IDENTIFICATION AND CHARACTERISATION OF SOUTH AFRICAN STRAINS OF CUCUMBER MOSAIC VIRUS

by

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ABBREVIATIONS

A_{405}	Absorbance reading at indicated wavelength.
AMV	Alfalfa mosaic virus.
BBMV	Broad bean mottle virus.
BMV	Brome mosaic virus.
BSA	Bovine serum albumin.
CARNA 5	Cucumber mosaic virus associated ribonucleic acid 5.
CCMV	Cowpea chlorotic mottle virus.
CMV	Cucumber mosaic virus.
cv.	Cultivar.
DAS-ELISA	Double antibody "sandwich" - ELISA.
DEP	Dilution endpoint.
DIECA	Diethyldithiocarbamate.
EDTA	Ethylenediaminetetraacetic acid.
ELISA	Enzyme-linked immunosorbent assay.
FA	Formaldehyde.
GA	Glutaraldehyde.
IEB	Immunoelectroblotting.
IEM	Immunoelectron microscopy.
IgG	Gamma globulin fraction of serum.
Kd	Kilodalton.
LIV	Longevity in vitro.
PAGE	Polyacrylamide gel electrophoresis.
PBS	Phosphate buffered saline.
PSV	Peanut stunt virus.
RCF	Relative centrifugal force.
RNA	Ribonucleic acid.
SDI	Serological differentiation index.
SDS	Sodium dodecyl sulphate.
SSV	Soybean stunt virus.
TAV	Tomato aspermy virus.
TEMED	NNN'N' tetramethylethylenediamine.
TIP	Thermal inactivation point.
TMV	Tobacco mosaic virus.
WMV	Watermelon mosaic virus.

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A. BIOLOGY

I. Introduction

The Cucumovirus group comprises three sero-groups: cucumber mosaic virus (CMV); tomato aspermy virus (TAV) and peanut stunt virus (PSV). The viruses infect an extremely wide variety of herbaceous and woody plant species. The most widespread and most studied of the viruses is the type member, CMV (Kaper and Waterworth, 1981).

Cucumber mosaic virus causes numerous diseases in a wide variety of plants. Its known host range numbers some 200 dicotyledonous and monocotyledonous plant species in approximately 40 families. It can be transmitted by more than 60 aphid species (Francki et al., 1979).

In a recent review of viruses affecting the cucurbits (Lovisolo, 1980), 775 plant species belonging to 85 families have been listed as hosts for CMV infection, with the *Cruciferae, Solanaceae, Compositae, Leguminosae,* and *Cucurbitaceae* being the families (in decreasing order) with the highest number of susceptible species.

Whereas the physical, chemical and antigenic properties of CMV-strains are very similar, biological properties such as host range and symptomatology are very variable. Numerous strains of the virus have been described, mainly on the basis of symptoms they produce on selected indicator plants (Francki and Hatta, 1980).

In earlier years, CMV was identified by its biological properties and physicochemical properties of infected sap such as thermal inactivation point (TIP), longevity in vitro (LIV) and dilution end point (DEP). Diagnosis based on chemical, biophysical and serological criteria as outlined by Hamilton et al., (1981) is now regarded as being more reliable.

2. Epiphytology

Weeds and aesthetic plants play a very significant role in the incidence of virus disease in economic crops. In order to cause epidemics in crops, Cucumoviruses, like most plant viruses, must have host plants in which they can multiply and survive throughout the year, and also rely on the presence of aphid vectors for efficient spread (Kaper and Waterworth, 1981).

Lovisolo (1980), quotes Tomlinson as having proposed an epidemiological model for CMV, illustrating some features common to all regions. The model relates especially to annual crops, which are initially healthy and only become infected from external sources. The local interpretation of the model has to be based on a thorough knowledge of common regional crop practice, a detailed examination of the local flora in which the virus survives (either inside or outside the diseased crops), and the presence and behaviour of the aphid vectors. Bruckart and Lorbeer (1976), for instance, demonstrated that certain ubiquitous weeds growing near lettuce (*Lactuca sativa L.)* and celery (*Apium Graveolens L. verdulce Pers.*) fields in New York, are commonly infected with CMV. They concluded that only by indexing weeds growing near lettuce and celery plantings would it be possible to determine the natural weed hosts of CMV. This knowledge is valuable for epidemiological studies of CMV and may be useful in virus control programs.

Several workers have reported that dozens of other perennial weed species have been shown to harbour CMV through the winter in Europe (Tomlinson & Carter, 1970) and in the USA (Barnett & Baxter, 1973). Removal of overseasoning weeds has helped reduce virus incidence but is not a final solution to the source of inoculum. At least 19 weed species are known to carry the virus through their seed, some of which remain viable in the soil for several years (Francki et al., 1979). Their germination creates scattered infection foci for spread of CMV by aphids in subsequent years. In South Africa, CMV was reported to be epidemic in cucurbits (Van Regenmortel, 1960).

3. Strains

Cucumber mosaic virus exists as a myriad of strains, both real and apparent. For example Lovisolo and Conti (1969) did not find any two identical isolates based upon a host range and symptoms among 60 obtained from different localities. However, the word 'strain' has often been used loosely in the literature without sufficient comparative testing of isolates. Some authors have used the term 'strain' where 'culture', 'serotype', 'variant', or 'isolate' might have been more appropriate (Kaper & Waterworth, 1981).

According to Francki et al., (1979), many symptom variants occur, making the virus often difficult to identify from symptoms alone. Nevertheless, there are strains which have been well characterised by their biological properties. These include strains such as Price's Y-strain, and the M-strain (Mossop et al., 1976). Lovisolo and Conti (1969) distinguished 6 strains by their biological properties. Some strains such as the Y-strain (Kaper et al., 1965), the Q-strain (Francki et al., 1966; Habili & Francki, 1974a) and the S-strain (Van Regenmortel, 1967; Van Regenmortel et al., 1972) have been characterised physicochemically. Table 1 lists some of the better known strains showing the origin of the abbreviated letter-codes and country of origin.

TABLE 1 : SOME BETTER KNOWN STRAINS OF CMV

Name of Strain	Country	Reference
M (Mossop isolate)	Australia	Mossop et al., (1976)
Q (Queensland)	Australia	Francki et al., (1966)
S (Squash)	South Africa	Van Regenmortel (1967)
Y (Yellow)	USA	Price (1934)
Ix (Ixora)	USA	Waterworth and Povish (1975)
Com (Commelina)	El Salvador	Waterworth et al. (1978)
P (Spinach)	Australia	Gonda & Symons (1978)
WL (White Leaf)	USA	Gonsalves et al. (1982)

According to Kaper and Waterworth (1981), apparent differences among isolates result from numerous causes such as laboratory practices, experimental growing conditions, specific cultivars under test, and interpretation of symptoms observed. Another aspect influencing symptom expression in host range studies is the choice of the plant used as the inoculum source because it is known to affect the relative proportions of the different RNA's associated with the virus genome. Under certain conditions, CMV exists in the host as a mixture of two replicating entities, the virus and an unrelated virus-dependent satellite-like RNA that interact with each other and the host and influence viral multiplication, symptom expression and infectivity (Kaper and Waterworth, 1977; Waterworth et al., 1979).

The emphasis has shifted in recent years to utilizing biochemical techniques to differentiate CMV isolates. It has been discovered that differences occur among isolates in the amino-acid sequence and composition of their coat proteins (Van Regenmortel, 1967; Habili & Francki, 1974a; Kaper & Waterworth, 1981).

Coat protein differences could cause serological differences among isolates and affect the electrophoretic mobility of the virions and their protein subunits. Such differences were shown by Lot and Kaper (1976). Isolates also differ in vector specificity, in the ease with which they are transmitted by vectors and in the severity of symptoms they cause in their hosts (Francki et al., 1979).

Researchers in several laboratories have compared genomic nucleic acid sequences of CMV strains with the aid of hybridization techniques (Gonda & Symons, 1978; Piazzolla et al., 1979). They demonstrated the feasibility of using these techniques to estimate sequence homologies between plant viral RNA's. Piazzolla et al. (1979) grouped eighteen CMV isolates into two categories, depending on the percentages of sequence homology.

Although symptomatology and serology are still widely used to differentiate CMV-isolates and strains, there is a gradual re-orientation towards characterization of viruses by using molecular biological techniques. For instance, Hanada and Tochihara (1982) investigated properties of a CMV isolate found in soybean seeds. In this investigation these workers used agar gel double-diffusion tests, symptomatology and hybridization techniques. Several single lesions were isolated from inoculated leaves of *Chenopodium quinoa* for all combinations of RNA's. The serotype of each single isolate was determined by gel diffusion tests and symptoms on soybean (*Vigna unguiculata* subspecies *sesquipedalis*). The pseudorecombination experiments confirmed a close relatedness between soybean stunt virus (SSV) and CMV. Edwards et al. (1982) used hybridization and genetic analysis to study location of determinants for pathogenicity in two naturally occurring strains of CMV; however, they could not detect any serological differences when comparing the naturally occurring strains and their pseudorecombinants in Ouchterlony gel diffusion tests with antisera to all four strains.

Variations of enzyme-linked immunosorbent assay (ELISA) have been compared with respect to their ability to detect and to differentiate serologically related plant virus species (Devergne et al., 1981; Koenig & Paul, 1982).

Recently, Edwards and Gonsalves (1983) categorised seven CMV isolates by comparing their coat proteins using peptide mapping and serological relationships using double antibody sandwich ELISA (Clark & Adams, 1977). Subjecting coat-proteins of the seven CMV isolates to limited digestion with three proteases, they were able to classify them into two subgroups according to the patterns the digests formed on polyacrylamide gels. Comparison of the seven isolates using ELISA, supported the peptide mapping analysis. They concluded that classification through peptide mapping is a sensitive and consistent means of supplementing symptomatological or serological classification.

*CARNA5 or satellite RNA's (see I.B.II) can affect symptomatological tests (Gonsalves et al., 1982). These workers, investigating how the RNA species of CMV-WL affect symptoms in tomato plants, found that RNA's 1+2+3 were necessary for infectivity. They also found that tomato plants inoculated with these RNA's developed a downward leaf curling along the midvein and the leaf lamina was greatly reduced, but white leaf symptoms did not develop. However, when the plants were inoculated with RNA 1+2+3+5, white leaf symptoms did develop.

4. Transmission

CMV is transmissible mechanically, by aphid vectors (Bouwen et al., 1978), through seeds (Bos and Maat, 1974), and by dodder (Bouwen et al., 1978; Francki et al., 1979). Although CMV is easily transmissible mechanically, it has been noted that certain buffers are more suitable than others (Gibbs and Harrison, 1976). Additives such as sodium sulphite and mercaptoethanol and chelating agents (EDTA and diethyldithiocarbamate) have been shown to be useful. Adverse conditions of ionic strength, pH and certain cations such as Ba^{2+}, Ca^{2+} and Mg^{2+} can be inhibitory to infection with CMV (Kaper & Waterworth, 1981).

CMV is transmitted in the non-persistent manner by more than 60 aphid species of which *Myzus persicae* and *Aphis gossypii* are the most commonly known. The efficiency of transmission varies with the aphid species and host plant species, and some virus strains are more readily transmitted than others (Bouwen et al., 1978; Francki et al., 1979; Kuwite and Purcifull, 1982).

* CUCUMBER MOSAIC VIRUS-ASSOCIATED RNA

Mossop and Francki (1977) showed that transmissibility by *M. persicae* can be lost. By constructing pseudorecombinants of the two strains, M and Q, they demonstrated that RNA3, which also codes for the viral coat protein, determines the ability of CMV to be transmitted by aphids. CMV-M is not transmitted by *M.persicae* whereas CMV-Q is. Pseudorecombinants which contained RNA3 of CMV-M were not transmitted by aphids whereas those with RNA3 of CMV-Q were. Although the authors conclusively demonstrated the role of RNA3 in aphid transmissibility of CMV, they say that the mechanism of non-persistent aphid transmission of plant viruses is not understood.*

Transmission of CMV through seed embryo occurs, to varying degrees, in different plant species, including some weed species such as *Stellaria media* (Tomlinson & Carter, 1970). A CMV strain that is seed-transmitted in *Phaseolus vulgaris* was reported in Spain by Bos and Maat (1974). Davis et al. (1981) also reported seedborne CMV in French beans (*P. vulgaris*) in USA. Seed-transmission of CMV in barley (*Hordeum vulgare*) and maize (*Zea mays*) have been reported in South Africa (Von Wechmar et al., 1984; Knox and Von Wechmar, 1984).

CMV is known to be transmitted by at least ten species of dodder (*Cuscuta* spp; Francki et al., 1979). Bouwen et al. (1978) found that CMV was readily transmitted from diseased *N. tabacum L.* and white barley plants to healthy plants of the same cultivar by *Cuscuta subinclosa*. They noted that symptoms appeared two weeks after shoots of dodder parasitising diseased plants had established on the healthy plants.

*According to Francki et al. (1979), instars can acquire the virus in 5-10 seconds. Acquisition decreases after 2 minutes and diminishes after 2 hours.

5. Comparative Studies on Cucumoviruses

The biological properties of the three Cucumoviruses and their strains, overlap to such an extent that only properties reflecting chemical differences are sufficiently reliable for use in the final identification (Kaper and Waterworth, 1981). Comparative studies on nucleic acid homologies of several CMV isolates (Q, M and P) have been carried out to confirm these differences (Gonda & Symons, 1978).

Piazzola et al. (1979) classified eighteen CMV isolates into two main groups according to nucleic acid homology. They also made serological studies and determined electrophoretic mobilities of the isolates to corroborate sequence homology studies. The results using these two techniques were consistent with those of hybridization studies. Isolates belonging to different groups formed spurs in Ouchterlony tests and had different electrophoretic mobilities whereas isolates from the same group, according to hybridization studies, did not cross-react and had identical electrophoretic mobilities.

Most comparative studies in Cucumoviruses have been between CMV and PSV or CMV and TAV. Habili and Francki (1974b) found that TAV is more resistant to degradation by pancreatic RNase and to precipitation by NaCℓ than CMV. Moreover, TAV is stabilised by the cation Mg^{2+} whereas CMV precipitates in the presence of the same cation. EDTA stabilizes CMV while TAV is degraded. Both viruses dissociate in low sodium dodecyl sulphate (SDS) concentrations, indicating that their capsid structures depend on RNA-protein interactions. Hull (1976) reported similarity in behaviour of CMV and TAV in cesium sulphate gradients with both forming a single broad band at density = 1,304 g/mℓ.

Lot and Kaper (1976) performed differentiation studies on physical and chemical properties of three CMV strains and PSV and found that the four viruses exhibited clear differences in electrophoretic mobility, in reactivity with respect to the lysyl-specific reagent trinitrobenzenesulphonic acid, and in their stability with respect to high concentrations of LiCℓ.

Gonda and Symmons (1978) used hybridization analysis with complementary DNA to determine sequence homology between three strains of CMV (Q,M and P) and a single strain of TAV. They found that the four RNA's of CMV-P were indistinguishable from the corresponding CMV-Q RNA's whereas they found only partial sequence homology with the RNA's of CMV-M and TAV with the latter showing the least sequence homology with the CMV-Q RNA's.

6. Serology

The major problem that has been encountered in serological studies on CMV is its poor immunogenicity due to virus instability (Francki et al., 1966; Scott, 1968; Francki & Habili, 1972; Francki et al., 1979; Francki & Hatta, 1980; Kaper & Waterworth, 1981; Kuwite and Purcifull, 1982). Francki et al. (1966) could not raise a titer above 1:2 with the CMV-Q strain. Scott (1968) working with the CMV-Y strain could produce a serum with antibody titer of only 1:128 in spite of injecting rabbits with as much as 53 mg of virus.

Francki and Habili, (1972) found that they could enhance the immunogenicity of the CMV-Q strain by treatment with formaldehyde (FA). The FA-treatment stabilized the capsid

structure of the virus. They reported that the protein shells of FA-treated virus retained their structure during digestion of the viral RNA with pancreatic ribonuclease while those of untreated virus degraded rapidly. They concluded that the observed changes in the physical properties of CMV-Q by FA-treatment are the result of cross-linking of reactive sites on the adjacent subunits of the protein shells rendering the structural integrity of the capsids less dependent on bonding between the viral RNA and protein. Thus the ability of the antigen to retain its structural integrity in the tissues of the animal was responsible for the enhanced immunogenicity of the virus. The stabilising forces apparently differ between strains as Scott (1968) failed to achieve the same effect on the CMV-Y strain. In both investigations (Francki et al., 1966; Scott, 1968) it was found that the virus precipitates when exposed to physiological salt solutions and mild heating and therefore serological tests had to be carried out in agarose double-diffusion plates with buffers of low molarity or in water. Francki and Hatta (1980) have reported the use of glutaraldehyde-fixed virus for routine preparation of anti-CMV sera, they find glutaraldehyde to be more satisfactory than formaldehyde.

The double antibody "sandwich" enzyme-linked immunosorbent assay (DAS-ELISA) technique introduced to plant virus serology in 1977 by Clark and Adams has found widespread use in detecting virus infections because of the sensitivity and other advantages. Gera et al. (1978) used the technique to detect CMV in single aphids. Sako et al. (1980) found it specific enough to distinguish between watermelon mosaic virus (WMV) and CMV even when the plants were infected doubly with the two viruses. Using crude plant extracts, they were able to detect both WMV and CMV in various parts of the plants one week after mechanical inoculation with crude extracts. Their calculations showed that over ten thousand samples could be processed per millilitre of anti-

serum by ELISA test. They furthermore state that an additional advantage of the procedure was the lack of interference by non-specific precipitations of healthy materials from cucurbitaceous plants used in the experiments.

Devergne et al. (1981), demonstrated that closely related strains of CMV could be detected by both DAS- and compound indirect ELISA when antibodies to a single strain were used. However, they found that only the indirect ELISA procedure was capable of detecting distantly related viruses such as TAV, PSV and CMV. Davis et al. (1981), used DAS-ELISA for detecting CMV in bean seeds when they were investigating its possible application in monitoring seedborne CMV in bean plasma in the United States.

In a review by Kaper and Waterworth (1981), the ELISA procedure is considered to be 100 and 1 000 times more sensitive than the radial-immuno diffusion and the double-diffusion tests, respectively, in detecting CMV in crude plant-juice extracts.

The large number of strains of CMV that have been described by various researchers (Francki et al., 1979), have been assigned to two main groups, designated TORS and DTL, on the basis of their antigenic properties. According to Devergne et al. (1981), the serological relationship between these two CMV groups is fairly close and corresponds to a serological differentiation index (SDI) of 1-2 (the SDI corresponds to the number of twofold dilution steps separating homologous from heterologous precipitin titres). They also mention a new strain, CMV-C_o (origin not given), which was recognised later than other CMV strains and seems to be more distantly related to both TORS and DTL groups than these are to each other. Certain members of each of the two serological subgroups of CMV are related to some strains of TAV and PSV albeit more distantly (Francki et al., 1979). Some members of each group

show no such relationship (Habili & Francki, 1975). But subsequent work by the same group of workers using the ELISA technique showed a serological relationship that could not be detected in the earlier investigation (Rao et al., 1982).

