PROTEINS SYNTHESIZED IN MAIZE LEAVES AND MESOPHYLL PROTOPLASTS INFECTED WITH MAIZE CHLOROTIC MOTTLE VIRUS

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ABBREVIATIONS

cm - Centimeter

Ci - Curie

o - Degree centigrade

E - Einstein

GLC - General laboratory centrifuge

g - Gram

g - Gravitational unit

^{3}H - Tritium

hr - Hour

KDa - Kilodaltons

lb/sq inch - Pounds per square inch

mg/ml - Milligram per milliliter

mm - Millimeter

m M - Millimolar

M - Molar

min - Minutes

MES - 2(N-Morpholino) ethane sulfonic acid

mRNA - Messenger ribonucleic acid

nm - Nanometer

% - Percent

rpm - Revolutions per minute

RNA - Ribonucleic acid

sec - Second

SS - Single stranded

ul - Microliter

uCi - Microcurie

ug - Microgram

v/v - volume per volume

w/v - weight per volume

CHAPTER I

INTRODUCTION

MCMV:Isolation and Properties

Maize chlorotic mottle virus (MCMV) was first isolated from diseased maize plants (Zea mays L.) in Peru (Castillo and Herbert, 1974). It was subsequently found in Kansas in 1976 (Niblett and Claflin, 1976). MCMV is presently found in southern Nebraska and northern Kansas. The virus interacts synergistically with other viruses such as maize dwarf mosaic virus (MDMV) or wheat streak mosaic virus (WSMV) to cause corn lethal necrosis (CLN) an economically important disease of maize. Symptoms of this disease include severe chlorotic bleaching followed by necrosis. Infected plants usually die and those surviving are severely stunted and/or sterile. Inoculation of corn seedlings with any one of the viruses stated above causes only mild mosaic symptoms. MDMV and WSMV are endemic to many regions of North America and as MCMV spreads into new areas more widespread outbreaks of CLN are likely to occur. Since MCMV persists in decaying plant material from year to year, some control of MCMV has been achieved by rotating maize with crops that are not hosts of MCMV (Uyemoto, 1983). Research on the modes of replication and gene expression of MCMV may contribute an insight into the possible mechanisms of the synergistic interaction of MCMV with other viruses.

The Peruvian isolate of MCMV (MCMV-P) has an isometric particle of

about 30 nm in diameter (Castillo and Herbert, 1974). The MCMV-K (Kansas isolate) had a similar size and morphology (Niblett and Claflin, 1976). Reciprocal serological comparison of the two isolates show that they are distinguishable (Niblett and Claflin, 1976). The host range of both isolates is restricted to a few species of grasses (Niblett and Claflin, 1976).

Six species of the chrysomelid beetles including both larval and adult forms of Oulema melonopa transmit MCMV (Nault et al., 1978). Of the six chrysomelid vector species, however, O.melanopa does not occur in Kansas. Its initial spread in Kansas could be due to Chaetocnema pulicaria or Diabotrica undecimpuctata both of whose adults overwinter and appear in corn fields during the growing season (Metcalf, Flint and Metcalf, 1962).

MCMV has a single strand of RNA with a molecular weight of about $1.4x10^6$ daltons (4360 nucleotides), as determined by agarose gel electrophoresis in 2.2 M formaldehyde (Gardner, unpublished). The properties of MCMV suggest that it may belong to the southern bean mosaic virus (SBMV) group (Hull, 1977a). Members of the SBMV (sobemovirus) group have the following properties; 1) sedimentation velocity 110 to 120s, 2) molecular weight of the virus around 6.0 to $6.5x\ 10^6$, 3) molecular weight of coat protein around 30 kDa, and 4) molecular weight of the single-stranded RNA around $1.4x10^6$ daltons. Each of the viruses in this group have their structure stabilized by divalent cations, a pH dependent bond and protein-RNA interaction (Hull, 1977b). Sobemoviruses also form crystalline arrays in the cytoplasm of the plant cell (Milne,1967; Bakker,1974; and Plaskitt, unpublished).

The major translation products of MCMV RNA in a wheat germ system are 46, 45, 36, 29, 18 and 16 kDa polypeptides (Gardner unpublished). Some minor proteins of molecular weight 60, 49, and 25 kDa are also detected. The total molecular weight of all these polypeptides far exceeds the coding capacity of the MCMV RNA.

Other Sobemoviruses

Comparison with the proteins synthesized by other sobemovirus RNAs may help elucidate the translational strategy followed by MCMV. The translation of turnip rosette virus (TRosV) RNA in nuclease-treated rabbit reticulocytes (Morris-Krsinich and Hull, 1981), showed the appearance of four major products of molecular weights 105, 67, 35, and 30 kDa. The 30 kDa was identified as the virus capsid protein as indicated by peptide mapping and immunoprecipitation. Peptide analysis after partial proteolysis, showed that the 105, 67 and 35 kDa were related. The time course analysis suggested that the 67 kDa is derived from the 105 kDa by post-translational cleavage. Viral coat protein seemed to be most efficiently synthesized from a virus encapsidated subgenomic RNA.

Translation of southern bean mosaic virus (SBMV) RNA, another sobemovirus, in wheat embryo and rabbit reticulocyte lysate (Salerno-Rife, Rutgers and Kaesberg, 1980) gave rise to four major polypeptides, two of which seemed to be related as shown by tryptic digestion analysis. These polypeptides had molecular weights of 105 and 75 kDa. One polypeptide of 29 kDa resembled the coat protein and a lower molecular weight polypeptide of 14 kDa also appeared in some analyses. The molecular weight of SBMV RNA is 1.4×10^6 (Diener, 1965),

which is not sufficient to encode all four of the above proteins. So it seems as if their cistrons overlap (Salerno-Rife, Rutgers and Kaesberg, 1980), or read-through products are synthesized.

Other sobemovirus are known to produce a subgenomic mRNA which codes for the coat protein (Ghosh et al., 1981; Morris-Krsnich and Hull, 1981; and Morris-Krsnich and Forster, 1983). It is possible that this strategy is followed by MCMV also. However several other mechanisms have been proposed for the synsthesis of multiple proteins from genomic RNAs of sobemoviruses such as 1) post translational cleavage of a large polypeptide (Morris-Krsnich and Hull, 1981), 2) translation of subgenomic messages (Ghosh et al., 1981), and 3) proteins synthesized by a read through of "leaky" (i. e. continuation of translation beyond a normal termination site) termination codons (Salerno-Rife, Rutgers and Kaesberg, 1980; Morris-Krsnich and Forster, 1983).

To identify the mode of gene expression of a virus genome, it is necessary to analyze the viral genome and its translation products both in vivo and in vitro. The objective of this research was to identify proteins induced by MCMV infection, utilizing the excised leaves of maize seedlings and maize mesophyll protoplasts.

