1 v/v), followed by lead citrate (0.4%), NaOH (0.02N), and rinsed several times in water. Occasionally, thin sections were stained with 2% aqueous potassium phosphotungstic acid and then rinsed with water. Sections were examined in a Philips EM300 electron microscope.

The callus clumps were taken at various periods after inoculation and infectivity of callus homogenate was determined by bioassay on half leaves of Pinto beans as described in Part I.

RESULTS

Localization of virus particles. Virus like particles were first detected 5 days after inoculation. They were smooth outlined spherical particles with diameters of 22-27 nm in thin sections. Their images were projected onto a screen and size determined with a known standard. They were observed in cytoplasm (Figure 3) and nucleus (Figure 5) at this time. Similar particles were absent in healthy cells. The virus-like particles occurred scattered or in loose aggregations. No crystalline arrangements were seen at this stage. Ten days after inoculation, the virus particles were also found in vacuoles as well as in the nuclei and cytoplasm. Particles found in vacuoles were most often in irregular clumps (Figure 1). They were not associated with dense vacuolar inclusions which were frequently found adhering to the inner face of the tonoplast (Figure 1A). The virus clumps were most often associated with irregular diffuse fibrillar substances in the vacuoles (Figure 1B-D). Sometimes the virus was in close association with or included in electron dense vacuolar inclusions (Figure 1C-E). The inclusions were irregular in shape and varied in their reaction to the strains. Occasionally, the virus particles were present in an invaginated structure in the vacuoles (Figure 1F), where they were intermixed with ribosomes and were not readily distinguishable. This invaginated structure probably originated as a protuberance of swelling endoplasmic reticulum (ER) lumen

into vacuoles (Figure 1G). The ER membranes protruding into vacuoles may later have collapsed and included virus particles as well as ribosomes into the protuberances.

The presence of virus aggregations in the vacuoles were most prominent in later stages of infection (15 and 20 days). The vacuoles were the main locations having large numbers of virus particles. Cultured soybean cells usually possessed a large vacuole which occupied most of the areas in cells. This is a common feature of cultured cells (Street et al., 1972), and was probably one of the reasons that virus aggregates were most often found in vacuoles.

Crystalline arrangements of virus particles were first observed 10 days after inoculation (Figure 2) and were also seen 15 days after inoculation but not at 20 days after inoculation. The crystalline aggregates of virus particles were only observed in cytoplasm. Nuclei and vacuoles did not contain such crystals. Intensive search in healthy cells did not reveal any crystal form of particles.

Sections through crystals revealed some regular patterns of crystal lattice. As shown in Figure 2A, the view was approximately through a threefold axis and in Figure 2B, resembled tetragonal arrays. These were somewhat similar to some of the crystal patterns of TYMV illustrated by Hatta (1976). The virus aggregates were sometimes associated with moderately-stained vesicles and the background texture of cytoplasm which contained virus crystals was difficult to characterize because of its diffuse nature (Figure 2C; 4E).

The viruses were usually scattered as small groups in the cytoplasm and were occasionally associated with a mass of diffuse ground substances and ribosomes (Figure 3). Five days after inoculation, the polyribosomes were frequently seen (Figure 4A). They were in close proximity to smooth ER and were observed in control cells but much less frequently than in infected cells. It seemed that the frequency of polyribosomes present in infected cells decreased as incubation time increased. The cytoplasm of infected cells sometimes filled with rough ER (Figure 4B) and occasionally the ER fused, encircling a "pocket" which contains a mass of membrane-like materials, electron-dense to electron-opaque substances and ribosomes (Figure 4C). Large osmiophilic globules were often observed in both infected and control cells (Figure 4D). Although highly vesiculated cells were occasionally seen in control cells they are more often observed in infected cells 5 days after inoculation (Figure 4F). When the cytoplasm of infected cells contained small pieces of double membranes associated with virus particles (Figure 4H). It appeared that the virus particles were being released from a double membrane vesicle into cytoplasm. Membranous substances were observed to associate with cell walls and plasmodesmata (Figure 4G). Particles, possibly a mixture of virus particles and ribosomes were present in large numbers in the vicinity of the membranous substances. Since the virus particles were not in discrete groups and were intermixed with large amounts of ribosomes they were not readily identified. The presence of virus particles was best determined by observing virus aggregates in vacuoles.

Particles found in nuclei were similar in size to virus but were less regularly outlined than those found in cytoplasm and vacuoles (Figure 5A, B). The nuclei of control cells frequently contained numerous irregular particles about 15 nm in diameter. These particles were concentrated in the nucleoli and sometimes found adjacent to nuclear membrane areas (Figure 5). Because of their smaller size and rougher outlines, they appear to be distinguishable from virus particles. The virus particles were often observed in the vicinity of these granular areas or mixed with them. They were often freely dispersed which increased the uncertainty in identifying virus particles in the nuclei. Fine diffuse fibrillar substances were abundant in nucleoplasm in cultured soybean cells. The nucleoli of infected cells were occasionally vacuolated (Figure 5C) in which particles suspected to be virus and irregular fine fibrils were observed. The vacuolated structure of nucleoli was probably an inherent nature of soybean callus cells rather than a cytopathic structure imposed by virus infection. Only rarely was virus associated with it, and control cells were observed to have similar structures.

The nuclear membranes of infected cells were often highly lobated (Figure 5A, D; Figure 7G). Occasionally, the nuclear membrane was convoluted (Figure 5D). In one occasion, curved fibrous clusters were intermingled with ribosomes, mitochondria, granular nuclear substances, and nuclear pores which appeared opaque (Figure 5E).

Cytoplasmic inclusions. The cytoplasm of infected cells contained inclusions of various types. Five days after inoculation, amorphous inclusions composed of fine granules and fine fibril-like substances were observed (Figure 6A, D). They were most often spherical-like and surrounded by several large electron-dense deposits. Occasionally, the central region of the amorphous body had a vacuolated area which contained virus-like particles and probably ribosomes as well. The presence of amorphous bodies was always closely associated with rough ER in their adjacent areas, and occasionally beaded particles about 60 nm in size were present in their vicinities (Figure 6D).

An inclusion consisting of diffuse coarse fibers and a bundle of particles in its center was seen in cells inoculated after 10 days (Figure 6B).

Those central particles were measured to be approximately 15 nm in diameter which was much smaller than virus particles. Whether they were actually particles or cross-sections of well-defined coarse fibers was not determined, but since no longitudinal view of coarse fibers with well-defined outlines were seen in their surrounding areas or elsewhere, they were most likely particles. Some parts of these inclusions were seen to associate with pieces of double membrane structures and mitochrondria (Figure 6B). Virus particles were not seen in surrounding area nor were they present in the inclusion.

In early stages of infection (5 days), inclusions containing clusters of dark diffuse spots were frequently seen in the cytoplasm (Figure 6E, F). They were also seen in cells 10 days after inoculation

but not later. Few virus-like particles were seen scattered in it. Striated diffuse fibers mixed with the dark irregular spots formed a network in the inclusions. This pattern of inclusion seems similar to those dark diffuse materials observed in tomato spotted wilt virus infected cells (Ie, 1971).

Microfilaments. Microfilaments were frequently observed in infected callus cells 10 days after inoculation (Figure 7). They usually appeared as parallel or curved bundles. The number of filaments per bundle varied from a few to hundreds. The microfilaments occurred in both cytoplasm and nuclei. The directions of microfilament bundles were sometimes frequently changed (Figure 7E) or waved (Figure 7A, G). In cytoplasm, the microfilaments were always closely associated with rough ER (Figure 7A). The mitochondria in the vicinity of microfilaments were often darkly stained (Figure 7E). The cross section of microfilaments appeared as slightly beaded structure having 6-9 nm in diameter (Figure 7D). The microfilaments in nucleoplasm were either straight or waved in their directions (Figure 7F, G). Occasionally, they went accross nuclear membrane and extended into cytoplasm leaving behind disrupted nuclear membranes (Figure 7G, H).