B. BIOCHEMISTRY

I. Physico-Chemical Properties of the Cucumoviruses

(a) Morphology

The three major members of the Cucumovirus group are morphologically similar. The virus particles are polyhedral, about 28 nm in diameter, with icosahedral T = 3 surface lattice symmetry (Matthews, 1982). Although all particles have the same sedimentation rate, three different (isocapsidic) particles exist containing four species of single stranded RNA. RNA 1 is encapsidated in one particle, RNA 2 in another and RNA 3 + 4 in a third. Finch et al. (1967), made an in depth study of the structure of CMV and broad bean mottle virus (BBMV) using electron microscopy. They negatively stained the specimens with uranyl acetate and took electron micrographs of fields on carbon film and over holes in the film. Over the latter they could recognize two-side images, which are characteristic of the hexamer-pentamer grouping of 180 sub-units in the T = 3 icosahedral surface lattice. No details could be distinguished between the particles of the two viruses except that images of CMV appeared larger. They concluded that the common pattern in which the subunits of the two viruses pack may be dictated by the common length of the RNA molecules, which are presumably folded in a similar way in both cases.

When Habili and Francki (1974a) studied electron micrographs of TAV and CMV stained with uranyl acetate, they revealed that the particles were similar. Computing the mean diameters of the particles based on samples of 100 particles, the determinations were 29,0 ± 0,28 nm for TAV and 28,8 ± 0,22 nm for CMV. On mixing the two viruses and examining in the electron microscope, they could not distinguish the particles into two different populations, and their mean diameter was 28,4 ± 0,20 nm.

(b) Physical-Chemical Properties

For a detailed structural and biochemical study of a virus, it is of primary importance to have knowledge of viral particle weight, relative proportions of nucleic acid and protein, number and molecular weight of protein subunits, and number and molecular weight of nucleic acid components. These properties are a prerequisite for classification of viruses into groups. Among the cucumoviruses, CMV, being the most widely studied, is the only member whose complete set of physical-chemical properties have been assembled (Kaper and Waterworth, 1981).

Table 2 gives the molecular properties of cucumoviruses approved by the International Committee on Virus Taxonomy (Matthews, 1982).

TABLE 2 MOLECULAR PROPERTIES OF CUCUMOVIRUSES

Virus Particles		
Shape - isometric		
Size - ~28 nm diameter (by electron microscopy)		
Symmetry - icosahedral (T = 3 surface lattice)		
Sedimentation coefficient ($S_{20,w}$) = 99S		
Molecular weight - = 5,8 x 10^6 daltons		
Nucleic acid content - 18%		
Viral Protein		
Molecular weight of peptide - ~ 2,4 x 10^6 daltons		
Amino acids - ~ 236		
Polypeptides per particle - 180		
Viral Nucleic Acid		
Type - linear positive sense single stranded RNA		
Molecular weight -	RNA_1 1,27 x 10^6	(genomic)
	RNA_2 1,13 x 10^6	(genomic)
	RNA_3 0,82 x 10^6	(genomic)
	RNA_4 0,35 x 10^6	(coat protein m-RNA)

(c) Nucleic Acid

Kaper, Diener and Scott (1965) did pioneering work in determining the nature of CMV-RNA. Using the analytical ultracentrifuge, they found that the CMV-RNA preparations, obtained by phenol extraction, sedimented as paucidisperse mixtures of three components with sedimentation rates of 13,35, 19,65 and 22,95. They estimated the molecular weight of the 22,95S component to be in the range of 0,9-1,2 million.

Kaper and West (1972), combined analytical ultracentrifugation and polyacrylamide gel electrophoresis to determine the number and molecular weights of CMV-RNA species. The RNA samples that were used in the experiments were formylated. In the gel determinations they could detect a pattern of six CMV-RNA species. In this investigation, the authors found the components of CMV-RNA ranging from 0,91 x 10^6 daltons to 0,01 x 10^6 daltons by means of sedimentation velocity ultracentrifugation and then when components C_1 and C_2, were determined by polyacrylamide gel electrophoresis, had M.W.'s of 1,01 x 10^6 and 0,89 x 10^6 daltons, respectively.

Peden and Symons (1973), determined that CMV contains a functionally divided genome. Using the Q-strain, they separated the four main RNA species isolated from purified virus on 2,4% polyacrylamide gels. They determined the molecular weights of the RNA species using 16S and 23S *Escherichia coli* ribosomal RNA as molecular weight standards. In aqueous gels, they estimated the molecular weights of species 1-4 of CMV RNA to be 1,30 x 10^6, 1,13 x 10^6, 0,78 x 10^6 and 0,34 x 10^6 daltons and in gels run in 98% formamide to be 1,35 x 10^6, 1,16 x 10^6, 0,85 x 10^6, and 0,35 x 10^6 daltons. The pattern of the CMV-RNA species on polyacrylamide gels they observed, was similar to that shown by RNA isolated from brome mosaic virus (BMV), cowpea chlorotic mottle virus (CCMV) and broad bean mottle virus (BBMV), all of which are members of the

Bromovirus group. They found that the three largest species of CMV-RNA were essential for infectivity by inoculating cowpea (*Vigna sinensis*) plants with various combinations of all four species and counting the number of local lesions formed for each combination in several experiments.

Peden and Symons (1973) observed that the electrophoretic profile of CMV-RNA isolated from purified virus strain Q is similar to that found for CMV-RNA from strain S by Kaper and West (1972) except that molecular weights of RNA species isolated from the strain S were lower than for strain Q.

The concept of the divided genome of certain virus groups (Bromoviruses, Cucumoviruses and Alfalfa mosaic virus (AMV) stimulated experiments in which the three genomic RNA components were used in the construction of pseudorecombinants, and inoculated onto plants and the resulting infections analyzed for stability of hybrid characteristics (Habili & Francki, 1974c; Edwards et al, 1982).

Habili and Francki (1974c), constructed pseudorecombinants from genomic RNA's of CMV-Q and TAV. From these experiments they found that the coat protein cistron is on RNA 3, whereas the cistrons determining host reactions are on either RNA 1 or RNA 2 or on both RNA 1 and 2.

Comparative studies of parts of the genomic RNA sequences of BMV and CMV have revealed evolutionary relationships between these two viruses (Murthy, 1983). In these studies, this worker observed an extensive homology in the 3a protein sequences and almost no relationship between the coat protein sequences of the two viruses. He concluded that this shows the big variations in the rate of evolution of different genomic components.

2. The Virus - Associated Satellite-Like RNA's (CARNA 5)

The existence of a fifth major RNA component in CMV of low molecular weight (0,1 x 10^6 daltons) was detected by Kaper and West (1972) and by Lot et al. (1974). In 1976, Kaper, Tousignant and Lot investigated whether the component was a defective or satellite RNA. In their report, they demonstrated that RNA 5 can exist in varying proportions in the same CMV strain preparation, depending on the host in which CMV was propagated. They also demonstrated that RNA 5 is not a host RNA, but that its appearance in the virions of CMV is directly related to its presence in the inoculum, together with genomic CMV-RNAs 1,2 and 3.

Firstly, to demonstrate its host dependence, Kaper et al. (1976) propagated CMV-S in squash (*Cucurbita pepo L.* cv.Caserta Bush) and in tobacco (*Nicotiana tabacum L.* cv. Xanthi). After virus purification, the RNA species were separated on sucrose gradients and these were fractionated using a fraction recovery system. Some of RNA 5 was UV-irradiated. Polyacrylamide gel electrophoresis (PAGE) was used for estimation of RNA component proportions.

The patterns obtained by PAGE of RNA components of CMV propagated in squash (S) exhibited four major CMV-RNA's, and only a small amount of RNA 5. In CMV propagated in tobacco (T), there was a decrease in the major RNA's, in particular in RNA 1, and a dramatic increase in the proportion of RNA 5. The change, they observed, took place in a single passage of CMV from squash to tobacco and did not affect the properties of the virus. Further repeated passage of CMV in tobacco continued the shift in the RNA component proportions of CMV. The decrease in RNA 1, they felt, was probably primarily responsible for the fact that CMV preparations in tobacco were obtained in smaller yields, and have lower specific infectivities than squash CMV preparations. When T (CMV) was

passaged back to squash, the characteristic (S) CMV-RNA component proportions were almost immediately restored.

In studies of the role of CARNA 5 to modify symptom expression in plants, it was found to be responsible for lethal necrosis in tomato (*Lycopersicum esculentum*) by Kaper and Waterworth (1977). Waterworth et al. (1977) isolated CMV from *Commelina diffusa* which did not contain any detectable amounts of CARNA 5. However, the CMV-Com strain produced substantial amounts of CARNA 5 when CARNA 5 from the CMV-S strain was added to the inoculum. When they inoculated CMV-Comm without detectable CARNA 5 in *L.esculentum* cv Rutgers tomato plants, the symptoms were a mild mosaic and a fern-leaf condition without necrosis or death of the plants. When the plants were inoculated with CMV-Com that had CARNA 5, they developed necrosis, collapsed and died.

The improvements in nucleic and sequencing techniques have made it possible to characterize the nucleotide sequences of viral RNA molecules. Kaper and Tousignant (1978) elucidated the extensive nucleotide sequence homology that exists among CARNA 5 preparations of five strains of CMV.

Richards et al. (1978) found limited variations in sequences of CARNA 5 from different strains of CMV.

CHAPTER II

INTRODUCTION

Cucumber mosaic virus is probably one of the most commonly occurring plant-viruses. This is not surprising in view of it being transmitted so freely by aphids in the non-persistent manner, and by seed-transmission in many different plant species. Added to this is the ease with which it is mechanically transmitted and that many horticultural host plants are propagated vegetatively.

A vast library of literature exists describing diseases in many different horticultural and agricultural crops, as well as wild plants (weeds) serving as reservoir hosts to maintain a continuous cycle of infection.

In contrast to the detailed research attention this virus received elsewhere, CMV escaped notice as an important disease agent in South Africa. However, a few reports exist. Van Regenmortel (1960) reported it on squash plants (*Cucurbita pepo* L.) and called it the "ringspot" isolate because of the typical symptoms it caused on squash plants. Later, when this strain was fully characterised it became known as the CMV-S (squash) strain and was taken up in the international collections of CMV (Van Regenmortel, 1964, 1967, 1972; Finch et al., 1967 and Habili and Francki,1974a).

Bekker (1967) investigated serological methods for plant virus identification and showed that a CMV-isolate from naturally infected Lupin plants (CMV-Lupin-K5) was serologically related to a British strain and a Dutch tomato aspermy strain. Hendry (1969) followed this up by biophysical studies on the CMV-S strain and the CMV-K5 and a CMV-R isolate. Comparing them serologically, he could detect no differences.

In spite of the earlier research activities, nothing is on record on more recent research. Supplies of antiserum to this important virus were also not available when this project was started. It is particularly regrettable that lyophilised CMV-S infected leaf tissue could not be revived and that antisera supplies to this well-characterised strain were deplete.

This project was then aimed at finding naturally occurring isolates of CMV, characterising them, producing much needed antisera and to use such antisera in a comparison with other well characterised strains by the use of new contemporary sensitive serological techniques.

As CMV was found to occur frequently in lupins in the earlier work of Bekker (1967), and observations by Professor von Wechmar (personal communication), the presence of this virus was investigated in contemporary lupin cvs used commercially.

In participation with other departmental research projects, some work was also done to determine whether maize could serve as host to several CMV strains as symptom expression appears to be masked.

CHAPTER III

MATERIALS AND METHODS

A. BUFFERS AND REAGENTS

1. Phosphate Buffers

All phosphate buffers were prepared by the method of Williams and Chase (1968), recipe No. 33A. All the buffers presented were made with double distilled water to a final concentration of 0,1M molarity. The pH of the buffers was measured after dilution and adjusted when necessary. Unless otherwise stated, all chemicals used were supplied by Merck and were GR grade.

Solution A : NaH_2PO_4, 0,2M
(Dissolve 27,6 g of $NaH_2PO_4 \cdot H_2O$ to a final volume of 1 litre in distilled water).

Solution B : Na_2HPO_4, 0,2M
(Dissolve 28,4 g of Na_2HPO_4 to a final volume of 1 litre in distilled water).

1(a) 0,1M Sodium phosphate pH 7,0

Mix 195 mℓ of solution A with 305 mℓ of solution B. Make up to 1 litre with distilled water.

1(b) 0,1M Sodium phosphate, pH 7,4

Mix 95 mℓ of solution A with 405 mℓ of solution B. Make up to 1 litre with distilled water.

1(c) 0,1M Sodium phosphate, pH 8,0

Mix 26,5 mℓ of Solution A with 473,5 mℓ of solution B. Make up to 1 litre with distilled water.

2. Phosphate Buffered Saline (PBS)

Phosphate buffered saline was made at two pH values, namely pH 7,0 and pH 7,4. The composition of these buffers was as follows:

2(a) PBS pH 7,0

Mix 1 volume of 0,15M NaCℓ (8,7 g/litre) with 1 volume of 0,1M Sodium phosphate, pH 7,0 (III.A.1.a).

2(b) PBS pH 7,4

Mix 1 volume of 0,15M NaCℓ (8,7 g/litre) with 1 volume of 0,1M Sodium phosphate, pH 7,4 (III.A.1.b).

3. Virus Inoculation, Extraction and Storage Buffers

3(a) 1% Sodium Sulphite

Dissolve 1 g of sodium sulphite (Na_2SO_3) in distilled water and make up to 100 mℓ.

3(b) 0,05M Phosphate/2-Mercaptoethanol/DIECA Buffer pH 7,0

Mix 500 mℓ of 0,1M Sodium phosphate, pH 7,0 (III.A.1.a) with:
0,72 mℓ of 2-Mercaptoethanol (0,02M) and
2,25 g of diethyl dithiocarbonate (DIECA, 0,02M)
Adjust pH to 7,0 with either 0,1M HCℓ/0,1M NaOH and add distilled water to make 1 litre.

Both the buffers described above were used for crushing infected leaves for mechanical inoculation of CMV on plants.

3(c) 0,1M Sodium phosphate/Thioglycollate/DIECA pH 8,0

Make 1 litre of 0,1M Sodium phosphate pH 8,0 (III.A.1.c) containing 0,1% Thioglycollic acid and 0,1% diethyldithiocarbamate.

The above buffer was made according to the recipe of Mossop et al. (1976). This buffer was used for the extraction of virus from infected plant tissue.

3(d) 0,2M Borate buffer pH 9,0

This buffer was prepared by the method of Williams and Chase (1968), recipe No. 15. The buffer was diluted with double distilled water to give the desired molarity.

Stock Solutions

Solution A : H_3BO_3, 0,2M and KCℓ, 0,2M
(Dissolve 12,4 g of H_3BO_3 and 14,91 g of KCℓ to a final volume in 1 litre distilled water).

Solution B : NaOH, 0,2M
(Dissolve 8 g of NaOH to a final volume of 1 litre in distilled water).

The composition of the buffer was as follows:
Mix 250 mℓ of Solution A with 104 mℓ of Solution B.
Dilute to 1 litre with distilled water.

4(a) Buffers used for Double-Diffusion Gel Precipitin Tests

Difco Noble agar, 0,7% was used.

The agar gel was buffered with the following buffer:

0,1M Potassium phosphate, pH 7,0

This buffer was made according to the specifications of Williams and Chase (1968), recipe No. 27.

Solution A : KH_2PO_4, 0,5M
(Dissolve 68,04 g of KH_2PO_4/ℓ)

Solution B : K_2HPO_4, 0,5M
(Dissolve 87,09 g of K_2HPO_4/ℓ)

Mix 78 mℓ of Solution A with 122 mℓ of Solution B. Dilute to 1 litre with distilled water.

4(b) 0,5M Sodium Phosphate, pH 7,0

This buffer was made according to the specifications of Miller and Golder (1950).

Solution A : 5,0M NaCℓ
(Dissolve 292,2 g/ℓ)

Solution B : 5,0M Na_2HPO_4
(Dissolve 70,98 g/ℓ)

Solution C : 4,0M NaH_2PO_4
(Dissolve 6,24 g/100mℓ)

Mix 32 mℓ Solution A and 22,7 mℓ Solution B and 1,6 mℓ Solution C and dilute to 2 litres with distilled water.

5. Virus Stabilization Buffers

5(a) 0,1M Sodium phosphate/Formaldehyde buffer, pH 8,0

37% Formaldehyde solution was mixed to a final concentration of 1% with 0,1M Sodium phosphate, pH 8,0 (buffer in III.A.1.c).

To stabilize virus for Ouchterlony double diffusion tests, infected leaves were crushed in the sodium phosphate/formaldehyde buffer.

5(b) 0,005M Borate/Formaldehyde buffer, pH 9,0

37% Formaldehyde solution was mixed to a final concentration of 2% with 0,005M Borate buffer, pH 9,0 (III.A.3.d).

This buffer was used to stabilize virus for rabbit injections and serological relationship studies in Ouchterlony tests.

B. PROPAGATION OF VIRUS ISOLATES

1. Virus Origin

CMV-Tobacco (CMV-Tob) was isolated from flowering tobacco plants (*Nicotiana tabacum L.* garden form) obtained from a garden in Rosebank, Cape Town. CMV-Nelspruit (CMV-Nel) was collected at Nelspruit (Transvaal) on a wild tobacco tree (*Nicotiana glauca*) by Dr. M. Bar-Joseph.

CMV-Impatiens (CMV-Imp) was isolated from Impatiens plants by Mrs. F. Van der Meer at the Horticultural Research Institute at Roodepoort, Pretoria. CMV-Gladiolus-South Africa (CMV-Glad-SA) was also isolated by Mrs. F. Van der Meer from gladiolus plants grown from corms imported from Holland.

CMV-Lupine-K5 (CMV-Lup K5) and CMV-Y were obtained as lyophilized samples from Professor M.B. Von Wechmar. The isolate CMV-Lup-K5 was isolated in 1965 from field grown lupins by Mrs. Hendry (neé Bekker). CMV-Israel (CMV-Is) was obtained from Dr. M. Bar-Joseph in Israel in the form of dried material. This isolate also originated from naturally infected *N. Glauca.*

CMV-K and CMV-Q were obtained from Dr. R.I.B. Francki in Australia in the form of dried leaf material.

2. Multiplication, Harvesting and Storage

2(a) Hosts

All isolates were propagated in either tobacco (*Nicotiana tabacum* cv Soulouk, and *Nicotiana glutinosa*) or squash (*Cucurbita pepo* cv Long green bush) plants.

2(b) Inoculation and Multiplication

Virus isolates were multiplied from fresh or dried virus-infected leaves. The dried leaf tissue was first ground to a fine powder with a mortar and pestle, followed by the addition of 1% Na_2SO_3 or sodium phosphate/mercaptoethanol/DIECA buffer (IIIA.3a and 3b). Celite (diatomaceous earth) was added as an abrasive. The fresh leaves were ground with the buffer and squeezed through cheesecloth. The inoculum was always kept on ice.

Squash plants were inoculated at the dicotyledonous stage, 3-4 days after germination and tobacco plants were inoculated at the 4-leaf stage. The plants were inoculated by rubbing the leaf surface with cotton wool dipped in the inoculum. The plants were rinsed with water immediately after inoculation and left in a plant growth room which was maintained at a relative humidity of 70%, a day temperature of 24°C, a night temperature of 21°C and a 14/10 hour day/night cycle.

2(c) Harvesting and Storage

Symptoms appeared usually 4 days after inoculation. After 7 days post-inoculation, the plants were harvested by cutting them off at soil level.

Short Term Storage

Harvested plants were stored at 0-4°C in plastic bags if virus extraction followed soon afterwards.

