PURIFICATION, PARTIAL CHARACTERIZATION, AND SEROLOGY OF THE CAPSID AND CYLINDRICAL INCLUSION PROTEINS OF FOUR ISOLATES OF WATERMELON MOSAIC VIRUS

BY

ROBERT H. BAUM

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate Council
of the University of Florida in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

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By

Robert H. Baum

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Chairman: Dan E. Purcifull
Major Department: Plant Pathology

Watermelon mosaic viruses (WMV), which are members of the potyvirus group, have been classified into two distinct types, WMV-1 and WMV-2, on the basis of host range, serology of the capsid proteins and morphology of virus-induced inclusions. Several isolates, however, are at variance with the two general types on the basis of host range or serology of the capsid proteins. The purpose of this study was to further clarify the distinctiveness of both WMV-1 and WMV-2 and an isolate from Morocco (WMV-M) by the use of serology and peptide maps of the capsid and cylindrical inclusion proteins.

Virus isolates used extensively in this study were WMV-M, WMV-1 and WMV-2 from Florida, and WMV-1 from Jordan. Viruses were increased in pumpkin, *Cucurbita pepo* L. "Small Sugar," and purified by clarification of sap with a mixture of chloroform and carbon tetrachloride followed by concentration with polyethylene glycol and fractionation on either cesium chloride or cesium sulfate isopycnic density gradients. Cytoplasmic cylindrical inclusions were purified using the same clarification procedure followed by fractionation on sucrose step gradients or preparative polyacrylamide gels.

Antisera to both formaldehyde fixed and unfixed virus and to purified inclusions were produced in New Zealand white rabbits. Sera collected following immunization up to approximately one year were checked for immunochemical specificity.

The serological relationships of selected potyviruses were determined by enzyme linked immunosorbent assay and sodium dodecyl sulfate (SDS) immunodiffusion. In reciprocal SDS immunodiffusion tests, heterologous reactions were obtained between WMV-2 and: bean common mosaic virus (BCMV), blackeye cowpea mosaic virus (BlCMV), and potato virus Y (PVY), but not WMV-1 (Jordan or Florida), WMV-M, or papaya ringspot virus (PRSV). WMV-1 Florida and WMV-1 Jordan, hereafter collectively referred to as WMV-1, were shown to be serologically identical by intragel absorption in SDS immunodiffusion tests. WMV-1 reacted heterologously with PRSV infected sap but not in reciprocal immunodiffusion tests with WMV-2, WMV-M, BCMV, BlCMV, or PVY. Sera from early bleedings of WMV-M did not react with WMV-1 or WMV-2, but bleedings after four months did give reactions of partial identity with WMV-1. This was confirmed by intragel absorption and ELISA.

Antisera specific for the cylindrical inclusions of WMV-M, WMV-1, and WMV-2 in SDS immunodiffusion tests with sap and purified inclusions were obtained. Antisera to WMV-2 inclusions reacted heterologously with BCMV, dasheen mosaic virus (DMV), soybean mosaic virus (SoyMV), and lettuce mosaic virus (LMV). WMV-M inclusion antiserum reacted heterologously with WMV-1, WMV-2, DMV, and PRSV and with some isolates of LMV. The WMV-1 inclusion antiserum did not react with either WMV-M or WMV-2 inclusions.

Analysis of peptide fragments after cleavage of capsid proteins with cyanogen bromide (CNBr) showed distinct patterns for WMV-1, WMV-2, and WMV-M. *Staphylococcus aureus* V-8 protease digests of the cylindrical inclusions of WMV-1, WMV-2, and WMV-M were analyzed by disc polyacrylamide gel electrophoresis. While there were some similarities between patterns, significant differences existed.

This study provides additional evidence that WMV-1 and WMV-2 are distinct viruses on the basis of inclusion serology and analysis of the peptides formed by partial cleavage of capsid and cylindrical inclusion proteins. Evidence is also provided that WMV-M is a distinct virus.

INTRODUCTION

Watermelon mosaic viruses, which are members of the potyvirus group (Brandes, 1964; Harrison *et al.*, 1971), cause economically important diseases of cucurbits throughout the world (van Regenmortel, 1971). The watermelon mosaic viruses have flexuous anisometric particles 700-800 nm long, induce cylindrical inclusions in their hosts, and are transmitted in a nonpersistent manner by aphids (van Regenmortel, 1971; Edwardson, 1974a).

Symptoms induced by WMV are highly variable, depending on the virus strain, the host, and on environmental conditions. Symptoms range from mild mottling to severe shoestring distortion and blistered leaves (Anderson, 1954; Webb and Scott, 1965; Greber, 1969; Milne *et al.*, 1969; Bakker, 1971; Thomas, 1971a; van Regenmortel, 1971; Webb, 1971; Demski and Chalkley, 1974; Fischer and Lockhart, 1974; Horvath *et al.*, 1975). Due to variability, this characteristic cannot be readily used to distinguish watermelon mosaic from other viral diseases of cucurbits. Serological tests have been used to distinguish six potyviruses that infect cucurbits (Purcifull and Hiebert, 1979). Other viruses that infect cucurbits are distinguished from WMV on the basis of the host range (Lindberg *et al.*, 1956; Grogan *et al.*, 1959), mode of transmission (Greber, 1969), absence of cylindrical inclusions in the cytoplasm of infected plants (Christie and Edwardson, 1977), physical properties (Lindberg *et al.*, 1956), serology, and virus morphology (van Regenmortel *et al.*, 1962).

Watermelon mosaic virus was first described by Anderson (1954) and it was first purified by van Regenmortel (1961). The North American isolates of the virus were divided into watermelon mosaic virus 1 (WMV-1) and watermelon mosaic virus 2 (WMV-2) on the basis of the host range (Webb and Scott, 1965). WMV-1 isolates were limited to the Cucurbitaceae, whereas WMV-2 had a wider host range covering some 17 plant families (van Regenmortel, 1971). WMV-1 and WMV-2 were reported to be serologically distinct (Webb and Scott, 1965) and reciprocal tests indicated that neither could cross-protect against a challenge by the other virus (Webb and Scott, 1965). It was concluded, therefore, that WMV-1 and WMV-2 are distinct viruses. The serological results have been confirmed (Purcifull and Hiebert, 1979; Greber, 1978; Baum *et al.*, 1979). However, Milne and Grogan (1969) reported a close serological relationship between WMV-1 and WMV-2. On the basis of serology, cross-protection, indicator hosts, and host range, Milne and Grogan concluded that their WMV-1 and WMV-2 isolates were strains of the same virus. Webb and Scott (1965) suggested the possibility of a third virus or virus group because they were unable to get a positive serological reaction between WMV-1 or WMV-2 and van Regenmortel's South African WMV antisera. The presence of a third member of the watermelon mosaic virus subgroup was also suggested by Schmelzer (1969) and by Horvath *et al.* (1975) based on differences in host range and symptoms and by Purcifull and Hiebert (1979) based on serological experiments. They were unable to detect a serological relationship between a Moroccan isolate of WMV (WMV-M) (Fischer and Lockhart, 1974) and antisera to Florida isolates of WMV-1 and WMV-2.

Although antisera to the cylindrical inclusions have been useful in studying relationships of other potyviruses (Hiebert *et al.*, 1971; Purcifull *et al.*, 1973; McDonald and Hiebert, 1975), the serological relationships of WMV-induced inclusions have not been studied previously.

The objectives of this dissertation were to: (i) study the serological relationship of WMV-M to Florida isolates of WMV-1 and WMV-2 and to a Jordan isolate of WMV-1, (ii) test the use of WMV cylindrical inclusion antisera in determining relationships among isolates of WMV, and (iii) analyze the relationships among WMV isolates by comparing peptide maps of capsid and cylindrical inclusion proteins.

LITERATURE REVIEW

Watermelon mosaic virus was described by Anderson (1951a, 1951b, 1954), who recognized that it was distinct from other cucurbit viruses. WMV was first noted to be distinct from cucumber mosaic virus (CMV) in spite of what appeared to be partial cross-protection (Anderson, 1951a). These distinguishing factors were: a synergistic effect resulting in a more severe disease when both viruses were present in cucumber (*Cucumis sativus* L.), increased numbers of primary lesions induced by CMV on watermelon (*Citrullus lanatus* [Thunb.] Matsun. and Naki) when inoculation and infection occurred with both viruses, and no evidence that CMV predominated over WMV (Anderson, 1951a).

The symptoms observed by Anderson on systemically infected leaves of cucurbits were mild chlorosis, mottle, green vein banding, raised blisters, distortion, and shoestringing. These have generally been the symptoms observed on WMV infected cucurbits, but fruit distortion, stunting, and mottling are often noted--especially on plants infected early in the season (Thomas, 1971b; Demski and Chalkley, 1974; Fischer and Lockhart, 1974).

WMV-1 and WMV-2 Defined

Webb and Scott (1965) divided WMV into two distinct viruses, partly on the host range of North American isolates. WMV-1, as defined by Webb and Scott (1965) infects only the Cucurbitaceae while WMV-2 can infect plants in 21 additional families (Edwardson, 1974b; Molnar and Schmelzer, 1964; Webb and Scott, 1965; Greber, 1969; Provvidenti

and Schroeder, 1970; Webb, 1971). WMV-2 usually produces milder leaf symptoms with less distortion and blistering than WMV-1 (Webb and Scott, 1965). Both viruses have a worldwide distribution (Table 1), though WMV-1 may have been recently introduced into Europe (Horvath *et al.*, 1975) and Australia (Greber, 1978) from the western hemisphere.

Transmission and General Characteristics

Seed transmission of WMV was not demonstrated in a test using several hundred seeds from infected Summer Crookneck Squash (*Cucurbita pepo.* var. *melopepo* Alef.) and cantaloupe (*Cucumis melo* L.) (Anderson, 1951b). Seed transmission of WMV has not been detected by others using different isolates of WMV and other cucurbit hosts (Anderson, 1951b; Grogan *et al.*, 1959; Greber, 1969; Thomas, 1971a; Fischer and Lockhart, 1974; Bhargava, 1977). WMV may therefore be distinguished from viruses which are seed-borne in cucurbits. These include squash mosaic virus (SqMV) (Campbell, 1971), and melon necrotic spot virus (MNSV) (Gonzalez-Garza *et al.*, 1978).

WMV-1 and WMV-2 have been transmitted in a nonpersistent manner by several aphid species (Table 2). *Aphis gossypii* Glover transmits WMV, single aphids being capable of transmitting WMV to about 50% of test plants after an acquisition period of 18-36 seconds (Anderson, 1951b). Thomas (1971a) was unable to transmit WMV-2 from infected to healthy plants of Buttercup Squash (*Cucurbita maxima* Duch.) using *A. gossypii* or *Myzus persicae* (Sulzer). Bakker (1971) also reported the nontransmissibility of a Kenyan isolate of WMV by *Aphis fabae*.

The physical properties observed by Anderson (1954) have been confirmed for both WMV-1 and WMV-2. The physical properties are: a

Table 1. Geographical distribution of watermelon mosaic viruses.

Australia	WMV-1	Greber, 1978
	WMV-2	Greber, 1978
Canada	WMV-2	Gates and Bronskill, 1976
Chile	WMV-2	Auger *et al.*, 1974
Cuba	WMV-1	Schmelzer, 1969
Czechoslovakia	WMV-2	Schmelzer and Milicic, 1966
Eastern Europe	WMV-1	Molnar and Schmelzer, 1964
	WMV-2	Molnar and Schmelzer, 1964
	WMV-?	Molnar and Schmelzer, 1964
France	WMV-2	Arteaga *et al.*, 1976
Germany	WMV-1	Hein, 1977
Guadeloupe	WMV-1	Quiot *et al.*, 1971
Hungary	WMV-2	Horvath *et al.*, 1975
India	WMV-1	Bhargava, 1977
	WMV-1	Ghosh and Mukhopadhyay, 1979
	WMV-2	Ghosh and Mukhopadhyay, 1979
Iran	WMV-1	Ebrahim-Nesbat, 1974
	WMV-2	Ebrahim-Nesbat, 1974
Iraq	WMV-2	Shawkat and Fegla, 1979
Israel	WMV-2	Cohen and Nitzany, 1963
Italy	WMV-1	Ragozzino and Stefanis, 1977
	WMV-2	Ragozzino and Stefanis, 1977
Japan	WMV-2	Inouye, 1964
Kenya	WMV-Kenya	Bakker, 1971
Mexico	WMV-1	Milne and Grogan, 1969
	WMV-2	Milne and Grogan, 1969
Morocco	WMV-Morocco	Fischer and Lockhart, 1974
New Zealand	WMV-2	Thomas, 1971a
South Africa	WMV-SA*	van Regenmortel, 1961
United States		
Arizona	WMV-2	Nelson and Tuttle, 1969
California	WMV-1	Milne and Grogan, 1969
	WMV-2	Milne and Grogan, 1969
Florida	WMV-1	Adlerz, 1969
	WMV-2	Adlerz, 1969
Georgia	WMV-2	Demski, 1968
Hawaii	WMV-1	Shanmugasundaram *et al.*, 1969
	WMV-2	Shanmugasundaram *et al.*, 1969
	WMV-Kauai**	Shanmugasundaram *et al.*, 1969
Massachusetts	WMV-2	Komm and Agrios, 1978
New York	WMV-1	Provvidenti and Schroeder, 1970
	WMV-2	Provvidenti and Schroeder, 1970
U.S.S.R.	WMV-2	Schmelzer and Milicic, 1966
Venezuela	WMV-1	Lastra, 1968
	WMV-2	Lastra, 1968
Yugoslavia	WMV-2	Stakic and Nikolic, 1966

*WMV-SA = South African isolate

**WMV-Kauai isolates from Kauai were distinct from WMV-1 and WMV-2.

Table 2. Aphid transmissibility of watermelon mosaic viruses.

Aphid	Isolate	References
Myzus persicae Sulzer	WMV-1	Webb and Scott, 1965; Lastra, 1968; Milne and Grogan, 1969; Quiot *et al.*, 1971; Webb, 1971; Hein, 1977; Shawkat and Fegla, 1979.
	WMV-2	Webb and Scott, 1965; Lastra, 1968; Greber, 1969; Milne and Grogan, 1969; Webb, 1971; Auger *et al.*, 1974;Horvath *et al.*, 1975; Arteaga *et al.*, 1976; Bhargava, 1977; Greber, 1978; Shawkat and Fegla, 1979.
	WMV-M	Fischer and Lockhart, 1974.
	WMV-Kenya	Bakker, 1971.
	WMV-South Africa	van Regenmortel *et al.*, 1962.
Aphis gossypii Glov.	WMV-1	Lastra, 1968.
	WMV-2	Lastra, 1968; Greber, 1969, 1978; Bhargava, 1977.
	WMV-Kenya	Bakker, 1971.
Aphis fabae Scop.	WMV-2	Shawkat and Fegla, 1979.
	WMV-Morocco	Fischer and Lockhart, 1974.
Macrosiphum euphorbiae Thomas	WMV-2	Greber, 1969, 1978.
	WMV-Kenya	Bakker, 1971.
Aphis craccivora Koch.	WMV-1	Greber, 1978.
	WMV-2	Greber, 1969, 1978.

dilution end-point of approximately 10^{-3} to 10^{-5}, a thermal inactivation point of about 55 C to 65 C, and a longevity *in vitro* of about 6 to 20 days (van Regenmortel *et al.*, 1962; Webb and Scott, 1965; Milne and Grogan, 1969; Greber, 1978).

Differential Systemic and Local Lesion Hosts for WMV-1 and WMV-2

Pinpoint, brown-bordered, local, circular, paper-white necrotic lesions with minute dark brown centers developed on cotyledons and leaves of muskmelon (*Cucumis melo* L. var. *reticulatus* Naud.) selection P.I. 180280) inoculated 4-6 days earlier with two isolates of WMV (Webb, 1963). Some plants that developed these local lesions remained free of virus in secondary leaves whereas others died after developing stem and top necrosis. Muskmelon cotyledons inoculated with other isolates of WMV developed a systemic mottle but not local lesions. In reciprocal tests, no cross-protection occurred with local-lesion and systemic mottle isolates (Webb, 1963), suggesting that WMV might consist of at least two unrelated viruses. Further study showed that isolates that induced local lesions on muskmelon were also restricted to the Cucurbitaceae; these isolates were hereafter classified as belonging to the WMV-1 group. Those isolates which induced a systemic mottle in muskmelon also had wider host ranges, including certain species in the families Leguminosae, Chenopodiaceae, and Euphorbiaceae, and were designated members of the WMV-2 group (Webb and Scott, 1965). The WMV-2 isolates were maintained free of WMV-1 by culturing the former in non-cucurbitaceous hosts (Webb, 1965).

Toba (1962) used *Chenopodium amaranticolor* Coste and Reyn. in a host range study of WMV isolates in Hawaii. All of his isolates were

restricted to the Cucurbitaceae and did not form local lesions on *C. amaranticolor*. Cohen and Nitzany (1963) studied cucurbit viruses in Israel. Based on its ability to induce lesions in *C. amaranticolor*, to infect legumes, and on its physical properties, the melon mosaic virus they reported was of the WMV-2 type. Molnar and Schmelzer (1964) in an extensive study of the host range of two Eastern European isolates of WMV, found that several members of the Chenopodiaceae formed local lesions in response to inoculation with one of these strains but not with the other. Both strains, however, infected many families outside the Cucurbitaceae, indicating that not all WMV-2 isolates will form local lesions on *C. amaranticolor*. This inability of some isolates of WMV to induce local lesions on *C. amaranticolor* led to the identification of a virus which was latent in cucurbits and named cucurbit latent virus (CLV) by Webb and Bohn (1961). CLV formed local lesions in *C. amaranticolor* and had a host range similar to WMV-2. Lack of cross-protection in tests involving CLV infections challenged by several other viruses (SqMV), CMV, tobacco ringspot virus [TRSV] and WMV) indicated that CLV was unrelated to these other viruses. Separation of WMV into WMV-1 and WMV-2 was not recognized in 1961 and Webb and Bohn did not indicate what isolate of WMV was used in the cross-protection tests. That CLV was probably synonymous with WMV-2 was suggested by studies in which it was impossible to separate or differentiate WMV-2 from CLV (Milbrath and Nelson, 1968; Demski, 1968; Milne *et al.*, 1969). All of Milne and Grogan's WMV-2 isolates formed local lesions on *C. amaranticolor* and both WMV-2 and CLV were systemic in the malvaceous plant *Lavatera trimestris* L. (Milne *et al.*, 1969).