Use of Protoplasts in the Study of Plant Viruses

Protoplasts are plant cells which have had their cell walls removed by dissecting plasmolysed plant tissue or by enzymes (Cocking, 1960). The first successful isolation of mesophyll protoplasts (Takebe et al., 1968), and their infection by a plant virus (Takebe and Otsuki, 1969) were accomplished using tobacco leaves. Conditions for the

synchronous infection of protoplasts were established and a new avenue opened to the study of plant viruses. Synchronous infection has been obtained with cowpea (Hibi, Rezelman and Van Kammen 1975) tomato (Motoyoshi and Oshima,1975), turnips (Morris-Krsnich, Hull and Russo, 1979), and several other plants.

Yields of mesophyll protoplasts are influenced considerably by the age and the physiological state of the source of leaves. Environmental conditions such as temperature, humidity, soil type and even method of watering can affect the yield. These conditions have to be standardized for every plant and laboratory, in order to obtain consistent yield throughout the year (Kubo et al., 1975b).

Two methods are currently followed in the preparation of protoplasts. The two step procedure and the one step procedure. The two step procedure is the original technique (Takebe, Otsuki and Aoki, 1968). In this technique the leaves, are stripped of their lower epidermis, followed by a bath in a mannitol solution of polygalacturonidase, and then a cellulase solution. This takes a longer time for release of protoplasts, but the yield is generally better than that obtained by the one step procedure. Incubation time is cut by half in the one step procedure (Kassanis and White, 1974; Hibi, Rezelman, and Van Kammen, 1975), as the tissue is incubated with both enzymes simultaneously. However the preparation is heterogenous due to release of subprotoplasts (protoplasts lacking part of their cellular content). Kanai and Edwards (1973), prepared maize mesophyll protoplasts by the one step procedure and introduced a liquid-liquid two phase separation technique to remove damaged cells and chloroplasts from the preparation. Several variables affect the proper partitioning of

particles in the two phase system; 1) effect of centrifugal force (the optimum for intact protoplast isolation is 300 g to 500 g), 2) molecular weight of dextran (the optimum is 40 kDa), 3) salt concentration (the optimum is 10 mM sodium phosphate, it is by far the most important (Kanai and Edwards, 1973).

Morris-Krsnich, Hull and Russo (1979) studied the translation of TRosV RNA (a sobemovirus) in protoplasts by infecting the protoplasts in vitro. Hull's study showed that protoplasts could be infected in vitro by TRosV, thus facilitating the study of TRosV protein synthesis in isolated cells

The study of proteins synthesized in protoplasts of tobacco (Nicotiana tabacum cv. Xanthi nc.) made from leaves infected with cucumber mosaic virus (CMV) was carried out by Roberts and Woods (1981) in the hope that virus-induced polypeptides would be more easily detected. Two polypeptides of low molecular weight (23, and 21 kDa), were detected in the infected protoplasts. Also larger polypeptides of 32 kDa and 120 kDa were detected in the cellular particulate fractions. These polypeptides could be virus-induced plant proteins or synthesized from viral RNA using the host's protein synthesizing machinery.

Protein Synthesis in Intact Leaves

Intact leaves have been used for the study of virus-induced proteins since the early studies of tobacco mosaic virus (TMV) started. Hirai and Wildman (1969) developed a method by which intact third leaves of uninfected and infected plants were supplied with radioactive amino acids for a period of 8 hr. The chloroplasts from the infected leaves had a reduced capacity for nucleoside triphosphate incorporation

into RNA and amino acid incorporation into proteins. Gianinazzi et al., (1970) studied the changes in soluble leaf proteins in a tobacco plant as a result of infection. They demonstrated the appearance of four new leaf protein components (b1, b2, b3, and b4) not seen among the uninfected leaf proteins. Zaitlin and Hariharasubramanian compared protein synthesis in TMV infected leaf tissue with that of uninfected leaves by exposing leaf strips to (^{3}H)-leucine following a treatment with actinomycin D. They were able to identify at least four polypeptides found in infected tissue and not in the uninfected.

Protein changes in wheat infected with wheat streak mosaic virus (WSMV) or barley stripe mosaic virus (BSMV) were studied by comparing proteins labelled in infected leaves to those labelled in uninfected leaves (White and Brakke, 1983). Polypeptides of molecular weight 100, 98, 72, 66, 43, and 29 kilodaltons were synthesized and were identified as possible viral-coded proteins as they were synthesized in the presence of chloramphenicol (an inhibitor of chloroplast protein synthesis), alpha-amanitin (an inhibitor of host RNA polymerase II) and actinomycin D (an inhibitor of DNA-dependent RNA synthesis). In BSMV infected leaves, polypeptides of molecular weights 87, 83, 62, and 35 kDa were increased in the 1000 g pellet; 83, 70, 23 kDa in the 12,000 g pellet; 35 and 23 kDa in the 275,000 g pellet and 83, 81, and 43 kDa in the 275,000 g supernatant. The polypeptide of molecular weight 23 kDa is the BSMV coat protein and was a major protein in extracts of BSMV infected leaves.

Roberts and Wood (1981), studied protein synthesis in cucumber mosaic virus (CMV) infected tobacco leaf discs which were radiolabelled with (^{3}H)-leucine. The proteins detected in the infected leaves were

compared to those from the mock inoculated tobacco leaf discs. Coat protein of molecular weight 27 kDa was identified along with protein of higher molecular weight 120 kDa to 160 kDa and possibly 32 kDa was detected in the particulate fractions.

Since infection of maize protoplasts with MCMV in vitro would involve the establishment of a number of parameters, protoplasts made from infected leaves were chosen for studying the proteins synthesized in MCMV infected tissues. Excised leaves of maize infected with MCMV were also chosen for this purpose. Experiments were designed after the work of other researchers working with preinfected protoplasts and whole leaves as mentioned above. (^{3}H)- leucine was used to label the newly synthesized proteins in both systems. The aim of this research was to identify the proteins synthesized in the two systems in response to viral infection, and to compare the proteins synthesized in the two systems.

CHAPTER II

MATERIALS AND METHODS

Materials

Seeds of maize cultivar N28Ht were obtained from Dr. C. O. Gardner, University of Nebraska. Inoculum of MCMV-K was obtained from Dr. Myron Brakke, USDA, Lincoln, Nebraska. Rabbit anti-MCMV serum (titer 1/64) was prepared by Dr. J. Uyemoto, and goat anti-rabbit Fc fragment was prepared by Dr. U. Melcher.