Long Term Storage

Some of the harvest from the propagation was stored in two other ways:

(i) Infected leaf tissue from the first multiplication cycle of the original inoculation material was dried desiccated over self-indicating silica gel in a desiccator under vacuum and stored in vials containing silica gel at 4°C. This served as a source stock of virus isolates. Virus stored in this fashion remains infectious for several years. CMV-Lup-K5 was stored as dried material in a sealed vial and was still infectious after twenty years (von Wechmar, personal communication). This storage method also serves as a means of preventing the emergence of mutants resulting from serial passage of virus over a long period. Virus for purification was occasionally propagated from dried material as purified virus was found to lose infectivity when stored in suspension for a period longer than five days.

(ii) Some of the infected plants were also put in plastic bags and stored at -20°C for medium-term storage. These plants were used routinely for further multiplication of the CMV isolates when required.

3. Symptomatology

Comparison of symptoms was done for the various CMV-isolates, i.e. CMV-Tob, CMV-Imp, CMV-Glad, CMV-Nel and CMV-Is. The virus isolates were inoculated on the following plants: *Cucumis sativus* cv National pickling; *Cucurbita pepo* cv Long white; *Chenopodium quinoa; Datura stramonium; Nicotiana glutinosa; Nicotiana tabacum* cv Soulouk; *N. Tabacum* cv White barley; *Petunia hybrida*

cv Nana compacta, *Zea mays* cv Kalahari Early Pearl; *Zinnia elegans* cv Dahlia coloured mix.

The plants were maintained in a plant growth room in the same manner as in CH.III.B.2.b. The symptoms on plants were inspected regularly from 4 days after inoculation and recorded for all isolates under study. Photographs of the virus infected plants were sometimes taken.

4. Virus Extraction and Purification

4(a) Centrifugation of Viruses

In the text the terms (1) 'low speed' (LS) and (2) 'high speed' (HS) centrifugation refer to centrifugation at 8 000 rpm for 10 minutes and 34 000 rpm for 90 minutes, respectively. Low speed centrifugation was performed in the Sorvall RC2-B or RC5 refrigerated centrifuge, using the Sorvall SS-34, GSA and GS3 rotors. High speed centrifugation was performed in the Beckman L3-50 and L5-65 ultracentrifuges, using the Beckman type 35 and 60 Ti rotors.

The relative centrifugal forces (RCF), in gravities, exerted by the Sorvall SS-34, GSA and GS3 rotors at 8 000 rpm are 7 265 g, 10 444 g and 10 825 g, respectively.

The RCF of the 35 rotor at full speed is 142 800 g, and the 60 Ti rotor has a relative centrifugal force at 35 000 rpm of 144 800 g. Virus was always pelleted by centrifugation at 35 000 rpm, and not at a higher speed, because the particles tended to break up if packed too tightly. Rate zonal centrifugation was performed in a Beckman 28 SW rotor which, at 27 000 rpm, has an RCF of 130 000 g.

4(b) Extraction Procedure

The extraction method of Mossop et al. (1976) was used. All the steps were carried out at 4°C or on crushed ice. The CMV-infected plants were homogenized in a Waring blender in the presence of 0.1M Sodium phosphate/thioglycollate/DIECA buffer pH 8,0 (III.A.3.c). A leaf to buffer ratio of 1:3 (w/v) was used. The homogenate was filtered through cheesecloth to obtain a filtrate free of plant debris.

After low speed centrifugation of the CMV homogenate, the supernatant was decanted and Triton X100 added to a final concentration of 2% (v/v). This was stirred continuously on a magnetic stirrer for 15 minutes to facilitate the separation of host material from the virus particles. The mixture was subsequently subjected to a cycle of high speed centrifugation. The virus pellet that was obtained was resuspended in 0.1M sodium phosphate buffer pH 8,0. (The pellets were resuspended by pouring enough buffer into the tubes to cover the pellets, and leaving them at 4°C overnight). The resuspended pellets were centrifuged at LS. This centrifugation step removed all denatured insoluble components, leaving the supernatant as a straw-coloured semi-purified and concentrated virus suspension. The virus suspension was subjected to a further cycle of high and low speed centrifugation to ensure greater purity. The final pellets were resuspended in 0,005M borate buffer pH 9,0 (III.A.3.d).

4(c) Further Purification

The purified virus suspension mentioned above was considered to be fairly pure. However, when a CMV preparation of higher purity was needed, further purification of the partially purified virus suspension involved:

(i) rate zonal centrifugation on 10-40% sucrose gradients in a Beckman 28 SW rotor at 27 000 rpm (131 000 g) for 2½ hours. The contents of the tubes were scanned by ultraviolet absorption at 254 nm on an Isco fractionator. The CMV fraction was collected and dialysed against 0,005M borate buffer, pH 9,0 (III.A. 3d(ii)) to remove sucrose and to change suspending buffer. This was followed by HS centrifugation.

The virus pellets were resuspended in a small volume of 0,005M borate buffer, pH 9,0.

(ii) The virus suspension was further purified by zone electrophoresis (III.K) through a sucrose density gradient. The virus zone was collected, dialysed, centrifuged at high speed and resuspended in 0,005M borate buffer pH 9,0.

4(d) Quantitation

The concentrations of purified virus were determined by using the ultraviolet absorption method. The virus solutions were diluted in 0,1M sodium phosphate buffer, pH 8,0 or 0,005M borate buffer, pH 9,0 and scanned in 1 cm quartz cuvettes in the range 220-320 nm on a Beckman Model 25 spectrophotometer. The extinction coefficient of $E^{1\%}_{260}$ = 5,0 (Francki et al., 1979) was used.

C. ELECTROPHORETIC TECHNIQUES

1. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The system described by Laemmli (1970) was used for the detection and calculation of the protein subunit molecular weights of CMV isolates. Electrophoresis was carried out in a Hoefer SE 600 vertical slab gel electrophoresis unit.

SDS-PAGE Buffers

These buffers were made according to the specifications of Laemmli (1970). All chemicals used were Merck brand Analar grade.

(i) Monomer solution

30% acrylamide : 0,8% N,N'-methylene bis-acrylamide (w/v)

(ii) Resolving gel buffer

1M Tris/HCℓ pH 8,8

(iii) Stacking gel buffer

1M Tris/HCℓ pH 6,8

(iv) Bath buffer (pH 8,3)

Dissolve 3,03 g Tris
14,10 g Glycine
1,00 g SDS

in a final volume of 1 litre distilled water.
Store at room temperature.

(v) Dissociation mixture

10% SDS (w/v)/10% β-mercaptoethanol (v/v)/
15% glycerol (v/v)/0,01% bromophenol blue (w/v)/
0,125M Tris/HCℓ pH 6,8

(vi) 10% SDS (w/v), aqueous solution

(vii) Initiator

1,5% (w/v) aqueous ammonium persulphate

(viii) Coomassie blue stain

45% (v/v) methanol
10% (v/v) glacial acetic acid
0,2% (w/v) Coomassie blue R250
45% (v/v) water

(ix) Destaining solution

25% (v/v) methanol
10% (v/v) glacial acetic acid
65% (v/v) distilled water

1(a) Making of Gels

The apparatus comprised two glass plates, two side spacers, a bottom spacer and a comb. These were assembled using vacuum grease as a sealant. A 12% polyacrylamide resolving gel was poured in. The resolving gels used had a length of 110 mm, a width of 140 mm, and a thickness of 1,5 mm. A 4% polyacrylamide stacking gel was poured in after the resolving gel had gelled to the top of the glass plates ($\sim$ 70 mm long) and a comb inserted to make ten wells.

1(b) Sample Preparation

Healthy and virus-infected leaf extracts were prepared by grinding freshly collected leaves in 0,05M sodium phosphate buffer, pH 7,0 in a mortar and pestle. The extracts were partially clarified by LS centrifugation. In some cases the clarified extracts were concentrated by ultracentrifugation at 35 000 rpm for 180 minutes.

Appropriate dilutions of samples were made in dissociation mixture (III.A.6a(v)). In the case of samples concentrated by ultracentrifugation the pellets were directly resuspended in dissociation mixture. The sample mixtures were then heated for 5 minutes in a heating block at 95°C, cooled, and stored sealed at -20°C until needed.

1(c) Sample Application and Electrophoresis

Treated samples (10-25 µl) were loaded into the wells with a 25 µl Hamilton syringe, and electrophoresis was performed at a constant current of 8 mA/gel for 17 hours until the bromophenol blue front was about 1 cm from the bottom of the gel.

1(d) Staining and Destaining of the Gels

The gels were removed from the glass plate sandwiches, and stained overnight with a 0,2% (w/v) Coomassie blue stain. The gels were destained in destaining solution for 12-15 hours. The destained gels were dried on a Hoefer SE 540 slab gel dryer for 3 hours. The dried gels were photographed using a red filter.

2. Electrophoresis of RNA on Agarose Gels

The system used for characterization of RNA was that described by Grierson (1982) for the eletrophoresis of RNA on agarose slab gels. Electrophoresis was carried out in a vertical slab gel electrophoresis unit with 2 mm thick spacers 8,18 cm x 14 cm long).

I. RNA Electrophoresis Buffers

Two types of electrophoresis buffers were used:

(i) Tris-EDTA/Phosphate buffer

Tris-base 180 mM
$NaH_2PO_4 \cdot 2H_2O$ 150 mM
EDTA $Na_2 \cdot 2H_2O$ 5 mM

(ii) Tris-EDTA/Borate buffer

Tris-base 90 mM
Boric acid 90 mM
EDTA $Na_2 \cdot 2H_2O$ 2,5 mM
SDS 0,1% (w/v)

Either of the two buffer systems were used in RNA electrophoresis. The buffers served both as gel and tank buffers.

(iii) Denaturing solutions

The denaturing solutions were made as described by Hull and Lane (1973).

(a) 0,2% SDS/1M Urea/5% sucrose/0,05M β-mercaptoethanol

This buffer was used to denature phenol extracted RNA preparations.

(b) 1% SDS/1M Urea/5% sucrose/0,05M β-mercaptoethanol

This buffer was used to denature whole viruses.

Procedure

Agarose gels of concentration 1,5-2,0% (w/v) were prepared by dissolving agarose in electrophoresis buffer by boiling on a heating mantle. The hot gel solution was allowed to cool to a temperature of about 60°C. Meanwhile, an apparatus of two glass plates, two side spacers and a bottom spacer were assembled using vacuum grease as a sealant. The assembled apparatus was warmed to 45°C and the cooled gel solution poured in. Combs were quickly inserted and the assembly allowed to cool for 1 hour at room temperature for Agarose C, and at 4°C for low gelling temperature (LGT) agarose. The gels were cast in glass plate assemblies with a sheet of Gelbond (Marine Colloids, USA) stuck onto one of the gel plates with the hydrophilic side up. Combs were removed gently to avoid tearing the gels. Gels were then connected to buffer reservoirs and pre-electrophoresed, with the anode to the bottom at 10-20 mA for 30-60 minutes before use.

2(b) Sample Preparation

Virus RNA was prepared in two ways: (i) The RNA was obtained by phenol extraction in the presence of 1% bentonite. After ethanol precipitation, it was heated for 10 minutes at 90°C in 0,2% SDS, 1M Urea, 5% sucrose and 0,05M mercaptoethanol and immediately cooled in an ice bath. (ii) Alternatively, the whole virus, freshly purified, was heated for 10 minutes at 90°C in electrophoresis buffer containing 1% SDS, 1M Urea, 5% sucrose, and 0,05M mercaptoethanol and immediately cooled in an ice bath.

2(c) Sample Application and Electrophoresis

Treated samples (5-10 µℓ) were loaded into the wells with a 10 µℓ Hamilton syringe, and electrophoresis was carried out at a constant current of 20 mA for 3-4 hours.

2(d) Staining of Gels and Detection of RNA

The gels were removed from the glass plate sandwiches on gelbond and stained with 5 µg/mℓ ethidium bromide dissolved in distilled water for 30-60 minutes. The gels were destained by washing briefly in water. The RNA was visualized by its fluorescence when the gels were viewed on a transilluminator (ultraviolet) at a long-wave (320 nm) or medium-wave (260 nm). The gels were photographed with a fixed-exposure (1 second), fixed-distance Polaroid camera with a red filter. Positions of fluorescent bands on photographs were measured, and used to determine apparent molecular weights of nucleic acids with reference to known standards.

D. ZONE ELECTROPHORESIS

This technique was used for the separation of plant viruses from plant proteins and has been fully described by Van Regenmortel (1964, 1972) and Polson and Russel (1967). Zone electrophoresis was performed on semi-pure preparations of CMV in a sucrose density-gradient prepared in a 0,1M borate buffer pH 8,6. Phenol red was used as a standard reference to indicate the progress of electrophoresis and to determine the R_{ϕ} values of the various components (Van Regenmortel, 1968).

E. STABILIZATION OF VIRUS

Because of the unstable nature of CMV particles, the purified virus preparations were subjected to formaldehyde treatment in order to stabilize the virus particles. The virus pelleted from sucrose density gradient fractions was resuspended in 0,005M sodium borate buffer pH 9,0 and dialyzed against 0,005M sodium borate buffer pH 9,0 containing 2% formaldehyde (FA). These virus preparations were then subsequently dialysed against 0,005M sodium borate buffer pH 9,0 containing 0,2% FA. The stabilized virus preparations were used to immunize rabbits and for serology.

F. ANALYTICAL ULTRACENTRIFUGATION

A stabilized preparation of CMV-Tob was subjected to analytical ultracentrifugation in order to determine the sedimentation coefficient.

Centrifugation was performed on a Beckman model E analytical ultracentrifuge, equipped with Schlieren optics and electronic speed control. The sample was run in a cell with 12 mm aluminium single-sector centre piece with 4° sector angle and quartz windows. The standard single sector counter balance was used in the second rotor position. An AN-D Beckman rotor was used for the run. The sample was centrifuged at 30 000 rpm at 20°C. Rotor temperature was maintained by the centrifuge heating and cooling systems.

Photographs of Schlieren patterns were taken with an exposure time of 20-30 seconds at 4 minute intervals. The phase-plate angle of 60° was used. Virus concentration was 2-4 mg/mℓ.

The radial position of the Schlieren peaks in the photographs were determined by measurements made on photographic images projected onto graph paper. The images were brought to the correct scale by precalibrating the enlarger image with a photograph in which both the reference cell lines were clearly defined.

Sedimentation coefficients were determined by the graphical method described by Chervenka (1969). Alternatively, the gradient was obtained using a statistical calculator pre-programmed for least squares regression coefficient determination (Statistician, Compucorp).

G. SEROLOGICAL TECHNIQUES

1. Production of Antisera

1(a) Antigen preparation

Prior to infection, virus suspensions were diluted to a concentration of 1-3 mg/mℓ. Care was taken to ensure that no sample for injection contained more than 0,2% formaldehyde, as higher concentrations were painful to rabbits when injected.

The virus suspensions were mixed 1:1 with Freund's incomplete adjuvant, and the mixtures emulsified by repeated uptake into a 10 mℓ syringe through a 20 gauge needle.

1(b) Rabbit antisera

Rabbits of 10 weeks and older were initially immunised with an intramuscular injection of 2 mℓ antigen emulsion at weekly intervals for 3 weeks. Thereafter, the rabbits were boosted at 4-6 week intervals. Rabbits of 2 years or older, which had been used continuously, were boosted at longer intervals. For each different antigen used in this work, one or two rabbits were immunised.

Rabbits were bled from a marginal vein in the ear at weekly intervals, commencing at least three weeks after the first injection of antigen. Ten to twenty mℓ of blood were collected in sterile screwcap bottles at each bleeding. This was allowed to clot, and was then centrifuged at low speed to separate the serum fraction. The serum samples were sealed in sterilised bottles, and either frozen at -20°C for long-

term storage, or kept at 4°C for immediate use. Each serum was numbered with the cumulative bleeding number, rabbit identification, and date of bleeding for record purposes. Bleedings obtained up to six weeks after the initial immunisation, or after three injections, were designated as 'early' bleedings. Bleedings obtained after two months, or after six or more injections, were designated 'late' antisera. Antiserum titres were determined for selected bleedings by the method of double diffusion in agar gel (Ouchterlony).

2. Enzyme-Linked Immunosorbent Assay (ELISA)

2(a) Buffers

(i) Antigen dilution buffer

Phosphate buffered saline pH 7,4 (III.A.2.b) was used. (Tween and BSA were deliberately omitted).

(ii) Rinsing buffer (PBS-Tween)

Phosphate buffered saline pH 7,4 containing 0,05% (v/v) Tween-20.

(iii) Post-coating and conjugate dilution buffer

Phosphate buffered saline, pH 7,4 containing 0,05% (v/v) Tween-20 and 0,2% (w/v) Bovine serum albumin (BSA) (PBS-T-BSA).

(iv) Substrate buffer (10% Diethanolamine)

This buffer was made according to the specifications of Clark and Adams (1977).

2(b) Procedure

The double antibody-sandwich ELISA (DAS-ELISA) as described by Clark and Adams (1977) was used for the determination of serological relationships among the CMV-isolates and for detection of CMV in field samples.

2(c) Preparation of Reagents

(i) Purification of Gamma-Globulin

The procedure of Clark and Adams (1977) was used for the purification and enzyme-labelling of the gamma-globulin serum fraction.

One millilitre of antiserum (see Table 6) was diluted with 9 mℓ of distilled water, and mixed 1:1 (v/v) with saturated ammonium sulphate solution. After leaving to stand at room temperature for 15 minutes, the precipitated globulin fraction was collected by low-speed centrifugation (10 000 rpm for 10 minutes). The precipitate was dissolved in 2 mℓ half-strength PBS, pH 7,4 (III.A.2.b) and dialysed overnight at 4°C against 2 litres of half-strength PBS pH 7,4 to remove excess ammonium sulphate. The gamma globulin fractions were purified by ion-exchange chromatography by eluting through a glass column (15 cm x 1 cm) packed with diethylaminoethyl (DEAE) cellulose (Whatman DE 52 anion-exchange), pre-equilibrated with half-strength PBS, pH 7,4. The gamma globulin was eluted through the column with half-strength PBS pH 7,4. One millilitre fractions were collected and the effluent was monitored by ultra-violet (U.V.) absorption at 280 nm. The fractions with an absorbance (A_{280}) of 1,4 and above were pooled. The A_{280} of the pooled fraction was measured and adjusted to 1,4. The concentration of the gamma globulin fraction after adjusting was 1 mg/mℓ. The extinction coefficient of gamma globulin was taken as $E^{0,1\%}_{280\ nm} = 1,4$ (Clark and Adams, 1977).

(iii) Conjugation with Alkaline Phosphatase

Alkaline phosphatase from beef mucosa (Miles) was used for antibody labelling. The enzyme was obtained as a salt-free, freeze-dried powder and had a specific activity of 1 000 units per mg protein.

Two milligrams of enzyme was dissolved directly in 2 mℓ of purified gamma globulin at a concentration of 1 mg/mℓ. The mixture was dialysed overnight at 4°C against 2ℓ of half-strength PBS pH 7,4. The mixture was placed in a vial and glutaraldehyde (25% (w/v) solution (from Fluka AG, Sweden) was added to give a final concentration of 0,05% by addition of 40 µℓ of 2,5% glutaraldehyde in half-strength PBS pH 7,4 to 2 mℓ of the dialysed globulin-enzyme mixture. The fresh mixture was left at room temperature for four hours. This was dialysed overnight at 4°C against 2ℓ of PBS pH 7,4, to remove unreacted glutaraldehyde. The enzyme conjugated gamma globulins were stored at 4°C in sealed vials in the presence of 0,5% (w/v) bovine serum albumin (BSA).

The conjugated gamma globulins were diluted in PBS-T-BSA buffer (III.A.7.d) before each assay.