Separation of WMV-1 from WMV-2 was achieved by using *Luffa acutangula* Roxb., which was found to be susceptible to WMV-1, but immune to the WMV-2 isolates tested (Webb, 1965). Symptoms induced by inoculation of cotyledons with WMV-1 were expressed as mild to severe chlorotic spots with stunting of leaves and runners. This use of *L. acutangula* to separate WMV-1 from WMV-2 has been questioned by Milne *et al.* (1969), who found it to be susceptible to 16 of 46 isolates of WMV-2 tested. Several other workers have since used *Luffa* to separate WMV-1 from WMV-2 without difficulty (Greber, 1969; Quiot *et al.*, 1971; Arteaga *et al.*, 1976; Purcifull and Hiebert, 1979). Bhargava (1977) found that certain cultivars of *Luffa acutangula* were susceptible to two different isolates of WMV-2. These two isolates, however, were primarily limited to the Cucurbitaceae. Many species outside the Cucurbitaceae were tested by Bhargava, but only *Vigna sinensis* Savi ex Hasski var. Black Turtle was susceptible to both isolates and *Zinnia elegans* Jacq. was a symptomless carrier of one of the isolates. By the use of reciprocal tests, Webb (1971) was unable to achieve infection with 17 isolates of WMV-2 in *L. acutangula* by approach grafting to watermelon or cantaloupe.

Purification of WMV Isolates

Several schemes have been developed or modified for purifying WMV. van Regenmortel (1961) developed a purification procedure for several South African isolates of WMV, testing different methods for clarifying and concentrating infective plant sap. Freezing or treatment of sap with ethanol greatly reduced infectivity and antigenicity. The use of *n*-butanol or a mixture of chloroform and *n*-butanol was less

effective than chloroform alone for clarification of sap in phosphate buffer. Three cycles of differential centrifugation were superior in removing host material as compared with perosmosis, pervaporation, salting out with ammonium sulfate or acid precipitation with 10% acetic acid. Following the removal of most host material, the virus was further purified by zone electrophoresis (van Regenmortel, 1960). Even though this method was superior to DEAE chromatography, aggregation sometimes occurred. Preparations of WMV obtained by this method were satisfactory for the production of antisera but were too aggregated for determination of particle lengths. An alternative method was developed (van Regenmortel *et al.*, 1962), in which the leaf sap was clarified with chloroform in sodium citrate buffer followed by three cycles of differential centrifugation. The concentrated, clarified virus suspension was filtered through a column consisting of 4% granulated agar in sodium citrate buffer. This method removed all visible impurities and resulted in a nonaggregated virus preparation suitable for particle length determination, as well as for the production of antisera.

Webb and Scott (1965) clarified infective sap in sodium phosphate buffer by centrifugation at 5000 *g* followed by two cycles of differential centrifugation. The resuspended virus had been concentrated thirty times based on an infectivity dilution end point of 10^{-6} after purification of WMV-1. They were successful in purifying WMV-1 and producing an antiserum against it, but were unable to purify sufficient virus for the production of an antiserum against WMV-2.

Milne and Grogan (1969) used potassium phosphate buffer to homogenize pumpkin (*Cucurbita pepo* L. 'Small Sugar') leaves infected

with either WMV-1 or WMV-2. The resulting sap was clarified using *n*-butanol followed by two cycles of differential centrifugation. The virus was further purified by zone electrophoresis (van Regenmortel, 1960). Neither the WMV-1 nor WMV-2 preparations reacted with an antiserum made against concentrated healthy pumpkin sap. Antisera were made against the purified WMV-1 and WMV-2. The virus was severely aggregated after zone electrophoresis, and leaf dips (Hitchborn and Hills, 1965) were used in normal length determinations of virus particles.

Purcifull and Hiebert (1979) clarified WMV-1 sap from infected Small Sugar Pumpkin with a chloroform-carbon tetrachloride mixture in phosphate buffer. Butanol was used as the organic solvent for clarifying sap from WMV-2 infected pumpkin leaves. The virus was precipitated from the aqueous phase with polyethylene glycol and subjected to equilibrium density gradient centrifugation in CsCl ($\bar{\rho}$ = 1.28 g/ml). The virus-containing zone was diluted with buffer and subjected to one cycle of differential centrifugation. These purified virus preparations were used for determinations of the molecular weights of the capsid proteins and for the production of antisera. The molecular weights of undegraded capsid proteins of WMV-1 and WMV-2 were about 36,500 daltons.

Several other workers have used simplified or shortened procedures to purify WMV-2. Thomas (1971a) and Auger *et al.* (1974) used modifications of Milne and Grogan's purification scheme using *n*-butanol clarification to purify WMV. Bakker (1971) and Bhargava (1977) used chloroform clarification to purify isolates of WMV. Fischer and Lockhart (1974) modified the procedure of Damirdagh and Shepherd

(1970) to partially purify a Moroccan isolate of WMV. Thomas (1971a) used the purified WMV-2 to produce an antiserum. None of these workers used zone electrophoresis or cesium chloride equilibrium density gradient centrifugation in their purification schemes.

Inclusions of WMV

Potyviruses induce the formation of distinctive cylindrical inclusions in the cytoplasm of infected host cells (Edwardson, 1966; Purcifull and Edwardson, 1967; Hiebert *et al.*, 1971; Edwardson, 1974a). Cylindrical inclusions are composed of protein (Shepard, 1968) whose monomeric subunits have estimated molecular weights of approximately 67,000 to 70,000 daltons (Hiebert and McDonald, 1973) and are immunologically distinct from the viral capsid protein (Shepard and Shalla, 1969; Purcifull *et al.*, 1973; McDonald and Hiebert, 1975) and host proteins (Purcifull *et al.*, 1973). Edwardson (1966) proposed that the presence of cytoplasmic cylindrical inclusions was diagnostic for infection by members of the potyvirus group. Differences in morphology of the inclusions as determined by ultrastructural studies (Edwardson *et al.*, 1968) led to the separation of potyviruses into three subdivisions (Edwardson, 1974a). Viruses in Subdivision I, to which WMV-1 belongs, induce tubular inclusions attached to the central portion of the cylindrical inclusion. In cross section, the inclusions appear as scrolls, while in longitudinal section they appear as tubes. Subdivision II viruses form laminated aggregate inclusions attached to the central portion of the cylindrical inclusion. The laminated aggregates are usually observed in negatively stained preparations as roughly triangular or rectangular plates appressed together for part

or all of their length. Viruses in Subdivision III, to which WMV-2 belongs, induce both tubes and laminated aggregates in their host cells.

Martelli and Russo (1976) found several isolates of WMV which infected *C. amaranticolor* and *C. quinoa*. They therefore classified these isolates as WMV-2 since their host ranges extended outside the Cucurbitaceae. These isolates, however, induced tubular inclusions (as reported for Subdivision I types) and amorphous cytoplasmic inclusions, as reported for WMV-1 (Edwardson, 1974; Christie and Edwardson, 1977). Purcifull and Hiebert (1979) tested two of Martelli and Russo's isolates and found that they were serologically identical to WMV-1 but not to WMV-2, gave a systemic reaction when inoculated to *Luffa acutangula*, and did not infect either *Nicotiana benthamiana* (Christie and Crawford, 1978) or *Pisum sativum*. Hence the question of the reliability of using the Chenopodiaceae for typing WMV isolates is probably more relevant than considering Martelli and Russo's isolates as exceptions to Edwardson's scheme for separating potyviruses on the basis of inclusion morphology.

Other types of inclusions are associated with certain potyviruses (Edwardson, 1974a; Christie and Edwardson, 1977). In addition to tubular inclusions, WMV-1 induces amorphous inclusions similar to those induced by papaya ringspot virus (Edwardson, 1974a). A thin plate-like nuclear inclusion has been detected in cells infected with WMV-2, by both light and electron microscopy (Christie and Edwardson, 1977). These nuclear inclusions have not been observed in plants infected with WMV-1 isolates (Edwardson, 1974a; Christie and Edwardson, 1977).

Many virus induced inclusions can be observed and identified with the light microscope after relatively simple staining techniques (Christie, 1967; Christie and Edwardson, 1977). These procedures allow for the rapid screening of infected tissue at minimal time and expense. Often, accurate identification to potyvirus subgroup or even to a specific virus is possible. The cytoplasmic and nuclear inclusions induced by WMV isolates can be detected by these techniques (Christie and Edwardson, 1977).

The serological properties and relationships of some potyvirus cylindrical inclusions have been investigated (Hiebert *et al.*, 1971; Purcifull *et al.*, 1973; Batchelor, 1974; McDonald and Hiebert, 1975; Purcifull and Batchelor, 1977; Lima, 1978; Zurawski, 1979). Antisera produced against partially purified inclusions of tobacco etch virus (TEV) and potato virus Y (PVY) gave strong homologous reactions but did not cross react with each other (Hiebert *et al.*, 1971).

Five potyviruses (TEV, PVY, turnip mosaic virus, bidens mottle virus, and pepper mottle virus) were found to induce cylindical inclusions that were serologically distinct (although some were related) and the propagative hosts did not affect the antigenic specificity of the inclusions (Purcifull *et al.*, 1973). Antigenic differences between strains of turnip mosaic virus (TuMV) (McDonald and Hiebert, 1975) were detected in the capsid proteins but not the cylindrical inclusions even though one of the three strains studied had distinctly different laminated aggregates. This work supported the concept that cylindrical inclusions are coded for by the viral nucleic acid and that serological studies of inclusions could be useful in determining the taxonomic relationship between potyviruses.

Direct evidence for the hypothesis that cylindrical and some nuclear inclusions are products of the potyvirus genome come from *in vitro* translation of pepper mottle virus (PeMV) and TEV RNAs (Dougherty, 1979). In these studies, molecular weight determinations utilizing SDS polyacrylamide gel electrophoresis and serology indicate cylindrical inclusion and capsid proteins are synthesized *in vitro* from PeMV and TEV RNA.

Serological Relationships of WMV Isolates

There have been several points of controversy concerning serological relationships among WMV isolates. Specifically, there have been disagreements about the serological relationships between WMV-1 and WMV-2 (Webb and Scott, 1965; Milne and Grogan, 1969; Purcifull and Hiebert, 1979), and about the serological relationship of papaya ringspot virus to WMV-1 and WMV-2 (Milne and Grogan, 1969; Purcifull and Hiebert, 1979). There also have been indications that at least one serotype distinct from either WMV-1 or WMV-2 may exist (Webb and Scott, 1965; Purcifull and Hiebert, 1979). Complicating interpretation of the various results is the use of different virus isolates for the preparation and testing of antisera, and the use of various types of serological tests.

Webb and Scott (1965) divided WMV into two groups (WMV-1 and WMV-2), partly on the basis of serological differences between the two types. Milne and Grogan (1969), however, reported that WMV-1 and WMV-2 were serologically very closely related and they concluded that WMV-1 and WMV-2 should be considered as strains of the same virus. Several subsequent workers have been unable to find a close serological

relationship between WMV-1 and WMV-2 (Bakker, 1971; Greber, 1978; Purcifull and Hiebert, 1979). Purcifull and Hiebert (1979) produced antisera to Florida isolates of WMV-1 and WMV-2. In SDS double immunodiffusion tests, the WMV-1 antiserum gave a positive reaction only with isolates of WMV-1. Likewise, the WMV-2 antiserum reacted only with WMV-2 isolates. Martelli and Russo (1976) found several isolates of WMV from the Mediterranean region which were limited in host range to the Cucurbitaceae and Chenopodiaceae. Purcifull and Hiebert (1979) tested two of these Mediterranean isolates and found that they reacted with WMV-1 antiserum but not with WMV-2 antiserum.

In addition to serological differences between WMV-1 and WMV-2, there has been conflicting evidence on the serological relationship between WMV-1 or WMV-2 and several African isolates of WMV. Some of this evidence has led to the suggestion that WMV may consist of three or more serologically distinct viruses. Webb and Scott (1965) suggested the presence of a third serologically distinct virus in the WMV group when they were unable to obtain positive serological reactions in tests with an antiserum to a South African isolate of WMV and antigens of North American WMV-1 and WMV-2 isolates. Lastra (1968) obtained positive reactions in microprecipitin tests between Venezuelan isolates of WMV-1 and WMV-2 and antiserum obtained from Grogan and thought to have been made against WMV-1. He was, however, unable to get a positive reaction using the same isolates and antisera specific for the South African isolates. Bakker (1971) found that a Kenyan isolate of WMV, which was limited in host range to the Cucurbitaceae and Chenopodiaceae, reacted with Milne's WMV-2 antiserum and van Regenmortel's South African

WMV antiserum but not with Milne's WMV-1 antiserum. Milne and Grogan (1969), however, state that their WMV-1 and WMV-2 isolates gave positive reactions with the South African WMV antiserum. Purcifull and Hiebert (1979) got negative reactions between WMV-M and antisera specific for Florida isolates of WMV-1 or WMV-2. They were unable to produce an antiserum to WMV-M and therefore considered the possibility of WMV-M representing a third serotype as tentative. Schmelzer (1969) suggested the possibility of a third type of WMV based on host range differences and symptom expression of the South African isolates but did not present serological evidence.

Serological relationships between WMV and other potyviruses have been established though some confusion has resulted due to lack of agreement as to whether WMV-1 and WMV-2 are serologically distinct. Milne and Grogan (1969) found WMV-1 and WMV-2 to be closely related to papaya ringspot virus, while Purcifull and Hiebert (1979) obtained reactions of identity between papaya ringspot virus and WMV-1 antiserum but did not get a reaction with WMV-2 antiserum. Purcifull and Hiebert also showed in reciprocal immunodiffusion tests that soybean mosaic virus was closely related to but distinct from WMV-2. Van Regenmortel *et al.* (1962) demonstrated a serological relationship using an antiserum specific for the South African isolate of WMV and both BYMV and potato virus Y (PVY). Several other potyviruses which have been reported to infect cucurbits, viz. LMV, TuMV, and the severe strain of BYMV, were found to be serologically distinct from WMV-1 and WMV-2 (Florida isolates) in reciprocal gel immunodiffusion tests performed by Purcifull and Hiebert (1979). No cross-reactions were observed in reciprocal

tests between WMV-1 and soybean mosaic virus. Blackeye cowpea mosaic virus (BlCMV) was shown in reciprocal immunodiffusion tests to be related to but distinct from WMV-2. No cross-reactions were observed in reciprocal immunodiffusion tests between BlCMV and WMV-1 (Lima *et al.*, 1979).

Milne and Grogan (1969) obtained precipitin bands when detergent-treated, partially purified papaya ringspot virus (PRSV) was tested with either WMV-1 or WMV-2 antisera. They observed no detectable serological difference between PRSV and WMV-1 or WMV-2 antigens treated in the same manner. Purcifull and Hiebert (1979) did not get a detectable reaction in gel immunodiffusion tests with PRSV in sap and WMV-2 antiserum but PRSV gave a reaction of serological identity when compared with the Florida isolate of WMV-1.

MATERIALS AND METHODS

Source of Virus Isolates

The isolates were subcultures of those used in a previous study (Purcifull and Hiebert, 1979). The Florida strain of WMV-1 (WMV-1 Florida) was obtained originally from W. C. Adlerz. The Florida strain of WMV-2 was isolated from watermelon in Alachua County by D. E. Purcifull. The Jordanian isolate of WMV (WMV-1 Jordan) was obtained from G. Martelli, and the Moroccan isolate of WMV (WMV-M) was received from B. Lockhart.

Maintenance and Propagation of Virus Isolates

Watermelon mosaic viruses, WMV-1, WMV-2, and WMV-M were propagated in *Cucurbita pepo* L. var. Small Sugar Pumpkin. WMV-2 was also propagated in *Nicotiana benthamiana* Domin. All watermelon mosaic virus isolates were maintained in separate screened cages. Powdery mildew was a serious problem on pumpkin, and it was controlled by weekly sprayings with Dinocap. Frogeye spot, caused by *Cercospora nicotianae* Ell. and Ev., was controlled on *N. benthamiana* by spraying with Benomyl when symptoms appeared. Pesticides were used at concentrations recommended by their manufacturers.

Five to six seeds of pumpkin were sown per six inch pot. *Nicotiana benthamiana* were seeded in Jiffy pots consisting of peat moss and were transferred to six inch pots after 30 days. Seedlings were germinated in a greenhouse from

which known virus-infected plants were excluded. About one week after planting and just prior to virus inoculation, pumpkin seedlings were transferred either to screened cages or greenhouse benches. Pumpkin seedlings in the cotyledonary stage were mechanically inoculated with either WMV-1, WMV-2, or WMV-M. Inocula were prepared by grinding infected pumpkin leaf tissue in water with carborundum. One month old seedlings of *N. benthamiana* were mechanically inoculated as above with WMV-2 infected pumpkin tissue. After inoculation, seedlings were routinely fertilized every two weeks with a 20-20-20 soluble fertilizer until harvested.

Virus and Inclusion Purification

Extracts from plants infected with WMV-2 were clarified either with *n*-butanol or with a combination of chloroform ($CHCl_3$) and carbon tetrachloride (CCl_4) (Lima, 1978; Lima *et al.*, 1979). WMV-1 and WMV-M were unstable in *n*-butanol and, therefore, extracts containing them were clarified only with the $CHCl_3$-CCl_4 combination (Lima, 1978). Systemically infected pumpkin leaves were harvested 21 to 45 days after inoculation, whereas infected *N. benthamiana* leaves were harvested 30 to 90 days after the plants were inoculated. The leaves were kept in plastic bags up to four days at 4 C before purification of the virus.

n-Butanol Clarification Method

One hundred to 700 g of leaf tissue were homogenized in a blender with two parts (w/v) of a buffer (homogenization buffer) consisting of 0.5 M potassium phosphate, 0.01 M Na_2EDTA, and 0.5% Na_2SO_3 (pH of the mixture was 7.5 to 7.7). The homogenate was filtered through two leaves

of cheesecloth and centrifuged at 13,200 *g* (max) for 10 min. The supernatant, containing virus, was decanted from the pellet, which contained inclusions. The inclusions were purified from the pellets as described below. The supernatant was stirred while *n*-butanol (8 ml per 100 ml supernatant) was added slowly. The mixture was stirred for 4 hr at 4 C. The coagulated material was removed by a low speed centrifugation at 13,200 *g* for 10 min. The virus was precipitated (Hebert, 1963) from the aqueous phase by adding 8 g of polyethylene glycol MW 6000 (PEG) per 100 ml of supernatant at 4 C with stirring until the PEG dissolved, followed by centrifugation at 10,400 *g* for 10 min. The pellets were resuspended in 0.02 M potassium phosphate, 0.01 M Na_2EDTA, pH 8.2 (virus buffer) and subjected to equilibrium density gradient centrifugation in CsCl ($\bar{p}$ = 1.28 g/ml of virus buffer) in a Beckman SW 50.1 rotor at 150,000 *g* (max) for 13 to 18 hr. The virus zone, located 8 to 11 mm from the bottom of the tube, was collected dropwise through a hole punched in the bottom of the tube. The collected zone was then diluted with three volumes of virus buffer. The virus preparation was further clarified by a low speed centrifugation at 12,000 *g* and then precipitated as before with PEG. The final pellet was resuspended in a buffer consisting of 0.02 M Tris, 0.01 M Na_2EDTA, pH 8.0 to 8.2. The virus concentration was determined spectrophotometrically using an extinction coefficient of 2.4 per cm for a 0.1% solution at 261 nm (Purcifull, 1966). The optical density readings at 260 and 280 nm were corrected for light scattering by least squares linear regression analysis of readings taken at 320, 330, 340, 350, and 360 nm.