Macerase (3000 u/g) and Panasorbin were obtained from Calbiochem, Behring Corp., La Jolla, Ca. Cellulase (34 u/g) was obtained from Worthington Biochemical Corp. L-(4,5-^{3}H)-leucine (56.5 Ci/mmol, 1 mCi/ml) was obtained from New England Nuclear. Protein standards for electrophoresis (phosphorylase B, 92.5 kDa, bovine serum albumin, 66.2 kDa, ovalbumin, 45 kDa, carbonic anhydrase, 31 kDa, soybean trypsin inhibitor, 21.5 kDa, lysozyme, 14.4 kDa) were obtained from Bio-Rad.

Growth of Plants

Zea mays L. cv. N28Ht was used as the source of leaf tissue for all the in vivo translation experiments. Plants were grown in a growth chamber under 16 hr of light and 8 hr of darkness with a day temperature of 30° and a night temperature of 24.4° . Light was provided by a combination of incandescent and fluorescent lamps giving a quantum flux density of 40 u E/cm^{2}sec (measured using foot-candle

meter) between 400 and 700 nm. The N28Ht maize seeds were sown in one pint plastic containers filled with sterile vermiculite (grade III). Plants were fertilized two times a week with Stern's miracle-gro (15-30-15) 0.05% w/v solution.

Inoculation

Plants were inoculated at the two leaf stage. About 1 g of inoculum (infected leaves frozen at -70^0) was ground in 50 ml of 20 mM potassium phosphate buffer pH 7.0. The extract was filtered through miracloth and 1 g of celite added to the filtrate. The extract was then gently rubbed (using the thumb and index finger) onto one leaf of each plant. After 30 min the leaves were sprayed with distilled water to wash off the excess celite. All inoculum contaminated glassware were autoclaved. The first symptoms appear in less than seven days after inoculation.

Protoplast Isolation

At least 48 hr prior to protoplast preparation, selected healthy and infected plants were placed in the dark (to reduce the rate of photosynthesis and starch accumulation). All glassware required for protoplast isolation were washed in glass distilled water and then sterilized at 15 lb/sq inch pressure at 121^o for 20 min.

For protoplast isolation 2 to 3 g of young (two to four weeks old) expanded leaves were surface sterililzed in 1% v/v chlorox solution for 3 min, rinsed three times in sterile distilled water, followed by 70% v/v ethanol for 3 min and several washes in sterile distilled water. Sterilized leaves were cut vertically into segments less than one mm

wide. The leaf segments were vacuum infiltrated in 50 ml of enzymatic digestion medium (see below) and incubated on a slow shaker (50 rpm/min) at 30° (Kanai and Edwards, 1973).

The enzyme solution was made up of 100 ml of mannitol buffer (20 mM MES, 0.6 M mannitol pH 5.5), containing 0.5 g macerase (Calbiochem, 3000 u/gm), 0.2 g cellulase (Worthington, 34 u/mg). The two enzymes can be added simultaneously as in the one step procedure (Howell and Hull, 1978), or macerase followed by cellulase as in the two step procedure (Takebe, Otsuki and Aoki 1968). The incubation medium was changed every 2 hr for 4 hr and the chlorophyllous material released was collected and filtered through 80x, 100x, and 200x mesh screens. The filtrate was centrifuged at 300 g for 10 min and the precipitate suspended in 5 ml of mannitol buffer. This gave a crude preparation of mesophyll protoplasts from both infected and uninfected maize leaves (Kanai and Edwards, 1973).

The crude protoplast preparation contained a mixture of intact protoplasts, broken protoplasts and chloroplasts. Intact protoplasts were identified under a light microscope by their spherical shape. Also intact protoplasts excluded 1.25% Evan's blue stain, while broken protoplasts and chloroplasts did not (Gaff and Okong'o Ogloa, 1971). Intact protoplasts were separated from the other particles by a liquid-liquid two phase system (Kanai and Edwards, 1973). The system (Table I) was composed of high molecular weight polyethylene glycol (8000 Da) and dextran (40,000 Da) which help to partition the intact protoplasts from chloroplasts and broken protoplasts. The source of dextran is very important in the purification (Kanai and Edwards, 1973) because, greater yield of intact protoplasts is obtained using

TABLE I

COMPOSITION OF DEXTRAN-POLYETHYLENE GLYCOL SYSTEM USED FOR THE STANDARD PROCEDURE TO PURIFY MAIZE MESOPHYLL PROTOPLASTS

Stock solution	ml added	Final Concentration
Polyethylene Glycol 6000 30% (w/w)	1.1	5.5%
Dextran T_{40} (Pharmacia) 20% (w/w)	3.0	10.0%
Na_2HPO_4 - NaH_2PO_4 0.2 M pH 7.5	0.3	10 mM
Sorbitol 2.4 M	1.0	460 mM
Cell suspension containing		
$MgCl_2$ 5 mM		0.5mM
Tricine 50 mM, pH 8.0		5.0 mM
Sorbitol 0.6 M		

Kanai and Edwards (1973).

the Pharmacia brand dextran. The two phase solution was prepared as outlined in Table I. A 5.7 ml aliquot of the solution is thoroughly mixed in a 13x100 mm test tube by inversion prior to the addition of 0.6 ml of the crude protoplast suspension. The tubes were inverted again to mix well and centrifuged at 2° at 300 g for 6 min in a GLC centrifuge. The layer of green material which was collected at the interface were the protoplasts (which tend to stick to one another). They could be taken up easily with a sterile disposable pasteur pipette and transferred to a second two phase solution of the same composition. A single purification step using two phase system was usually sufficient.

Protoplasts collected from the interface had some dextran mixed with them, which had to be removed to maintain normal osmolarity. The dextran was diluted by adding 2 volumes of mannitol buffer and one volume of interface substance and centrifuged at 2^0, at 300 g for 10 min and the step was repeated. One to two ml of mannitol buffer were added to the precipitate and the protoplasts were stored in the dark at 4° for 2 to 3 days (protoplasts are sensitive to light).

A hemocytometer count of freshly isolated protoplasts was done to estimate the number of protoplasts present in one ml of solution. A hemocytometer has a 3 mm square which is divided into 9 squares of 1 mm each. The number of protoplasts was estimated by the following formula,

$$\#\text{ of protoplasts} = \frac{A+B+C+D}{4} \times 10^5.$$

where A,B,C,D represent 1 mm squares at the apices of the 3 mm square.