2(d) Enzyme Immunoassays

(i) Equipment used

Dynatech "Micro ELISA" flat-bottomed trays were used for all assays . Finn variable volume micropipettes (50-250 µℓ and 200-1 000 µℓ) and Gilson Repetman repeating, variable volume micropipettes (0-200 µℓ and 200-1 000 µℓ) were used for all pipetting operations.

During the assay, the microtitre trays were placed in a moist plastic container with a lid and incubated at 37°C.

The optical absorbance of the hydrolysed substrate in the wells was recorded on a Titertek Multiskan automatic read-out spectrophotometer (Type 310c, Flow Laboratories).

(ii) The double antibody "sandwich" ELISA (DAS-ELISA)

The wells were coated by the addition of 200 µl of purified gamma globulin (usually at 5 µg/ml concentration) diluted in coating buffer. The microtitre tray was incubated in a moist chamber at 37°C for 1½-2 hours. The wells were emptied and washed by flooding the tray with PBS-Tween, pH 7,4 buffer, and then immediately emptying it, followed by two longer washes of three minutes each. This was followed by flooding the tray with PBS-T-BSA buffer and incubating at room temperature for 15 minutes in order to allow the BSA to coat the areas in the wells left uncoated by the gamma globulins. This prevented non-specific adsorption of the antigens to the wells. The wells were drained and the trays were dried by gentle tapping on a paper towel.

200 µl volumes of each dilution of sample in PBS pH 7,4 were added in the appropriate rows of wells, and the tray was incubated for 1½-2 hours at 37°C or at 4°C for 18 hours. The tray was washed and drained as before. 200 µl volumes of the enzyme-conjugated gamma globulins diluted appropriately in PBS-T-BSA buffer were added to each well. The tray was incubated for 1½-2 hours at 37°C.

The wells were emptied, washed, drained and dried, then 300 µl of freshly prepared substrate solution 1 mg/ml p-nitrophenyl phosphate in substrate buffer was added to each well.

The trays were left at room temperature for enzyme-substrate reaction to occur. After 45-60 minutes the absorbance of the hydrolysed substrate in the wells was read at 405 nm in the Titertek Multiskan spectrophotometer.

Grid titrations with different antibody dilutions, virus concentrations, and conjugate dilutions were performed in order to select the best conditions for the assays. This involves grid titrations of purified CMV (serial four-fold dilutions) against coating concentrations of 0,1 µg/mℓ 1 µg/mℓ and 10 µg/mℓ and conjugate dilutions of $^{1}/_{250}$, $^{1}/_{400}$, $^{1}/_{500}$ and $^{1}/_{1000}$. The combination of coating IgG concentration and conjugate dilution that gave a wide range of A405 readings (A_{405} max of 1-2 units and A_{405} min of 0,1 unit) for CMV reactions and a low background O.D.405 nm reading with controls was taken as the optimal proportions. It was found that a coating concentration of 2,5 µg/mℓ was suitable for all antisera used, and a $^{1}/_{400}$ dilution of conjugate was found to be suitable for all antisera.

Controls that were used in the assays included CMV as a positive antigen control, BMV and healthy plant sap as negative antigen controls. Another negative control involved the coating of wells with gamma globulin of normal rabbit serum (NRS), instead of with anti-CMV gamma globulins.

3. The Enzyme-Immunoelectroblotting Technique

(a) Immunoelectroblotting buffers

These buffers were made according to the specifications of Rybicki and Von Wechmar (1982).

(i) Transfer buffer

The composition of the buffer was as follows:
25 mM Tris
192 mM Glycine
20% (v/v) Methanol
Make up in distilled water and adjust to pH 8,3.

(ii) Tris-saline BSA buffer

10 mM Tris HCℓ pH 7,4
2% (w/v) BSA
0,15 M NaCℓ

(iii) Enzyme substrate solution

The stock solution of the enzyme substrate was prepared as follows:
Dissolve 3 mg 4-chloro-1-napthol (Bio Rad) per mℓ of methanol. Store at 4°C.

Just before using, dilute the enzyme substrate with 5 volumes of the following solution:

50 mM Tris-HCℓ
200 mM NaCℓ, pH 7,4
and add 0,015% (v/v) H_2O_2

(iv) SDS polyacrylamide gel electrophoresis

This was carried out as already described in Section (III.H.1).

(v) Sample preparation

Sample preparation was also carried out as described in Section (III.H.1a).

(b) Electrophoretic transfer (Electroblotting)

Resolving gels were put on moist nitrocellulose paper (transfer buffer) (0,45 um pore, Schleicher & Schuell BA 85, NH, USA), and these were sandwiched between wetted sheets of filter paper (Whatman 3MM). These were then laid upon 15 x 20 x 0,8 cm

Scotch-brite scouring pads. Large carbon electrodes (20 x 15 x 1 cm) were secured on both sides of the scouring pads and elastic bands strung around the electrodes. The assembly was placed vertically in a 5 litre capacity tank containing transfer buffer. The electrodes were connected to a Shandon Southern 50v/iA powerpack with the electrode nearest to the nitrocellulose connected to the anode. A current of 0,8-1A was applied for 1-2 hours.

(c) The Enzyme-Immune Electroblotting Technique

This technique was performed according to Rybicki and von Wechmar (1982).

(d) Enzyme-assisted indirect immunoassay

Electroblots were soaked for 3-4 hours at 37°C or overnight at 22°C in a 1% (w/v) suspension of bovine serum albumin in 10 mM Tris HCℓ/saline pH 7,4 (Tris-saline-BSA buffer) to saturate free protein binding sites. Rabbit antisera were diluted $^{1}/_{25}$-$^{1}/_{80}$ in Tris-saline-BSA, and incubated with the blots in individual closed containers on a shaker at 22°C for 1-2 hours.

Blots were washed for 10 minutes on a shaker in at least four changes of saline. Goat anti-rabbit horseradish peroxidase conjugate was diluted $^{1}/_{1000}$-$^{1}/_{1500}$ in Tris-saline-BSA and incubated with the blots on a shaker for 1-2 hours at 22°C. After further washing in saline, the enzyme substrate solution (1 volume 3 mg/mℓ 4-chloro-1-napthol in methanol/0,015% H_2O_2/5 volumes 50 mM Tris HCℓ/ 200 mM NaCℓ, pH 7,4) was added and the blots left at 22°C until the colour reaction had sufficiently taken place. The colour reaction was stopped by washing in tap water. The blots were then dried in layers of paper towel and photographed.

H. ELECTRON MICROSCOPY

1. Negative Staining

Purified virus samples in 0,005M borate buffer pH 9,0 or 0,1M sodium phosphate buffer pH 8,0 were negatively stained with 2% uranyl acetate pH 6,5.

Procedure

A drop of sample was placed on a Formvar coated grid sitting on parafilm. The grid was covered with a Petri dish and left for 5 minutes at room temperature. The grid was washed with a series of drops of distilled water from a Pasteur pipette. Grids were stained for 1 minute with 2% uranyl acetate pH 6,5. The grid was drained by touching the edge to a filter paper, and allowed to dry. The grid was viewed in a Zeiss 109 transmission electron microscope at 80 kV.

2. Immunosorbent Electron Microscopy

This technique, also known as serologically specific electron microscopy (SSEM) or the Derrick method, was first described by Derrick (1973) for the assay of plant viruses. The technique has been used extensively in the detection, quantitation and characterisation of plant viruses (Milne, 1975, 1977; Derrick and Brlansky, 1976). It has been used successfully for the detection of viruses (Roberts and Harrison, 1979).

The technique described below is the short incubation method of Milne (1980):

The same virus samples used for negative staining were used. 5 µℓ drops of 1/1000 diluted antiserum were placed on Parafilm in a humid Petri dish. The grids were incubated face down on these drops for 5 minutes. The face of the grid was rinsed with 20 consecutive drops of 0,1M sodium phosphate buffer, pH 7,0. The grid was drained by touching the edge to filter paper. The grid was not allowed to dry at this stage. The grid was then incubated face down for 15 minutes on a drop of the virus sample. Next, the grid was rinsed with 30 consecutive drops of distilled water from a Pasteur pipette. The face of the grid was stained with 2% uranyl acetate for 1 minute and drained as before and allowed to dry. The grid was viewed in the electron microscope.

CHAPTER IV

RESULTS

A. SEPARATION OF CMV-TOB FROM FILAMENTOUS VIRUS (III.B.1)

1. Preliminary Investigation of a Mixture of Two Viruses from a Flowering Tobacco Plant

Two types of symptoms developed on *N. glutinosa* plants when inoculated with sap from the original plant, a systemic mosaic and necrotic lesions developed on the leaves.

The virus was crudely extracted from such plants (method of Mossop et al., 1976; see IV.D.3), and the preparations examined in the following ways:

Electron Microcopy: (see III.H.1) Negative staining with 2% Uranyl acetate, pH 6,5 revealed spherical and filamentous particles as illustrated in Figure 1.

Zone Electrophoresis: (see III.D). The extract separated into two bands after zone electrophoresis for 18 hours in a sucrose gradient at pH 8,6. The average R_{ϕ} values calculated from four separate runs were determined to be 0,55 for the upper band below the phenol red band, and to be 0,31 for the lower band (see also IV.K.1). These two bands were sampled and inoculated on *N. glutinosa* plants. Both bands gave rise to infections with the upper band (R_{ϕ} = 0,55) causing mosaic symptoms and the lower band (R_{ϕ} = 0,31) causing necrotic lesions.

Rate Zonal Centrifugation: (see IIIB.C.i). After 150 minutes of centrifugation at 27 000 rpm in 10-40% (w/v) sucrose density gradients two bands were observed at 4,2 and 5,9 cm from the meniscus. Clean separation of 2 components appeared to have occurred.

2. Separation of the Viruses

(a) Separation by host selectivity

Preliminary tests showed that the spherical particles present (see Fig.1) had an Rϕ value of 0,55 in zone electrophoresis, and subsequent studies (see III.E) showed the particles had a sedimentation coefficient of 98S. From these findings and symptoms in tobacco (*N. glutinosa*) plants, it was assumed that the spherical particles could possibly be cucumber mosaic virus (CMV). Certain plant species were selected in an effort to separate the putative CMV from the virus mixture. The plants were: Squashes (*Cucurbita pepo L.* cvs Long white bush, Long green bush and Caserta bush), Watermelon (*Cucumis melo* cv Sweetmeat), and cucumber plants (*Cucumis sativus* cvs Ashley, National pickling and Stono). Of these plants, all squashes, the watermelon, Ashley and National pickling cucumbers, developed mosaic symptoms with stunted growth when inoculated with sap from the original infected plant material.

Extracts from tobacco plants revealed only spherical particles when viewed with the electron microscope, similar to those in Figure 2(a) and (b). Zone electrophoresis and rate zonal centrifugation yielded a single zone. The Rϕ value of 0,55 of this single zone corresponded closely with Rϕ of 0,53 reported by Van Regenmortel (1968) for CMV strains S and Y.

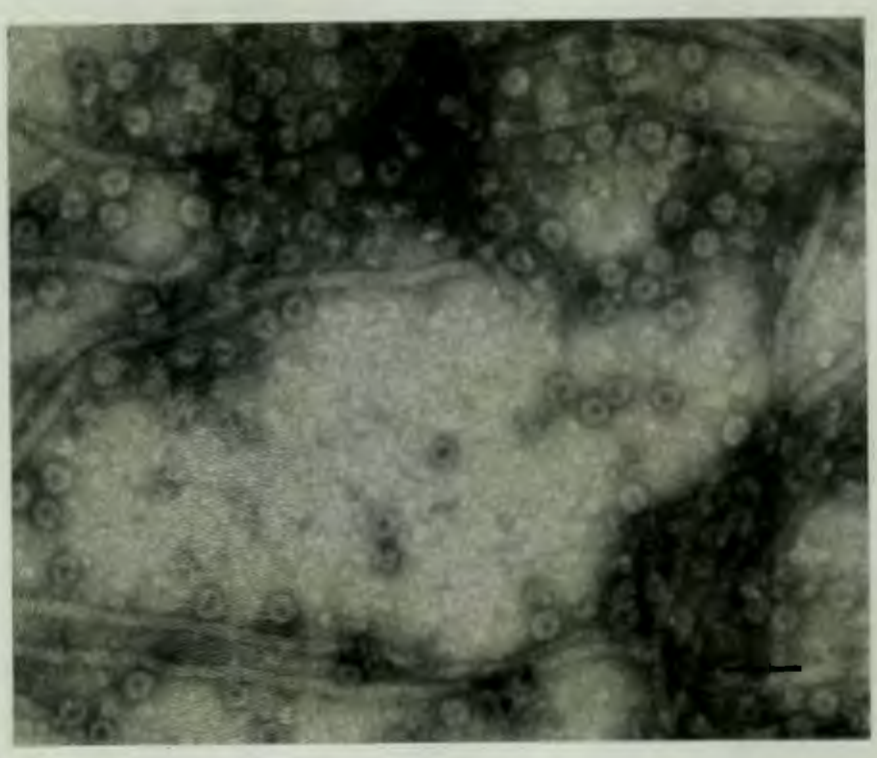
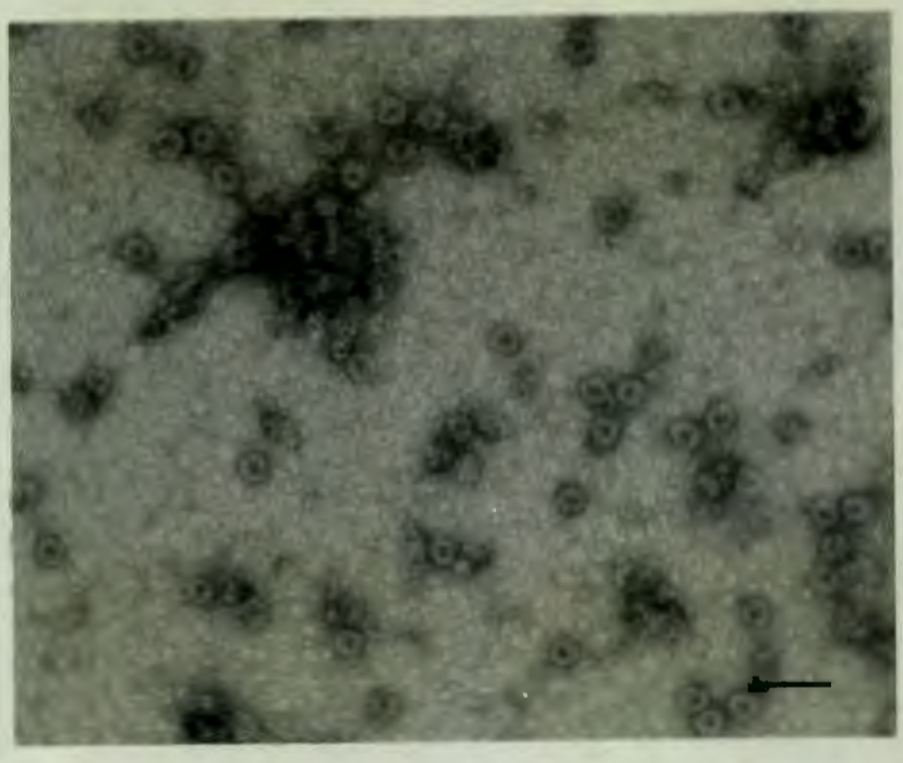
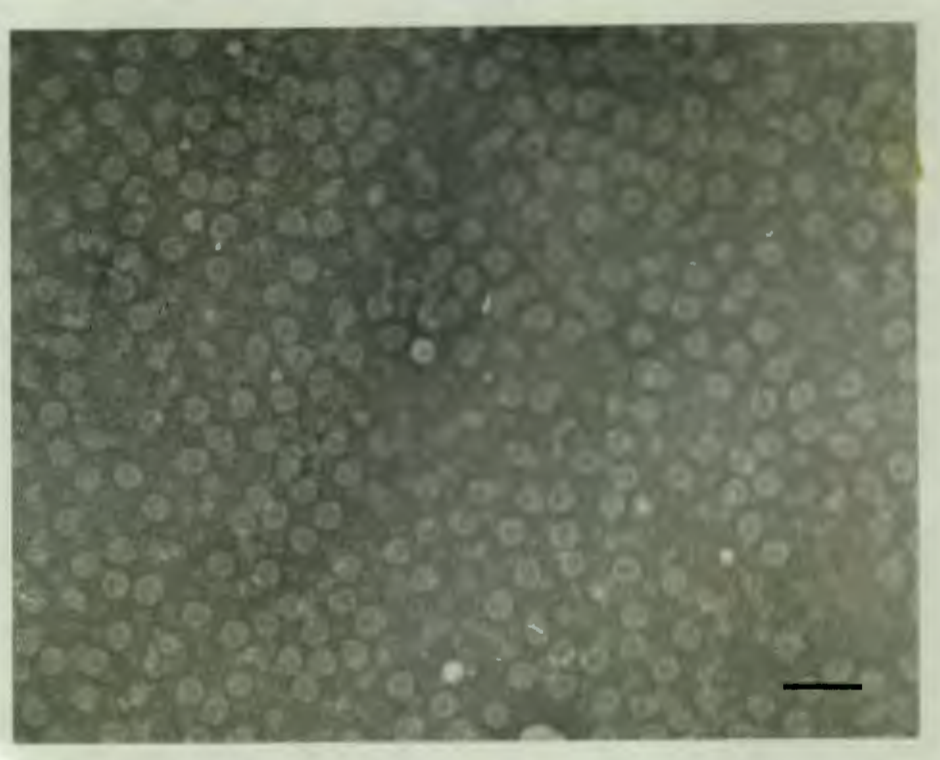


FIGURE 1 : Electron micrograph of the spherical and flexuous virus particles originally extracted from tobacco leaves. The particles were negatively stained with uranyl acetate. The bar represents 100 nm.



A



B

FIGURE 2 A & B : Electron micrographs of CMV-Tob extracted from tobacco plants and stabilised with 2% FA by dialysis. The same preparation was stained with:

A. Uranyl acetate and,
B. Ammonium molybdate.

The size bar represents 100 nm.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (see III.C.1) showed one protein band whereas three bands were observed before the inoculation of the virus mixture onto cucurbits. This is illustrated in Fig.3, where the single band is clearly visible in track 1 and three major bands are seen in track 2.

Back-inoculation of virus passaged through cucurbits onto *N. glutinosa* plants gave rise to mosaic symptoms only. Filamentous particles were absent from the virus extracts processed from *N. tabacum* cv Soulouk and *N. glutinosa* plants.

(b) Separation by dilution of inoculum

N. glutinosa plants were inoculated with the dilutions 10^{-3}, 10^{-4} and 10^{-5} of sap from tobacco plants infected with both viruses. 7-10 days, post-inoculation, the plants inoculated with the 10^{-3} dilution showed a few necrotic lesions and systemic mosaic symptoms. The plants inoculated with the 10^{-4} and 10^{-5} dilutions showed a mild systemic mosaic and no necrotic lesions. Further propagation and extraction of virus obtained from the plants exhibiting the mosaic symptoms only gave rise to single bands on zone electrophoresis (R_{ϕ} = 0,55) and rate zonal centrifugation.

Electron microscopic examinations of these fractions showed only spherical particles. On SDS-PAGE the fraction gave rise to one band at the 25 kilodalton (kd) position as illustrated in Figure 3.