Cylindrical Inclusion Purification

The first 13200 *g* pellets from the *n*-butanol purification method were resuspended in 100 to 300 ml of the homogenization buffer and emulsified in a blender with an organic solvent consisting of a 1:1 solution (v/v) of chloroform ($CHCl_3$) and carbon tetrachloride (CCl_4). The ratio of organic solvent to homogenization buffer was 1:3 (v/v). The emulsion was broken by centrifugation at 4,080 *g* for 5 min. The aqueous phase consisting of inclusions was decanted and filtered through glass wool and the inclusions were precipitated by centrifugation at 16,300 *g* for 10 min. The inclusion pellets were resuspended in 0.05 M potassium phosphate buffer, pH 8.2, containing 0.5% 2-mercaptoethanol (2-ME). Triton X-100 (TX-100) solution was added to the inclusion preparation to yield a final concentration of 5% TX-100. This was stirred for one hr at 4 C. The inclusions were pelleted by centrifugation at 17,300 *g* (max) for 15 min and resuspended in 0.02 M potassium phosphate buffer containing 0.1% 2-ME, pH 8.2 (inclusion buffer). The resuspended inclusions were layered on a fresh sucrose step gradient consisting of 6 ml of 80% sucrose, 10 ml of 60% sucrose, and 10 to 12 ml of 50% sucrose. All sucrose stock solutions were made up in 0.02 M potassium phosphate buffer, pH 8.2. Inclusions were centrifuged on the fresh sucrose gradients at 21,000 rpm for 1 hr in a Beckman SW 25.1 rotor. Inclusions were found layered on top of the 80% sucrose and were collected dropwise. The inclusion fraction was diluted with 3 volumes of inclusion buffer and precipitated by centrifugation at 17,300 *g* (max) for 15 min. The pellet was resuspended in 0.02 M Tris-HCl, pH 8.2, and the inclusion yield was estimated spectrophotometrically after being dissolved in 1% sodium dodecyl sulfate (SDS) (Hiebert *et al.*,

1971). Inclusions were examined in a Philips 200 electron microscope after negative staining with 2% aqueous uranyl acetate, ammonium molybdate, or 1% phosphotungstate. The inclusion preparations were either used immediately for immunization of rabbits or stored by one of the following methods: freezing with 0.02 M Tris-HCl buffer, pH 8.2 at -20 C, freeze drying in Tris buffer followed by storage at -20 C, solubilization by dissociation in either 1% SDS or in the Weber-Osborn dissociation buffer (Hiebert and McDonald, 1973), followed by freezing at -20 C. Inclusion preparations were tested for proteolytic degradation and contamination with viral capsid and host proteins by analytical polyacrylamide gel electrophoresis (PAGE) and by serology against the corresponding virus antisera.

Chloroform-Carbon Tetrachloride Clarification Method

One hundred to 700 g of leaf tissue were homogenized in a blender for 1 min with 2 parts (w/v) of the homogenization buffer. The homogenate was then emulsified by adding to 3 parts of the homogenate, 1 part (v/v) of a 1:1 solution of $CHCl_3$ and CCl_4 and blending at high speed for 2 min. Several hundred milliliters of shaved ice were added during emulsification. The emulsion was broken by centrifugation at 480 *g* for 5 min. The aqueous phase was decanted, filtered through glass wool and centrifuged at 12,100 *g* for 15 min. The supernatant containing virus was decanted from the pellet containing inclusions.

The supernatant was made 1% (v/v) in TX-100. The mixture was stirred for 1 hr and centrifuged at 12,100 *g* for 10 min. The virus was precipitated from the supernatant with PEG as described previously. The remainder of the virus purification was essentially the same as

that described for the *n*-butanol purification scheme except that an additional PEG precipitation was often required prior to density gradient centrifugation. When Cs_2SO_4 was substituted for CsCl during density gradient centrifugation of WMV-M the amount used was 10.5 g dissolved in 27 ml of virus buffer and adjusted to a final pH of 8.2 with 1 M KOH.

Purification of the inclusion pellets was identical to the procedure described previously for the *n*-butanol purification scheme beginning with the solubilization step employing TX-100.

Purification of Cylindrical Inclusions for Peptide Mapping

The procedure required 150-200 g of pumpkin leaves showing strong mosaic and distortion symptoms and free of powdery mildew. The tissue was homogenized at 4 C in 3 parts of homogenization buffer and 1 part (v/v) of a 1:1 chloroform-carbon tetrachloride mixture (see virus purification). The emulsion was broken by centrifuging at 480 *g* for 5 min. The supernatant was filtered through glass wool and the pellet resuspended in about 250 ml of homogenization buffer and homogenized a second time at 4 C. The emulsion was broken as before and the filtered supernatant was combined with the first supernatant and centrifuged at 12,100 *g* for 20 min. The pellets were resuspended with the aid of a glass tissue homogenizer, in 36 ml of a pH 8.2, 0.02 M potassium phosphate buffer containing 0.1% 2-mercaptoethanol (final concentration). Four milliliters of 20% TX-100 (v/v) were then added and the mixture was stirred at 4 C for 90 min. After centrifugation at 27,000 *g* (max) for 15 min, the green supernatant was discarded and the light green pellet was resuspended and washed in buffer two or three times to remove all green pigments. Occasionally a second exposure to 2% TX-100 was required. After several

washes, the gray inclusion pellet was resuspended in 4 ml of buffer and homogenized in the micro-homogenizer of a Sorvall Omnimixer at high speed for 2 min at 4 C. The inclusions were centrifuged at 270 *g* for 5 min. The supernatant was decanted and a soft pellet consisting primarily of inclusions was removed by gently resuspending it in buffer and aspirating. A hard white pellet was discarded. Inclusion protein concentrations of the supernatant and resuspended soft pellet were determined spectrophotometrically (Hiebert *et al.*, 1971). The partially purified preparations were further purified by preparative gel electrophoresis using 8% Weber-Osborn gels run in an Ortec 4217 cell with preparative well former (Ortec Inc., Oak Ridge, Tn.). After electrophoresis for 3 to 4 hr, the gels were removed, and immediately wrapped in clear plastic wrap and chilled overnight at 4 C. The opaque inclusion band was visible about 2/3 the distance (60 mm) from the bottom of the gel. The band could be further identified by staining with Coomassie brilliant blue. The opaque band was cut out by pressing down on a taut wire held in a coping saw. The band was then cut into slices approximately 1 mm thick and eluted in 5 to 7 ml of water overnight at 4 C. The eluate was then removed and saved and the bands again eluted in water for about 8 hr at room temperature. The eluates were pooled and small fragments of polyacrylamide gel were removed by passing the eluates through a 0.045 µm pore size membrane filter. The eluates were then dialyzed exhaustively against 0.02 M Tris-HCl, pH 8.0 or water. Concentrations of inclusion proteins were determined spectrophotometrically prior to pooling and again before freeze drying. The inclusion samples were tested serologically for antigenic specificity by double immunodiffusion tests. The dissociated

inclusions were also examined on Weber-Osborn (1969) or Laemmli (1970) analytical polyacrylamide gels to test for the presence of host or viral capsid protein contaminants. After freeze drying the inclusion proteins were stored at -20 C.

Cleavage of WMV Capsid and Cylindrical Inclusion Proteins

Capsid proteins of each virus were cleaved on the carboxyl side of methionyl peptides with cyanogen bromide (CNBr) (Gross and Witkop, 1962; Gross, 1967). The purified viruses were resuspended in either 0.02 M Tris, pH 8.2 or 0.02 M Tris, 0.01 M Na_2EDTA, pH 8.2, at concentrations between 1-7 mg/ml, and then were cleaved by an excess of CNBr (10-20 mg) in 70% formic acid. The reaction was carried out at room temperature for at least 18 hr. The cleaved peptides were concentrated by lyophilization, resuspended in 2 ml of water, and stored at 4 C. Each uncleaved virus preparation was examined on analytical Weber-Osborn gels just prior to CNBr cleavage to insure that the preparation contained only undegraded virus capsid proteins (Hiebert and McDonald, 1976; Hiebert *et al.*, 1979).

Partial proteolytic digestion and analysis on polyacrylamide gels of purified inclusion proteins dissociated in SDS was performed using *Staphylococcus aureus* V-8 protease (Miles Laboratories) as described by Cleveland *et al.* (1977). The method involved a slight modification of the procedure described by Cleveland *et al.* (1977). The acrylamide concentration of the stacking gel was increased from 3% to 5.6% for use on 1.5 mm thick slab gels.

Conditions were optimized based on a kinetic study in which purified inclusions at concentrations of 300 µg/ml were treated by

incubation at 37 C with either no protease or 25 µg/ml of *S. aureus* protease for times ranging up to 150 min. Proteolysis was stopped by adding SDS and 2-ME to final concentrations of 2% and 10%, respectively. The samples were heated at 100 C for 2 min. Fifteen, 30, or 60 µg of partially digested inclusions were added to each well. Electrophoresis was performed in a slab gel apparatus (Studier, 1973) at 100 volts, 21 mA for 4-6 hr. Gels were stained with Coomassie brilliant blue and photographed. Based on these kinetic studies, the standard conditions used were 15 µg of inclusion protein (per well) which had been incubated for 30 min at 37 C with 25 µg/ml of *S. aureus* protease.

Polyacrylamide Gel Electrophoresis of Viral and Inclusion Proteins

Polyacrylamide slab gel electrophoresis (PAGE) was performed according to the method of Weber and Osborn (1969) as modified by Hiebert and McDonald (1973).

Gel Electrophoresis of Cyanogen Bromide Cleaved Viral Capsid Proteins

WMV capsid proteins which had been cleaved by treatment with CNBr were electrophoresed on a low pH gel modified from Mauer's gel system #7 (1971). System #7 required three stock solutions: stock buffer #17 had a final phase of 4.3 and consisted of approximately 48 ml of 1N KOH and 17.2 ml of glacial acetic acid plus sufficient water to bring the total volume to 100 ml. Stock buffer #19 had a final pH of 6.7 and consisted of 48 ml of 1N KOH, 2.87 ml of glacial acetic acid and was brought up to a total volume of 100 ml with water. The electrode buffer stock had a pH of 4.5 and consisted of 31.2 g of

β-alanine, and 8 ml of glacial acetic acid brought up to one liter. The electrode buffer was a 10% aqueous solution of the electrode buffer stock.

Thirty milliliters of the running gel body consisted of 11.75 ml of water, 3.75 ml of buffer #17, 12.5 ml of the acrylamide stock (30 g monomer and 0.8 g Bis in 100 ml) and 0.2 ml of TEMED. The gel solution was degassed for 5 min, then 1.8 ml of freshly prepared ammonium persulfate solution (15 mg/ml) was added and the mixture was poured to a height of about 72 mm in an Ortec casting stand. The gel was overlaid with a solution consisting of 0.3 ml of stock #17 and 2.7 ml of water. Polymerization occurred after transferring the casting stand to a water bath set at 40 C. The casting stand with the polymerized gel was removed from the hot water bath and allowed to cool to room temperature. After cooling, the overlay was removed and a stacking gel of about 4.5 mm in height was added. The stacking gel consisted of 0.3 ml of buffer #19, 0.4 ml of acrylamide stock, 0.03 ml of TEMED and 1.5 ml of water. To this was added 0.18 ml of ammonium persulfate and the gel was mixed rapidly and poured on top of the running gel. The stacking gel was immediately overlaid with a solution consisting of 0.3 ml of buffer #19 and 2.1 ml of water. The cap and well gel solution was composed of 1 ml of undiluted electrode buffer stock, 2.7 ml of acrylamide stock, 6.3 ml water and 0.01 ml of TEMED. The combined gel was divided into two 5 ml portions for the well and capping gels, respectively. Polymerization of both well and capping gels was achieved by adding 0.7 ml of ammonium persulfate just prior to pouring. A twelve tined comb was used to form the wells.

The sample buffer was made fresh each time and consisted of 1.08 g of ultrapure urea (Schwartz/Mann), 0.25 g of sucrose, 0.25 ml of #19 buffer and 0.025 ml of a 1% solution of methylene blue. The sample buffer was brought up to a total volume of 2 ml with water. Approximately 50 µg of each CNBr cleaved and dissolved protein was dried on a spotting plate and redissolved in about 30 µl of sample buffer. Samples were immediately transferred to the appropriate wells. The capping gel was pipetted into the wells and the gel former was transferred to the Ortec vertical slab electrophoresis apparatus, Model 4010,4011. The anode was attached to the upper tank and the cathode was attached to the lower tank. Electrophoresis was performed for 3 to 4 hr at 200 volts, with a pulsed power of 300 pulses per second and a current of approximately 70 mA. After electrophoresis, the gel was stained overnight in a solution containing 50% methanol, 10% glacial acetic acid and 0.1% Coomassie brilliant blue stain R 250. The gel was destained in several changes of a solution consisting of 10% methanol and 7% glacial acetic acid. The gels were photographed and then scanned using an automatic gel scanner attached to a Beckman Model 25 recording spectrophotometer.

Serology

Anitsera for virus and cytoplasmic inclusions were obtained by injecting adult New Zealand white rabbits with either undegraded virus capsid or cylindrical inclusion protein or with SDS dissociated capsid or inclusion proteins obtained from preparative polyacrylamide gels. The WMV-M capsid immunogens used in the production of antisera were checked by analytical Weber-Osborn polyacrylamide gels to insure that

partial proteolytic cleavage (Hiebert and McDonald, 1976) had not occurred. All rabbits were bled for normal serum prior to immunization. The initial immunizations consisted of intramuscular injections of 1 to 2 mg of protein in 1 ml 0.02 M Tris buffer, pH 8.2, emulsified with 1 ml Freund's complete adjuvant (Difco). Rabbits were boosted with one or two subsequent intramuscular injections two to four weeks apart using similar quantities of protein emulsified in Freund's incomplete adjuvant (Difco). All other immunizations involved essentially the same procedure except that at least one toepad was injected with 0.15 to 0.2 mg of protein. Antisera to formaldehyde fixed antigens were obtained with only a single toepad and intramuscular injection. Rabbits were bled according to the procedure of Purcifull and Batchelor (1977) at approximately weekly intervals and for three to nine months beginning 10 to 15 days after the first injection.

Antisera also were made against formaldehyde fixed viruses. During virus purifications, antigens were fixed in 1.8% formaldehyde for 10 min at room temperature prior to equilibrium density gradient centrifugation in CsCl, and prepared for immunization as with unfixed viruses.

Initially, antisera were freeze-dried and stored at room temperature. After one year a loss in titer was detected in these antisera versus frozen antisera. Therefore, the freeze-dried sera were subsequently stored at 4 C or -20 C, and all new serum collections were frozen at -20 C.

Cross-Absorption of Antisera

Some antisera reacted with healthy plant antigens forming nonspecific precipitates in SDS-double immunodiffusion tests. These

antisera were cross-absorbed with concentrated healthy plant antigens according to the method of Purcifull *et al.* (1973). Sixty grams of frozen SSP were thawed and homogenized in 120 ml of 0.1 M potassium phosphate buffer, pH 7.4, which contained 1% Na_2SO_3. The homogenate was frozen for 3 hr, thawed, and centrifuged at 27,000 *g* for 10 min. The supernatant was centrifuged at 250,000 *g* (max) for 3 hr in a Beckman Ti 60 rotor. The resulting pellet was resuspended in 4 ml of 0.02 M Tris buffer, pH 7.4. Concentrated host antigens were combined with the antisera to be cross-absorbed according to the method of Purcifull and Zitter (1973). Host proteins were mixed with antisera (1:4, v/v) and incubated overnight at 4 C. The mixture was centrifuged at 81,000 *g* for 1 hr in a Beckman Type 40 rotor and the supernatant containing antisera was used immediately or frozen at -20 C.

Serological Tests

Double immunodiffusion tests in agar gels, microprecipitin tests, and enzyme linked immunosorbent assays (ELISA) were preformed during parts of this study.

Double immunodiffusion tests (Ouchterlony, 1962) were carried out in agar gels consisting of 0.8% Noble agar (Difco), 0.5% SDS (Sigma), and 1.0% sodium azide (NaN_3) (Sigma) in deionized water (Purcifull and Batchelor, 1977), or in a medium consisting of 0.8% Noble agar, 0.2% SDS, 0.1% NaN_3, and 0.7% NaCl in deionized water (Tolin and Roane, 1975). Reactant wells (7 mm in diameter) were arranged in a hexagonal array produced by an adjustable gel cutting device (Grafar Corp., Detroit, Mich.) with a spacing of 4.5 to 5 mm between wells. Plant

tissues were extracted by triturating with a mortar and pestle 1 g of tissue in either 2 ml of deionized water or in 1 ml of deionized water followed by the addition of 1 ml of 3% SDS. The extracts were expressed through two layers of cheesecloth. For routine tests, sap from 5 to 10 g of tissue were prepared in this manner and frozen in 3 to 4 ml aliquots. When titering an antiserum, either normal serum or bovine albumin was used as antiserum diluent (Purcifull and Batchelor, 1977).

Sometimes serological distinctions were demonstrated by the intragel cross-absorption technique (Lima, 1978; Lima *et al.*, 1979). Heterologous or homologous antigens were added to the center wells of a hexagonal array. The peripheral wells were cut but the agar was not removed until antigens in the center well had diffused into the agar. After 16 to 18 hr any remaining fluid in the center well was removed by aspiration and the agar in the peripheral wells was removed. Appropriate antiserum and antigens were then added in the usual manner to the center and peripheral wells, respectively.

Microprecipitin tests were sometimes used to titer antiserum according to the procedures of Ball (1974), except that uncoated plastic Petri dishes were used instead of Formvar-coated glass Petri dishes.

Fractionation of Gamma Globulin for ELISA

Enzyme linked immunosorbent assays (ELISA) were carried out using a modified procedure of Clark and Adams (1977). The gamma globulins (γG) used to coat microtiter plates and to conjugate with alkaline phosphatase were fractionated from antisera collected at least 2 months after the initial immunization as recommended by Koenig (1978). One milliliter of antiserum was diluted with 9 ml of deionized water and

stirred at room temperature while 10 ml of a saturated ammonium sulfate solution was slowly added. The precipitated gamma globulin fraction was allowed to incubate at room temperature for 30 min and was then collected by centrifugation at 10,000 *g* for 10 min. The pellet was resuspended in 2 ml of half strength phosphate buffered saline (PBS), pH 7.4, having a 1X concentration per liter of 8.0 g NaCl, 0.2 g KH_2PO_4, 2.51 g $Na_2HPO_4 \cdot 7H_2O$, 0.2 g KCl, and 0.2 g NaN_3. A 10X PBS stock solution was maintained at room temperature. The γG was dialyzed 3 times (approximately 4 hr each) against 500 ml of half strength PBS, and then filtered through a 5 ml DEAE Sephacel column previously equilibrated with half strength PBS. The γG was washed through the column with half strength PBS, and collected in 1 ml fractions in siliconized glass tubes. Each aliquot of the first protein to elute was read on a Beckman Model 25 recording spectrophotometer. Those tubes containing 0.8 O.D. or more were combined and the γG concentration adjusted to 1 mg/ml (1.4 $O.D._{280}$) with half strenth PBS (Clark and Adams, 1977). The γG was stored at -10 C in silicone-treated glass tubes.