Labelling of Proteins in Protoplasts

One ml of freshly isolated protoplasts was placed in a small petri dish kept cool in an ice bucket. Different substances were added to the protoplasts depending on the experiment. The effect of antibiotics on the synthesis of virus induced proteins was studied using actinomycin D and chloramphenicol. Both antibiotics were prepared in 0.01 M MES buffer containing 0.22 M mannitol pH 5.5. Under diffuse light 40 ug of actinomycin D (1 mg/ml) was added per ml of protoplasts (Lockhart and Semancik, 1968; Bancroft and Key, 1964). A stock solution of chloramphenicol (2.5 mg/ml) was prepared, and 100 ug of this was also added per ml protoplasts (Harrison et al., 1971). The petri dishes containing protoplasts and the antibiotics were wrapped in aluminium foil and incubated on a slow rotary shaker (50 to 75 rpm), for 8 hr, after which 20 to 30 uCi of (^{3}H)-leucine was added, again under diffuse light. The petri dishes were incubated in the dark for a total period of 24 hr. Similar additions were made to 1 ml of uninfected protoplasts which act as the control. After 24 hr of incubation the protoplasts were collected by centrifugation at 300 g and resuspended in 200 ul of dissolving buffer or phosphate buffer, in the case of immunoprecipitation studies.

Labelling of Proteins in Excised Leaves

Both uninfected and infected maize plants were submerged in a tub of water and the leaves were excised under water to prevent the entry of air bubbles into the vascular system. The leaves of the infected and control were approximately the same size, shape and weight for

comparison. The leaf petiole was immersed in a 1.5 ml Eppendorf tube containing 1 ml of 10 mM MES, pH 5.5 and 10 uCi of L-(4,5-^{3}H)-leucine. In some experiments, 40 ug of actinomycin D and 100 ug of chloramphenicol were also added. In the experiments where actinomycin D was added, (^{3}H)-leucine was added after the excised leaf was incubated for 8 hr in the dark with actinomycin D. The excised leaf was incubated for a total period of 16 hr or 24 hr (depending on the experiment), under growth chamber conditions. After the period of incubation the leaves were stored frozen at -70°.

The frozen leaves were ground under liquid nitrogen and the powder homogenized in Tris-sucrose buffer (50 mM Tris-HCl, 10 mM Na_2SO_3, 250 mM sucrose, 19 mM $MgCl_2$, 25 mM KCl, pH 7.3). To 1 g of leaf tissue, 10 ml of Tris-sucrose buffer was added. The homogenate was filtered through two layers of Miracloth and the filtrate was centrifuged in a 15 ml Corex tube at 30,000 g for 10 min. The pellet was resuspended in dissolving buffer (0.0625 M Tris, 5 % w/v SDS (sodium dodecyl sulphate), 5 M urea, 50% w/v glycerol, 0.001 % w/v bromophenol blue, 5% v/v 2-mercaptoethanol), or phosphate buffer (7 mM Na_2HPO_4, 3 mM KH_2PO_4, 0.015 M NaCl, pH 7.0), depending on whether the sample was being used for protein analysis on SDS-polyacrylamide gel electrophoresis (PAGE) or immunoprecipitation studies. The 30,000 g supernatant was centrifuged in a Ti-75 rotor, at 180,000 g for 60 min. The pellet was once again resuspended in dissolving buffer or phosphate buffer. To the 180,000 g supernatant 5 ml of 10% w/v trichloracetic acid (TCA) was added and the samples incubated in an ice bath for 30 min and centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and 5 ml of 10% TCA was added to the pellet and centrifuged

at 10,000 rpm in an SS-34 rotor for 10 min again. The procedure was repeated twice more. The pellet was then washed three times with ether, each time centrifuging the pellet down at 10,000 rpm for 10 min. After three ether washes the pellets were dried under vacuum for 2 hr or until completely dried. The dried pellet was resuspended in dissolving buffer or phosphate buffer. All the pellets suspended in dissolving buffer were heated in a water bath for 5 min, cooled and stored at -20° until further use.

Analysis of the Translation Products

The incorporation of (^{3}H)-leucine was determined by the method of Marcu and Dudock (1974). A 20 ul aliquot of the sample was added to 1 ml of 10% TCA and incubated in an ice bath for 30 min. Each sample was then passed through a glass fiber filter (Whatman GF/A) moistened with 10% TCA, rinsed three times in 5 ml of cold 10% TCA, followed by a rinse with absolute ethanol. The filters were dried on a hot plate and counted in a scintillation fluid (5 g 2-5 diphenyl oxazole (PPO) /1 L toluene) in a Beckman LS-3150T counter.

Proteins in the sample were resolved by SDS-polyacrylamide slab gel electrophoresis (Laemmli , 1970). Gels are prepared for fluorography by the method of Bonner and Lasky (1974). Dried gels were exposed to Kodak X-Omat-R film and incubated at -70° for as long as it takes to see a visible image. The sensitivity of detection of labelled proteins by autoradiography was slightly enhanced by following a method of preflashing (Laskey and Mills, 1975).

Immunoprecipitation

Samples for immunoprecipitation (either pelleted infected protoplasts or the 180,000 g pellet from infected leaves) were dissolved in 1 ml of phosphate buffer (7 m M Na_2HPO_4, 3 m M KH_2PO_4, 0.015 M NaCl, pH 7.0). The samples were divided into 3 equal aliquots of 0.33 ml each. Dissolving buffer (0.0625 M Tris, 5 % w/v SDS, 5 M urea, 50 % w/v glycerol, 0.001 % w/v bromophenol blue, 5 % v/v 2-mercaptoethanol) 0.67 ml was added to one aliquot and the sample was heated at 100° for 5 min and stored frozen. The other aliquots were adjusted to a final volume of 1 ml with 0.12 M KCl. Anti-MCMV rabbit serum (40 ul) was added to one aliquot and an equal amount of normal rabbit serum was added to the other. The mixtures were incubated at 37° water bath for 15 min after which they were further incubated for 2 to 3 hr on an ice bucket. Meanwhile 100 ul of Pansorbin (Staphylococcus aureus cells) were washed three times in wash buffer (6 ml of phosphate buffer + 14 ml of 0.12 M KCl). The samples containing the anti-MCMV serum or normal serum was added to the Pansorbin or to goat anti-rabbit Fc serum , either of which bind to the antigen-antibody complex and precipitate it. The samples were incubated on ice for 30 min, after which the pellet was collected by centrifugation (GLC) at 2,600 rpm. The pellet was washed two times, each time in 100 ul of dissolving buffer. After the addition of the dissolving buffer the samples were heated at 100° for 5 min centrifuged and supernatant collected. The supernatants were stored at -20° and analysed by SDS-PAGE.

Negative Staining

The negative staining technique was chosen to study the morphology of MCMV particles under the transmission electron microscope. In this method an electron dense material is deposited around a specimen so that the lighter specimen stands out from the dense background. Phosphotungstic acid (PTA) was chosen as the stain as it also acts as a fixative. PTA (2% w/v) was prepared and a drop of it mixed with a drop of MCMV virus loaded onto a 200 mesh copper grid. The excess liquid was drawn off by a piece of a tissue paper. The grid was dried and observed with a Phillips transmission electron microscope (TEM) at tap 22 (100,000 X) magnification.