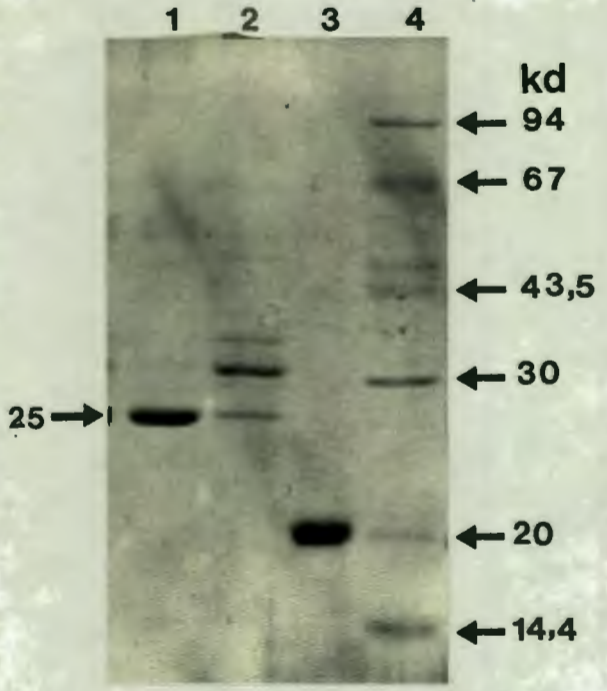


FIGURE 3 : SDS-polyacrylamide gel stained with coommassie brilliant blue.

Track 1: CMV purified from tobacco after separation from contaminating viruses by passage through cucurbits.

Track 2: Purified extract from tobacco plants before separation of viruses.

Track 3: Purified BMV-P marker.

Track 4: Pharmacia LMW molecular weight markers.

(c) Rate of multiplication

The third method used was the different rates of multiplication of the two viruses in *N. glutinosa* plants. The systemic mosaic symptoms appeared earlier than the necrotic lesions. The systemic mosaic became visible seven days post-inoculation and the necrotic lesions at 10 days. The youngest leaves of plants showing systemic mosaic were cut off before the appearance of the necrotic lesions and were used to inoculate healthy plants.

After five passages of the virus in this manner, no necrotic lesions were observed 14 days after inoculation. The virus extracted from these plants showed no evidence of the filamentous particles in the analyses used in the previous two methods of separation (IV.A.2(a) and (b)).

This isolate from now on will be referred to as CMV-Tob.

B. PROPAGATION OF CMV ISOLATES AND STRAINS

1. Hosts

The CMV isolates used in this investigation were CMV-Tob, CMV-Is, CMV-Nel, CMV-Imp, CMV-Glad-SA and CMV-Lup-K5. The CMV strains were CMV-Q and CMV-K. Their origins are as described in III.B.1.

The hosts that were routinely used for the propagation of the CMV isolates and strains were tobacco (*Nicotiana tabacum L.* cultivars Soulouk and Samsum, and *Nicotiana glutinosa*) and squash (*Cucurbita pepo L.*, CV Long white bush) plants. *N. glutinosa* was used for maintaining CMV isolates and strains. This host was

deliberately chosen as it is unattractive to aphids, and inoculated plants survive for months when kept under plant room tables, away from direct light.

The tobacco plants were inoculated at the 6-leaf stage and the squash plants at the dicotyledonous stage. The different isolates and strains multiplied at different rates in the above-mentioned hosts. For this reason the propagation hosts were not the same, as shown in Table 3.

Tobacco plants showed symptoms 7-10 days post-inoculation and squash plants 4-6 days post-inoculation. Tobacco plants were harvested 10-14 days after inoculation and squash plants 7-10 days after inoculation. The harvested plants were stored at 4°C before purification.

2. Buffers used for Sap Inoculation

Three buffers were compared for crushing infected leaves for sap inoculation, namely (a) 1% sodium sulphite (III.A.3.a); (b) 0,05M potassium phosphate, pH 7,0 (III.A.4.a), and (c) 0,05M sodium phosphate buffer, pH 7,0 containing 0,02M mercaptoethanol and 0,02M diethyldithiocarbamate (III.A.3.b). When buffers (a) and (b) were used, two observations were made: (i) no symptoms were observed when plants different from virus inoculum source plants were inoculated. (ii) After serial passages of isolates CMV-Is, CMV-Nel, and CMV-Tob on either Tobacco or squash plants, virus symptoms progressively became milder until they disappeared. When buffer (c) was used, the CMV-isolates retained their infectivity in both cases. The idea of additives in inoculation buffers was obtained from Gibbs and Harrison (1976) and Kaper and Waterworth (1981) who used the additives to inactivate virus inhibitors.

TABLE 3: Propagation Hosts used for CMV Strains and Isolates

Virus Isolate	Plant Host
CMV-Tob	*N. tabacum* cvs Soulouk, Samsum; *N. glutinosa*
CMV-Is	*N. glutinosa*; *C. pepo* cv Long white bush
CMV-Nel	*N. glutinosa*; *N. tabacum* cv Soulouk
CMV-Imp	*N. glutinosa*; *N. tabacum* cv Soulouk; *C. pepo* cv Long white bush
CMV-Glad-SA	*N. glutinosa*; *C. pepo* cv Long white bush
CMV-Lup-k5	*N. glutinosa*; *C. pepo* cv Long white bush
CMV-Q	*N. glutinosa*; *N. tabacum* cv Soulouk; *C. pepo* cv Long white bush
CMV-k	*C. pepo* cv Long white bush

C. SYMPTOMATOLOGY

A comparative study of symptoms caused by CMV-Tob; CMV-Is; CMV-Imp, and CMV-Glad-SA on 10 selected host plants was carried out. Figure 4A-H shows some of the symptoms observed on certain host plants.

Judging by symptoms only, CMV-Tob and CMV-Nel could not be distinguished. In most plants CMV-Is and CMV-Imp caused similar symptoms. The virus symptoms of CMV-Glad-SA on *Datura stramonium, N. Glutinosa, N. tabacum* cv White barley and *Zinnia elegans* were different from those caused by the other isolates. CMV-Imp caused necrotic spots on *Zea mays*. The symptoms of these isolates on the selected host range are tabulated in Table 4 .

TABLE 4: Symptoms of CMV-Isolates on a Selected Host Range

Host	CMV-Tob	CMV-Is	CMV-Nel	CMV-Imp	CMV-Glad
1. *Cucumis sativus* cv National pickling	Mild systemic mosaic, yellow spots	Leaf yellowing; stunted growth	Mild systemic mosaic, yellow spots	Leaf distortion; vein clearing; stunted growth	Necrotic lesions; etching; death of plant
2. *Cucurbita pepo* cv Long white bush	Mild systemic mosaic; yellow spots	Severe mosaic; yellow spots	Mild mosaic; yellow spots	Severe mosaic; yellow spots	Severe mottling; tiny yellow spots; stunted growth
3. *Chenopodium quinoa*	Local lesions,	Necrotic lesions	Local lesions, chlorotic	Necrotic lesions, etching	Necrotic local lesions
4. *Datura stramonium*	No symptoms	Necrotic lesions	Local lesions,	Leaf yellowing; yellow spots	Systemic mosaic; vein chlorosis
5. *Nicotiana glutinosa*	Systemic mosaic pale green spots	Chlorosis;dark green islands on leaf; leaf narrowing	Systemic mosaic; tiny brown lesions; leaf distortion	Systemic yellowing; concentric yellow rings; vein chlorosis	Systemic mosaic pale green islands
6. *N. tabacum* cv Solouk	Systemic mosaic	Systemic mosaic; dark green islands	Symptomless	Vein clearing; etching; dark green islands	Systemic mosaic
7. *N. tabacum* cv White barley	Mild mosaic	Systemic mosaic	Mild mosaic; leaf mottling	Severe necrosis; etching	Alternate dark green and pale green islands; mild mottling
8. *Petunia hybrida* cv Nana Compacta	Systemic mosaic	Systemic mosaic; leaf mottling	Systemic mosaic	Necrotic lesions; etching and dessication	Leaf yellowing; mottling
9. *Zea mays* cv Kalahari Early Pearl	No symptoms	No symptoms	No symptoms	Necrotic spots; rolling of youngest shoot; plant death	No symptoms
10. *Zinnia elegans*	No symptoms	Vein clearing	Not done	Leaf yellowing	Systemic mosaic

FIGURE 4 : Symptoms of various CMV isolates on tobacco, squash and quinoa plants.

A.(i) A healthy squash (*C. pepo*) plant and

(ii) a squash plant infected with CMV-Nel.

B. A tobacco (*N. tabacum* cv Soulouk) plant infected with CMV-Nel.

C

D

FIGURE 4 : C. Tobacco (*N. glutinosa*) infected with CMV-Nel.

D. Tobacco (*N. tabacum* cv Soulouk) infected with CMV-Imp.

E

F

FIGURE 4 : E. Squash *(C.pepo)* infected with CMV-Imp.
F. *N. glutinosa* infected with CMV-Is.

G

H

FIGURE 4 : G. Squash infected with CMV-Tob.

H. *Chenopodium quinoa* infected with CMV-Is - note the local lesions.

D. PURIFICATION OF CUCUMBER MOSAIC VIRUS

During the early stages of this investigation, various purification methods were compared.

1. Scott's Method (1963)

The purification method was carried out at 4°C. The infected tissue was ground in 0,5M citrate buffer pH 6,5 containing 0,1% thioglycollic acid and chloroform in the ratio 1:2:1 (leaves:buffer:chloroform).

The aqueous phase was subsequently dialysed against 0,005M borate buffer pH 9,0.

The dialysate was then subjected to three cycles of differential centrifugation.

The pellet of each purification step was inoculated onto *N. glutinosa* plants. The LS precipitates were more infective than the HS pellets showing that most of the virus was lost during LS centrifugation, probably due to aggregation.

2. Murant's Method (1965)

This method was also carried out at 4°C. The infected leaf material was homogenized in a Waring blender with 0,5M potassium phosphate buffer, pH 7,5 containing 0,1% thioglycollic acid in the ratio 1g:2mℓ. The pulp was squeezed through cheesecloth and the filtrate clarified by the addition of an equal volume of ether and stirred for 20 minutes. The mixture was then subjected to 2 cycles of differential centrifugation. The pellets were resuspended in 0,06M phosphate buffer pH 7,5.

The L.S. precipitates were highly infectious at $^1/_1$ and $^1/_{10}$ dilutions. The resuspended H.S. pellets were mildly infectious at $^1/_1$ dilution and no detectable symptoms were formed at $^1/_{10}$ dilution when inoculated onto *N. glutinosa* plants. This indicated that a large proportion of the virus was lost during L.S. centrifugation, probably due to aggregation as in the previous method.

3. Mossop et al's Method (1976) (III.4.a,b, & c)

The method was also carried out at 4°C. The infected material was homogenized in a Waring blender with 0,1M sodium phosphate buffer, pH 8,0 containing 0,1% thioglycollic acid and 0,1% diethyldithiocarbamate. The leaf tissue to buffer ratio was 1:3.

The pulp was squeezed through cheesecloth and the filtrate subjected to L.S. centrifugation. Triton X-100 was added to the supernatant to a final concentration of 2% and the mixture was stirred constantly for 15 minutes at 4°C. The mixture was then subjected to two cycles of differential centrifugation. The H.S. pellets were highly infectious on *N. glutinosa*. No virus symptoms were observed on *N. glutinosa* plants inoculated with dilutions of resuspended L.S. precipitates.

The yields of virus purified by this method were the highest, ranging from 115-360 mg/kg of leaf tissue. The method was adopted for routine purification. The average yields obtained for all isolates are shown in Table 5. The only modification made in the method was that 5mM sodium borate buffer pH 9,0 was used as final resuspending buffer instead of 10 mM sodium borate buffer pH 7,5 as the virus particles were infectious for longer periods in the former than in the latter buffer.

The suitability of 2 buffers in terms of the ability to retain infectivity of 3 CMV isolates was tested. Aliquots of CMV-Tob, CMV-Is, and CMV-Nel, were divided into two batches each and resuspended in each of the two buffers. After storage for 3, 7 and 14 days at 4°C, samples of the different treatments were inoculated onto *N. glutinosa* plants. CMV-Tob in 10 mM sodium borate buffer pH 7,5 had lost infectivity at 3 days while the same virus isolate in 5 mM sodium borate, pH 9,0 was still infective at 3 days but had lost infectivity at 7 days. Both CMV-Is and CMV-Nel in 10 mM borate pH 7,5 had lost infectivity at 7 days while the virus in 5 mM borate, pH 9,0, was still infective, but lost infectivity at 14 days. The virus preparations of all three isolates became turbid, indicating that degradation was faster in 10 mM borate pH 7,5 than in 5 mM borate pH 9,0.

TABLE 5 : Average Virus Yields for CMV Isolates (mg/kg) when Propagated in *N. glutinosa* and Purified According to the Method of Mossop et al., 1976

Isolate	*Approx. Virus Yield (mg/kg leaf tissue)
CMV-Tob	350
CMV-Is	120
CMV-Nel	230
CMV-Imp	360
CMV-Glad-SA	115
CMV-Lup-K5	340

*Average virus yield calculated from six separate virus extractions.

The ultraviolet absorption spectra of the purified CMV-Tob preparation as shown in Figure 5 is representative of the strains and isolates purified, and had a similar profile to those obtained by Scott (1963), for CMV-Y and Francki et al. (1966) for CMV-Q. The absorbance ratios of 260:280 ranged from 1,68-1,72 which was in good agreement with the corrected value of 1,70 from Francki et al. (1979).

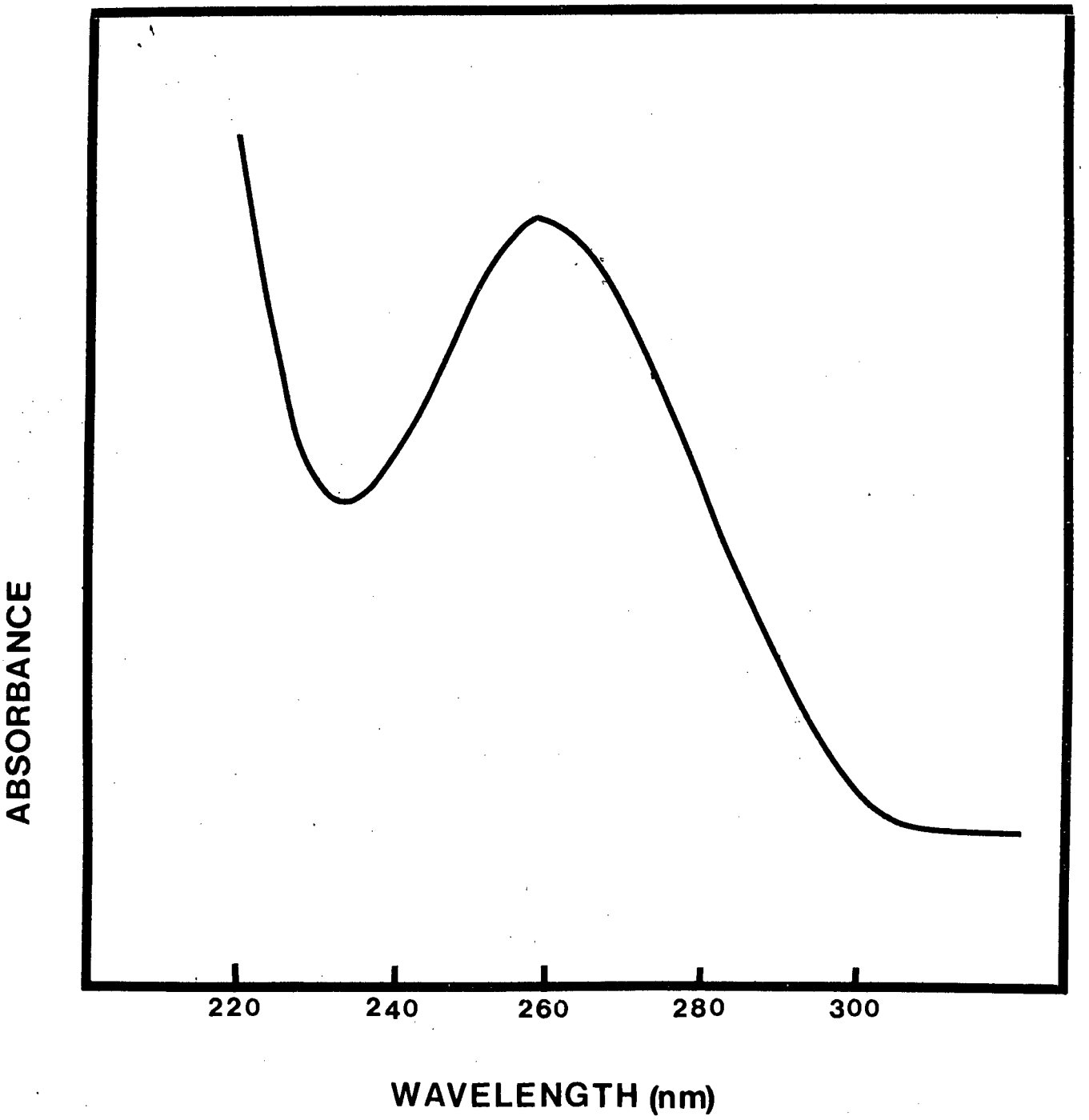


FIGURE 5 : An ultra-violet absorption scan of CMV-Tob purified by the method of Mossop et al., (1976).

Electron microscopic examination of purified preparations showed spherical particles with dark staining cores. The ultra-violet absorption profiles after separation by gradient centrifugation in 0,1M sodium phosphate buffer pH 8,0 showed a single homogenous peak which sedimented faster than brome mosaic virus (BMV) as shown in Figure 6.

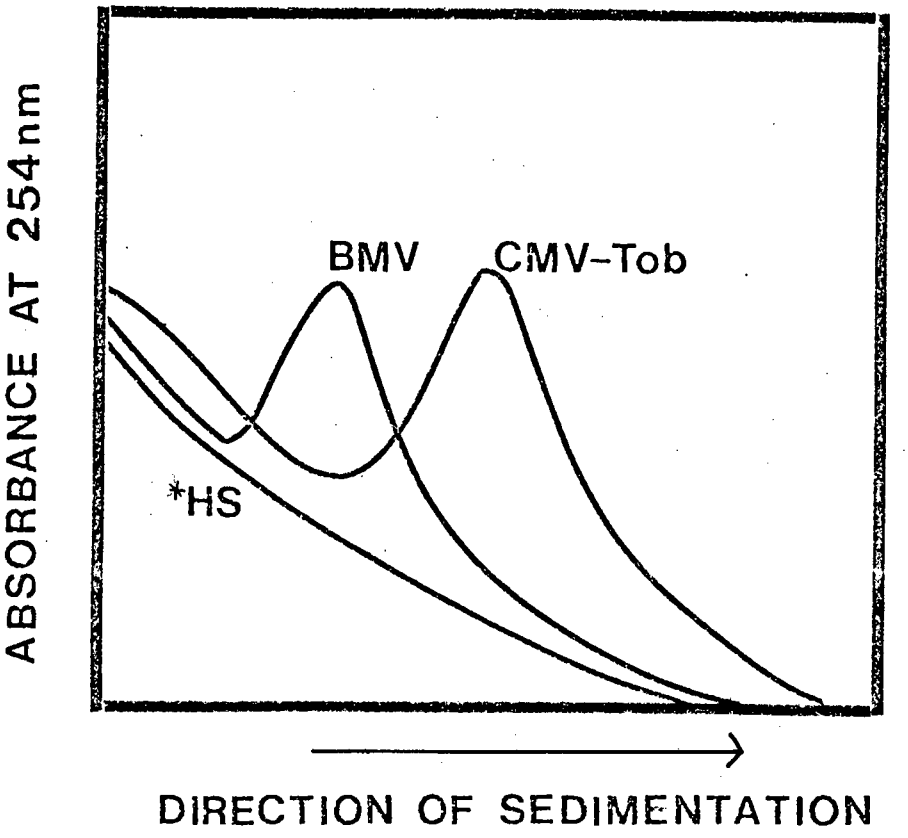


FIGURE 6 : A sucrose density gradient profile of BMV, CMV-Tob and healthy tobacco sap extracted as for CMV, at pH 6,5. *HS = Healthy sap.