Conjugation of Alkaline Phosphatase with Gamma Globulin

A crystalline suspension (usually 2.5 mg) of alkaline phosphatase (Sigma No. P4502, 1000 units/mg), in $(NH_4)_2SO_4$ was centrifuged at 10,000 *g* for 10 min. The pellets were dissolved in 1 ml (1 mg) of purified γG and dialyzed 3 times against 500 ml of half strength PBS. A 25% (v/v) solution of glutaraldehyde was added to the mixture to yield a final concentration (v/v) of about 0.1%. The solution was incubated at room temperature for 4 hr during which a slight yellow-brown color

developed. The conjugated γG was then dialyzed 3 times against half strength PBS as described previously. Five milligrams of bovine serum albumin was then added per milliliter of conjugate and the mixture stored at 4 C.

Preparation of ELISA Plates

Two hundred microliters of coating buffer containing purified gamma globulin was added to all except the peripheral wells of round bottom microtiter plates (Cook MicroELISA Substrate Plates #1223-24, Dynatech Labs, Inc.). The coating buffer contained 1.5 g Na_2SO_3, 2.93 g $NaHCO_3$, and 0.2 g NaN_3 per liter and had a pH of 9.6. The optimum concentration of γG had to be determined for each conjugate, but it was usually in the range of 1 to 2 µl/ml. The plates were usually incubated 2-6 hr at room temperature though overnight incubation gave similar results. Plates were washed three times with PBS-Tween (PBST) which consisted of the PBS buffer to which had been added 0.5 ml of Tween 20 per liter. The PBST was dispensed from a wash bottle with great care to prevent contamination from one well to the next, particularly during all first washes. Wells were filled with PBST and after at least 3 min the PBST was removed by shaking the wash solution into a sink. Plates were blotted on paper towels and washed two more times.

The test antigens were made by triturating leaf tissue in a buffer consisting of PBST plus 2% (w/v) polyvinyl pyrrolidone (Sigma PVP-40), using 9 ml buffer per g tissue. Two hundred microliters of each test antigen usually were added to duplicate wells. Plates were incubated at 4 C overnight or at 37 C or room temperature for 4-6 hr.

The optimum concentration of enzyme-labelled conjugate was determined for each conjugate.

Plates were washed 3 times with PBST, and 250 µl of freshly prepared substrate buffer containing 0.6 to 1.0 mg/ml of *p*-nitrophenyl phosphate (Sigma #104-105 tablets) was added to each well. Each liter of substrate buffer contained 97 ml diethanolamine, 0.2 g NaN_3, and sufficient HCl to give pH 9.3. The plates were incubated at room temperature for 30 to 60 min, and the reactions were stopped by the addition of 50 µl of 3 M NaOH to each well.

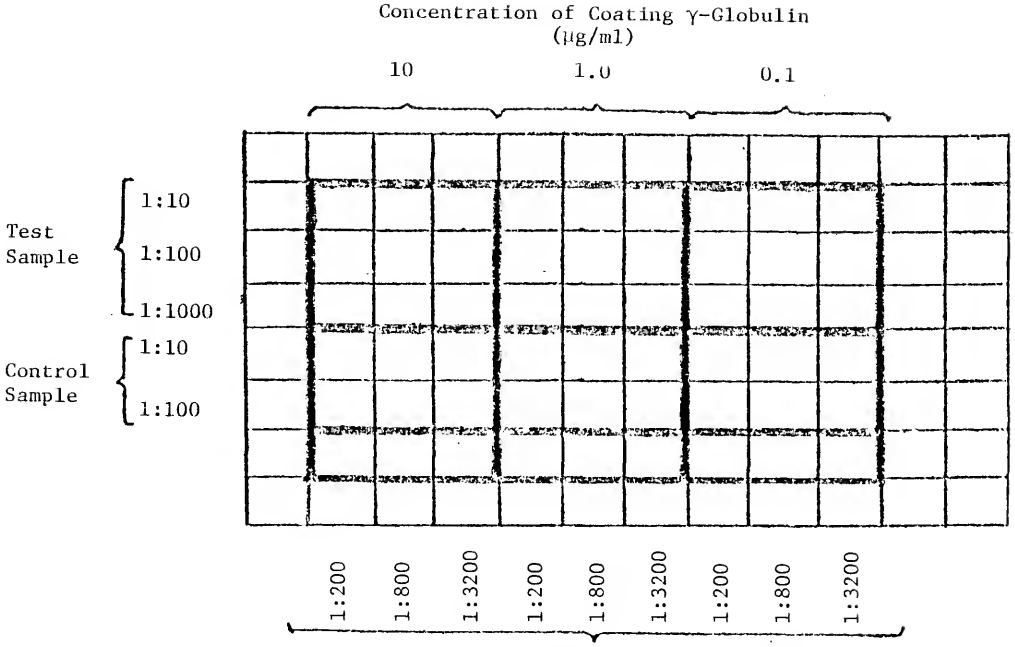
Reactions were assessed visually by rating on a 0, +, ++, +++ scale and by measurement of absorbance at 405 nm with a Beckman Model 25 spectrophotometer. Absorbance measurements required a dilution (1:2) with water. Results were sometimes recorded photographically, using backlighting and Kodak Verachrome 64 Film.

The determination of optimum concentrations of coating gamma globulin, test and control samples and dilutions of enzyme-labelled conjugates was similar to the general procedure except that the concentrations and setups were as shown in Figure 1.

Figure 1. Scheme for determining optimum concentrations of coating and enzyme labelled γ-globulin. (From Clark and Adams, unpubl.)

Optimum concentrations were determined spectrophotometrically by selecting the lowest concentrations of the following, in order: enzyme labelled conjugate, coating γ-globulin, and test sample, which gave the highest ratio with the controls at the same concentration. Ideally, concentrations were such that the control samples approached zero absorbance at OD_{405} and the test samples had OD_{405} = 1.00 to 2.00.

For this scheme the outside rows of wells are not used, as these sometimes give rise to spurious results, particularly at the corners.


Concentration of Coating γ-Globulin
(μg/ml)
10
1.0
0.1
Test
Sample
1:10
1:100
1:1000
Control
Sample
1:10
1:100
1:200
1:800
1:3200
1:200
1:800
1:3200
1:200
1:800
1:3200
Dilutions of Enzyme-Labelled Conjugate

RESULTS

Purification and Properties of Watermelon Mosaic Viruses and Inclusions

Purification schemes for virus and virus-induced inclusions are shown diagramatically in Figures 2, 3, and 4. Virus yields were determined using an extinction coefficient for TEV of $E_{261}^{0.1\%} = 2.4$ (Purcifull, 1966). The typical ultraviolet absorption curve (Figure 5) obtained for all purified WMV isolates had a maximum absorbance between 260 and 262 nm and a minimum at about 245 nm. The 260/280 ratio was approximately 1.2 after correction for light scattering. This value is consistent with those found for other potyviruses (*e.g.*, Lima, 1978). WMV-2 infected pumpkin or *N. benthamiana* tissue yielded approximately 10 mg of virus per 100 g fresh weight when *n*-butanol (Figure 2) was used in the clarification process, as opposed to only 3-4 mg per 100 g fresh weight when a 1:1 (v/v) mixture of carbon tetrachloride-chloroform (Figure 3) was used. Both the Florida and Jordan isolates were unstable in *n*-butanol and pumpkin infected with these viruses routinely yielded approximately 10 mg virus per 100 g fresh weight with the carbon tetrachloride-chloroform clarification method. WMV-M, also unstable in *n*-butanol, gave maximum yields of only 3-4 mg per 100 g fresh weight with the carbon tetrachloride-chloroform method.

To prevent irreversible aggregation of WMV-2, it was necessary to add 0.01 M Na_2EDTA (final concentration) to each solution throughout the procedure. Neither WMV-1 nor WMV-M required Na_2EDTA though it was

Figure 2. Flow diagram outlining the procedure of purification of WMV-2 using *n*-butanol as the clarifying agent, polyethylene glycol (PEG) for virus concentration, and CsCl equilibrium density gradient centrifugation for separation of virus from host components. See description in Materials and Methods for further details.

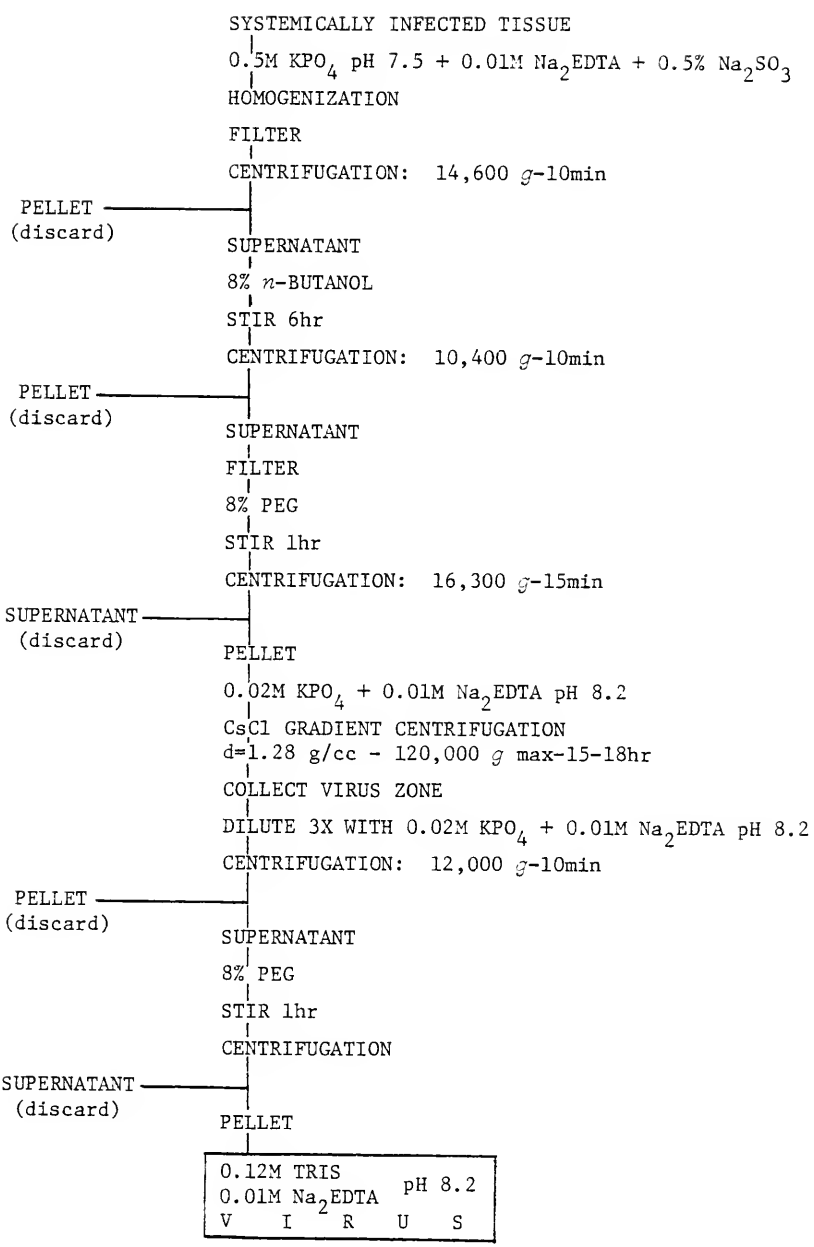

SYSTEMICALLY INFECTED TISSUE
0.5M KPO_4 pH 7.5 + 0.01M Na_2EDTA + 0.5% Na_2SO_3
HOMOGENIZATION
FILTER
CENTRIFUGATION: 14,600 g-10min
PELLET (discard)
SUPERNATANT
8% n-BUTANOL
STIR 6hr
CENTRIFUGATION: 10,400 g-10min
PELLET (discard)
SUPERNATANT
FILTER
8% PEG
STIR 1hr
CENTRIFUGATION: 16,300 g-15min
SUPERNATANT (discard)
PELLET
0.02M KPO_4 + 0.01M Na_2EDTA pH 8.2
CsCl GRADIENT CENTRIFUGATION
d=1.28 g/cc - 120,000 g max-15-18hr
COLLECT VIRUS ZONE
DILUTE 3X WITH 0.02M KPO_4 + 0.01M Na_2EDTA pH 8.2
CENTRIFUGATION: 12,000 g-10min
PELLET (discard)
SUPERNATANT
8% PEG
STIR 1hr
CENTRIFUGATION
SUPERNATANT (discard)
PELLET
0.12M TRIS pH 8.2
0.01M Na_2EDTA
V I R U S

Figure 3. Flow diagram outlining the procedure of purification of WMV isolates and the initial separation of the cytoplasmic cylindrical inclusions from the virus. The procedure uses chloroform and carbon tetrachloride as the clarifying agents and a low speed centrifugation to pellet the inclusions (see Figure 4). Polyethylene glycol (PEG) is used to concentrate the virus, Triton X-100 (TX-100) for solubilization of the pigments, CsCl or Cs_2SO_4 for separation of the virus from host components. For detailed description, see Materials and Methods.

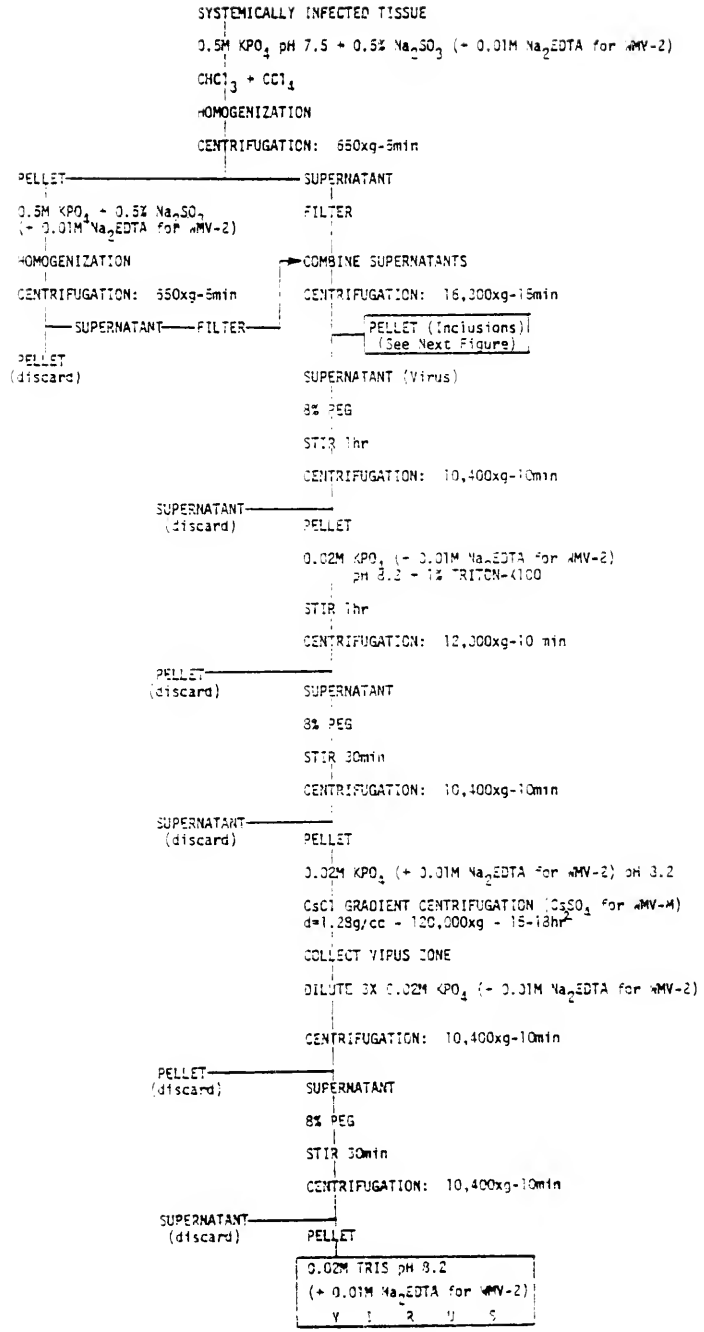

SYSTEMICALLY INFECTED TISSUE
0.5M KPO4 pH 7.5 + 0.5% Na2SO3 (+ 0.01M Na2EDTA for MMV-2)
CHCl3 + CCl4
HOMOGENIZATION
CENTRIFUGATION: 650xg-5min
PELLET
SUPERNATANT
0.5M KPO4 + 0.5% Na2SO3 (+ 0.01M Na2EDTA for MMV-2)
FILTER
HOMOGENIZATION
COMBINE SUPERNATANTS
CENTRIFUGATION: 650xg-5min
CENTRIFUGATION: 16,300xg-15min
SUPERNATANT
FILTER
PELLET (Inclusions) (See Next Figure)
PELLET (discard)
SUPERNATANT (Virus)
8% PEG
STIR 1hr
CENTRIFUGATION: 10,400xg-10min
SUPERNATANT (discard)
PELLET
0.02M KPO4 (+ 0.01M Na2EDTA for MMV-2) pH 8.2 + 1% TRITON-X100
STIR 1hr
CENTRIFUGATION: 12,300xg-10 min
PELLET (discard)
SUPERNATANT
8% PEG
STIR 30min
CENTRIFUGATION: 10,400xg-10min
SUPERNATANT (discard)
PELLET
0.02M KPO4 (+ 0.01M Na2EDTA for MMV-2) pH 8.2
CsCl GRADIENT CENTRIFUGATION (Cs2SO4 for MMV-M) d=1.28g/cc - 120,000xg - 15-18hr
COLLECT VIRUS ZONE
DILUTE 3X 0.02M KPO4 (+ 0.01M Na2EDTA for MMV-2)
CENTRIFUGATION: 10,400xg-10min
PELLET (discard)
SUPERNATANT
8% PEG
STIR 30min
CENTRIFUGATION: 10,400xg-10min
SUPERNATANT (discard)
PELLET
0.02M TRIS pH 8.2 (+ 0.01M Na2EDTA for MMV-2)
V I R U S

Figure 4. Flow diagram outlining second stage of purification of cytoplasmic cylindrical inclusions (for first stage, see Figure 3). Remaining pigments were solubilized with Triton X-100 (TX-100) and inclusions separated by centrifugation. Large aggregates of inclusions were broken up by homogenization at 9,000 rpm 3 min (max.) in a Sorvall omnimixer micro-homogenizer.

Inclusions to be purified as dissociated protein subunits were separated from a hard starchy-like pellet by very low speed centrifugation. The inclusions found in the resulting soft pellet and supernatant were dissociated in 1% SDS and purified by preparative PAGE. Inclusion subunits were eluted from the gel and freeze dried.