No virus aggregates were associated with microfilaments. Scattered particles similar in size to virus were frequently seen associating with microfilaments, but they were most probably ribosomes because they were not smoothly outlined as those of crystalized virus particles. The microfilaments occurred most often in later stages of infection (10 days after inoculation and later), but in rare cases,

they were also observed in earlier stage of infection and also in control cells.

Mitochondria. Mitochondria are sensitive organelles in response to plant virus infection in many host plants (Martelli and Russo, 1977), and to injuries in animal cells (Trump and Ericsson, 1965). They undergo a spectrum of morphological modification as a result of pathological effects. In order to see whether this is true in callus cells infected with SBMV, the mitochondria were carefully examined. No virus particles were present in or associated with mitochondria but the morphology of mitochondria had undergone many alterations upon virus infection. The cristae of many mitochondria in SBMV infected callus cells were concentric (Figure 8A, F-I) or firmly packed (Figure 8B-E). The densely packed cristae occurred as a large bundle in the center (Figure 8B) or side (Figure 8C). They were usually oriented in the same direction as the elongated mitochondria. Frequently, large bundles of diffuse microfibril-like substances occupied a large area of mitochondria and extended into the cytoplasm where they appeared as electron-opaque materials (Figure 8D, E). Sometimes they were confined in vacuolated canals which occurred in the central part of a mitochondria and their longitudinal ends fused with matrices of mitochondria (Figure 8J). An extremely electron-dense body within a vacuole of mitochondria frequently found in cowpea plants infected with SBMV (Weintraub and Ragetli, 1970a), were also observed in SBMV-infected callus cells (Figure 8K). It seemed likely that at times this dense body elongated into filaments and appeared

as a bundle of fibrous substances as shown in Figure 8J, or conversely, they may occur in such a way that the bundle of fibrous substances (Figure 8J) condensed and when cross-sectioned, it appeared as a dense body in a vacuole (Figure 8K).

The cross-sections of mitochondria in infected cells revealed the discoid structure of mitochondria (Figure 8F-I). They possessed a cup-shaped central area where the invaginated cytoplasmic substances were included. The connections between concentric cristae can be seen when sections were in proper angles (Figure 8F, G). Engulfment of mitochondria by encircled rough ER and ribosomes (Figure 9A, B) were seen in cells inoculated after 15 days and later. Degradation of mitochondria in such engulfment structures were observed (Figure 9B). The mitochondria seemed to be undergoing division which was indicated by two constriction regions of a mitochondrian (Figure 9A). It was interesting that both of the constricted areas of a mitochondrian had a bundle of fine filaments. It appeared that each bundle of fine filaments was related to one constricted region of the mitochondria. Both sides of the mitochondria after constriction were engulfed by encircled rough ER and ribosomes, and the engulfment appeared to end at the constricted region of mitochondria.

Electron-opaque deposits were sometimes seen in motochondria (Figure 9C) and densely stained matrices of mitochondria were frequently observed (Figure 9D) in infected cells 10 days and later after inoculation. Almost all the mitochondria having this densely stained nature of matrices were rather spherical in their shapes and they appeared to be in condensed states.

Chloroplasts. Aggregates of phytoferritin particles were frequently observed in chloroplasts of callus cells 15 days after inoculation and later (Figure 10). These particles measured to be 6-9 nm in diameter and they were usually in straight or slighly curved rows with center-to-center spacing of 11.0 nm. They were arranged alternately in dense lines and regularly spaced particles, very similar to the pattern described by Wildman and Hunt (1976). When the phytoferritin particles were in favorable orientations at high magnification (Figure 10E) they were shown to consist of several subunits. The central core appeared to be a small translucent area similar to those described by Wildmar and Hunt (1976). Occasionally, the chloroplasts disintegrated releasing phytoferritin aggregates (Figure 10D).

Virus particles were not seen in chloroplasts throughout the study. However, the chloroplasts showed some unusual structures and various degrees of deterioration in the later stages of infection (15 days and later). The grana of chloroplasts were disorganized (Figure 11). Some chloroplasts were either constricted at the middle and microfilaments (Figure 11B) or pocket-like structures discharging their contents into cytoplasm (Figure 11C) were observed in the constricted areas. The chloroplasts in infected cells were often highly vesiculated in the adjacent areas of chloroplast membranes (Figure 11C, D). Those vesicles are single membrane-bounded. Large vacuoles and grana which had swelled were observed in chloroplasts (Figure 11D), and occasionally chloroplasts did not develop fully and prolamellar

bodies were retained (Figure 11E, F). Highly osmophilic substances or light-stained globules were often surrounded or in the vicinity of prolamellar bodies.

Cell wall. The callus aggregates were vortexed in test tubes with virus suspension as an inoculation procedure in the present study. The vortexing resulted in damage of cell wall and produced cell debris (Figure 12A). At high magnification, the viruses were often seen to intimately associate with the cell debris (Figure 12B). Affinity between the virus and cell debris was apparent from the present observations.

Plasmodesmata were frequently seen during this study and virus-like particles were occasionally observed in plasmodesmata of infected cells (Figure 12C). DeZoeten and Gaard (1969) reported that SBMV has not been seen in plasmodesmata of bean meshophyll cells, but they also mentioned that plasmodesmata were seldom observed in their sections. SBMV probably existed in the plasmodesmata for only a short time so that they were not readily observed. The size of plasmodesmata in soybean callus cells were sometimes unusually large, and large lumina occurred inside the cell wall, perpendicular and connecting to the openings of plasmodesmata (Figure 12D). The lumen may be branched and it contained striated substances (Figure 12E).

Membrane-bound vesicular bodies accumulated between cell wall and plasmolemma in infected cells at 10 days after inoculation and later. They were also found in earlier stages of infection but to a lesser extent. They are known as paramural bodies or plasmolemmasomes, and have been related to the infection of many plant viruses

(Martelli and Russo, 1977). It was also suggested that they are the likely site of viroid RNA replication (Semancik and Vanderwoude, 1976). Plasmodesmata were usually not seen in the areas that paramural bodies accumulated. The destruction of plasmodesmata adjacent to paramural bodies observed by Spencer and Kimmins (1971) as a response of healthy cells to adjacent infected cells in TMV lesion areas was not seen in the present study. Since the virus was observed in cells that contained large amounts of paramural bodies, it was unlikely that the formation of paramural bodies was related to a localization mechanism as they suggested.

Microtubules were observed in both infected and control cells. They may occur parallel, perpendicular, or with an angle to the cell wall (Figure 12G, H). They were observed adjacent to the cell wall or associated with the cell wall and were not seen elsewhere. The microtubules were probably related to the synthesis of cell wall as reported by Packard and Stack (1976). Their relation with the SBMV infection is not known.

DISCUSSION

On the basis of present observations, it appears that the distribution and cytopathological effects of SBMV in soybean callus cells are different from those reported in the intact plant cells (Edwardson et al., 1966; DeZoeten and Gaard, 1969; Weintraub and Ragetli, 1970a). From their reports, the distribution of virus in mesophyll cells of bean plants was mainly in cytoplasm and nuclei, and the virus particles occurred in a scattered manner, no true crystalline arrays was seen and few cytopathic effects of SBMV on cell organielles were observed. The present study has shown that in SBMV-infected cells, the virus particles occurred in cytoplasm, nuclei, and vacuoles. It was interesting that the virus was not seen in vacuoles at 5 days after inoculation but was most often observed in vacuoles at 15 days after inoculation and later. Since no comparable results on the time-course of virus localization in bean plants were available, it is not known whether the presence of virus predominantly in vacuoles is an unique feature of SBMV-infected callus cells. The cultured soybean callus contained various electron-dense vacuolar inclusions and SBMV sometimes were associated with them (Figure 1). SBMV was able to survive in soybean callus for up to six months as described before (Part I). It is possible that the longevity of virus in callus cells was related to its favorable localization in vacuoles

during later stages of infection. A proteinaceous substance "Inhibitor-I" which is an inhibitor of protein-digesting anzymes, has been characterized from many plants (Ryan and Shumway, 1970; Green and Ryan, 1972). The vacuole is known to contain such substances (Gunning and Steer, 1975). It is possible that vacuoles of soybean callus cells also contain similar substances which may protect SBMV from being degraded by intracellular protease. This may relate to the longevity of SBMV in callus cells. Although polyphenolic compounds which are deleterious to many plant viruses (Hampton and Fulton, 1961; Mink, 1965), may be present in vacuoles (Withers, 1976), SBMV is very insensitive to high concentrations of phenolic substances (Mink and Diener, 1971). This could also explain the stability of SBMV in the vacuoles of callus cells.