E. VIRUS STABILIZATION

As expected from the literature (Kaper & Waterworth, 1981), it was found that the CMV isolates were highly unstable. To preserve particle structure in purified virus preparations used for immunization, serological tests and electron microscopy, it was necessary to chemically stabilize the particles.

A modified method based on the work done by Francki et al. (1966) for CMV stabilization was used (III.E).

Briefly, the purified virus preparations were dialysed against 5 mM borate buffer, pH 9,0 containing 2% formaldehyde (FA) for 12 hours at 4°C followed by another cycle of dialysis in 5 mM borate buffer, pH 9,0, containing 0,2% FA for 12 hours at 4°C. The stability of the FA-treated CMV-Tob was compared with that of the untreated CMV-Tob in electron microscopic studies. After 2 weeks no intact virions were detected in samples of untreated CMV-Tob whereas intact particles could be detected after the same period in FA-treated samples of CMV-Tob as illustrated in Figs. 7a and 7b.

FA-treated preparations of CMV-Tob and CMV-Is formed curved precipitin bands in double immunodiffusion test against anti-CMV-Tob antiserum. The various antisera used in this project are listed in Table 6. The untreated virus preparation of CMV-Tob formed a thick protein band in the same test as illustrated in Figure 8.

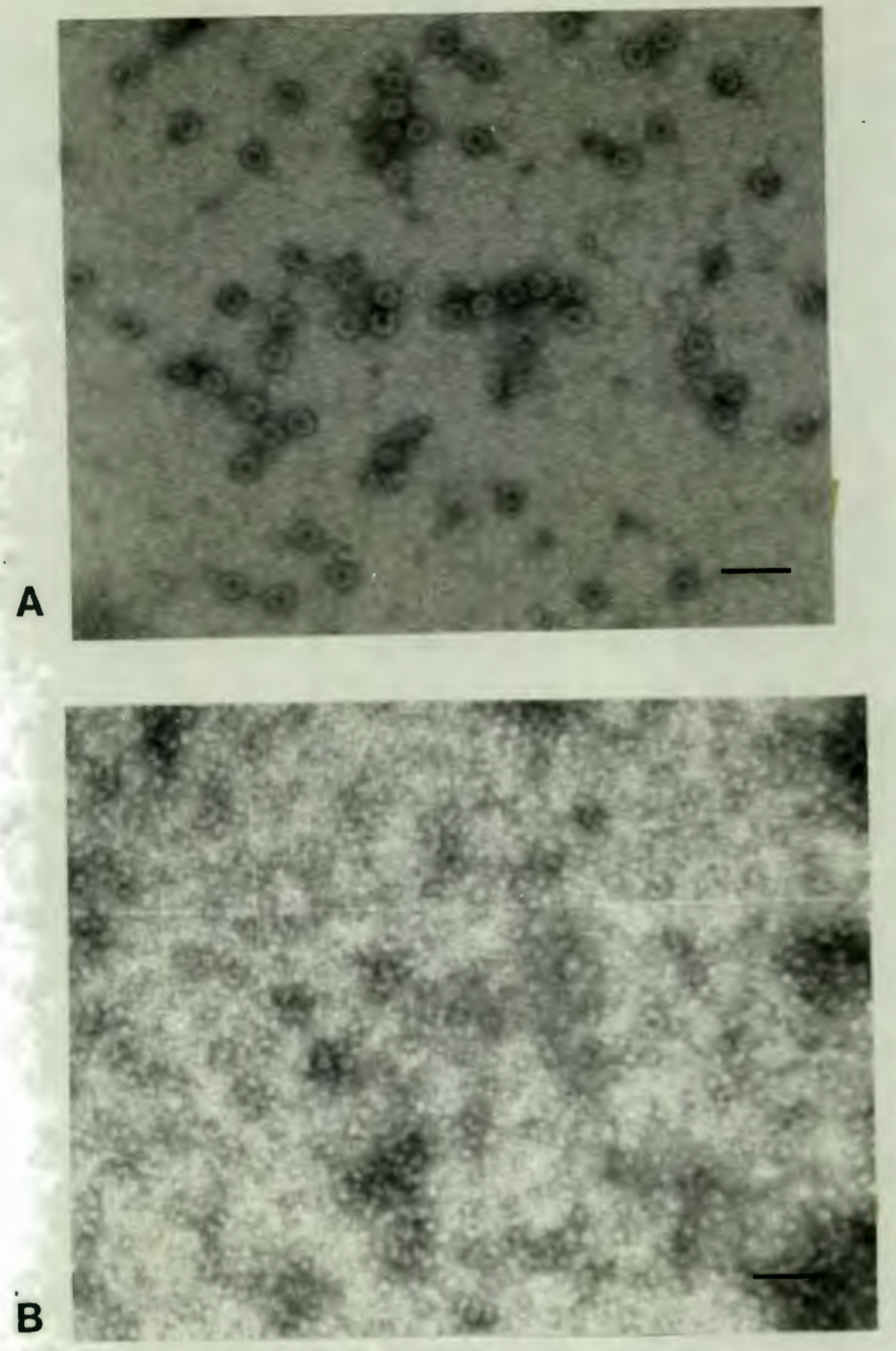


FIGURE 7 : Electron micrographs of purified CMV-Tob in 0,005M borate buffer, pH 9,0:

A. Stabilised with 2% formaldehyde, and

B. Same virus preparation unstabilised two weeks after extraction. The specimens were stained with 2% uranyl acetate. The size bar represents 100 nm.

FIGURE 8 : An Ouchterlony test showing the effect of formalinisation on CMV particles.

Wells a & b: Non-formalinised CMV-Tob in 0,005M borate buffer, pH 9,0.

Wells c & d: Formalinised CMV-Is in 0,005M borate buffer containing 2% FA.

Wells e & f: Formalinised CMV-Tob in 0,005M borate buffer containing 2% FA.

Well g : Antiserum to CMV-Tob.

F. ANALYTICAL ULTRACENTRIFUGATION

CMV-Tob was purified on sucrose density gradients and the particles stabilized by dialysing against 0,005 M sodium borate buffer pH 9,0, containing 0,2% formaldehyde. The concentration of the virus preparation was adjusted to 3 mg/mℓ and subsequently examined in the analytical ultracentrifuge.

The virus preparation sedimented as a single major component as shown in Figure 9. The sedimentation coefficient was determined to be 98 S. The value was close to 99 S reported by Francki & Hatta (1980).

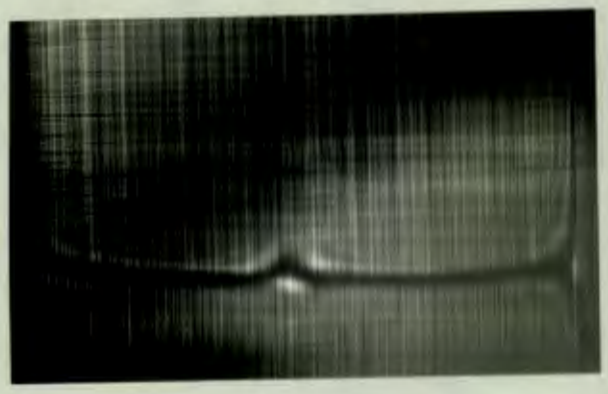


FIGURE 9 : Schlieren photograph of formalinised CMV-Tob in 0,05M sodium phosphate buffer, pH 8,0, sedimented in a Beckman An-D rotor at 30 000 rpm. The photograph was taken 16 minutes after starting. Phase plate angle was 60°.

G. ELECTRON MICROSCOPY

Three types of negative stains were used in electron microscopic studies of CMV, namely, 2% uranyl acetate pH 6,5, ammonium molybdate and phosphotungstic acid pH 7,0. Uranyl acetate gave better negative staining than ammonium molybdate, as illustrated in Figs.2a and b. Phosphotungstic acid was not a suitable negative stain for CMV particles since nothing was detected when CMV preparations were stained with it. The disruption of CMV particles in phosphotungstic acid and the suitability of uranyl acetate has been previously reported (Tolin, 1977; Francki et al., 1979; Francki & Hatta, 1980).

H. ANTISERUM PRODUCTION

For the production of antisera, rabbits were immunized (III.H.1a) with FA-treated virus preparations (see IV.c). The virus preparations were injected at two levels of purity, namely (i) purified by two cycles of differential centrifugation (III.B.b and IV.B.2(iii)) and by rate zonal centrifugation (III.B.c and IV.B.2(iii). Details of antisera produced are listed in Table 6.

In addition to the antisera produced in our own animal unit, the following antisera were made available to me:

1. Anti-CMV-Y obtained from Professor M.B. Von Wechmar (Stellenbosch).

2. Anti-CMV-Q was obtained from Dr. R.I.B. Francki, University of Adelaide, Australia.

3. Anti-CMV-antiserum that was unidentified was also obtained from Dr. R.I.B. Francki.

4. Anti-TAV was obtained from the American Type Culture Collection (ATCC 127)

5. Anti-CMV-S was also obtained from ATCC (ATCC 242A)

TABLE 6 : Details of Antisera Produced in Rabbits

Antiserum	Immunogen Used	Reciprocal titre Determined in Ouchterlony double-diffusion gels	Reaction with healthy sap	
			Ouchterlony	ELISA
Anti-CMV-Tob 1[7]	CMV-Tob (purified by D.C[4]&S.G.C[5])	256 (36)[1]	N.R.[2]	N.R.
Anti-CMV-Tob 2[7]	- do -	256 (36)	N.R.	N.T.[3]
Anti-CMV-Is	CMV-Is, D.C.	128 (30)	N.R.	R[6]
Anti-CMV-Imp-1	CMV-Imp D.C.	64 (5)	N.R.	N.R.
Anti-CMV-Imp-2	CMV-Imp D.C.	64 (4)	N.R.	N.T.
Anti-CMV-Glad-SA	CMV-Glad-SA. D.C.	64 (4)	N.R.	N.T.
Anti-CMV-Nel	CMV-Nel, D.C.	32 (4)	N.R.	N.T.

1. ( ) time after initial immunisation in weeks.
2. N.R. No reaction.
3. N.T. Not tested.
4. D.C. Differential centrifugation.
5. S.G.C. Sucrose gradient centrifugation.
6. R. Positive reaction .
7. 1 & 2 refers to 2 different rabbits immunised with the same antigen.

I. SEROLOGY

1. Double Immunodiffusion in Agar Gel (Ouchterlony)

Agar plates for tests were made with a variety of buffers and were used to determine the optimum conditions for immune precipitate (precipitin) formation. The following factors were varied: pH, buffer composition, salt concentration and agar concentration. Table 7 shows the various buffer combinations that were tested using CMV-Is and anti-CMV-Is antiserum.*

The precipitin reaction in agar plates made with buffers 2, 3, 4, 5 and 7 were indistinguishable from one another. Any of the five could be used for the Ouchterlony test. No bands were detected in agar plates made up in Tris/HCℓ (1), phosphate/Leonil (6) and double distilled water (8). The most favoured combination was No.5 containing 0,1% sodium azide as preservative.

(a) Titre determinations

Antisera to the CMV isolates had titres of between $^1/_{32}$ and $^1/_{256}$ in Ouchterlony tests when tested at optimal proportions. The optimal proportions were determined for the relationship studies. These did not vary for the antisera against the different CMV isolates. For antisera with an endpoint titre of $^1/_{128}$, optimal ratios for precipitin formations were normally at 2 mg/mℓ virus concentration and a $^1/_{16}$ serum.

* Ouchterlony tests were performed according to van Regenmortel (1966).

TABLE 7 : Double Immunodiffusion in Various Agar Gel Plates (Ouchterlony)

Buffer	pH	NaCℓ concentration	Agar concentration
1. 0,05 M Tris/HCℓ	7,0	0,074 M	0,70%
2. 0,025 M sodium borate	8,0	0,074 M	0,70%
3. 0,05 M sodium phosphate	6,0	0,074 M	0,70%
4. 0,05 M sodium phosphate	7,0	0,074 M	0,75%
5. 0,05 M potassium phosphate	7,0	0,074 M	0,70%
6. 0,05 M potassium phosphate/ 0,05% leonil	7,0	0,074 M	0,70%
*7. 0,5 M sodium phosphate	7,0	0,074 M	0,70%
8. Double distilled water	7,0	Nil	1% Agarose

*Miller & Golder (1950)

(b) Relationship studies

An attempt was made to demonstrate serological similarities and differences among CMV isolates and strains by investigating "spur" formation in agar double diffusion tests. This technique has been used previously by Rao et al. (1982) for a similar purpose. Mixtures of two antisera were reacted with the homologous virus preparations loaded in separate wells adjacent to the antiserum well. These tests showed a distinct serological difference between virus isolates CMV-Is and CMV-Nel by the formation of a single spur. However, CMV-Tob, CMV-Imp and CMV-Glad-SA were indistinguishable from each other. CMV-Is and CMV-Nel could not be differentiated from any of the other three isolates. CMV-Q and CMV-K had a tendency to degrade in agar gels thus making it difficult to study relationships between the individual isolates and the two strains. Figure 10 illustrates precipitin reactions between CMV isolates and heterologous antisera in Ouchterlony tests.

2. Double Antibody "Sandwich" Enzyme-Linked Immunosorbent Assays (DAS-ELISA)

DAS-ELISA as described by Clark and Adams (1977), was used to investigate serological relationships among the virus isolates CMV-Tob, CMV-Is, CMV-Nel, CMV-Imp and CMV-Glad-SA and strains CMV-K and CMV-Q. Coating antibodies and enzyme-conjugated antibodies to four of these viruses were used, namely, CMV-Tob, CMV-Is, CMV-Imp and CMV-Q.

Anti-CMV-Tob and anti-CMV-Is antisera were used at a concentration of 2,5 µg/mℓ for both coating and enzyme-conjugated antibodies. Anti-CMV-Imp antibodies were used at 10 µg/mℓ and anti-CMV-Q antibodies at 5 µg/mℓ. The CMV antigens under investigation were used at a starting concentration of 1 mg/mℓ followed by

10(a):

Well 1 : CMV-Tob
Well 2 : CMV-Is
Well 3 : anti-CMV-Tob
+ anti-CMV-Is

10(b):

Well 1 : CMV-Tob
Well 2 : CMV-Nel
Well 3 : anti-CMV-Tob
+ anti-CMV-Nel

10(c):

Well 1 : CMV-Tob
Well 2 : CMV-Imp
Well 3 : anti-CMV-Tob
+ anti-CMV-Imp

10(d):

Well 1 : CMV-Tob
Well 2 : CMV-Glad
Well 3 : anti-CMV-Tob
+ anti-CMV-Glad

10(e):

Well 1 : CMV-Nel
Well 2 : CMV-Glad
Well 3 : anti-CMV-Nel
+ anti-CMV-Glad

10(f):

Well 1 : CMV-Is
Well 2 : CMV-Nel
Well 3 : anti-CMV-Is
+ anti-CMV-Imp

10(g):

Well 1 : CMV-Is
Well 2 : CMV-Imp
Well 3 : anti-CMV-Is
+ anti-CMV-Imp

10(h):

Well 1 : CMV-Is
Well 2 : CMV-Glad
Well 3 : anti-CMV-Is
+ anti-CMV-Glad

10(i):

Well 1 : CMV-Imp
Well 2 : CMV-Nel
Well 3 : anti-CMV-Imp
+ anti-CMV-Nel

10(j):

Well 1 : CMV-Imp
Well 2 : CMV-Glad
Well 3 : anti-CMV-Imp
+ anti-CMV-Glad

FIGURE 10 (a)-(j) : Ouchterlony tests between CMV isolates. The virus antigens were adjusted to a concentration of 1,5 mg/mℓ. The antisera were adjusted to optimal proportions with the homologous antigens. (See Table 6)

6-fold serial dilutions. Tobacco mosaic virus (TMV) and extracts prepared from healthy tobacco plants (extracted as for CMV) were used for negative controls.

The enzyme-substrate reaction step was left to develop for an hour at room temperature. The absorbances of the well contents were subsequently read using a 405 nm filter in a titertek Multiskan plate reader (Flow Laboratories). The results were expressed graphically as semilogarithmic plots of A_{405} versus reciprocal antigen dilution factors as illustrated in Figs.11, 12, 13 and 14. Table 8 below highlights the extent of relationships among the CMV isolates and between the Australian strain Q and the CMV isolates. The graphs were drawn from results of several trays.

TABLE 8 : Serological Relationships among CMV Isolates and Strains

	ANTISERA			
ANTIGENS	Anti-CMV-Tob	Anti-ACMV-Is	Anti-ACMV-Imp	Anti-CMV-Q
CMV-Tob	5	4	3	4
CMV-Is	2	5	4	2
CMV-Imp	3	4	5	3
CMV-Q	1	3	2	5
CMV-Nel	4	3	4	3
CMV-Glad-SA	4	2	2	1

*FOOTNOTE: Rating: 1 = Weak; 5 = Strong. Based on colour intensities of the reactions. Stabilised virus was used in all tests.

From the data it could be concluded that CMV-Tob, CMV-Is and CMV-Imp were closely related. CMV-Nel was closely related to CMV-Tob and CMV-Imp, and CMV-Q was distantly related to all the isolates. CMV-Glad-SA was also distantly related to all the CMV isolates and strain Q. The CMV isolates and strain Q are different as the homologous reaction is markedly better in all cases.