Whole inclusions were further purified on a sucrose step gradient, washed, concentrated and stored at 4 C in 0.02 M Tris buffer, pH 8.2.

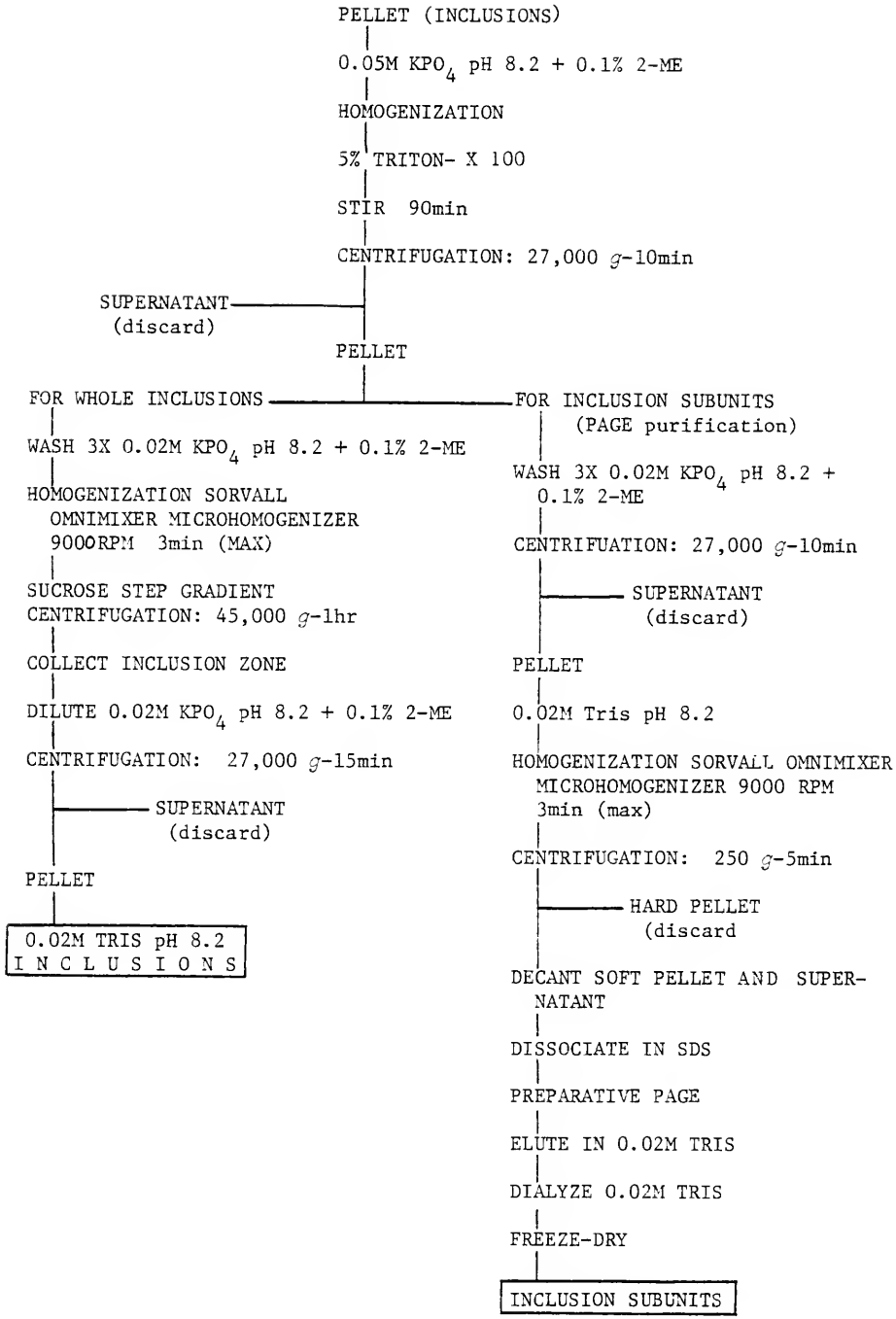
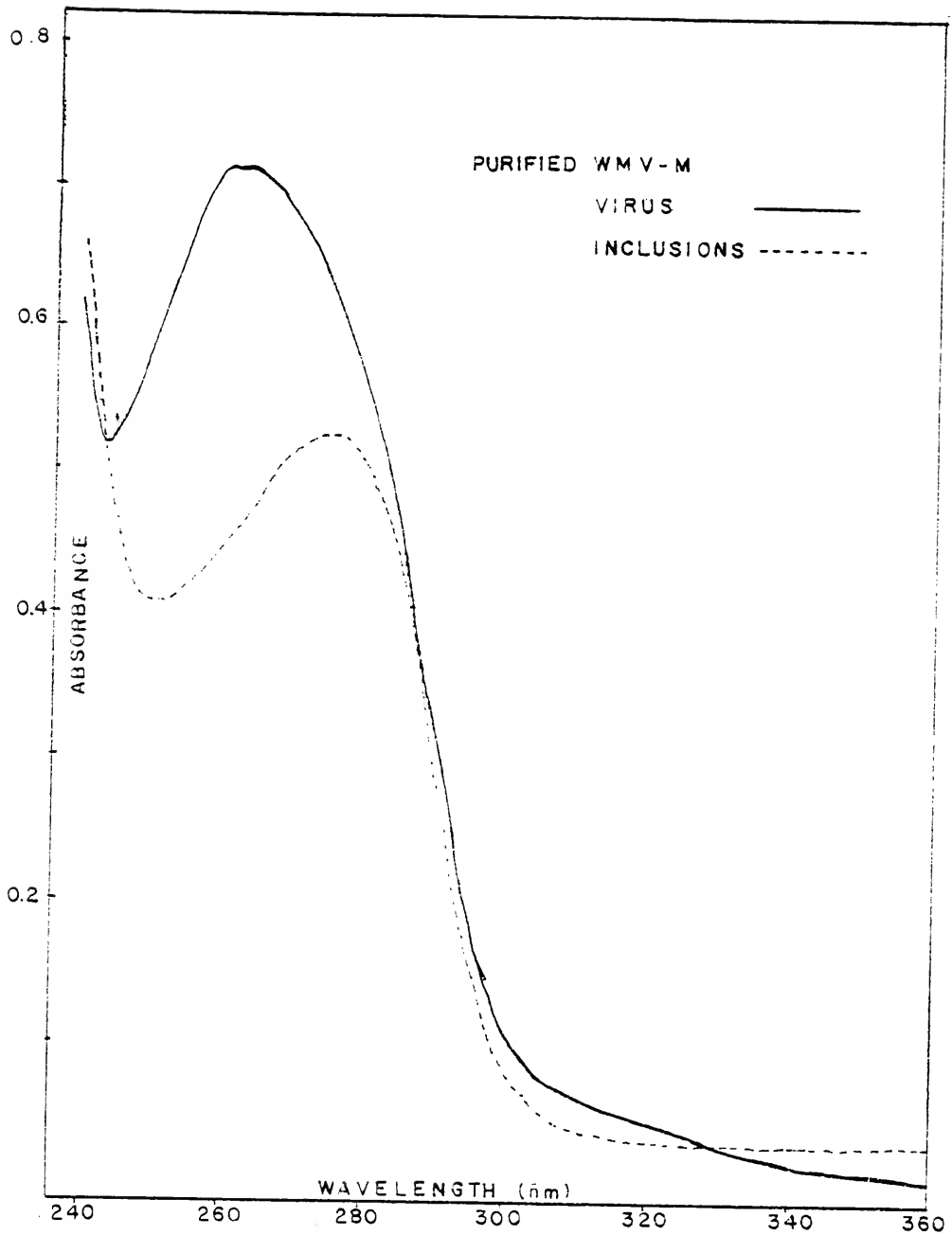

PELLET (INCLUSIONS)
0.05M KPO4 pH 8.2 + 0.1% 2-ME
HOMOGENIZATION
5% TRITON- X 100
STIR 90min
CENTRIFUGATION: 27,000 g-10min
SUPERNATANT (discard)
PELLET
FOR WHOLE INCLUSIONS
WASH 3X 0.02M KPO4 pH 8.2 + 0.1% 2-ME
HOMOGENIZATION SORVALL OMNIMIXER MICROHOMOGENIZER 9000RPM 3min (MAX)
SUCROSE STEP GRADIENT CENTRIFUGATION: 45,000 g-1hr
COLLECT INCLUSION ZONE
DILUTE 0.02M KPO4 pH 8.2 + 0.1% 2-ME
CENTRIFUGATION: 27,000 g-15min
SUPERNATANT (discard)
PELLET
0.02M TRIS pH 8.2 INCLUSIONS
FOR INCLUSION SUBUNITS (PAGE purification)
WASH 3X 0.02M KPO4 pH 8.2 + 0.1% 2-ME
CENTRIFUATION: 27,000 g-10min
SUPERNATANT (discard)
PELLET
0.02M Tris pH 8.2
HOMOGENIZATION SORVALL OMNIMIXER MICROHOMOGENIZER 9000 RPM 3min (max)
CENTRIFUGATION: 250 g-5min
HARD PELLET (discard
DECANT SOFT PELLET AND SUPERNATANT
DISSOCIATE IN SDS
PREPARATIVE PAGE
ELUTE IN 0.02M TRIS
DIALYZE 0.02M TRIS
FREEZE-DRY
INCLUSION SUBUNITS

Figure 5. Absorption spectra of purified preparations of WMV-M in 0.02 M Tris-HCl buffer, pH 8.2, and WMV-M dissociated cylindrical inclusions in 0.02 M Tris, pH 8.2, containing 1% SDS.


PURIFIED WMV-M
VIRUS
INCLUSIONS
ABSORBANCE
0.8
0.6
0.4
0.2
WAVELENGTH (nm)
240
260
280
300
320
340
360

usually included. All virus isolates at about 1 mg/ml in Tris or phosphate buffer showed strong stream birefringence. Most proteains were not detected by PAGE (Figure 6).

Cylindrical inclusions were unstable in *n*-butanol and were always purified using chloroform-carbon tetrachloride for clarification of homogenized plant saps (Figure 3). Yields of up to 40 A_{280} units (1 A_{280} = 1 mg protein) per 100 g fresh tissue weight were obtained. A typical ultraviolet absorption is given in Figure 5. Highly purified inclusion proteins were obtained by elution from preparative polyacrylamide gels. Yields from such gels averaged about 1 mg purified eluted inclusion subunits for every 3-4 mg of crude inclusion preparation. These highly purified inclusion proteins which reacted with antisera to untreated inclusions were used as immunogens and for partial digestion by *S. aureus* protease.

Infectivity of Purified Viruses

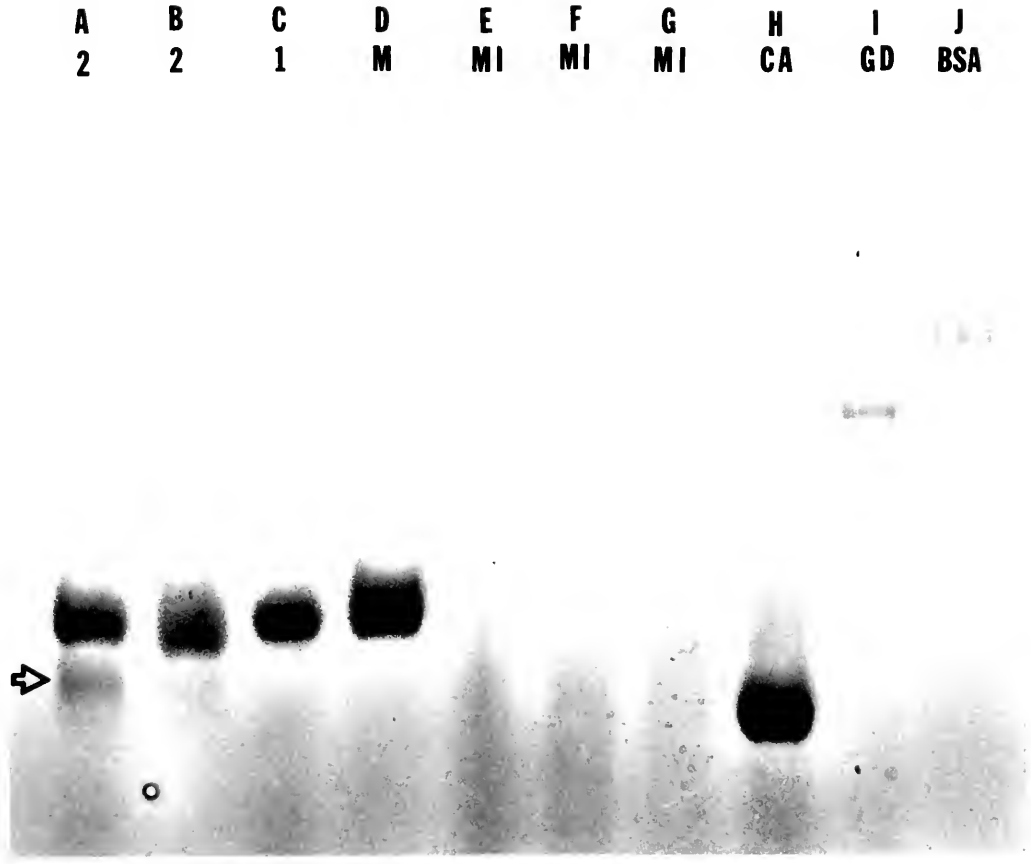
WMV-1 (Jordan) and WMV-2 (Florida) purified by these methods and freeze-dried with 0.02 M Tris and 0.01 M Na_2EDTA at a final concentration of 1 mg/ml were infective to a dilution of about 10^{-3} mg/ml after lyophilization and storage for up to one month at room temperature. Infectivity of WMV-2 was determined by inoculation to a local lesion host, *C. amaranticolor*, whereas WMV-1 (Jordan) infectivity was determined by mechanical inoculation of pumpkin at various dilutions and observing systemic symptom development.

Figure 6. Electrophoretic analysis of purified WMV capsid and cylindrical inclusion protein subunits and marker proteins in an 8% polyacrylamide gel containing 0.1% SDS and sodium phosphate buffer (pH 7.2).

Each capsid protein is from a different purification and shows the purity and nondegraded state required of all capsids used for cleavage by cyanogen bromide. The arrow points to the band indicating limited degradation of WMV-2 capsid protein to lower molecular weight form (approx. 30,000 daltons).

Proteins are as follows: WMV-2 Florida, lanes A and B; WMV-1 Florida, lane C; WMV-M, lane D; Moroccan cylindrical inclusions, lanes E, F, and G; marker protein carbonic anhydrase 29,000 daltons, lane H; glutamate dehydrogenase 53,000 daltons, lane I; bovine serum albumin 67,000 daltons, lane J.

Capsid lanes were purposely overloaded in order to detect the presence of degraded lower molecular weight forms. The molecular weights of the undegraded capsid proteins are about 36,500 daltons and that of the inclusion proteins are about 69,500 daltons. Molecular weight values were calculated by least squares linear regression analysis.


A
2
B
2
C
1
D
M
E
MI
F
MI
G
MI
H
CA
I
GD
J
BSA

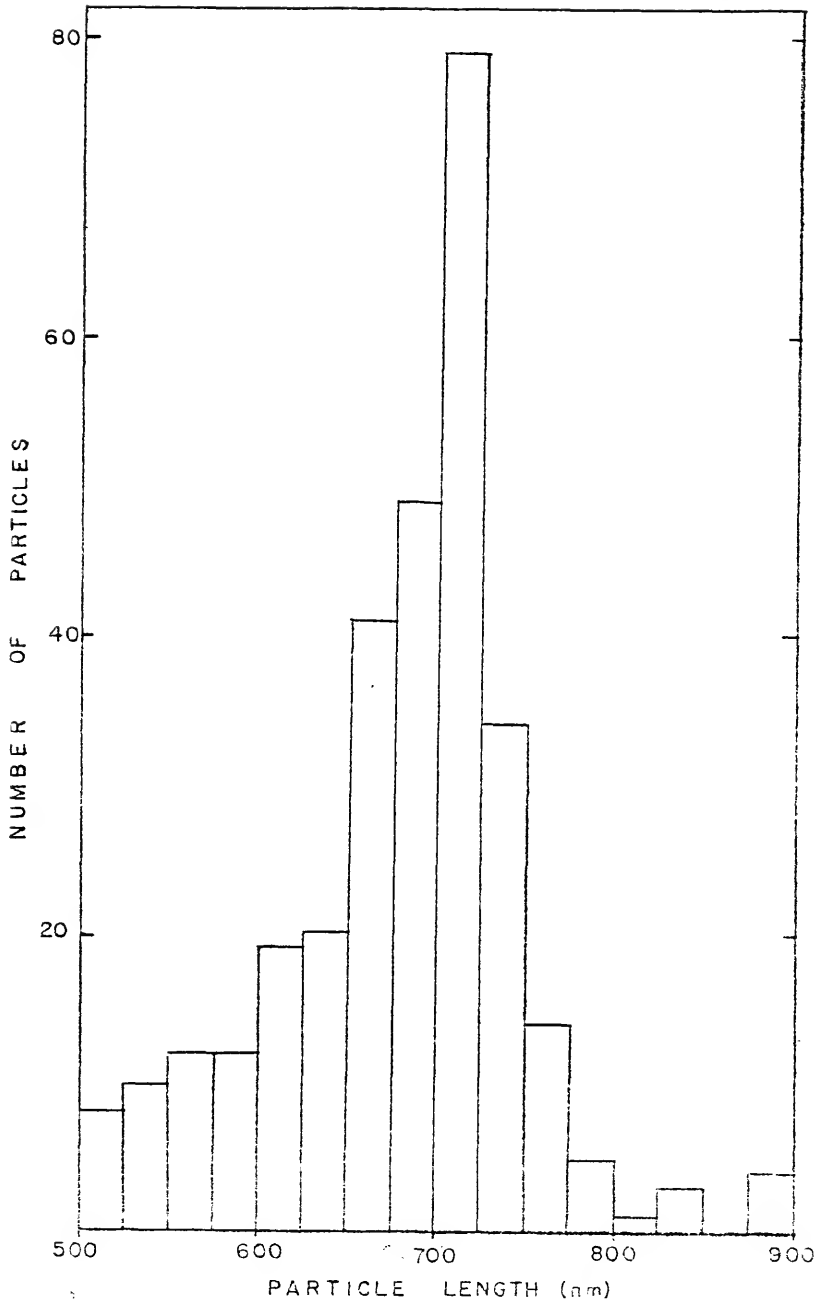
Particle Length Determination of WMV-M

Measurement of 350 purified WMV-M virus particles gave a normal length of 713 nm (Figure 7). Approximately 50% of the measured particles were between 675 and 750 nm. Approximately 5% of the examined rods were clustered between 1400 and 1440 nm as an apparent result of dimer formation due to end-to-end aggregation.