Crystallization of SBMV was frequently seen in cowpea plants but not in bean plants as reported by Weintraub and Ragetli (1970a). They attributed the difference to virus strains, but made the suggestion with uncertainty because of the lack of a common host for both strains. The soybean callus cells used for the present study are not only genetically but are also culturally different from those of intact bean plants. Infection by the same virus strain in these two different host systems revealed that crystallization of bean strain SBMV was host cell-dependent.

The virus particles present in thin sections of soybean callus cells were measured to be 22-27 nm, which is slightly less than the 25-30 nm particles reported by Weintraub and Ragetli (1970a), and the

28.4 nm maximum diameter particles measured by x-ray diffraction (Johnson et al., 1974) but larger than those of 18.5-22.5 nm reported by Edwardson et al. (1966). The virus particles can be readily identified in vacuoles because of the lack of ribosomes and the prominent clusters of virus aggregation. In cytoplasm, the crystalline arrangement of virus particles was easily identified but when the virus particles scattered freely they were identified with considerable uncertainty as described by Weintraub and Ragetli (1970b). However, virus particles in cytoplasm can be identified with confidence when they are at least in small groups (Figure 3) because of their smooth outlines and the fact that they do not attach to ER.

Various forms of cytoplasmic inclusions were observed in the present study. The virus-like particles were associated with some types of inclusions but not with others. The composition of the inclusions and their role in virus infection are still not well understood. It is interesting that such inclusions have never been observed in bean plants whereas they occurred frequently in soybean callus cells.

The association of double membrane structures with SBMV (Figure 4H) suggested the possibility that they may be the site of virus replication. It has been shown that double-stranded RNA is membrane-associated in plant cells infected with TYMV (Matthews, 1973) and CPMV (DeZoeten, 1974). Intracytoplasmic membranes were also demonstrated to be the site of synthesis of virus-specific RNA and proteins (Caliguiri and Tamm, 1970; Boulton and Westaway, 1976).

These findings suggest the same may apply to SBMV as well. The association of membranous structures with plasmodesmata in SBMV-infected callus cells (Figure 4G) also suggested possible transport of viral specific RNA in the form of replicative form (RF), replicative intermediate (RI), or minus strand viral RNA from cell to cell as suggested by DeZoeten (1976). This hypotheses agreed with my suggestion made in the finding of a possible RF + RI accumulation in infected cells incubated at 6°C, that RF and RI could move from cell to cell during the low temperature incubation periods (Part II). The striated substances observed in plasmodesmata of infected cells (Figure 12E) could indicate that nucleic acid substances were being transported. Autoradiographical studies would be necessary to support this speculation.

Microfilaments have been observed in various plants and are thought to relate to cytoplasmic streaming (Helper et al., 1974). Carrot tissue culture cells also contained "multifibrillar bundles" similar to the microfilaments reported here (Wilson et al., 1974). The microfilaments were frequently observed in infected cells but only rarely in control cells. This suggested that their amount of production was related to virus infection. Of special interest was the observation that microfilaments extended from nucleus into cytoplasm, breaking through the nuclear membranes (Figure 7G, H). Fibrillar bundles were closely associated with virus-like particles in green algae (Hoffman and Stanker, 1976). In the present observations, virus particles were not associated with microfilaments.

Therefore, they could be the products of host cells stimulated by virus infection rather than the sites of virus synthesis.

The mitochondria of soybean callus cells had undergone many changes after infection with SBMV. Mitochondrian was the only abnormal organelle in cucumber green mottle mosaic virus infected cells (Hatta et al., 1971). The outer membrane of mitochondria was associated with tobacco mottle virus (Harrison and Roberts, 1968). Giant mitochondria occurred as a result of an apple virus infection (Weintraub and Ragetli, 1971). These reports indicated that mitochondria are closely related to virus infection. The most significant alterations of mitochondria in SBMV-infected soybean callus were the firmly packed cristae and the presence of microfibril bundles. It is not known whether the latter was a result of compact stacking of fused cristae or a structure of other origin. The densely stacked cristae did not seem connect with each other at either of their longitudinal ends, and sometimes they extended beyond the mitochondria membranes.

The "densely packed cristae" was named with a degree of uncertainty, since the possibility that they were bundles of microfibrils, which were not natural components of mitochondria, cannot be excluded. This was in view of the fact that "normal cristae" were also observed in the mitochondria that possessed the densely packed cristae. Similar packing pattern of cristae in mitochondria was reported in Warthin's tumor cells (Tandler and Shipkey, 1964), but they appeared to have inter-connected cristae and were not stacked so firmly as observed in the present study. The functional significance

of this abnormality in relation to virus infection is not known. It seems possible that they were not directly related to virus synthesis. They were probably a cytopathic structure superimposed on mitochondria upon virus infection which changed the metabolic activities of host cells. This is based on the fact that no association of virus particles with mitochondria was observed throughout the study.

The association of phytoferritin particles with virus infection has been reported in plant cells infected with TYMV (Ushiyama and Matthews, 1970); beet yellows virus (Cronshaw et al., 1966); and citrus vein-enation virus (Hooper and Niblett, 1969). The present study also revealed the occurrence of phytoferritin particles in SBMV-infected callus cells. Phytoferritin inclusion were suggested to be iron that was stored in a nontoxic form in chloroplasts (Robards and Humpherson, 1971). It was also proposed that phytoferritin was a breakdown product which resulted from disrupted chloroplast structure and photosynthetic activity in yellowing leaves of plants (Wildman and Hunt, 1976). Based on these reports and the present observations, it may be concluded that the accumulation of phytoferritin in chloroplasts resulted from cell growth under stress conditions such as metabolic interruption by virus infection.

Double membrane-bound vesicles were reported in plant cells infected with TYMV (Matthew, 1973), belladonna mottle virus (Moline, 1973), and wild cucumber mosaic virus (Allen, 1972). Similar vesicles were not observed in the present study but single membrane-bound vesicles adjacent to chloroplast outer membranes were observed

frequently in infected cells. Whether they were the reflection of chloroplast aberrations caused by virus infection or were directly related to virus replication is not known. However, it is more likely that they were the result of secondary effects of virus infection, and not the sites of virus replication, because of the lack of virus particles in chloroplasts.

In conclusion, the present study provides evidence that the virus produced multiple alterations on mitochondria chloroplasts and cell wall associated structures and most of the aberrations resulted from the metabolic alterations upon virus infection and were not directly related to the virus replication process. It also should be noted that the response of soybean callus cells to SBMV infection was considerably different from those of intact bean plants, and that host cell-dependent cytopathic alterations have been shown in this study.

SUMMARY

Soybean (Glycine max. "H63") callus tissue grown in liquid medium was inoculated with southern bean mosaic virus (SBMV) and examined by transmission electron microscopy. Particles similar in size and shape to SBMV were found in cytoplasm, vacuoles and nuclei. Such particles were present in infected cells and lacking in control cells. They were spherical with a diameter of 24 nm in thin sections, occurring scattered, or in loose aggregations in cytoplasm or vacuoles. Crystalline arrangement of virus particles was sometimes seen in cytoplasm but never in vacuoles or nuclei. Particles found in vacuoles were most often in irregular clumps. Such particles were somewhat less regular in outline when present in nuclei. No virus-like particles were seen in any other cell organelles, but certain organelles had undergone cytopathic changes. Chloroplasts of infected cells frequently contained large amounts of phytoferritin, and occasionally chloroplasts did not develop fully and prolameller bodies were retained. Distinctive electron-opaque deposits occurred in mitochondria, and at times these inclusions were elongated with a filamentous nature. Infected cells also had rather extensive, amorphous cytoplasmic inclusions. Microfilaments were much more common in infected cells than in control cells and occurred in both cytoplasm and nuclei.