CHAPTER III

RESULTS

Electron Microscopy.

Negative staining of a partially pure preparation is ideal for the study of virus morphology, as unstained virus particles stand out against a densely stained background. Maize chlorotic mottle virus appeared to be an icosahedral particle (Figure 1), measuring 32.5 $\pm$ 0.5 nm in diameter.

Morphology of Protoplasts from Uninfected and Infected Leaves

Partially purified protoplasts (Figure 2) prepared from maize N28Ht showed some intact protoplasts, broken protoplasts along with single plant cells. Most of the debris was removed using the two phase purification method (Figure 3). No differences in morphology were seen between protoplasts prepared from uninfected and infected leaves under the light microscope. Protoplast yield varied for each preparation however, on an average a count of 34×10^5 was usually obtained.

Incorporation of Radiolabel and the Effect of Actinomycin D in Protoplasts

Protoplasts prepared from infected leaves show consistently (in 6 experiments) greater incorporation of (^{3}H)-leucine into TCA

Figure 1. Electron micrograph of MCMV Negative staining technique using 2% PTA. Total magnification on the micrograph is 312,500.

1

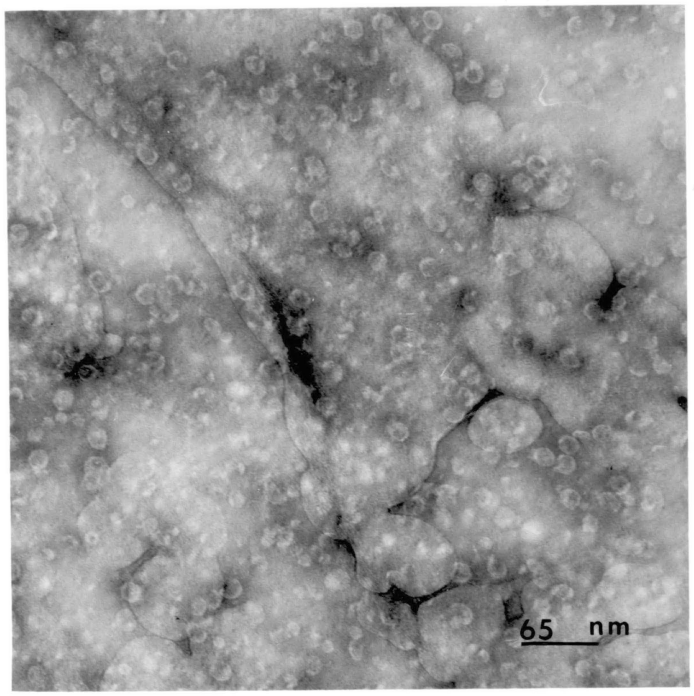
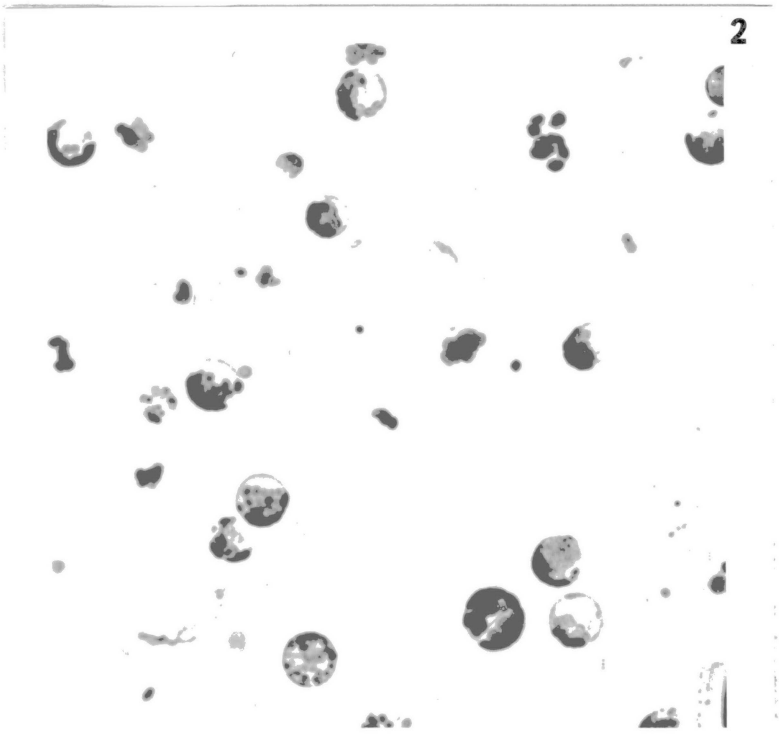


Figure 2. Partially purified maize protoplasts. Light micrograph taken at 75x.

Figure 3. Maize protoplasts after purification. Light micrograph taken at 300x.



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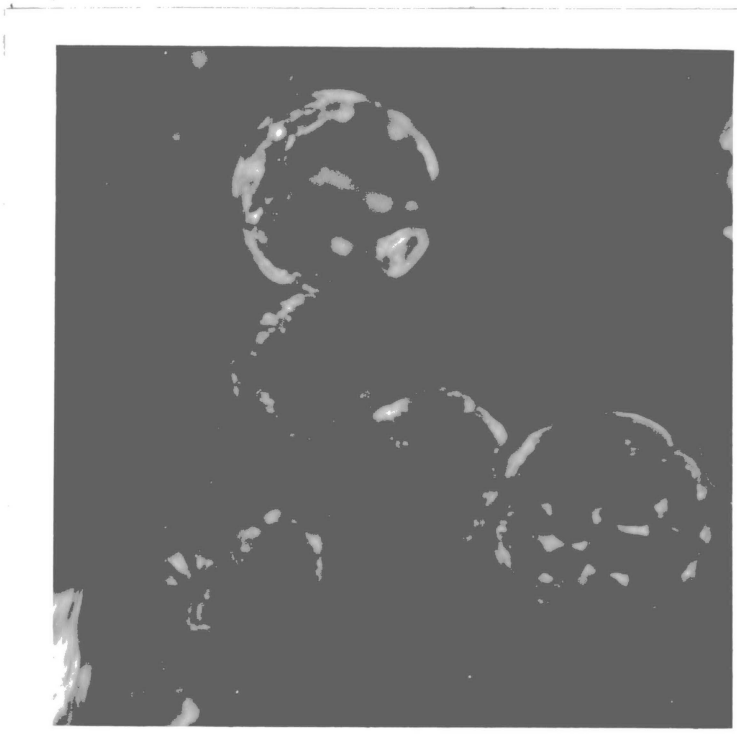


3

precipitable material as compared to the protoplasts prepared from uninfected leaves (Table II) under identical conditions.