Molecular Weight Determination

Molecular weights of capsid and inclusion proteins dissociated in SDS were determined from 8% polyacrylamide analytical gels (Weber-Osborn, 1969) employing proteins of known molecular weights as standards (Figures 8 and 9). The undegraded capsid proteins of WMV-1 (Florida and Jordan isolates), WMV-2 (Florida), and WMV-M, each tested at least four times, gave an average molecular weight for undegraded capsid protein of 34,000 daltons with a range from 32,500 to 36,000 daltons. Molecular weights of the cylindrical inclusion subunits of all four WMV isolates ranged from 68,000 to 71,000 daltons based on 18 determinations, with an average of 69,000 daltons. WMV-1 and WMV-2 virus preparations which were stored at 4 C showed typical proteolytic cleavage with time (Hiebert *et al.*, 1979; Hiebert and McDonald, 1976) to lower molecular weight forms of approximately 26,000 to 30,000 daltons (Figure 8). Purified preparations of WMV-M did not have lower molecular weight forms resulting from proteolytic cleavage on any of the preparations run on polyacrylamide gels. Other than the apparent resistance of WMV-M to proteolytic cleavage, significant differences in the molecular weights of the four isolates were not observed.

Figure 7. Histogram of lengths of WMV-M particles from purified preparation negatively stained in phosphotungstate. Normal length was 713 nm based on measurement of 350 particles. Class interval is 25 nm.


80
60
40
20
NUMBER OF PARTICLES
500
600
700
800
900
PARTICLE LENGTH (nm)

W M V - MOROCCO

Figure 8. Electrophoretic analysis on an 8% polyacrylamide gel of purified WMV isolate, which had been stored at 4 C for at least three weeks before being frozen, and marker proteins. The polyacrylamide gel contained 0.1% SDS and sodium phosphate buffer, pH 7.2. Lanes from left to right, with molecular weights of marker proteins in parentheses are as follows:

A - WMV-M, undegraded, 34,500 daltons;
B - WMV-1 Florida, only intermediate and fast forms present, 31,000 and 26,000 daltons, respectively;
C - WMV-1 Jordan, undegraded, 35,000 daltons and fast form 27,000 daltons;
D - WMV-2, undegraded, 34,000 daltons and a single fast form of 29,500 daltons;
E - Blank
F - Ovalbumin (43,000 daltons);
G - Yeast alcohol dehydrogenase (37,000 daltons);
H - Carbonic anhydrase (29,000 daltons);
I - WMV-M, undegraded, 34,500 daltons with a 40,000 dalton contaminant (not observed following any other WMV-M purification);
J - WMV-1 Florida, undegraded, 34,000 daltons and a fast form 27,500 daltons;
K - WMV-1 Jordan, intermediate form 32,000 daltons and a fast form 27,500 daltons;
L - WMV-2, undegraded, 34,000 daltons and fast form 29,000 daltons.

Molecular weights were determined by least squares linear regression analysis. Molecular weight calculations varied as much as 2,000 daltons for the same preparation run on different gels but all undegraded WMV isolates run on the same gel varied by only $\pm$ 500 daltons.

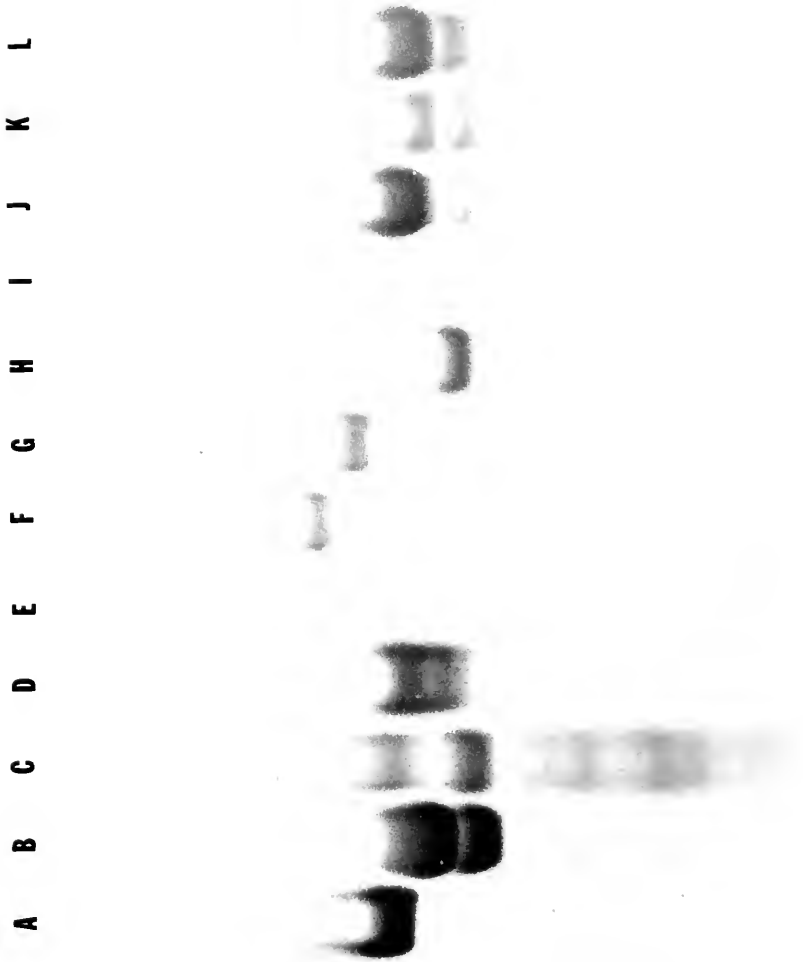
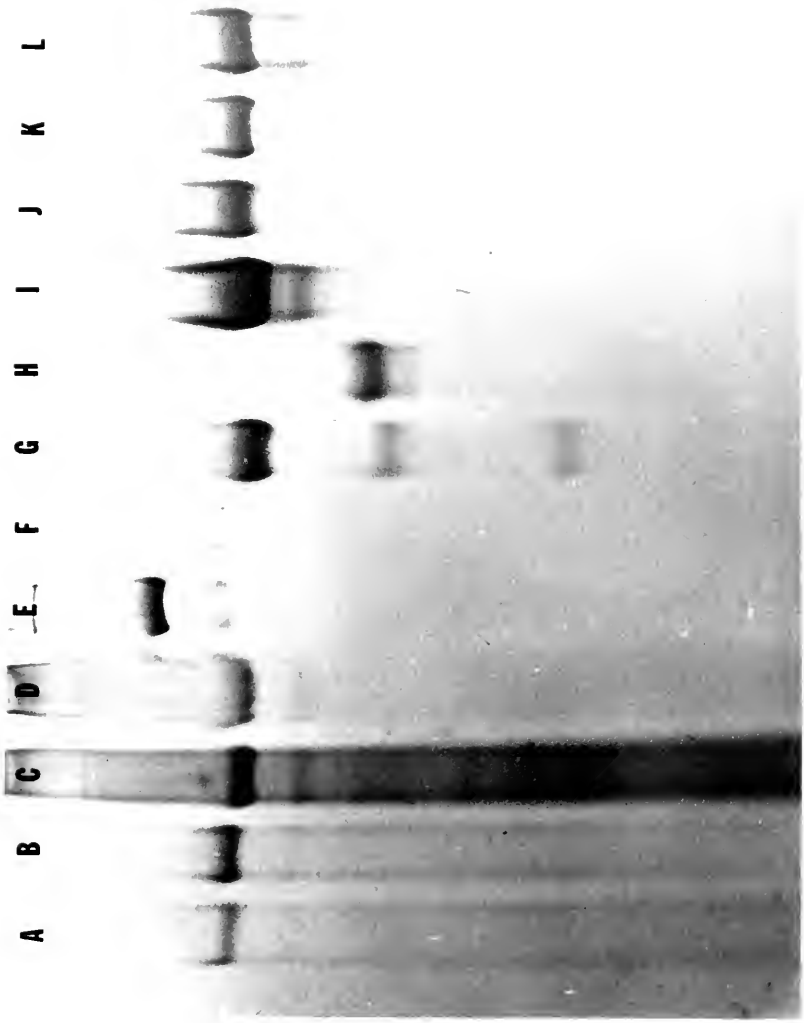

A B C D E F G H I J K L

Figure 9. Electrophoretic analysis of WMV cytoplasmic cylindrical inclusion subunits (1) in an 8% polyacrylamide gel containing 0.1% SDS and sodium phosphate buffer, pH 7.2. Lanes from left to right with molecular weights of marker proteins in parentheses are as follows:

A - WMV-MI, 71,000 daltons;
B - WMV-2I, 71,000 daltons;
C - WMV-MI, 69,500 daltons;
D - WMV-1 Jordan I, 70,000 daltons;
E - Phosphorylase a (94,000 daltons);
F - Blank;
G - Bovine serum albumin (67,000 daltons);
H - Ovalbumin (43,000 daltons);
I - WMV-MI, 69,500 daltons;
J - WMV-2I, 71,000 daltons;
K - WMV-1 Florida I, 71,000 daltons;
L - WMV-1 Jordan I, 71,000 daltons;

Molecular weights were calculated using least squares linear regression analysis and calculated weights of inclusion proteins varied as much as 1,500 daltons for the same preparation analyzed at different concentrations on the same gel (lane A and C are from the same purified preparation of WMV-M inclusions). Overloading was probably responsible for a portion of this variation, though overloading was intentionally done to ascertain purity of preparations which were selected for analysis by protease digestion.


A
B
C
D
E
F
G
H
I
J
K
L

Capsid Protein Digests by Cyanogen Bromide

Digests of capsid proteins of the four WMV isolates by CNBr (Gross and Witkop, 1962) revealed at least three distinctive patterns when analyzed by discontinuous PAGE. WMV-M and the two WMV-1 isolates (Florida and Jordan) formed similar patterns with Mauer's gel system #7 (1971)(Figure 10) while the position of bands from WMV-2 digests gave a consistently different pattern. All four isolates had two major bands and about seven to eight minor bands. The digest patterns were similar from gel to gel and with cleavages from different purifications of the same isolate. The two major bands, 6 and 7, of WMV-1 Florida showed a slightly slower electrophoretic mobility than the two corresponding major bands of WMV-M and WMV-1 Jordan. Band 9, which was present in WMV-M and WMV-1 Florida, was missing in WMV-1 Jordan. Band 8, which was detected as a shoulder in WMV-M and WMV-1 Florida, was not resolved clearly in WMV-1 Jordan. Analysis of peptide patterns on gels made with buffers having a lower pH than the system described here suggest that WMV-M is distinct from WMV-1 Florida and WMV-1 Jordan (data not shown).

Cylindrical Inclusion Digests

Purified cylindrical inclusion proteins eluted from preparative gels and cleaved by *Staphylococcus aureus* V-8 protease (Cleveland *et al.*, 1977) gave different peptide fragment patterns for WMV-1 Jordan, WMV-2, and WMV-M when analyzed on Laemmli discontinuous gels (Laemmli, 1970) (Figure 11). While the digest patterns for the inclusions of each isolate were distinctive, there was no apparent variation in the inclusion patterns of the same isolate purified on different dats.

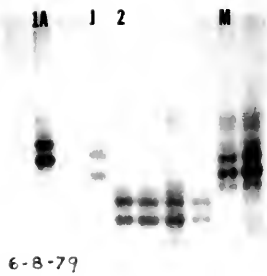
Serology

Antisera specific for both virus and cylindrical inclusions of WMV-1, WMV-2, and WMV-M were obtained (Tables 3 and 4). Unless

Figure 10. Electrophoretic analysis of cyanogen bromide cleaved WMV capsid protein. Undegraded capsid proteins were cleaved by cyanogen bromide and electrophoresed on a 12.5% polyacrylamide gel using a discontinuous buffer system (pH 4.5) of acetic acid and β-alanine. Gels stained with Coomassie brilliant blue were scanned at 565 nm. Lanes from left to right contain the following digests:

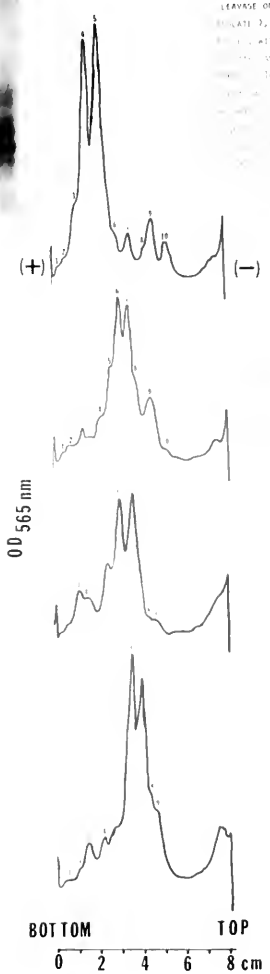
WMV-1 Florida	Lane "1A" and the next lane to its right
WMV-1 Jordan	Lane "J"
WMV-2	Lane "2" and the next three lanes to its right
WMV-M	Lane "M" and the next lane to its right.

CNBr Cleavage



CYANOGEN BROMIDE RESULTS

CLEAVAGE OF CAPSID PROTEINS OF WMV-M, WMV-1 (FLORIDA AND JORDAN ISOLATE), AND WMV-2 (FLORIDA) HAVE CONSISTENT RESULTS FROM GEL [illegible] WITH DIFFERENT PURIFICATIONS AND CLEAVAGES OF THE SAME [illegible] WITH INCUBATIONS STRETCHED FOR PERIODS OF UP TO FOUR [illegible] THE WMV-1 ISOLATES FROM FLORIDA AND JORDAN GAVE ALMOST [illegible] PATTERNS, THE MAJOR WHILE THE JORDANIAN FRAGMENTS [illegible] THE JORDANIAN FRAGMENTS HAVE THE SAME [illegible] WMV-1 ISOLATE. THE PEPTIDE FRAGMENT [illegible] EASILY DISTINCT WHEN IMPURE [illegible]



WMV-2 Florida (2)

WMV-M Morocco (M)

WMV-1 Jordan (J)

WMV-1A Florida (1A)

Figure 11. Comparison of *Staphylococcus aureus* V-8 protease digest patterns of WMV cylindrical inclusions with the undigested inclusions. Inclusions and peptide fragments were analyzed by SDS-PAGE. Lanes A through F are digest patterns, lanes H through M are respectively the same undigested inclusions. Lanes from left to right are as follows:

A - WMV-1 Jordan;
B - WMV-2;
C - WMV-1 Jordan;
D - WMV-M;
E - WMV-2
F - WMV-M;
G - enzyme alone;
H - WMV-1 Jordan;
I - WMV-2;
J - WMV-1 Jordan;
K - WMV-M;
L - WMV-2;
M - WMV-M.

Each digest is from a different inclusion purification.

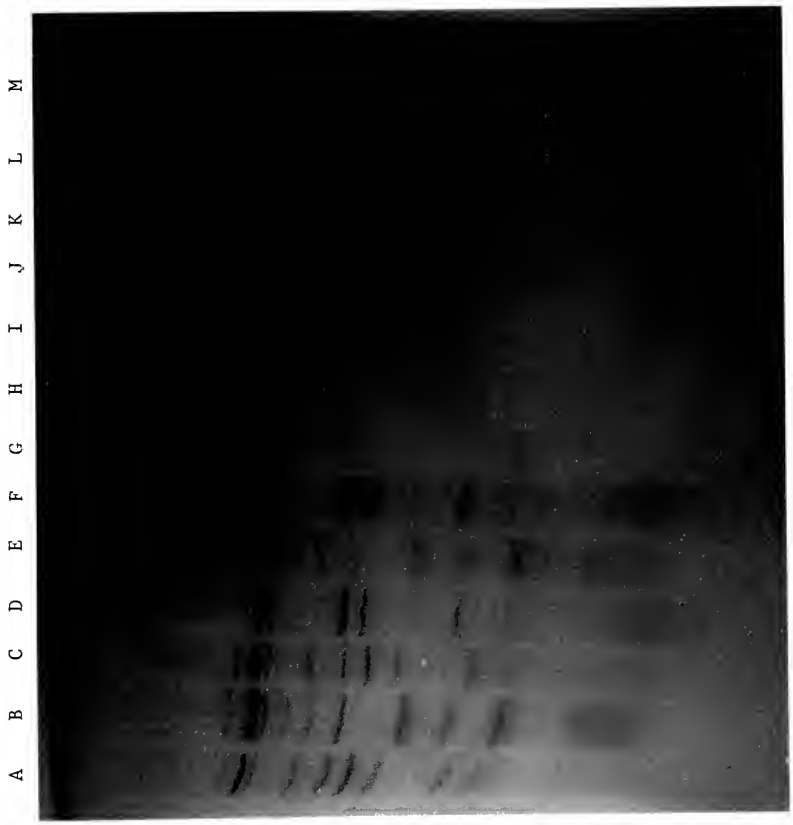

A B C D E F G H I J K L M

Table 3. Serological reactions with WMV virus antisera.

Antisera	WMV-M		WMV-1 Florida	WMV-1 Jordan		WMV-2 Florida	
Rabbit No.	914[a]		852	927	906	868	924[b]
Immunogen Treatment	None	Formaldehyde Fixed	None	Formaldehyde Fixed	None	None	Formaldehyde Fixed
State of Immunogen	Undegraded	Undegraded	Undegraded	Undegraded	Unknown[d]	Primarily Undegraded[c]	Unknown[d]
Antigen	Reactions with Anitgens[g]						
WMV-M	+	+	-	-	-	-	-
WMV-1 Florida	-	-(+[e])	+	+	+	-	-
WMV-1 Jordan	-	-(+[e])	+	+	+	-	-
WMV-2	-	-	-	-	-	+	+
PRSV	-	-	+[f]	0	0	-	0

Table 3. Continued.

Antisera	WMV-M		WMV-1 Florida	WMV-1 Jordan		WMV-2 Florida	
Rabbit No.	914[a]	917	852	927	906	868	924[b]
Immunogen Treatment	None	Formaldehyde Fixed	None	Formaldehyde Fixed	None	None	Formaldehyde Fixed
State of Immunogen	Undegraded	Undegraded	Undegraded	Undegraded	Unknown[d]	Primarily Undegraded[c]	Unknown[d]
				Reactions with Anitgens[g] (continued)			
BCMV	0	-	-	0	-	+[f]	0
BICMV	0	-	-	0	-	+[f]	0
SoyMV	0	-	-	0	-	+[f]	0
PVY	0	-	-	0	-	+[f]	0

[a] Antiserum only tested for first three months after immunization
[b] Anitserum gave only a weak reaction in SDS immunodiffusion tests - no comparison possible with WMV-1 isolates or WMV-M
[c] Determined by E. Hiebert (Purcifull and Hiebert, 1979)
[d] Reaction with antigens in SDS double immunodiffusion tests (sap and purified preps)
+ = positive reaction
- = negative reaction
0 = not tested
[e] Antiserum reacted with WMV-1 isolates beginning four months after immunization
[f] Only sap tested
[g] Immunogen not checked for degradation.

Table 4. Serological reactions with WMV inclusion antisera.