Figure 1. Vacuolar contents of SBMV-infected soybean callus cells.

A. Virus particles (V) loosely aggregated as irregular clumps in vacuoles. Arrow indicates the dense vacuolar inclusion frequently observed adhering to inner surface of tonoplast; n, nucleus. X6,900.

Insert: Virus clumps at higher magnification (X60,000). Note the irregular diffuse fibrillar substances.

B. Virus particles in vacuoles. The diameter of virus measured 22-27 nm. Bar = 100 nm.

C. Association of virus particles (V) with electron dense vacuolar inclusion (inc); ER, endoplasmic reticulum. X10,000.

D. Higher magnification of vacuolar inclusion from C. Note the association of virus with the inclusion. X60,000.

E. Electron-dense vacuolar inclusion (inc). Virus particles were included in the inclusion (arrow). X80,000.

F. Invaginated structure (arrow) in the vacuole. Virus particles and ribosomes were mixed and not readily distinguished. CYT, cytoplasm; VA, vacuole. X25,000.

G. A protuberance from swelling endoplasmic reticulum lumen (arrow). CYT, cytoplasm.

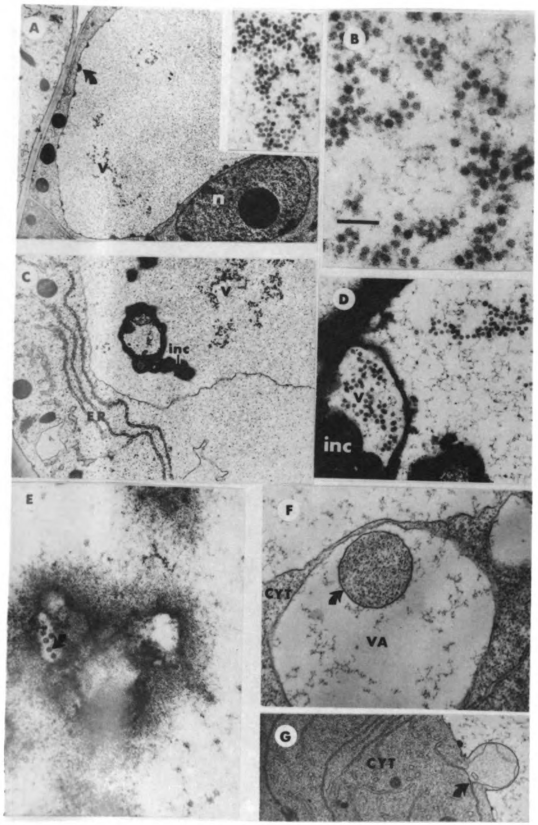


Figure 1

Figure 2. Crystalline arrangements of virus particles in cytoplasm of SBMV-infected callus cells. Bar represents 100 nm in all figures.

A. A hexagonal array of virus particles. View is approximately down a threefold axis. The black lines represent the position of a unit cell of the crystal.

B. A tetragonal array of virus crystals. View is approximately down a fourfold axis. The black lines represent the position of a unit cell of the crystal.

C. Background texture of cytoplasm which contained virus crystals. A crystalline arrangement of virus particles is in the lower part and a moderately stained vesicle (arrow) was associated with virus aggregates.

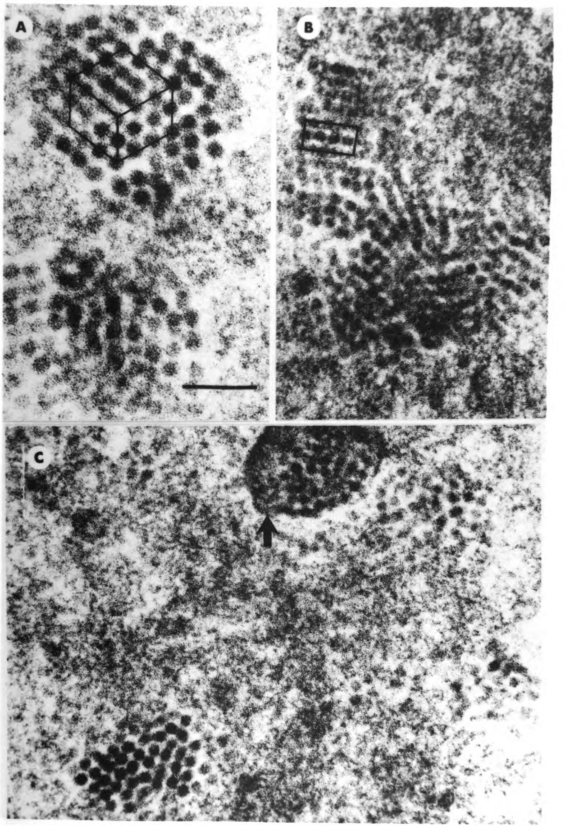


Figure 2

Figure 3. Cytoplasm of SBMV-infected callus cells (I). Virus particles were scattered as small groups (small curved arrows). A mass of diffuse ground substances (vicinity of asterisk). Insert: Squared area of Figure 3. The ribosomes (straight arrow) can be distinguished from virus particles by their irregular outlines and their attachment to endoplasmic reticulum. RER, rough endoplasmic reticulum; MB, microbody; GO, Golgi bodies; MT, mitochondria; CW, cell wall; V, virus particles. X21,350. (Insert: X80,000).