Actinomycin D caused a 22 % inhibition of (^{3}H)-leucine incorporation in protoplasts prepared from uninfected leaves as compared to the control under identical conditions (Table II). However actinomycin D causes a 27 % stimulation of (^{3}H)-leucine incorporation in protoplasts prepared from infected leaves as compared to the control. Similar results were obtained in several repetitions of the same experiment.

Analysis of the Translation Products.

The products labelled in vivo were analysed by SDS-PAGE on an 11% polyacrylamide gel. Comparison of the electrophoretic pattern of the samples showed the presence of some protein bands in samples obtained from infected leaves that were not observed in the samples obtained from uninfected leaves (Figure 4). The molecular weights of these protein bands were calculated based on the electrophoretic mobility of protein standards of known molecular weight. Two major bands and five minor bands were detected (Figure 4c and 4d). The major protein labelled in vivo had a molecular weight of 30 kDa. The 30 kDa protein had electrophoretic mobility similar to the MCMV coat protein. Other major proteins observed had a molecular weights of 37 and 24 kDa. Several other minor proteins bands were observed having molecular weights 47, 45, and 20 kilodaltons.

TABLE II

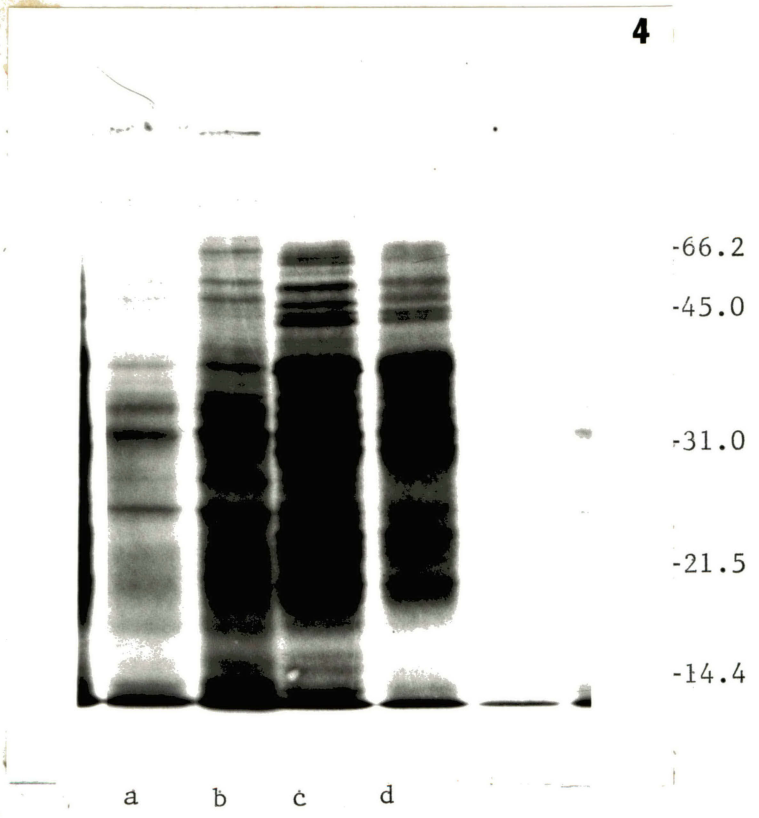
INCORPORATION OF (^{3}H)-LEUCINE IN PROTOPLASTS

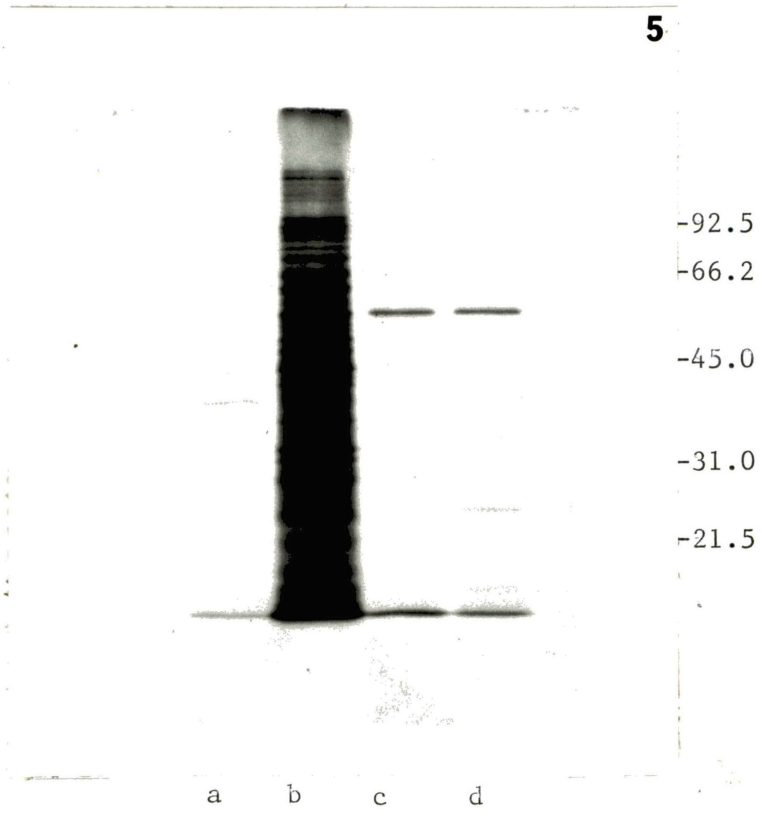
Sample	Incorporation in uCi/ml	% control.
Uninfected	0.615 uCi/ml	
Uninfected + actinomycin D	0.48 uCi/ml	78 %
Infected	9.62 uCi/ml	
Infected + actinomycin D	12.17 uCi/ml	127 %

Total (^{3}H)-leucine administered was 25 uCi/ml protoplast. Uninfected and infected protoplasts were divided into two groups, one was maintained as control and the other was treated with 40 ugs actinomycin D.

Figure 4. Fluorogram of an 11 % SDS-polyacrylamide gel of proteins obtained from uninfected and infected protoplasts samples. Lane c - Infected protoplasts treated with actinomycin D. Lane d - Infected protoplasts Lane a - Uninfected protoplasts treated with actinomycin D Lane b - Uninfected protoplasts.

Figure 5. Fluorogram of an 11.5 % SDS-polyacrylamide gel of protoplast samples treated with MCMV antiserum or normal serum. Lane a - Uninfected protoplasts. Lane b - Infected protoplasts. Lane c - + normal serum. Lane d - + MCMV-antiserum.