Antisera	WMV-1 Jordan	WMV-2 Florida			WMV-M	
Rabbit No.	903	904[a]	942	943	928	935[b]
Immunogen Treatment	None	None	None	Subunits[c] (PAGE)	None	Subunits[c] (PAGE)
Inclusion Antigens	Reaction with Antigens[d]					
WMV-M	-	-	-	-	+	+
WMV-1 Jordan	+	-	-	-	+	0
WMV-1 Florida	+	-	-	-	+	0
WMV-2	-	+	+	+	+[e]	0
DMV	-	0	0	+	+	0
BCMV	-	0	0	+	-	0
SoyMV	-	0	0	+	-	0
LMV	-	0	0	+	+	0
PRSV	-	0	0	0	+	0

Notes:

[a]Antisera from all bleedings gave strong healthy reactions
[b]Weak homologous reactions to all bleedings
[c]Subunits purified by SDS polyacrylamide gel electrophoresis (PAGE)
[d]Reaction with antigens in SDS double immunodiffusion tests:
+ = positive reaction
- = negative reaction
0 = not tested
[e]Reacted with purified inclusions, but not with sap.

specifically noted, antisera did not give a positive reaction with healthy antigens in gel immunodiffusion tests. All the antisera gave positive homologous reactions. In reciprocal SDS double immunodiffusion tests, reactions were negative between WMV-1, WMV-2, and WMV-M with virus antisera obtained during the first four months following immunization (Figure 12). The formaldehyde-fixed WMV-M antisera reacted with WMV-1 (Florida) and WMV-1 (Jordan) after four months (Figure 13). This antiserum was made from a virus which had a capsid protein molecular weight of approximately 34,000 daltons, indicating that it had not undergone proteolytic cleavage (Hiebert and McDonald, 1976). In SDS double immunodiffusion tests, the fixed antigen reacted with WMV-M antisera but not with WMV-1 antisera. WMV-M antisera (bleedings taken for three months following immunization) made against nondegraded capsid which was not formaldehyde fixed did not react with either isolate of WMV-1 (Figure 13). Antisera collected after four months formed only faint precipitin bands with WMV-M, precluding further studies of cross-reactivity with WMV-1 isolates. None of the other antisera to WMV-1, WMV-2, or WMV-M, representing bleedings taken up to one year after immunization, gave heterologous reactions.

Florida and Jordan isolates of WMV-1 gave reactions of identity in reciprocal double immunodiffusion tests. Intragel absorption tests failed to detect any serological differences between these two isolates (Figure 14).

In reciprocal SDS double immunodiffusion tests, WMV-2 reacted with bean common mosaic virus, blackeye cowpea mosaic virus, soybean mosaic virus (Figure 15). These viruses did not react with WMV-M or WMV-1 in reciprocal tests.

Figure 12. Reciprocal SDS-double immunodiffusion tests between WMV-1, WMV-2, and WMV-M, with antisera obtained during the first four months after initial injection of immunogen. Media contains 0.8% Noble agar, 0.5% SDS and 1% sodium azide.

Center wells were charged with:

1vs = WMV-1 Florida antiserum
2vs = WMV-2 Florida antiserum
Mvs = WMV-M antiserum.

Antigens in peripheral wells were as follows:

1 = WMV-1 Florida in sap
J = WMV-1 Jordan in sap
2 = WMV-2 in sap
M = WMV-M in sap
V = corresponding purified virus preparation, 50 µg/ml
H = healthy pumpkin sap.

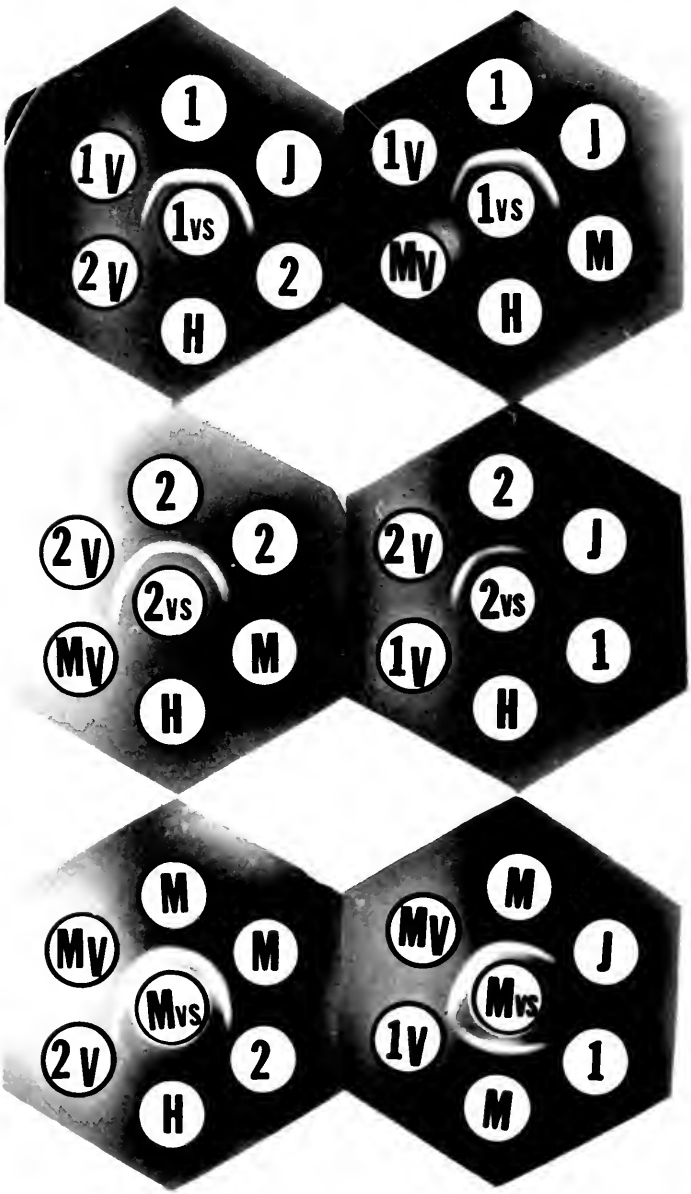

1
1V
J
1VS
2V
2
H
1
1V
J
1VS
MV
M
H
2
2V
2
2VS
MV
M
H
2
2V
J
2VS
1V
1
H
M
MV
M
MVS
2V
2
H
M
MV
J
MVS
1V
1
M

Figure 13. Double immunodiffusion serology of three WMV-M antisera showing heterologous reactions with WMV-1 isolates. Center wells were charged with the following: (A) WMV-M antiserum (Mvs) collected approximately three months after initial immunization of the rabbit with formaldehyde-fixed WMV-M; (B) WMV-M antiserum (Mvs) collected approximately six months after initial immunization of the rabbit with formaldehyde-fixed WMV-M; (C) WMV-M antiserum produced within six months of initial immunization of rabbit with unfixed WMV-M.

Media consisted of 0.8% Noble agar, 0.5% SDS, and 1% sodium azide.

Peripheral wells were charged with:

M = WMV-M in sap
1 = WMV-1 Florida in sap
1V = WMV-1 Florida, purified preparation (50 µg/ml)
2 = WMV-2 in sap
J = WMV-1 Jordan in sap
H = Healthy pumpkin sap.

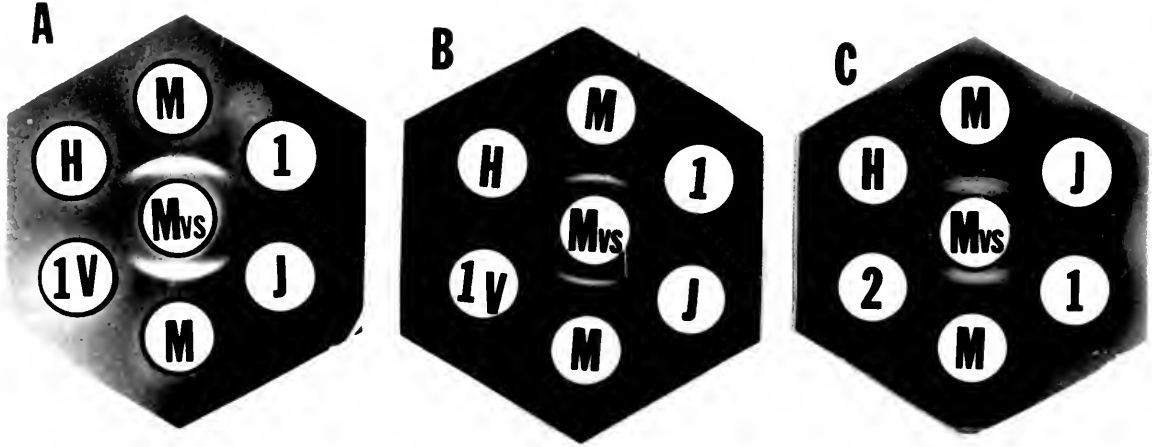

A
M
H
1
Mvs
1V
J
M
B
M
H
1
Mvs
1V
J
M
C
M
H
J
Mvs
2
1
M

Figure 14. Intragel cross-absorption tests with WMV-1 Jordan and WMV-1 Florida using SDS double immunodiffusion, media consisting of 0.8% Noble agar, 0.5% SDS, and 1% sodium azide.

A - Center well charged with purified WMV-1 Florida (1 mg/ml) and 18 hr later with WMV-1 Jordan antiserum.
B - Center well charged with purified WMV-1 Jordan (1 mg/ml) and 18 hr later with WMV-1 Jordan antiserum.
C - Center well charged with WMV-1 Jordan antiserum.
D - Center well charged with purified WMV-1 Florida (1 mg/ml) and 18 hr later with WMV-1 Florida antiserum.
E - Center well charged with purified WMV-1 Jordan (1 mg/ml) and 18 hr later with WMV-1 Florida antiserum.
F - Center well charged with WMV-1 Florida antiserum.

Peripheral wells were charged with the following antigens:

J = WMV-1 Jordan in sap.
1 = WMV-1 Florida in sap.
H = Healthy pumpkin sap.
V = Purified preparations of respective virus.

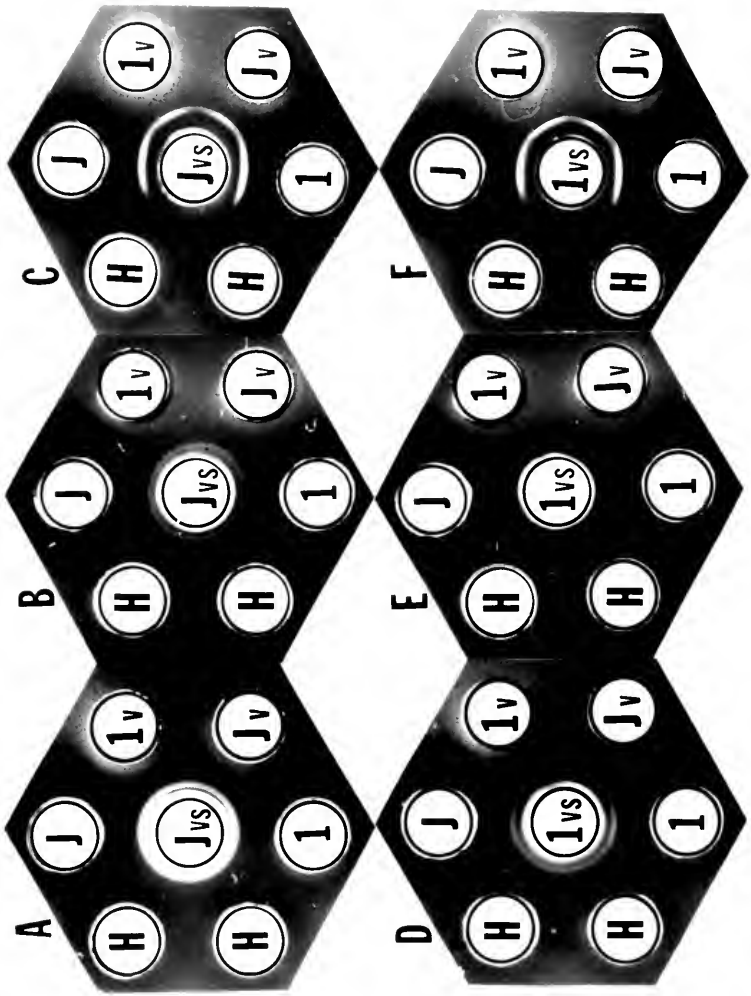

A
B
C
D
E
F
J
H
1
Jvs
1vs
Jv
1v

Figure 15. Reciprocal SDS double immunodiffusion tests between WMV-2 and BCMV, BlCMV, and SoyMV, and heterologous reactions between WMV-2 antiserum and DMV and PVY. Immunodiffusion medium consisted of 0.8% Noble agar, 0.5% SDS and 1% sodium azide. Arrow denotes spur formation.

Center wells were charged with the following:

A and B - WMV-2 antiserum.
C - BCMV antiserum.
D - BlCMV antiserum.
E - SoyMV antiserum.

Peripheral wells contained the following antigens in sap:

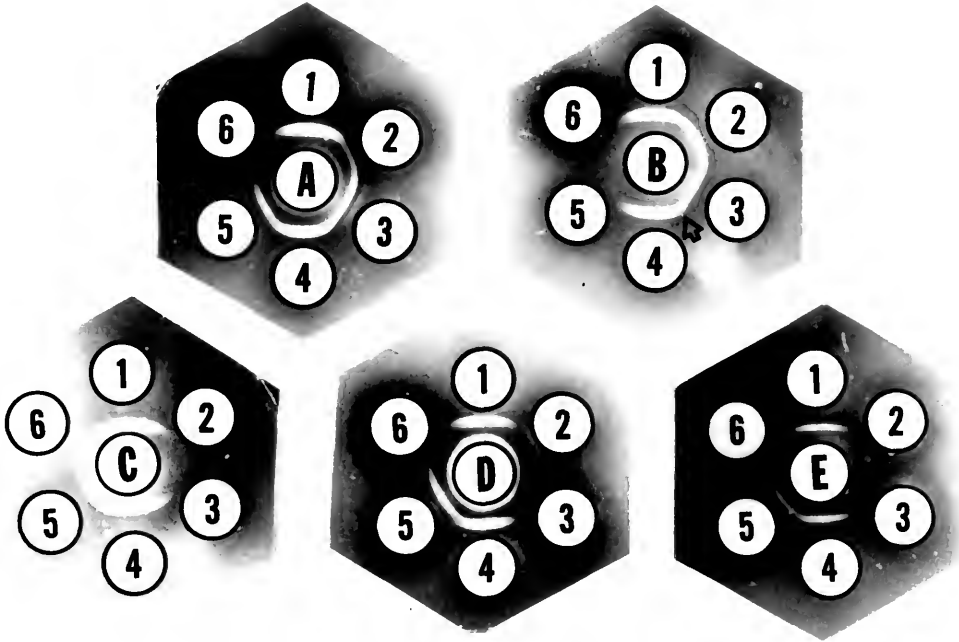
A.
1 WMV-2
2 DMV
3 BCMV
4 WMV-2
5 BlCMV
6 Healthy pumpkin

B.
1 WMV-2
2 Soy MV
3 PVY
4 WMV-2
5 TuMV
6 Healthy pumpkin

C
1 BCMV
2 WMV-M
3 WMV-1 Florida
4 BCMV
5 WMV-2
6 Healthy pumpkin

D.
1 BlCMV
2 WMV-M
3 WMV-1 Florida
4 BlCMV
5 WMV-2
6 Healthy pumpkin

E.
1 Soy MV
2 WMV-M
3 WMV-1 Florida
4 SoyMV
5 WMV-2
6 Healthy pumpkin


1
6
2
A
5
3
4
1
6
2
B
5
3
4
1
6
2
C
5
3
4
1
6
2
D
5
3
4
1
6
2
E
5
3
4

Antisera to inclusions induced by WMV-1 (Jordan), WMV-2 (Florida) and WMV-M each gave strong homologous reactions and none reacted with their respective purified viruses (at antigen concentrations of 50-100 µg/ml) in SDS double immunodiffusion tests (Figure 16). The WMV-1 and WMV-2 inclusion antisera reacted homologously only, whereas the WMV-M inclusion antiserum reacted heterologously with purified inclusion preparations (400 µg/ml) or crude extract preparations of either WMV-1 or WMV-2 (Figure 17, Table 4) and with sap containing DMV or PRSV (Figure 17). Intragel absorption of WMV-M inclusion antisera with purified inclusion preparations at 1 mg/ml of WMV-1, WMV-2, or WMV-M was performed. Intragel absorption wtih WMV-1 or WMV-2 inclusions resulted in the formation of a precipitin band between WMV-M inclusions and its homologous antiserum but not between WMV-M inclusion antiserum and the cross absorbing heterologous antigens. Intragel absorption by WMV-M inclusions prevented the formation of any precipitin bands by WMV-1, WMV-2, or WMV-M inclusions and WMV-M inclusion antisera (Figure 18). Intragel absorption of WMV-1 Jordan inclusion antisera with either WMV-1 Florida and WMV-1 Jordan purified inclusion preparations (1 mg/ml) was complete (Figure 19).

Enzyme-Linked Immunosorbent Assay (ELISA)

Reciprocal tests were conducted with enzyme labeled gamma globulins specific for WMV-M, WMV-1 (Florida), and WMV-2 (Florida) and their corresponding antigens (Table 5). The serum conjugates also were tested against samples containing the following viruses: WMV-1 Jordan, zucchini yellow fleck virus (kindly supplied by C. Volvas), turnip mosaic virus, potato virus Y, blackeye cowpea mosaic virus,

Figure 16. Reciprocal SDS double immunodiffusion serology of WMV cylindrical inclusions.

Center wells were charged with the following anitsera:

Jis = WMV-1 Jordan
2is = WMV-2
Mis = WMV-4

Peripheral wells contained the following antigens:

1 = WMV-1 Florida in sap
Ji = WMV-1 Jordan purified inclusions (400 µg/ml)
Jv = WMV-1 Jordan purified virus (50 µg/ml)
2 = WMV-2 in sap
2i = WMV-2 purified inclusions (400 µg/ml)
2v = WMV-2 purified virus (50 µg/ml)
M = WMV-M in sap
Mi = WMV-M purified inclusions (400 µg/ml)
Mv = WMV-M purified virus (50 µg/ml)
H = Healthy pumpkin sap

Arrow denotes spur formation. Medium consisted of 0.8% Noble agar, 0.5% SDS, and 1% sodium azide.