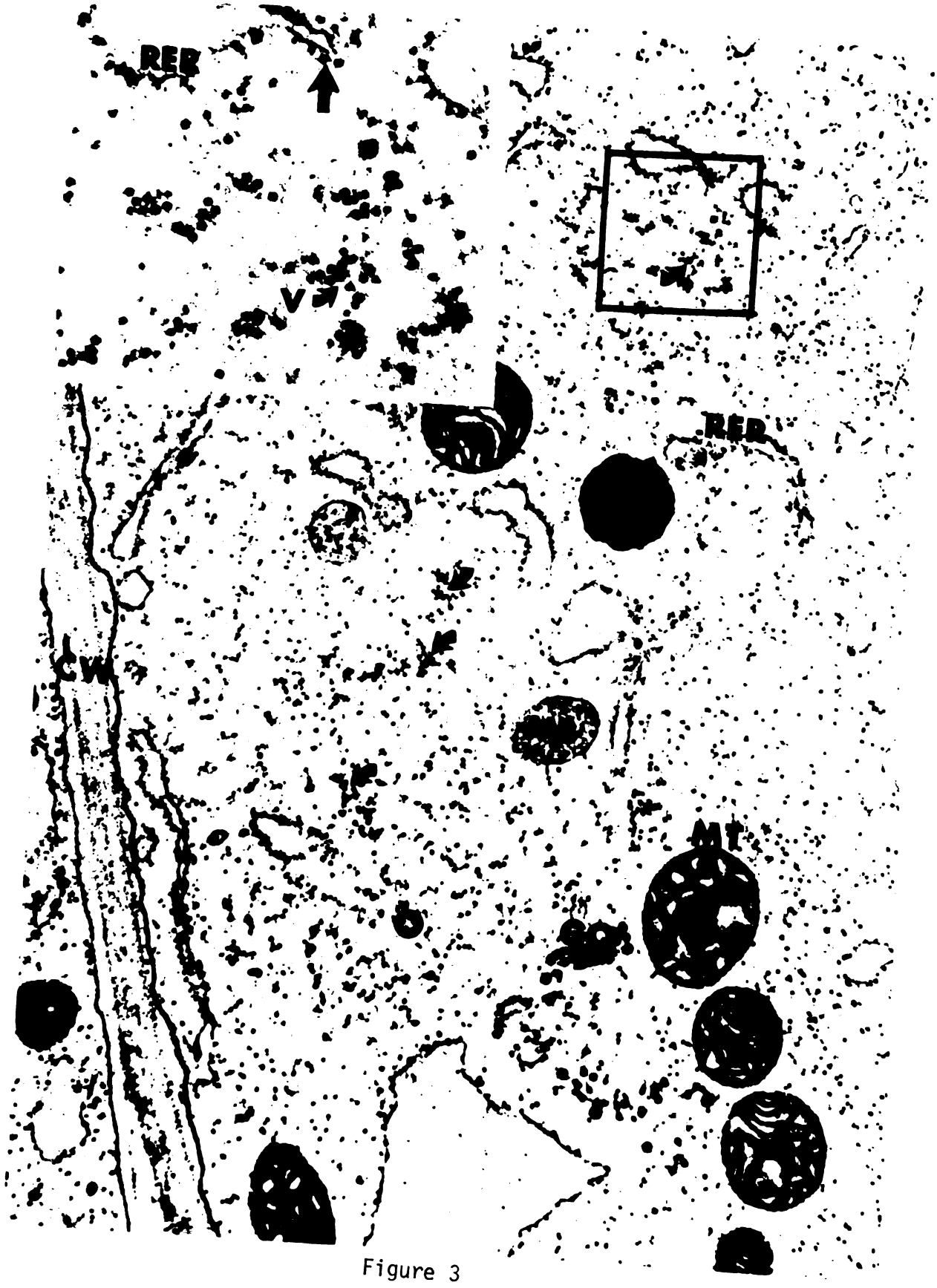


Figure 3

Figure 4. Cytoplasm of SBMV-infected callus cells (II).

A. Polyribosomes (arrow) in cells inoculated with SBMV after 5 days. They are in close proximity to smooth endoplasmic reticulum (SER). X52,000.

B. Rough endoplasmic reticulums filled the cytoplasm of cells 5 days after inoculation. X16,000.

C. A "pocket" formed by encircled endoplasmic reticulum of cells 10 days after infection. Membrane-like materials (arrow), electron opaque to electron dense substances (ed) and ribosomes can be seen in the pocket. X25,000.

D. A double-membrance vesicle (arrow) containing virus particles in the cytoplasm of cells 5 days after inoculation. X25,000.

E. Diffuse background texture of cytoplasm containing virus crystals (arrows). X25,000.

F. Highly vesiculated cytoplasm in infected cells 5 days after inoculation. X3,200.

G. Association (indicated by a black arrow) of membranous substances (M) with cell wall (CW) in cells inoculated after 5 days. Virus-like particles (white arrows) and ribosomes were richly present in the vicinity. X52,000.

H. A double membrane vesicle releasing virus particles into cytoplasm. X125,000.

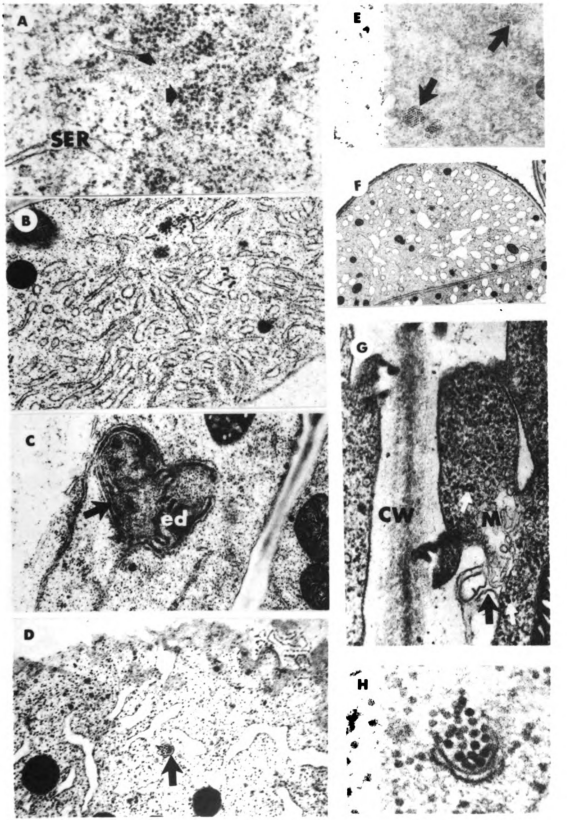


Figure 4

Figure 5. Nuclei of SBMV-infected cells.

A. Granular substances deposited adjacent to nuclear membrane (nm) in nucleus (nu). Virus particles (arrow) were freely dispersed in nucleus. Portions (black line--squared area) were enlarged in Figure 5B. Note the nuclear membrane is lobated. X21,000.

B. Virus particles (arrows) in nucleus. They were mixed with granular nuclear substances. Note the fine diffuse fibrillar substances which constitute the nucleoplasm. X60,000.

C. Vacuolated nucleolus (no) in cells 15 days after inoculation. X21,000.

D. Highly lobated nuclear membrane (arrow 1) in a SBMV-infected cell. Note the convoluted area (arrow 2); no, nucleolus; ch, chloroplast. X6,900.

E. Curved fibrous clusters (cfc) intermingled with ribosomes (ri), mitochondria (mt), granular nuclear substances (gns), and nuclear pores (np) in a SBMV-infected cell. X91,000.

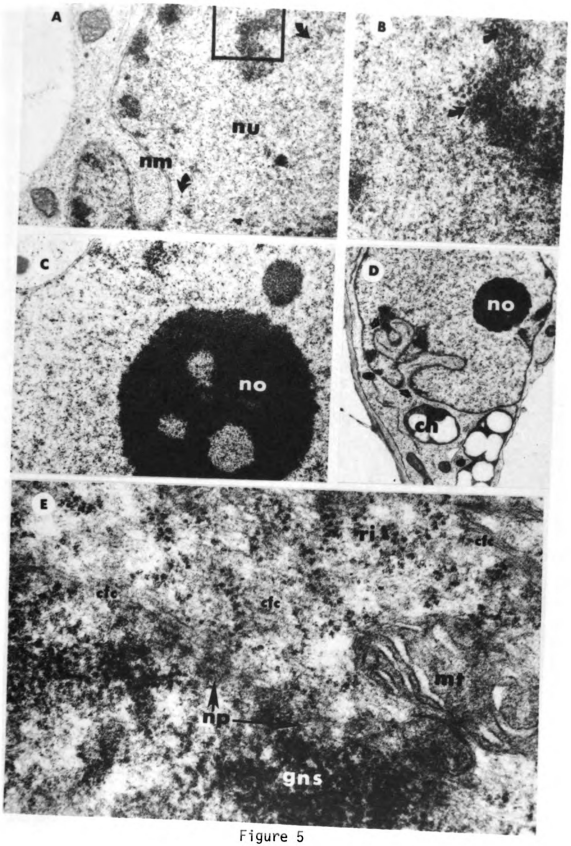


Figure 5

Figure 6. Cytoplasmic inclusions in SBMV-infected callus cells.

A. Amorphous inclusions (ai). Note the irregular electron-dense deposits (ed) in the surrounding areas. The endoplasmic reticulums (er) were also in close proximity to inclusions. Arrow indicated the vacuolated area within the inclusion where virus-like particles are present. X60,000.

B. An inclusion body (ib) consisted of diffuse coarse fibers and a bundle of particles (arrow 1) in its center present in a cell 10 days after inoculation. Note the double membrance (arrow 2) is associated with the inclusion, and the nucleus is highly lobated which resulted in 3 separated nucleus when cross sectioned; mt, mitochondria; n, nucleus; cw, cell wall.