4
-66.2
-45.0
-31.0
-21.5
-14.4
a
b
c
d


5
-92.5
-66.2
-45.0
-31.0
-21.5
a
b
c
d

Immunoprecipitation.

Immunoprecipitation experiments were done using normal rabbit serum as control (Figure 5). Goat anti-rabbit Fc serum was used to act as an immunoprecipitating agent. The lane 5d containing the sample treated with MCMV-antiserum shows the presence of a protein whose molecular weight is 27 kDa. A protein of similar electrophoretic mobility was seen in the lane 5c, containing the sample treated with normal serum. However this protein band is denser in the 5d lane as compared to the protein band in lane 5c. The major difference is in the protein of molecular weight 27 kDa which has similar mobility to the major protein seen in the lane 5b, containing sample of protoplasts prepared from infected leaves. Another protein of molecular weight 29 kDa was also seen in lane 5d and 5b. A lower molecular weight protein 17 kDa was also detected in lane 5d and 5b.

Effect of Antibiotics on Protein Synthesis In Excised Leaves.

The three fractions 30,000 g pellet,a 180,000 g pellet and a 180,000 g supernatant collected from uninfected and infected maize leaves were analyzed for incorporation of (^{3}H)-leucine after antibiotic treatment (Table III). The 30,000 g pellet fraction from the uninfected leaves treated with actinomycin D showed 52 % less incorporation of (^{3}H)-leucine as compared to the same fraction from uninfected leaf not treated with actinomycin D. The 180,000 g fraction from the uninfected leaf treated with actinomycin D showed 58% less incorporation of (^{3}H)-leucine as compared to the same fraction from the untreated uninfected leaf. The 180,000 g supernatant fractions from the

Table III

COMPARISON OF (^{3}H)-LEUCINE INCORPORTION IN ACTINOMYCIN D TREATED (+ACT D) AND UNTREATED (CONTROL) SAMPLES, IN INFECTED (I) AND UNINFECTED (UI) EXCISED LEAVES

Sample	Fraction	Control (uCi/ml)	+Actinomycin D (uCi/ml)	% of Control
UI	30,000 g	0.171	0.082	48
UI	180,000 g	0.166	0.069	42
UI	180,000 g(s)	0.497	0.290	58
I	30,000 g	0.138	0.081	59
I	180,000 g	0.101	0.163	161
I	180,000 g(s)	0.840	0.524	62

Fractions collected were 30,000 g, 180,000 g and 180,000 g supernatant.

uninfected leaves showed the minimum difference between actinomycin D treated and untreated samples. The actinomycin D treated uninfected leaf samples of this fraction showed 42 % less incorporation as compared to the same fraction from a untreated sample.

Similar analysis of infected samples were done (Table III). The 30,000 g pellet fraction from the infected leaves treated with actinomycin D showed a 41 % reduction in (^{3}H)-leucine incorporation as compared to the 30,000 g pellet fraction from the infected leaves not treated with actionmycin D. Similarly the 180,000 g supernatant fraction from the infected actinomycin D treated leaves showed 38 % reduction in (^{3}H)-leucine incorporation as compared to the same fraction from infected leaves not treated with actinomycin D. However, the 180,000 g pellet fraction from the infected leaves treated with actinomycin D showed the reverse effect of what was observed in other fractions. Here the actinomycin D treated sample showed 61 % greater incorporation as compared to the untreated.

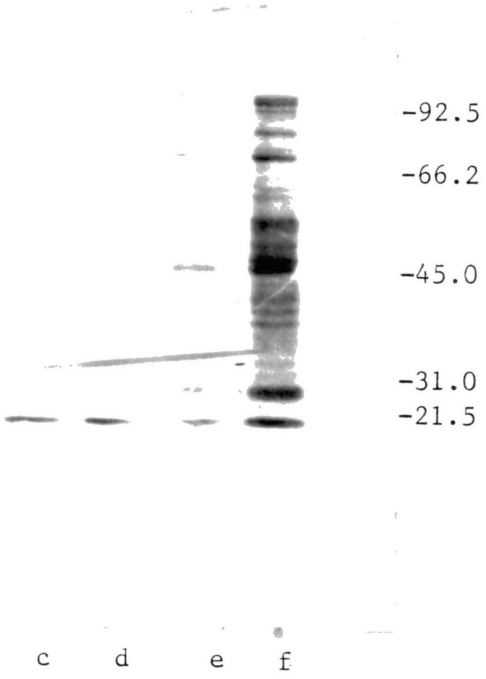
SDS-PAGE Analysis of the Fractions.

The 30,000 g pellet from the infected leaves showed no detectable difference in the electrophoretic pattern as compared to the same fraction from the uninfected leaf (Figure 6b and 6a resp.). The 180,000 g pellet from the infected leaf showed the presence of a 28 kDa protein not seen in the 180,000 g pellet of the uninfected leaf (Figure 6d and 6c resp.). Another protein of high molecular weight 187 kDa was detected in one experiment but not (Figure 6d) in any of the following experiments. Comparison of the 180,000 g supernatants show some differences between the uninfected and infected leaf samples. The

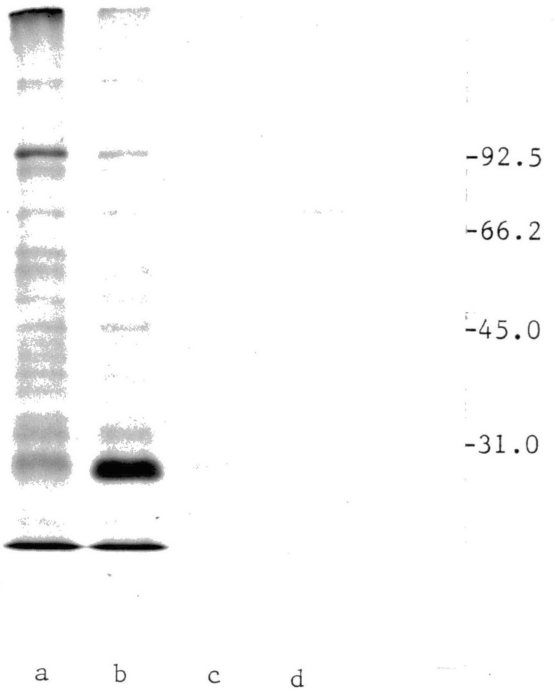
Figure 6. Fluorogram of an 11.5 % SDS-polyacrylamide gel of fractions of excised whole leaves. Lane a - 30,000 g pellet fraction of an uninfected leaf. Lane b - 30,000 g pellet fraction of an infected leaf. Lane c - 180,000 g pellet fraction of an uninfected leaf. Lane d - 180,000 g pellet fraction of an infected leaf. Lane e - 180,000 g(s) fraction of an uninfected leaf. Lane f - 180,000 g(s) fraction of an infected leaf.