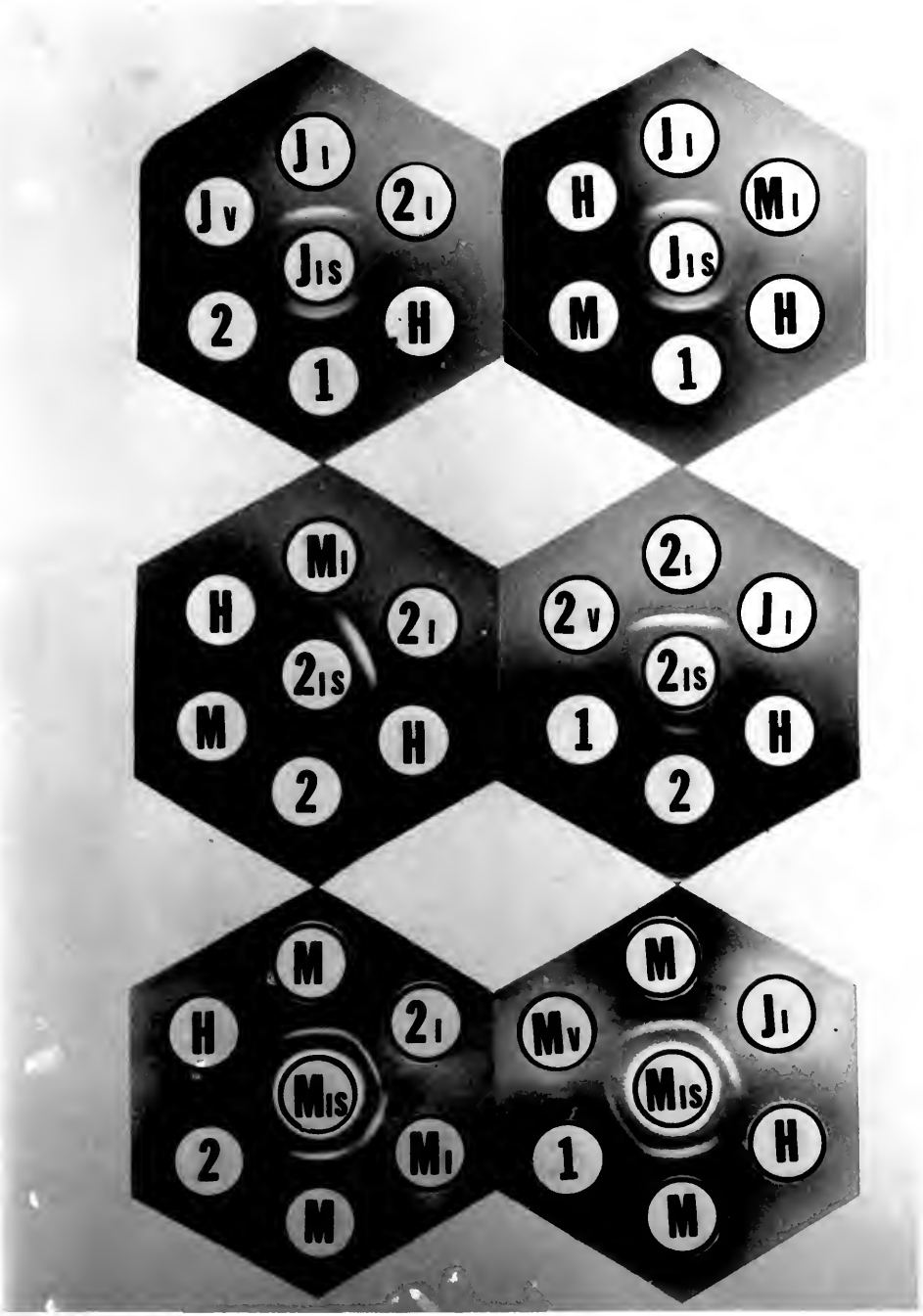

J1
Jv
2i
J1s
2
H
1
J1
H
Mi
J1s
M
H
1
Mi
H
2i
2is
M
H
2
2i
2v
J1
2is
1
H
2
M
H
2i
Mis
2
Mi
M
M
Mv
J1
Mis
1
H
M

Figure 17. Heterologous reactivity of WMV-M inclusion antiserum with other potyviruses by SDS double immunodiffusion serology employing media consisting of 0.8% Noble agar, 0.2% SDS, 0.1% sodium azide, and 0.7% sodium chloride.

Center wells were charged with WMV-M inclusion antiserum.

Peripheral wells were charged with the following antigens in sap:

A.
1 WMV-M
2 DMV
3 BCMV
4 WMV-M
5 BlCMV
6 Healthy pumpkin sap

B.
1 WMV-M
2 SoyMV
3 PVY
4 WMV-M
5 TuMV
6 PRSV

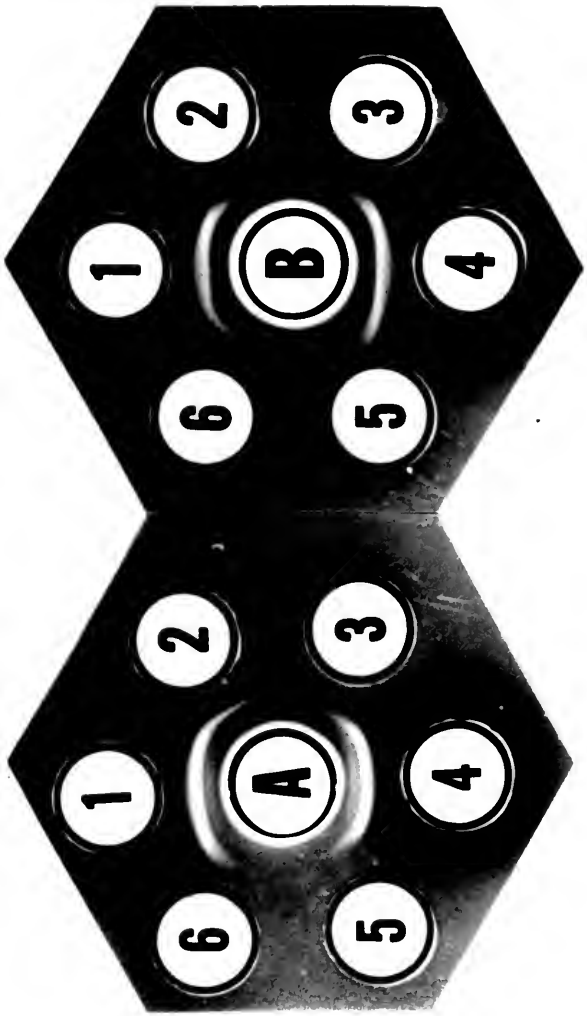

2
3
1
B
4
6
5
2
3
1
A
4
6
5

Figure 18. Intragel absorption of WMV-M inclusion antiserum with inclusions of WMV-1, WMV-2, and WMV-M in an SDS immunodiffusion medium consisting of 0.8% Noble agar, 0.2% SDS, 0.1% sodium azide, and 0.7% sodium chloride.

Center wells were charged as follows:

A - WMV-1 Florida purified inclusions (1 mg/ml) and 18 hr later with WMV-M inclusion antiserum
B - WMV-2 purified inclusions (1 mg/ml) and 18 hr later with WMV-M inclusion antiserum
C - WMV-M purified inclusions (1 mg/ml) and 18 hr later with WMV-M inclusion antiserum
D - WMV-M inclusion antiserum

The peripheral wells were charged with the following antigens at the same time as the antiserum was added to the center wells:

M WMV-M in sap
M_I WMV-M purified inclusions (400 µg/ml)
1 WMV-1 Florida in sap
J WMV-1 Jordan in sap
1_I WMV-1 Florida purified inclusions (400 µg/ml)
J_I WMV-1 Jordan purified inclusions (400 µg/ml)
2 WMV-2 in sap
2_I WMV-2 purified inclusions (400 µg/ml)
H Healthy pumpkin sap

A
1'
M
M'
H
J
1
M'S
B
2'
M
M'
H
H
2
M'S
C
H
M
M'
H
2'
J'
M'S
D
H
M
M'
H
J'
2'
M'S

Figure 19. Intragel absorption of WMV-1 inclusion antiserum with WMV-1 Jordan inclusions and WMV-1 Florida inclusions in an SDS immunodiffusion medium consisting of 0.8% Noble agar, 0.2% SDS, 0.1% sodium azide, and 0.7% sodium chloride.

WMV-1 Jordan inclusion antiserum was first cross-absorbed with healthy plant antigens.

Center wells were charged as follows:

A - WMV-1 Jordan purified inclusions (1 mg/ml) and 18 hr later by WMV-1 Jordan inclusion antiserum
B - WMV-1 Florida purified inclusions (1 mg/ml) and 18 hr later by WMV-1 Jordan inclusion antiserum
C - WMV-1 Jordan inclusion antiserum

Peripheral wells were charged with the following antigens:

J WMV-1 Jordan in sap
J_I WMV-1 Jordan purified inclusions (400 µg/ml)
1 WMV-1 Florida in sap
1_I WMV-1 Florida purified inclusions (400 µg/ml)
H Healthy pumpkin sap.

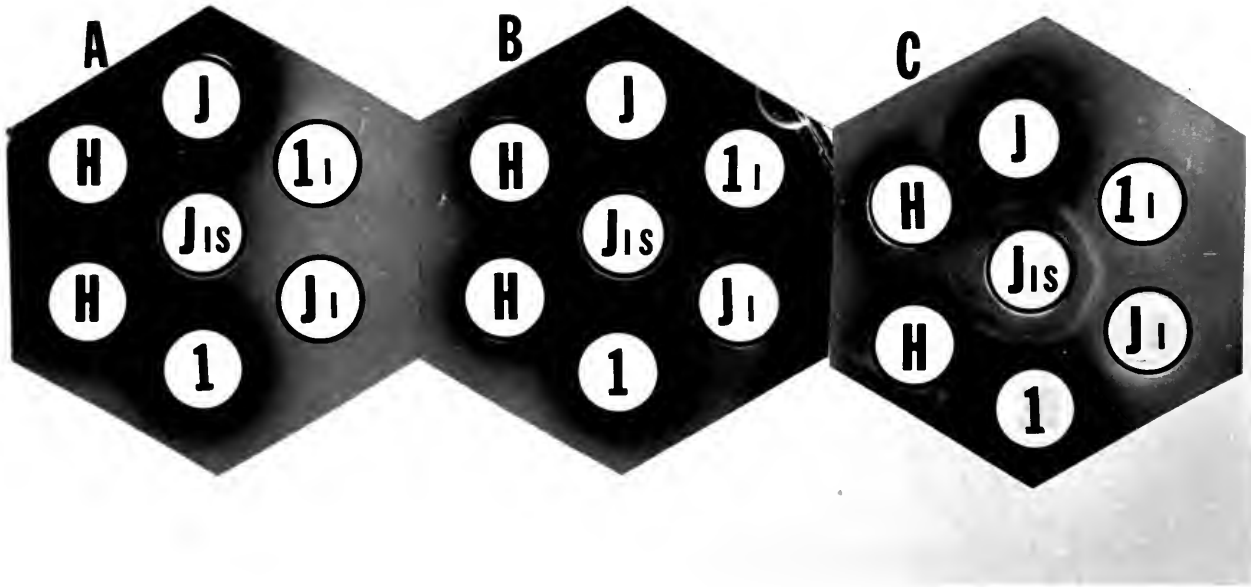

A
J
H
1I
JIS
H
JI
1
B
J
H
1I
JIS
H
JI
1
C
J
H
1I
JIS
H
JI
1

Table 5. ELISA serology of WMV isolates.

ELISA Test Antigens in Sap[a]	WMV-M[bd]	WMV-1(F1)[bd]	WMV-2(F1)[cd]
1. Healthy *N. benthamiana*	0.00	0.00	0.00
2. WMV-1 Jordan	0.16	2.40	0.00
3. WMV-1 Florida	0.10	0.88	0.00
4. WMV-2 Florida	0.00	0.01	5.20
5. Zucchini Yellow Fleck Virus	0.00	0.04	0.00
6. WMV-M	1.10	0.00	0.00
7. Healthy Cowpea	0.00	0.00	0.00
8. Turnip Mosaic Virus	0.00	0.00	0.00
9. Potato Virus Y	0.00	0.00	0.00
10. Healthy Bean	0.00	0.00	0.00
11. Healthy Pumpkin	0.00	0.00	0.00
12. PBS-Tween	0.00	0.00	0.00
13. Soybean Mosaic Virus	0.00	0.00	3.40
14. Bean Common Mosaic Virus	0.00	0.00	0.20
15. Healthy *Nicotiana edwardsonii*	0.00	0.00	0.00

[a]Used at dilutions (w/v) of 10% and 1% in each.

[b]Coating antiserum conc.: 1 µg/ml; antiserum-enzyme conjugate: 1/800 dilution.

[c]Coating antiserum conc.: 1 µg/ml; antiserum-enzyme conjugate: 1/300 dilution.

[d]At dilution of test antigens, value represents the average OD_{405} for four replications of each test antigen.

bean common mosaic virus (Siratro isolate; Lima, 1978), and healthy saps of pumpkins, bountiful bean, cowpea, *Nicotiana benthamiana* and *N. edwardsonii* Christie and Hall. Only the homologous reactions with the Moroccan conjugate was clearly positive, though a weak reaction at the threshold level (Rochow and Carmichael, 1979) occurred with the Florida and Jordan isolates of WMV-1. The WMV-1 (Florida) conjugate reacted only with the Florida and Jordan isolates of WMV-1. The Florida WMV-2 conjugate gave a strong homologous reaction, and heterologous reactions with soybean mosaic virus and bean common mosaic virus.

DISCUSSION

This study provides new evidence that the relationships among watermelon mosaic viruses are considerably more complex than was realized previously (Milne and Grogan, 1969; van Regenmortel, 1977). The evidence provided herein, however, supports the observations of Webb and Scott (1965) and Purcifull and Hiebert (1979) that WMV-1 and WMV-2 are serologically distinct.

Evidence also is presented in this dissertation that WMV-M is indeed serologically distinct from WMV-1 and WMV-2, as suggested previously by Purcifull and Hiebert (1979). Gel immunodiffusion tests with antisera specific for the capsid proteins of WMV-1, WMV-2, or WMV-M, showed that the three isolates are serologically distinct. These distinctions applied to antisera collected up to 4 months after the initial injections; one of the WMV-M antisera collected later than 4 months after immunization reacted with WMV-1. These results were generally upheld by the ELISA tests. The WMV-M antiserum showed a weak heterologous reaction with WMV-1, but failed to react either with WMV-2 or the zucchini yellow fleck virus from Italy in ELISA tests. Neither the WMV-1 nor WMV-2 antisera reacted with WMV-M in ELISA.

It is of limited value to define WMV-1 as isolates of WMV which have a host range confined to the Cucurbitaceae (Webb and Scott, 1965). Purcifull and Hiebert (1979) obtained serological reactions of identity using Florida WMV-1 antiserum and Mediterranean isolates of WMV whose host ranges included members of the Chenopodiaceae as well as the

Cucurbitaceae. In this study, antisera were made to the virus and the cylindrical inclusions of one of the Mediterranean isolates, WMV-1 Jordan. Intragel absorption in reciprocal double immunodiffusion tests failed to detect differences between the capsids of the two viruses. In addition, these two WMV-1 isolates produced similar capsid digest patterns following CNBr cleavage. Intragel cross-absorption of WMV-1 Jordan inclusion antisera with WMV-1 Florida purified inclusions was complete. These tests confirmed the close relationship between WMV-1 Florida, which has no known hosts outside the Cucurbitaceae (Purcifull and Hiebert, 1979), and the Jordan isolate, which has a broader host range (Martelli and Russo, 1976). The concept (Webb and Scott, 1965) that North American isolates of WMV-1 are limited to the Cucurbitaceae may also need revision, because an isolate of WMV has recently been found in South Carolina which caused local lesions on *C. amaranticolor* but which was closely related serologically to WMV-1 Florida (personal communication by O. W. Barnett).

The serological tests indicated that WMV-1, WMV-2, and WMV-M inclusions were distinct, although the Moroccan isolate was related to both WMV-1 and WMV-2. Antisera to inclusions gave stronger reactions in SDS double immunodiffusion tests with purified inclusions than with sap extracts. The medium of Tolin and Roane (1975) gave stronger reactions, but was more likely to result in nonspecific reactions, than the medium consisting of 0.8% agar, 0.5% SDS, and 1.0% sodium azide (Purcifull and Batchelor, 1977). These results suggest that it could be useful to investigate the efficacy of these media, in order to optimize results in serological detection of inclusion body proteins.

The WMV-1, WMV-2, and WMV-M isolates were compared in other ways. Unlike WMV-1 and WMV-2, the WMV-M was unstable in cesium chloride. Both WMV-1 and WMV-M were unstable in *n*-butanol, although this solvent was useful for WMV-2 purification. The CNBr derived peptide pattern of WMV-M capsid proteins had a degree of similarity with the peptide protein of WMV-1 isolates when electrophoresed at pH 4.5. However, the peptide pattern of WMV-M was distinct from those of the WMV-1 isolates when the pH of the electrode and separating gel buffers were lowered approximately one unit. The WMV-2 peptide pattern was always distinct from WMV-M and both WMV-1 isolates. Molecular weights of the cylindrical inclusion proteins of WMV-1 (both the Florida and Jordan isolates), WMV-2, and WMV-M all averaged 69,000 daltons. The peptide fragment patterns following digestion of inclusions with *S. aureus* protease indicated that WMV-M patterns were distinct from those of WMV-1 or WMV-2.

This dissertation points to the need for augmenting the standard techniques presently used to determine strain relationships among potyviruses. Peptide mapping of virus-specified proteins is one such approach. The recently evolved techniques for immunochemical analysis of CNBr cleaved fragments (Doyen and Lapresle, 1979; Vita *et al.*, 1979) may in the future be used to map antigenic sites and to expose new sites which may aid further in determining serological relationships.

In conclusion, this study supports the proposition that there are at least three serologically distinct viruses involved in the WMV complex. The three types are represented by the WMV-1, WMV-2, and WMV-M isolates. It would be of particular interest to determine the serological relationship of WMV-M to South African isolates (van Regenmortel *et al.*, 1962), which reportedly also may differ from WMV-1 and WMV-2 (Webb and Scott, 1965).

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BIOGRAPHICAL SKETCH

Robert H. Baum was born in Lynbrook, New York, on April 28, 1942, and grew up in South Carolina, New York, and Florida. He graduated with an A.B. degree from Goddard College in 1966 with majors in chemistry and biology. Following graduation, he worked in engineering positions at Cape Kennedy, Florida, and attended the University of Florida's Graduate Engineering Extension System on a part-time basis. After three years, he transferred to the Kwajalein Missile Range in the Marshall Islands, Central Pacific, where he worked on the Spartan Antiballistic Missile program. In 1972, he became a graduate student in the Botany Department where he held a teaching assistantship. In 1974, he received his M.S. degree under the supervision of Dr. H. C. Aldrich. He transferred to the Plant Pathology Department in 1975, where he was awarded a research assistantship under the supervision of Dr. Dan Purcifull. He is presently a Visiting Assistant Professor in the Plant Pathology Department at Clemson University. He is married to the former Diane Mundhenk and they are looking forward to the birth of their first child.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Dan E. Purcifull, Chairman
Professor of Plant Pathology

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J. R. Edwardson
Professor of Agronomy

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

E. Hiebert
Associate Professor of Plant Pathology

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D. R. Pring
Associate Professor of Plant Pathology

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D. A. Roberts
Professor of Plant Pathology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

R. E. Stall
Professor of Plant Pathology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

I. K. Vasil
Graduate Research Professor of Botany

This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

March, 1980

Jack L. Fry
Dean, College of Agriculture

Dean, Graduate School