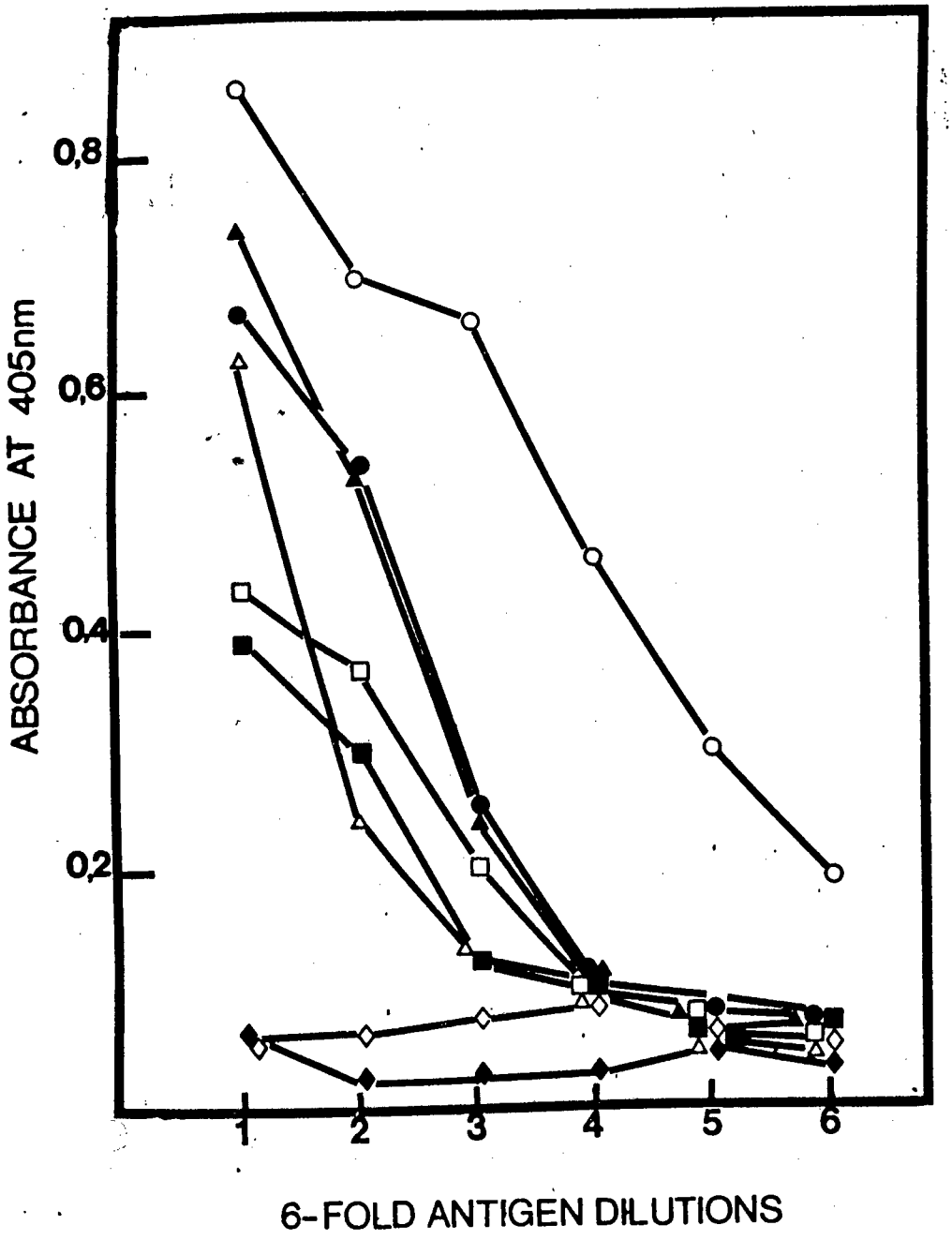


FIGURE 11 : Reactions of CMV isolates and the CMV-Q strain with anti-CMV-Tob antiserum in DAS-ELISA. Initial concentration of virus samples was 1 mg/mℓ, coating antibody and conjugate concentrations were 2,5 ug/mℓ.

◇ Represents healthy tobacco sap
◆ " TMV
○ " CMV-Tob
■ " CMV-Is
● " CMV-Nel
□ " CMV-Imp
▲ " CMV-Glad-SA
△ " CMV-Q

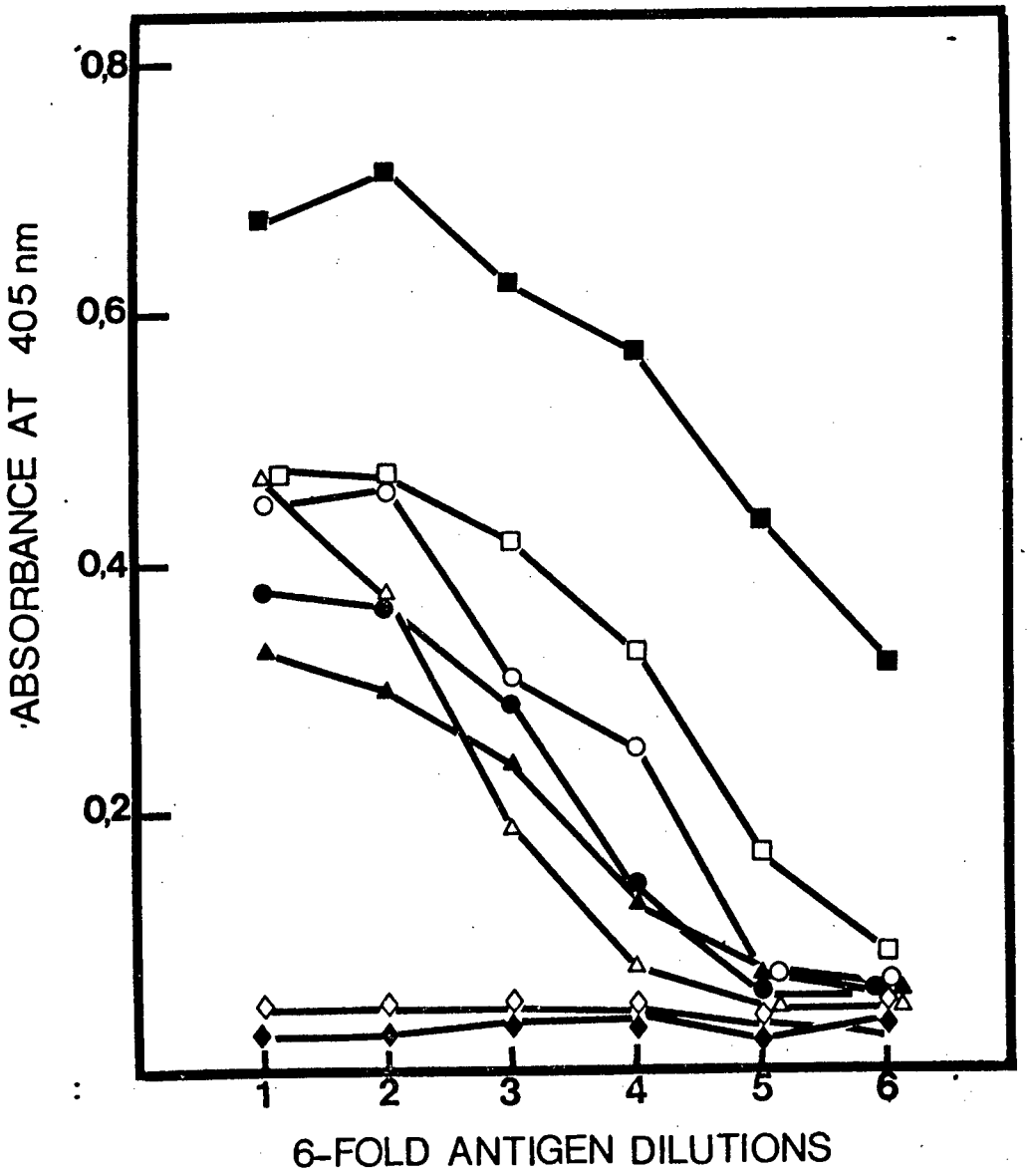


FIGURE 12 : Reactions of CMV isolates and the CMV-Q strain with anti-CMV-Is antiserum in DAS-ELISA. The starting concentration of the antigens was 1 mg/mℓ, and the coating/conjugate antibody concentration was 2,5 ug/mℓ.

◇ Represents healthy tobacco extract
◆ " TMV
○ " CMV-Tob
■ " CMV-Is
● " CMV-Nel
□ " CMV-Imp
▲ " CMV-Glad-SA
△ " CMV-Q

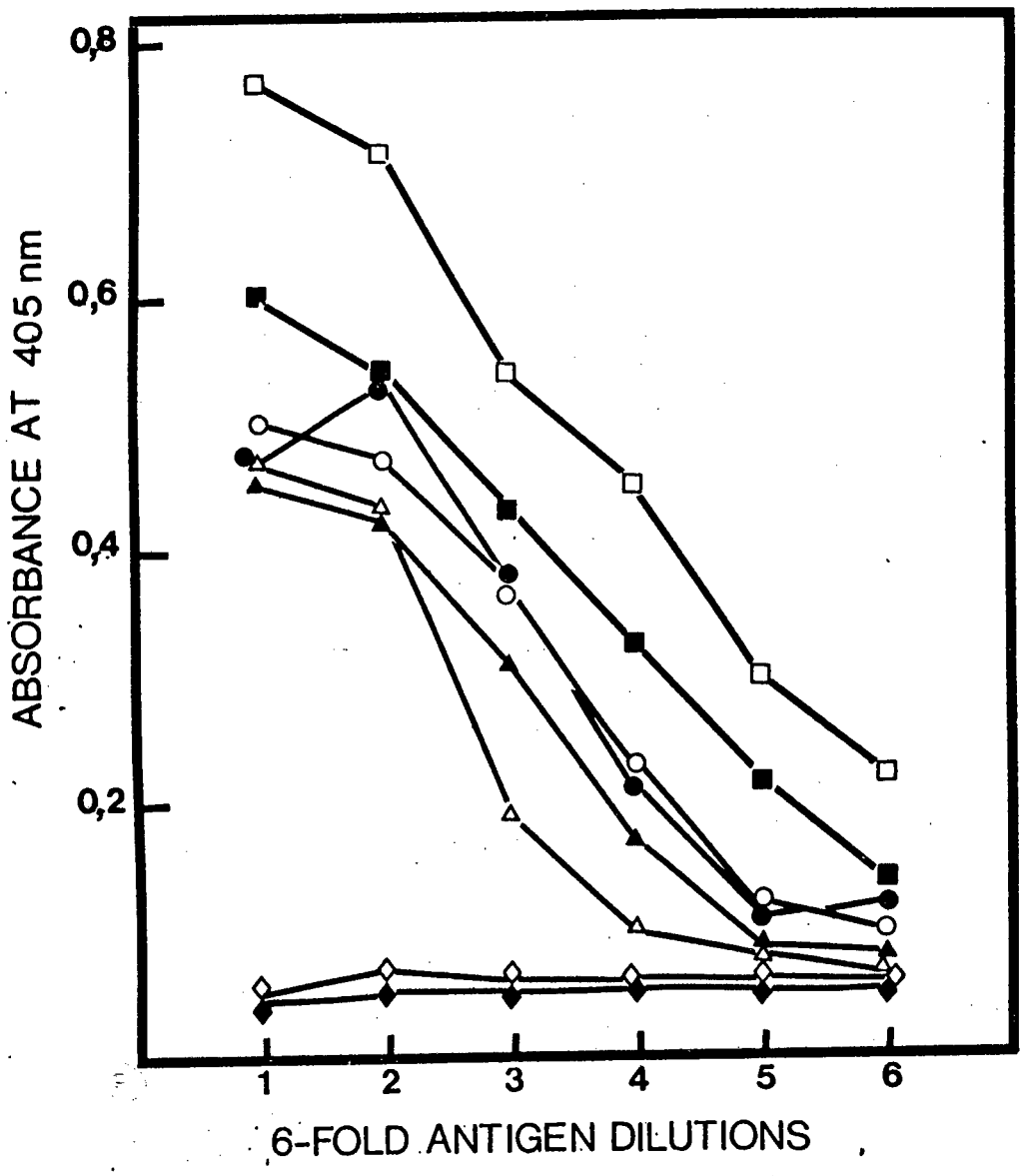


FIGURE 13 : Reactions of CMV isolates and the CMV-Q strain with anti-CMV-Imp antiserum in DAS-ELISA. The starting concentration of the antigens was 1 mg/mℓ, and the coating/conjugate antibody concentration was 6,7 ug/mℓ.

◇ Represents healthy tobacco extract
◆ " TMV
○ " CMV-Tob
■ " CMV-Is
● " CMV-Nel
□ " CMV-Imp
▲ " CMV-Glad-SA
△ " CMV-Q

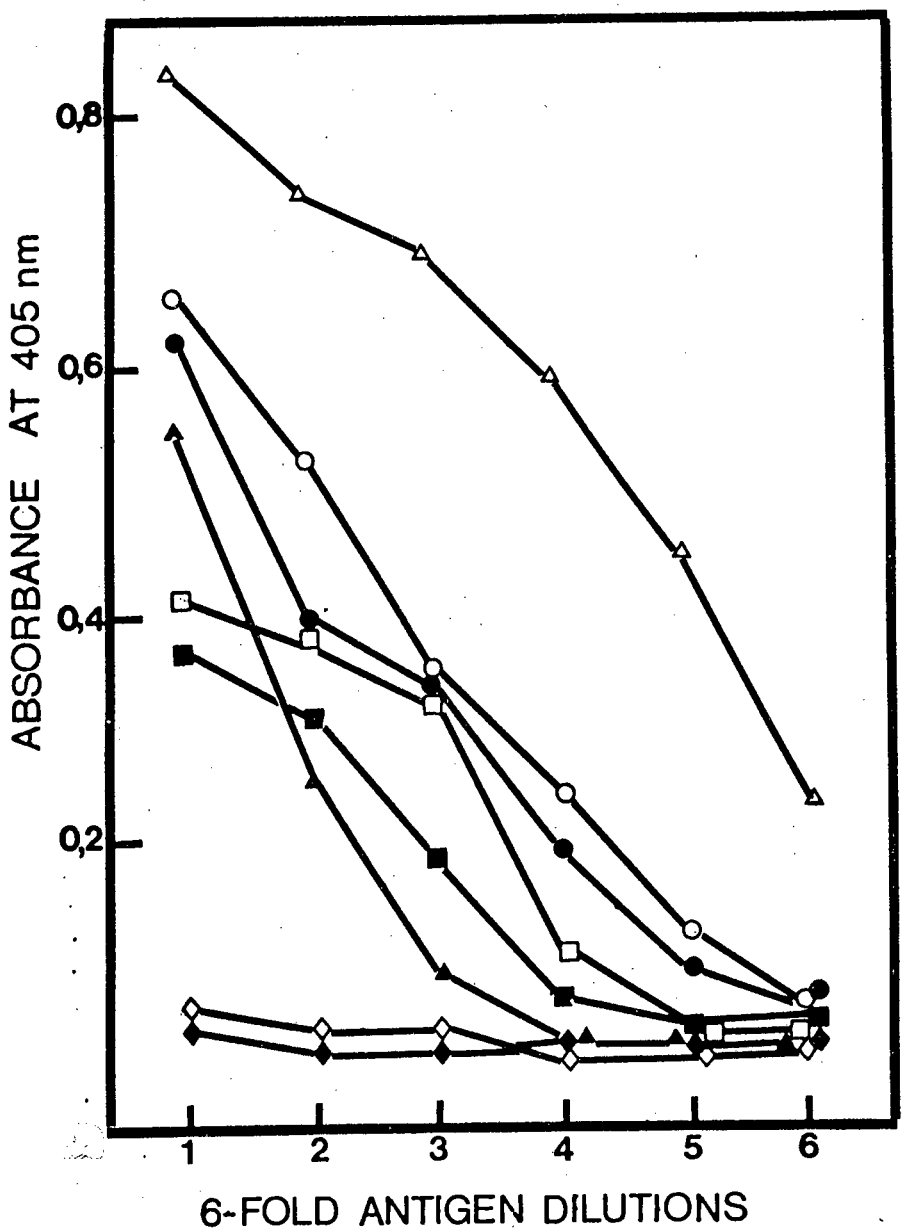


FIGURE 14 : Reactions of CMV isolates and the CMV-Q strain with anti-CMV-Q antiserum in DAS-ELISA. Initial concentration of antigens was 1 mg/mℓ. Coating/conjugate antibody concentration was 4,0 ug/mℓ.

◇ Represents healthy tobacco extract
◆ " TMV
○ " CMV-Tob
■ " CMV-Is
● " CMV-Nel
□ " CMV-Imp
▲ " CMV-Glad-SA
△ " CMV-Q

3. Immuno Electron Microscopy

Due to the instability of the CMV particle structure immuno-electron microscopy was found to be unsuitable as a method of studying serological relationships.

J. ELECTROPHORESIS

1. Zone Electrophoresis

CMV-Tob and CMV-Is were the only isolates to be purified by using zone electrophoresis. The R_{ϕ} values obtained for the two CMV isolates compared favourably with the R_{ϕ} values determined by van Regenmortel (1968) for CMV-S and CMV-Y as shown in Table 9.

TABLE 9 : Zone Electrophoresis R_{ϕ} Values of CMV

CMV-Isolate	Zone Electrophoresis R_{ϕ} Values
CMV-S	0,53 (Van Regenmortel, 1968)
CMV-Y	0,53 (Van Regenmortel, 1968)
CMV-Tob	0,55 ± 0,02*
CMV-Is	0,54 ± 0,02*

*FOOTNOTE: R_{ϕ} values for CMV-Tob and CMV-Is are average results obtained in four separate determinations.

2. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Virion Proteins

Semi-purified virus preparations after the second cycle of differential centrifugation (III.B.4b) were prepared as in III.D.1 and analysed on 12% acrylamide gels. Pharmacia LMW standards were used as molecular weight markers. Table 10 lists the standards together with their molecular weights.

TABLE 10 : Molecular Weights of Molecular Weight Markers

Standard*	Molecular Weight (daltons)
Phosphorylase B	94 000
Bovine Serum Albumin	68 000
Ovalbumin	43 500
Carbonic anhydrase	30 000
Soybean trypsin inhibitor	20 100
α-Lactalbumin	14 400

*Pharmacia LMW standards

20-25 µℓ of samples were usually loaded onto the gels and electrophoresis carried out at 8 mA/gel for 14-16 hours. The gels were stained, destained, dried and photographed as described in III.D.1.

Figure 15 shows the electrophoretic profiles of the South African CMV isolates. Table 11 lists the molecular weights of the various isolates. CMV-Nel had the lowest molecular weight at 24 800 daltons, CMV-Impatiens at 26 500 daltons had the highest molecular weight and all the other isolates had a molecular weight of 25 000 daltons. All the molecular weights were in good agreement with the value cited in literature (24 500 daltons) for CMV (Francki et al., 1979; Matthews, 1982) except for CMV-Imp.

a

b

c

d

e

f

g

h

i

j

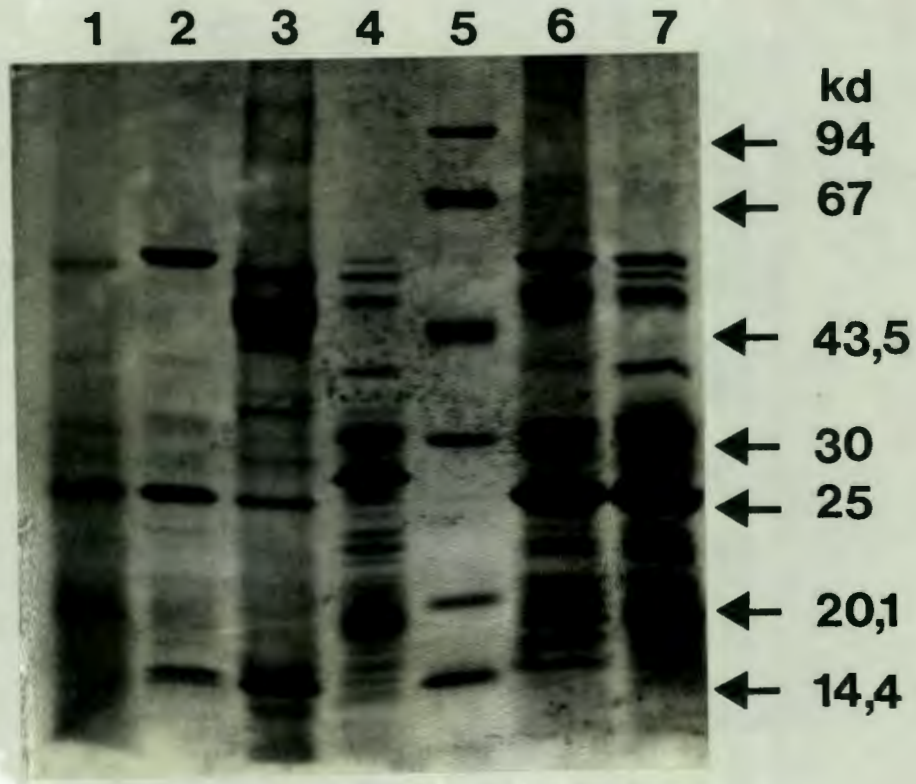


FIGURE 15 : SDS-polyacrylamide gel stained with Coommassie brilliant blue.