Figure 7. Fluorogram of an 11.5 % SDS-polyacrylamide gel of samples from excised whole leaves treated with MCMV antiserum or normal serum. Lane a - 180,000 g pellet of uninfected leaf Lane b - 180,000 g pellet of infected leaf. Lane c - 180,000 g pellet + MCMV antiserum. Lane d - 180,000 g pellet + normal serum.

6



7



major protein synthesized in the infected leaf fraction and the uninfected leaf fraction was 28 kDa in molecular weight (Figure 6f and 6e resp). Two other minor proteins of molecular weight 43 kDa and 34 kDa were detected in the infected leaf fractions in some experiments, but not in all. In one experiment two proteins of high molecular weight 87 and 67 kDa were detected in the 180,000 g supernatant fractions. The major protein synthesized in these fractions in this experiment was 30 kDa in molecular weight.

Identification of p28 as Coat Protein.

The 180,000 g pellet fraction from the infected leaf sample was used for immunoprecipitation studies. The lane (Figure 7b) loaded with 180,000 g pellet sample shows a protein band of molecular weight 31 kDa. A protein of similar molecular weight was identified in the lane (Figure 7c), loaded with the immunoprecipitate. Such a protein was not seen in the sample treated with normal serum. Hence this protein is probably the MCMV coat protein.

CHAPTER IV

DISCUSSION

The measurement obtained for the size of the virus particle agrees well with the literature values. Uyemoto (1983) reported a value of 30 nm.

The one step procedure for the isolation of protoplasts was found to be better than the two step procedure due to the following reasons: 1) The yield was better and 2) the total period of incubation was half that required for the two step procedure. The major drawback in the one step method, however, is that a heterogenous population of protoplasts is obtained, i.e. the intact protoplasts are contaminated with subprotoplasts, broken protoplasts and other plant debris, thus making purification by the dextran-polyethylene glycol system absolutely necessary.

Protoplasts prepared from infected leaves consistently gave higher incorporation of (^{3}H)-leucine as compared to those prepared from uninfected leaves. One or many factors could be responsible for this effect. Virus infection alters a number of physiological processes of the cell (Albershiem et al., 1984). The cell wall structure is known to change upon virus infection (Hiruki, 1977). Changes in wall structure may have affected the condition in which protoplasts were isolated and certain cell wall degradation products may have also affected protoplast physiology (Gollin et al., 1984), which may affect

(^{3}H)-leucine incorporation. Based on the above observation it seems as if maize protoplasts infected with MCMV could be a good system for protein synthesis studies.

Stimulation of (^{3}H)-leucine incorporation in protoplasts from infected leaves by actinomycin D, could be due to the fact that actinomycin D only inhibits DNA-dependent RNA synthesis but not RNA-dependent RNA synthesis. The inhibition of host RNA synthesis may make more substrates available for viral RNA synthesis or it could be that viral RNA translation is stimulated as the competition for ribosomes, tRNA, etc. is reduced .

Actinomycin D had a stronger effect on protein labelling in leaves than in protoplasts. The reason for this not known. Possible speculation was that the rate at which actinomycin D gets incorporated in an intact leaf could be more rapid than in protoplasts.

The proteins synthesized in infected but not uninfected protoplasts had estimated molecular weights of 47, 45, 37, 30, 24 and 20 kDa. These could possibly correspond to proteins synthesized from MCMV RNA in vitro in wheat germ extracts with estimated molecular weights of 46, 44, 36, 29, 25, and 18 kDa. Polypeptides of 24 kDa and 20 kDa seen in the protoplast system could represent coat protein degradation products.

A protein of 28 kDa was the only difference between infected and uninfected leaf fractions which was consistently detected. In some experiments minor proteins of 34 and 43 kDa could be detected. This could be due to better and more uniform polyacrylamide gels and/or greater sensitivity in the method of detection (some x-ray films were given a brief exposure from a flash before exposure to the fluorogram.

Flash activated X-ray film required less exposure time then the unactivated ones. The sensitivity of detection of minor proteins was greatly enhanced.

The evidence that p28 was the coat protein is as follows: 1) It had similar molecular weight as of the MCMV coat protein. 2) The protein was precipitated by anti-MCMV antiserum, and 3) this protein was present in the 30,000 g supernatant and the 180,000 g pellet of infected leaves. Mature MCMV particles sediment similarly. 4) The 180,000 g pellet from infected leaves showed increased incorporation of (^{3}H)-leucine. This was due to increased synthesis of p28 suggesting that it was translated from an RNA which was transcribed by an RNA-dependent RNA polymerase.

Results from experiments with strips of healthy and infected leaves were inconclusive, because (^{3}H)-leucine was poorly incorporated by these tissues.

Based on the results obtained so far, it is not yet possible to identify the mode gene expression of this virus. It is possible that the viral RNA gets translated into a large polypeptide which later gets processed. Some SDS-PAGE analysis of leaf fractions have shown the presence of a 187 kDa protein in the 180,000 g fraction. It is possible that this is the precursor to other proteins that have been identified. However such suggestions are at best speculative. The coat protein in SBMV (a sobemovirus) is synthesized from a subgenomic message. It is possible that the coat protein of MCMV is synthesized by a similar message. Future studies may answer these questions. Future experiments will be to infect the maize protoplasts in vitro with MCMV.

CHAPTER V

SUMMARY AND CONCLUSION

Proteins synthesized in maize mesophyll protoplasts and excised leaves infected with maize chlorotic mottle virus were detected by radiolabelling and SDS-PAGE and compared to the proteins synthesized in uninfected tissue.

MCMV infection caused the synthesis and accumulation of certain proteins. The major protein detected in both species had a molecular weight of 28 kDa. This protein is probably the MCMV coat protein. Polypeptides of molecular weights 47, 45, 37, 30, 24 and 20 kDa were detected only in the protoplasts isolated from infected leaves. The 30 kDa protein could be the 28 kDa detected in other analyses. No virus-induced proteins were detected in the 30,000 g pellet fraction of the excised infected leaf. Small amounts of proteins with molecular weights of 87, 67, 43, 34 kDa were detected in some analyses of the 180,000 g supernatant fractions of infected leaves. However no major virus-induced proteins were detected in this fraction. A protein of 29 kDa was detected consistently in the 180,000 g pellet fraction of the infected leaf.

Actinomycin D had an inhibitory effect on the incorporation of (^{3}H)-leucine on all uninfected tissues and on infected tissues except those samples containing large amounts of induced proteins (infected protoplasts and the 180,000 g pellet of excised leaves).

These and other results show that mesophyll protoplasts made from infected leaves are ideal for the detection of proteins induced by MCMV infection of maize.

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VITA 2

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