C. Portions of inclusion seen in Figure 6B. X103,000.

D. Amorphous inclusion (ai) and beaded particles (bp); er, endoplasmic reticulum. X40,000.

E. An inclusion containing clusters of dark diffuse spots observed 10 days after inoculation. X32,000.

F. Portion of Figure 6E. Striated diffuse fibers intermingled with the dark spots. X80,000.

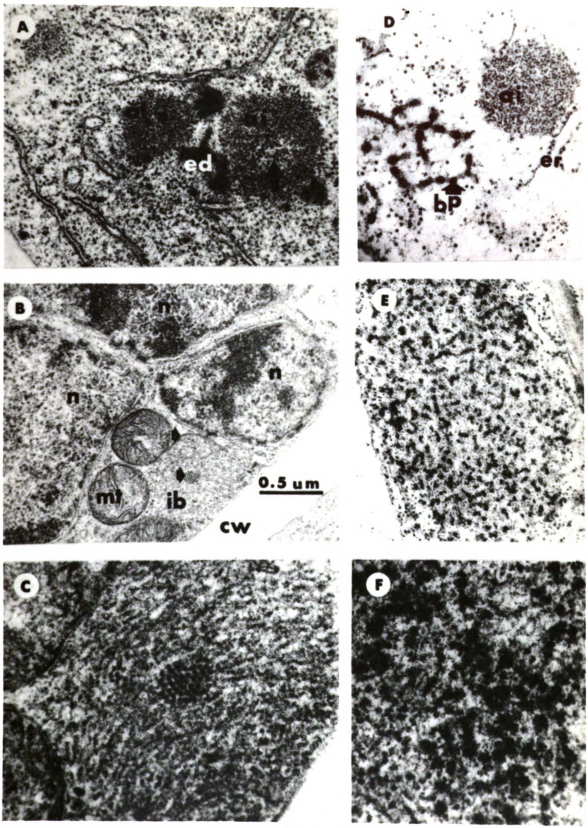


Figure 6

Figure 7. Microfilaments (mf) in SBMV-infected callus cells.

A. Microfilaments appeared as curved bundles. Rough endoplasmic reticula (rer) are closely associated with them. X21,000.

B. Slightly curved microfilaments; va, vacuole. X32,000.

C. Curved microfilaments at high magnification. X60,000.

D. Cross section of microfibrils. Phosphotungstic acid stained. X53,000.

E. Straight microfilament bundles which frequently change their directions; mt, mitochondria. X12,500.

F. Microfilaments in the nucleus. NU, nucleus; CYT, cytoplasm. X52,000.

G. Microfilaments extended from nucleus (NU) into cytoplasm breaking through nuclear membrance. Note the highly lobated nucleus. Arrow indicates the end of microfilaments. X12,500.

H. The "exit" of microfilaments from nucleus into cytoplasm (CYT). Arrow indicates the disrupted nuclear membrane. X40,000.

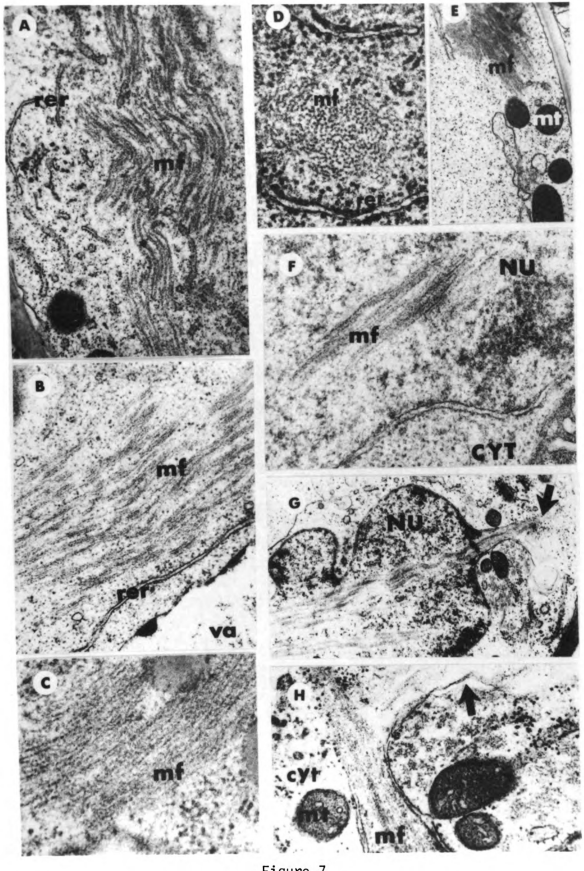


Figure 7

Figure 8. Alteration of mitochondria in SBMV-infected cells (I).

A. Concentric cristae in a longitudinal section of mitochondria. X32,000.

B. A large bundle of densely packed cristae occupied most of the central area of a mitochondrian; n, nucleus; nm, nuclear membrane. X40,000.

C. Densely packed cristae as a bundle of "microfilaments" occurred in one side of the mitochondria membrane. Note both of their longitudinal ends are not interconnected (the same as Figure 8B). Also note the cristae of other areas appeared "normal." Bar = 0.5 μm.

D, E. Bundles of diffuse microfibril-like substances in a mitochondrian extending into cytoplasm (bold arrow) where they appeared as electron-opaque materials (thin arrow). D = X52,000; E = X40,000.

F, I. Discoid structure of mitochondria in their lateral sections (F, G), and cup-shaped top sections (H, I). The connections between cristae can be seen (arrows). The asterisks indicate the invagination of cytoplasmic contents into the cup-shaped areas. F = X60,000; G = X40,000; H = X52,000; I = X52,000.

J. A bundle of microfibrial-like substance was confined in a vacuolated canal in the center of a mitochondrian. The arrow indicates the connection between matrices and the microfilament-like substances. X60,000.

K. A dense body in a small vacuole of mitochondria. X52,000.

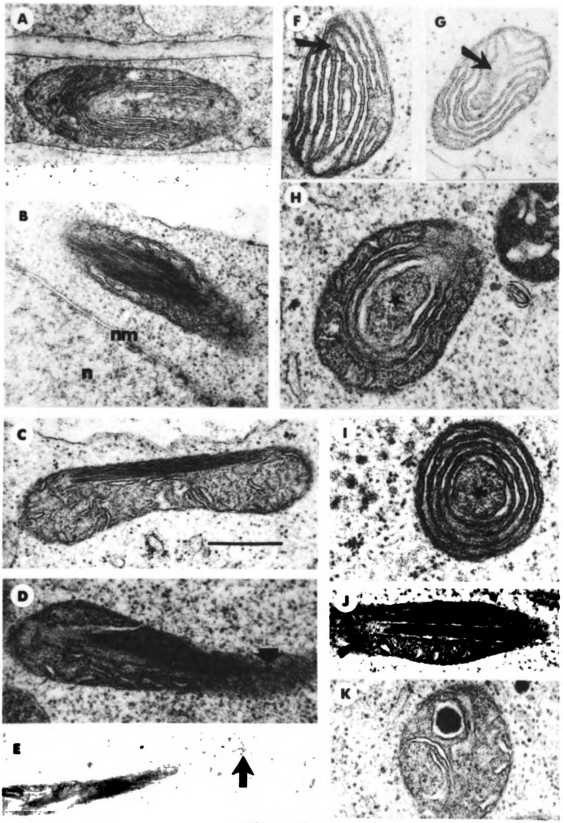


Figure 8

Figure 9. Alteration of mitochondria in SBMV-infected cells (II).

A. Engulfment of a mitochondrian by encircled rough endoplasmic reticula (rer) which are indicated by straight bold arrows. Two constricted regions in the mitochondria are indicated by curved arrows. Note two bundles of microfilament are present in the mitochondria; ai, amorphous inclusion; va, vacuole; mb, microbody; cw, cell wall; mt, mitochondria. X32,500.

B. Cross-section of Figure 9A. The mitochondrian is being degraded. X60,000.

C. Electron-opaque deposits in mitochondria; cw, cell wall; va, vacuole. X60,000.

D. Dense-stained matrices and condensed spherical mitochondria (mt). X32,000.

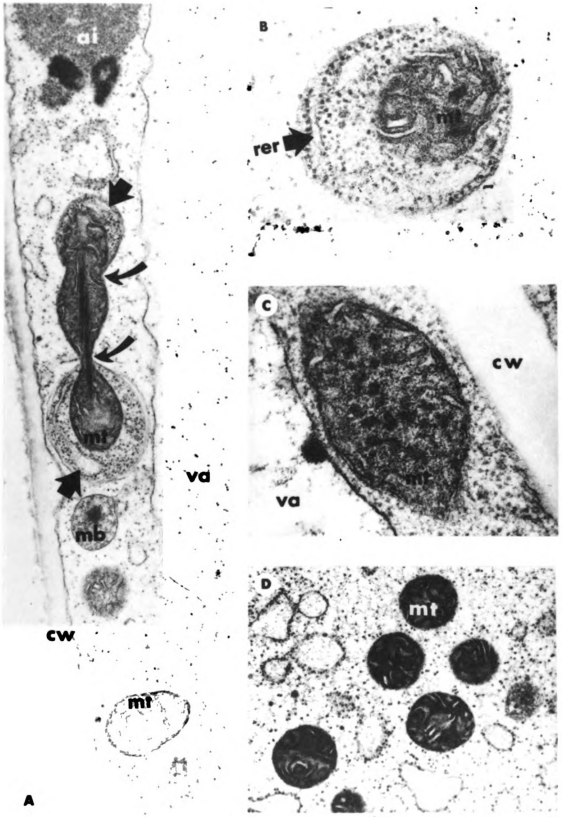


Figure 9

Figure 10. Aggregation of phytoferritin particles in chloroplasts of SBMV-infected callus cells.

A, B. Phytoferritin particles arranged in alternate straight dense lines (arrowed) and regularly spaced particles. A = X60,000; B = X160,000.

C. Phytoferritin particles arranged in slightly curved rows and alternate dense lines. Note the vesiculated structures adjacent to chloroplast membrance (also seen in Figure 10A). X80,000.

D. Phytoferritin released from a degraded chloroplast. X60,000.

E. A favorable orientation of phytoferritin showed their electron-dense subunit structures (arrow) and electron-translucent core. X320,000.

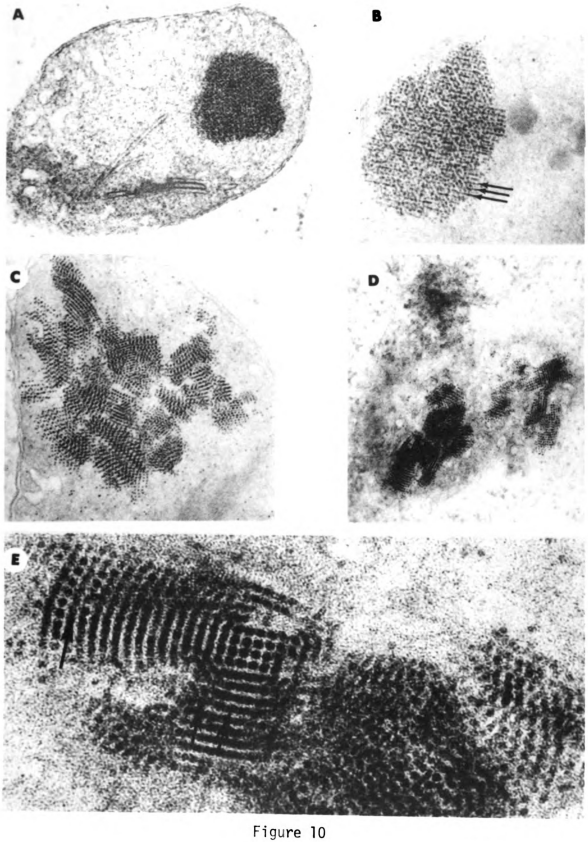


Figure 10

Figure 11. Cytopathic effects of chloroplast in SBMV-infected callus cells.

A. Disorganized grana (g) of chloroplasts. Microfibrils (mf) and an osmophilic globule (og) are adjacent to the chloroplast; va, vacuole; cw, cell wall. Bar = 2 μm.

B. Microfilaments (mf) in a constricted chloroplast. Starch grain (s) and disorganized grana (g) are present in chloroplast. Arrow indicates the constriction. X32,000.

C. A pocket-like structure (arrow) in a chloroplast discharging its contents into cytoplasm. Not the highly vesiculated structures (short arrow) adjacent to the chloroplast. X16,000.

D. Single-membrane bounded vesicles (short arrows) adjacent to chloroplast membrane. A large vacuole (V), swelled grana structure (g) and starch is present in the chloroplast. X32,000.

E, F. Prolamellar bodies (pb) in chloroplasts. Note the osmophilic substance (O) surrounded the prolamellar body and the light-stained globules present (lg) in the vicinity. E = X25,000; F = X80,000.

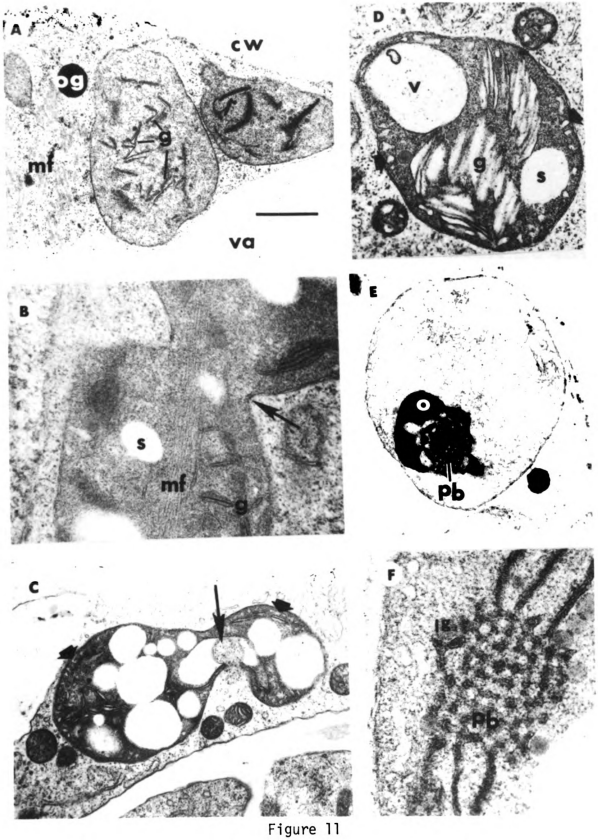


Figure 11

Figure 12. Cell walls (cw) and cell wall-associated structures in SBMV-infected callus cell.

A. Cell debris (open arrows) and damaged cell walls (solid arrows) resulted from vortexing during the inoculation of virus. X25,000.

B. Association of virus particles (arrows) with cell debris produced during the inoculation procedure. Bar = 100 μm.

C. Presence of virus particles (arrows) in plasmodesmata (pd). Bar = 100 μm.

D. Large lumina (open arrows) occurred inside the cell wall connecting the plasmodesmata (solid arrows). X52,000.

E. Branched lumen which contained striated substances in cell wall (open arrow). X80,000.

F. Accumulation of paramural bodies (pb) between cell wall and plasmolemma (arrowed). X10,000.

G, H. Microtubules (open arrows) intimately associated with cell wall. They oriented angularly (seen in G) in parallel, or perpendicularly (seen in H) to the cell wall. G = X25,000; H = X40,000.

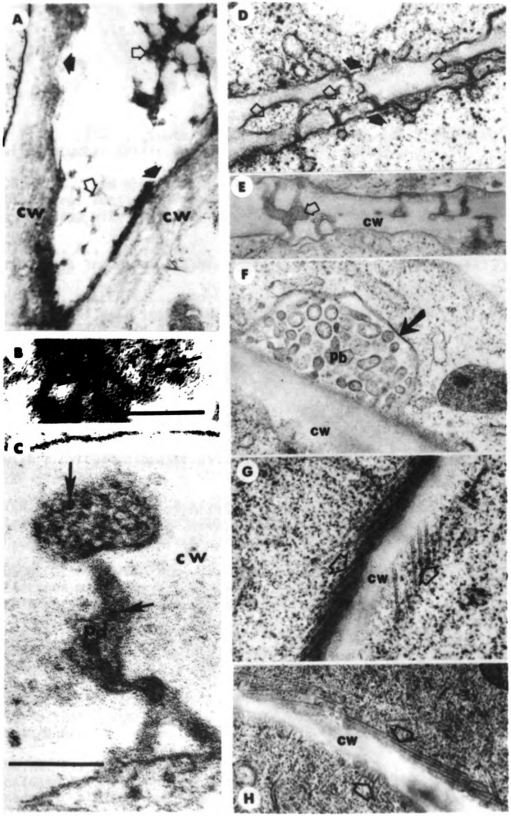


Figure 12

REFERENCES

ALLEN, T. C. (1972). Subcellular responses of mesophyll cells to wild cucumber mosaic virus. Virology 47: 467-474.

BOULTON, R. W., and WESTAWAY, E. G. (1976). Replication of the flavivirus kunjin: proteins, glycoproteins, and maturation associated with cell membranes. Virology 69: 416-430.

CALIGUIRI, L. A., and TAMM, I. (1970). The role of cytoplasmic membranes in poliovirus biosynthesis. Virology 42: 100-111.

CRONSHAW, J., HOEFERT, L., and ESAU, K. (1966). Ultrastructural features of Beta leaves infected with beet yellows virus. J. Cell. Biol. 31: 429-443.

DeZOETEN, G. A., and GAARD, G. (1969). Possibilities for inter- and intracellular translocation of some icosahedral plant viruses. J. Cell Biol. 40: 814-823.

DeZOETEN, G. A., ASSINK, A. M., and KAMMEN A. VAN. (1974). Association of cowpea mosaic virus-induced double-stranded RNA with a cytopathological structure in infected cells. Virology 59: 341-355.

DeZOETEN, G. A. (1976). Cytology of virus infection and virus transport. In "Physiological Plant Pathology" (R. Heitefuss and P. H. Williams, eds.), pp. 129-149. Springer-Verlas, New York.

EDWARDSON, J. R., PURCIFULL, D. E., and CHRISTIE, R. G. (1966). Electron microscopy of two small spherical plant viruses in thin sections. Can. J. of Bot. 44: 821-830.

GREEN, T. R., and RYNN, L. A. (1972). Wound-induced proteinase inhibitor in plant leaves: a possible defense mechanism against insects. Science, N.Y. 175: 776-777.

GUNNING, B. G. S., and STEER, M. W. (1975). Vacuole and tonoplast. In "Ultrastructure and the Biology of Plant Cells," pp. 33-38. Edward Arnold, London.

HAMPTON, R. E., and FULTON, R. W. (1961). The relation of polyphenol oxidase to instability in vitro of prune dwarf and sour cherry necrotic ringspot viruses. Virology 13: 44-52.

HARRISON, B. D., and ROBERTS, I. M. (1968). Association of tobacco rattle virus with mitochondria. J. Gen. Virology 3: 121-124.

HATTA, T. (1976). Recognition and measurement of small isometric virus particles in thin sections. Virology 69: 237-245.

HATTA, T., NAKAMOTO, T., TAKAGI, Y., and USHIYAMA, R. (1971). Cytological abnormalities of mitochondria induced by infection with cucumber green mottle mosaic virus. Virology 45: 292-297.

HEPLER, P. K., and PALEVITZ, B. A. (1974). Microtubles and microfilaments. Ann. Rev. Plant Physiol. 25: 309-362.

HOFFMAN, L. R., and STANKER, L. H. (1976). Virus-like particles in the green algae cylindrocapsa. Can. J. of Bot. 54: 2827-2841.

HOOPER, G. R., and NIBLETT, C. L. (1969). Phytoferritin and virus-like particles in citrus infected with citrus vein-enation virus. Phytopath. 59: 12 (Abstr.).

IE, T. S. (1971). Electron microscopy of developmental stages of tomato spotted wilt virus in plant cells. Virology 43: 468-479.

JOHNSON, J. E., ROSSMAN, M. G., SMILEY, I. E., and WAGNER, M. A. (1974). Single crystal X-ray diffraction studies of southern bean mosaic virus. J. Ultrastruct. Res. 46: 441-451.

MARTELLI, G. P., and RUSSO, M. (1977). Plant virus inclusion bodies. In "Advances in Virus Research" (M. A. Lauffer et al., eds.), Vol. 21, pp. 175-266. Academic Press, New York.

MATTHEWS, R. E. F. (1973). Induction of disease by viruses, with special reference to turnip yellow mosaic virus. Ann. Rev. Phytopathol. 11: 147-170.

MINK, G. I. (1965). Inactivation of tulare apple mosaic virus by o-quinones. Virology 26: 700-707.

MINK, G. I., and DIENER, T. O. (1971). The effect of tetrachloro-o-benzoquinone on southern bean mosaic virus and on its nucleic acid. Virology 45: 764-766.

MOLINE, H. E. (1973). Ultrastructure of Datura stramonium leaves infected with the physalis mottle strain of belladonna mottle virus. Virology 56: 123-133.

PACKARD, M. J., and STACK, S. M. (1976). The preprophase band: possible involvement in the formation of the cell wall. J. Cell. Sci. 22: 403-411.

RALPH, R. U., BULLIVANT, S., and WOJCIK, S. J. (1971). Evidence for the intracellular site of double-stranded turnip yellow mosaic virus RNA. Virology 44: 473-479.

ROBARDS, A. W., and HUMPHERSON, P. G. (1967). Phytoferritin in plastids of the cambial zone of willow. Plants (Berl.) 76: 109-178.

RYAN, C. A., and SHUMWAY, L. K. (1970). Differential synthesis of chymotrypsin inhibitor-1 in varigated leaves of a cytoplasmic mutant of tobacco. Pl. Phys. (Lancastor) 45: 512-552.

SEMANCIK, J. S., and VANDERWOUDE, W. J. (1976). Exocortis viroid: Cytopathic effects at the plasma membrane in association with pathogenic RNA. Virology 69: 719-726.

SHEPHERD, R. J. (1971). Southern bean mosaic virus. Description of plant viruses. Common. Mycol. Inst. Assoc. Appl. Biol. Kew, Surrey, England, 4 p.

SPENSER, D. F., and KIMMINS, W. C. (1971). Ultrastructure of tobacco mosaic virus lesions and surrounding tissue in Phaseolus vulgaris var. Pinto. Can. J. of Bot. 49: 417-421.

SPURR, A. R. (1969). A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26: 31-43.

STREET, H. E., DAVEY, M. R., and SUTTON-JONES, B. (1972). Ultrastructural features of cells and tissues in culture. Symp. Biol. Hung. 14: 145-159.

TANDLER, B., and SHIPKEY, F. H. (1964). Ultrastructure of warthin's tumor. I. Mitochondria. J. Ultrastruct. Res. 11: 292-305.

TRUMP, B. F., and ERICSSON, J. L. (1965). Some ultrastructural and biochemical consequences of cell injury. In "The Inflammatory Process" (B. N. Zweifach, L. Grant, and R. T. McCluskey, eds.), pp. 35-120. Academic Press, New York.

USHIYAMA, R., and MATTHEWS, R. E. F. (1970). The significance of chloroplast abnormalities associated with infection by turnip yellow mosaic virus. Virology 42: 293-303.

WEINTRAUB, M., and RAGETLI, H. W. J. (1970a). Electron microscopy of bean and cowpea strains of southern bean mosaic virus within leaf cells. J. Ultrastruc. Res. 32: 167-189.

WEINTRAUB, M., and RAGETLI, H. W. J. (1970b). Identification of the constituents of southern bean mosaic virus in crystals of infected cells, and their distribution within the virion. Virology 41: 729-739.

WEINTRAUB, M., and RAGETLI, H. W. J. (1971). A mitochondria disease of leaf cells infected with an apple virus. J. of Ultrastruct. Res. 36: 669-693.

WILDMAN, R. B., and HUNT, P. (1976). Phytoferritin associated with yellowing in leaves of Cocos nucifera (Arecaceae). Protoplasma 87: 121-134.

WILSON, H. J., ISRAEL, H. W., and STEWARD, F. C. (1974). Morphogenesis and the fine structure of cultured carrot cells. J. Cell. Sci. 15: 57-73.

WITHERS, L. A. (1976). Studies on the growth in culture of plant cells. J. of Exp. Bot. 27: 1073-1084.