Track 1 : CMV-Tob

Track 2 : CMV-Is

Track 3 : CMV-Nel

Track 4 : CMV-Imp

Track 5 : Pharmacia LMW molecular weight markers

Track 6 : CMV-Glad-SA

Track 7 : CMV-Lup-K5

TABLE11 : Molecular Weights of CMV Isolates and Strains

CMV Strain/ Isolate	Protein Coat Molecular Weight (Daltons)	Reference
CMV-S	24 500	Van Regenmortel (1972)
CMV-Q	24 500	Habili & Francki (1974a)
CMV-S	24 000	Matthews (1982)
CMV-Tob	25 500*	
CMV-Is	25 000*	
CMV-Nel	24 800*	
CMV-Imp	26 500*	
CMV-Glad-SA	25 000*	
CMV-Lupine K5	25 000*	

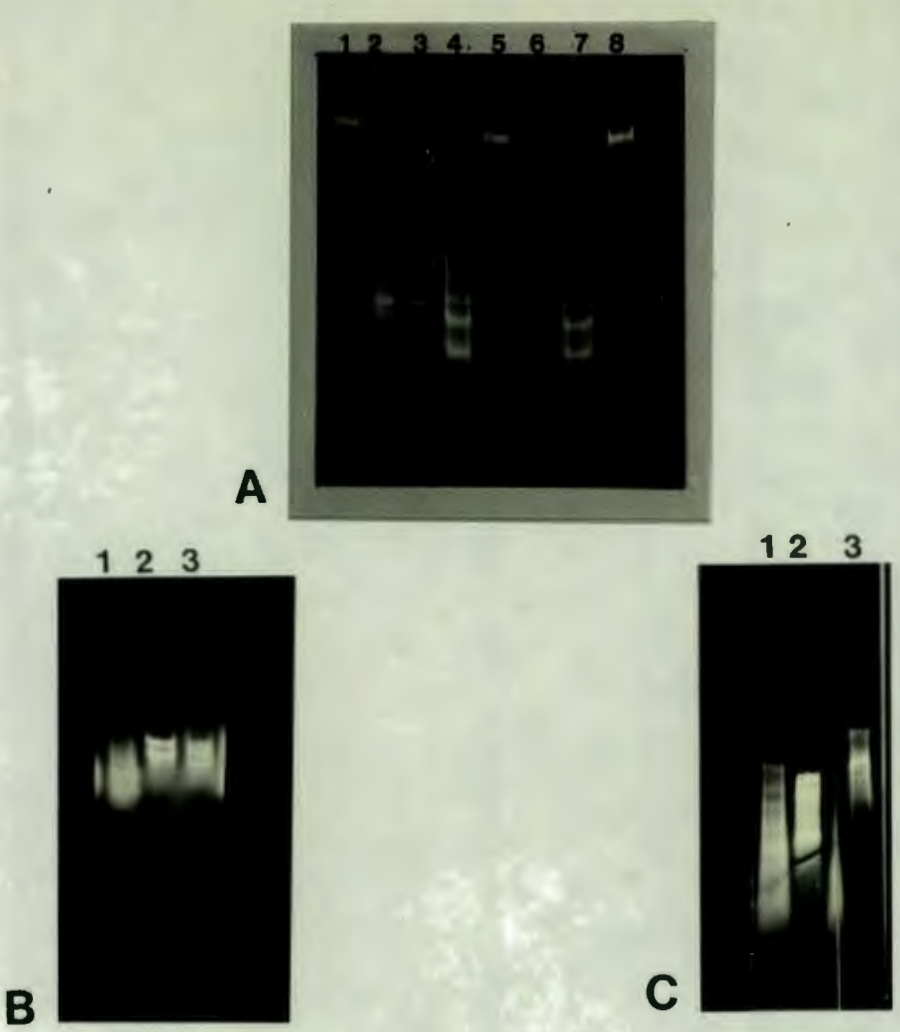
*Four molecular weight determinations were performed.

3. Electrophoresis of RNA on Agarose Gels

The virus used for the determination of RNA species in CMV was purified from both tobacco and squash plants immediately prior to electrophoresis in order to minimise nuclease degradation of virion RNA. CMV-Tob, CMV-Is and CMV-Imp were the virus isolates used in this analysis. The sample was pretreated (see III.D.2) before loading onto gels.
The RNA was analysed on 1,5% agarose gels as described in III.D.2. Tobacco mosaic virus (TMV), BMV and *E. coli* ribosomal RNAs 16S and 23S were used as internal markers.

CMV-Tob and CMV-Is, both of which were purified from squash (*C.pepo*) plants, produced four presumptive RNA bands in agarose gels as shown in Fig.16a. CMV-Imp, which was purified from Tobacco (*N. tabacum* cv Soulouk) produced five bands (Fig.16b). Sometimes more than five bands were observed as in Fig.16c.

The molecular weights of the CMV-RNA bands seemed to be almost similar to those of BMV-RNA bands on comparison. This would be in fairly good agreement with the values in literature. There was difficulty in measuring the molecular weights because a proper calibration curve from the RNAs used as standards could not be obtained.



FIGURES 16 A, B & C : Viral RNA electrophoresed on 1,5% agarose gels and stained with ethidium bromide. See Methods and Materials section C.2 for procedures. Cathode is to the top in each case.

A. Track 1,5 & 8 : TMV-RNA
Track 2 : CMV-Is-RNA
Track 3 : BMV-RNA
Track 4 & 7 : CMV-Tob-RNA

B. Track 1 : CMV-Imp-RNA
Track 2 : *E.coli* ribosomal RNA
Track 3 : BMV-RNA

C. Track 1 : CMV-Imp-RNA
Track 2 : BMV-RNA
Track 3 : TMV-RNA

K. ENZYME-IMMUNOELECTROBLOTTING OF VIRUS COAT PROTEINS

The enzyme-immune electroblotting technique as described by Rybicki and von Wechmar (1982) was used to investigate serological relationships among the CMV isolates used in this study. The method is described in III.H.5.

Proteins resolved in SDS-PAGE gels were transferred onto nitrocellulose sheets and these were subsequently used in the selective detection of the viral coat protein bands of CMV as described elsewhere (see III.H.5). When the substrate reaction was sufficiently intense, the nitrocellulose sheets were dried and photographed. Figure 17 shows some of the electroblots of CMV isolates comparatively detected using antisera to various CMV isolates and tomato aspermy virus (TAV).

Host adsorbed antisera to isolates CMV-Tob, CMV-Is, CMV-Imp, CMV-Glad-SA and strains CMV-S, and CMV-Y and an Italian tomato aspermy virus (TAV) strain were used for homologous and heterologous reactions with isolates, CMV-Tob, CMV-Is, CMV-Nel, CMV-Imp, and CMV-Glad-SA in all gels. Strains CMV-Q and CMV-K were used as standards for comparison. The virus preparations were used at a concentration of 2 mg/mℓ and 25 µℓ amounts of each isolate and strain were loaded into the wells. An extract from healthy tobacco plants was used as a control, but no protein bands were observed at the 25 kilodalton position for any of the antisera used. Table 12 illustrates the relative intensity of the reactions between the various CMV isolates and strains and the various antisera. This is further illustrated in Figs.17A-H.

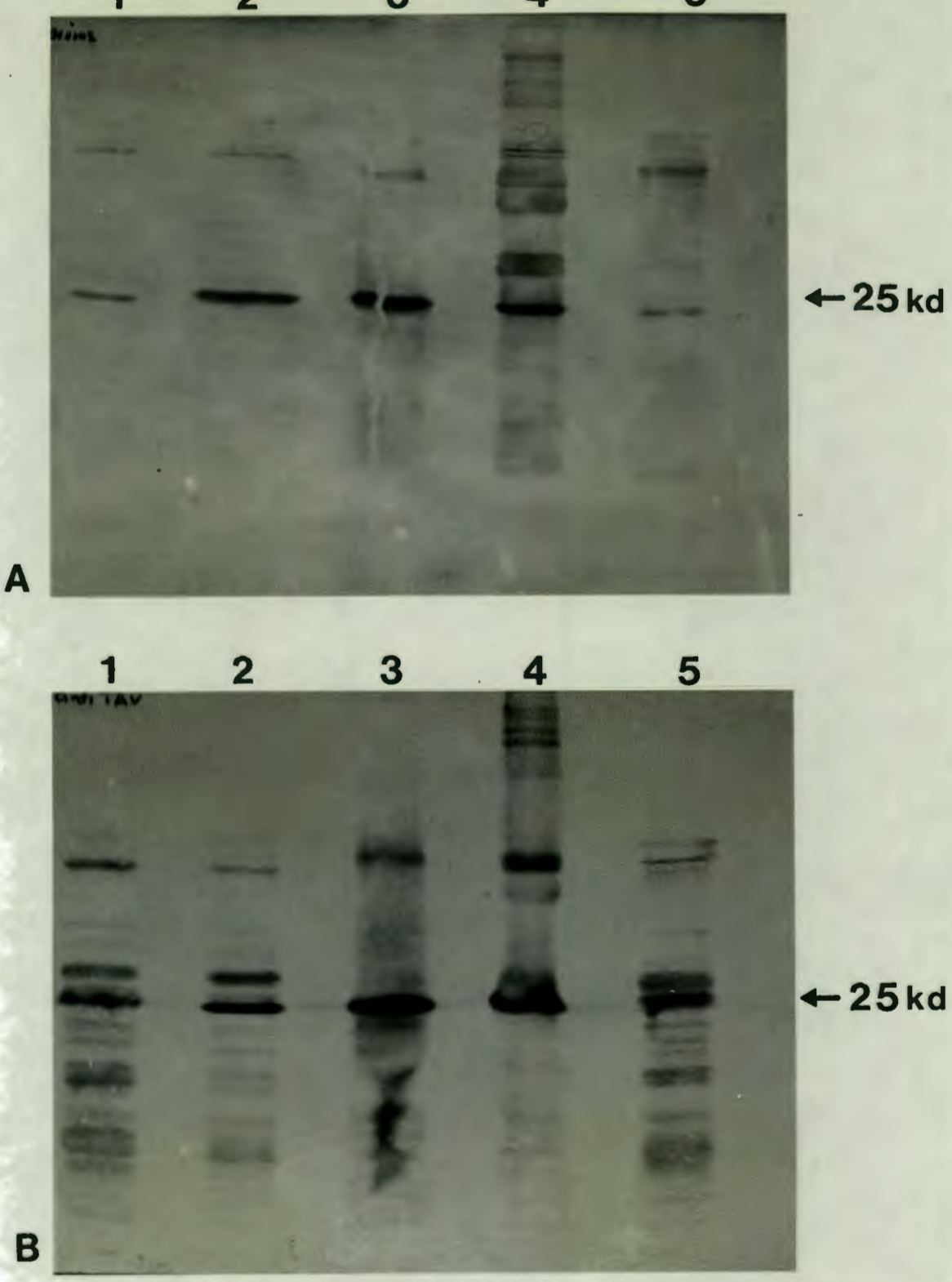


FIGURE 17 A & B : Blots of CMV strains and isolates probed with:
A. Anti-CMV-Tob antiserum B. Anti-TAV antiserum

Track 1 : CMV-K
Track 2 : CMV-Q
Track 3 : CMV-Tob
Track 4 : CMV-Nel
Track 5 : CMV-Is

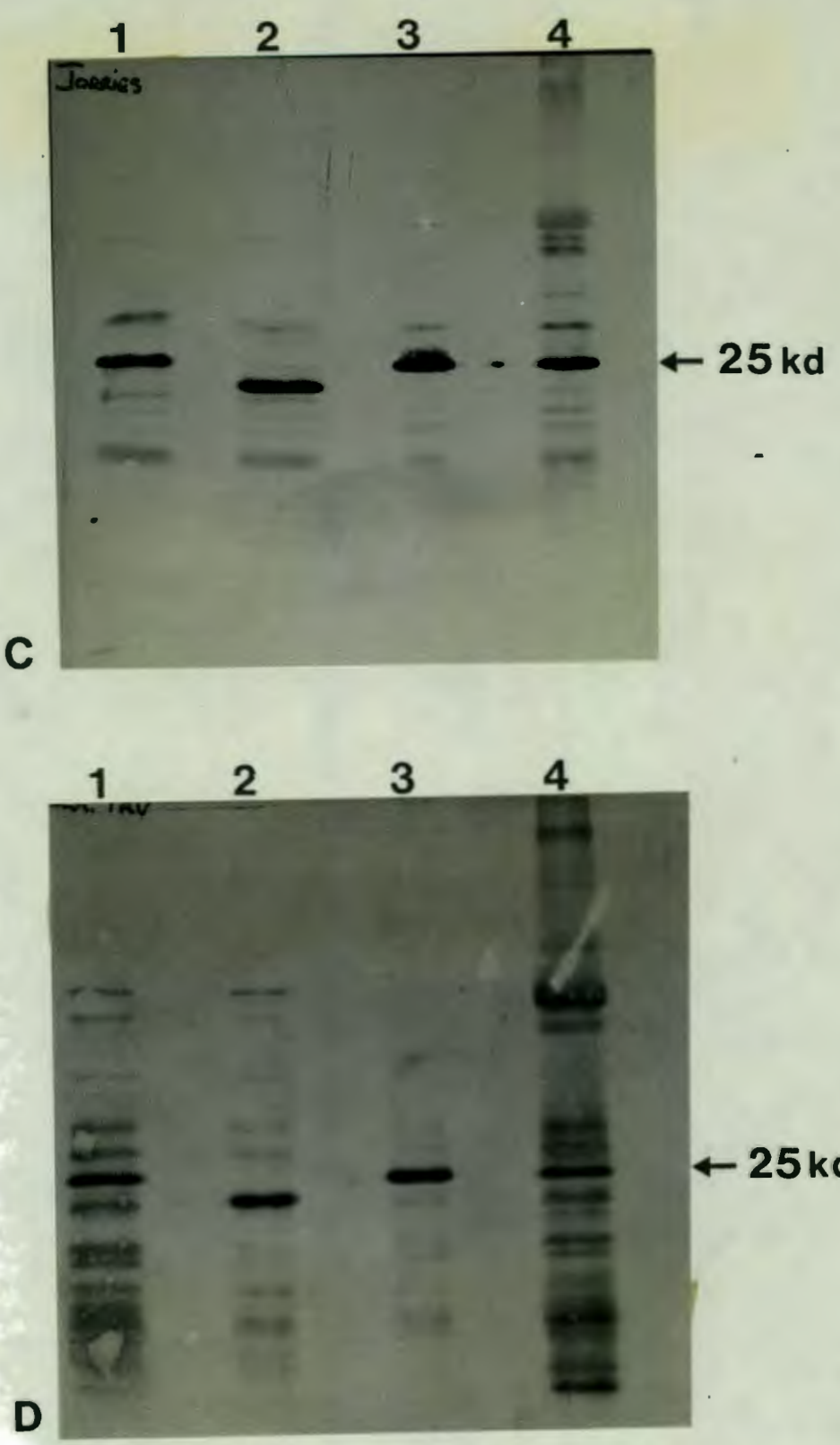


FIGURE 17 C & D : Blots of CMV strains and isolates probed with:
C. Anti CMV-Tob antiserum D. Anti-TAV antiserum.

Track 1 : CMV-K
Track 2 : CMV-Q
Track 3 : CMV-Imp
Track 4 : CMV-Glad-SA

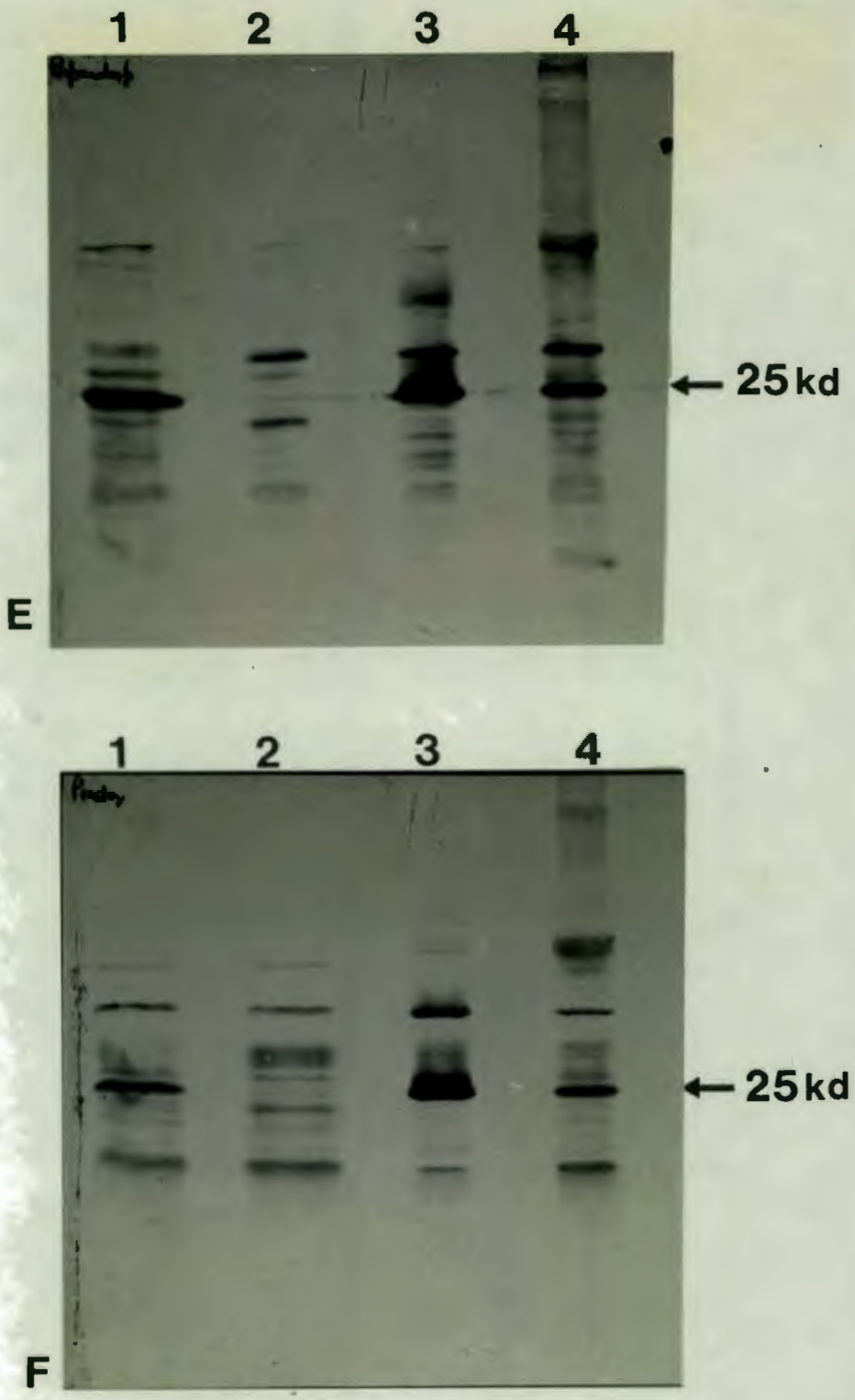
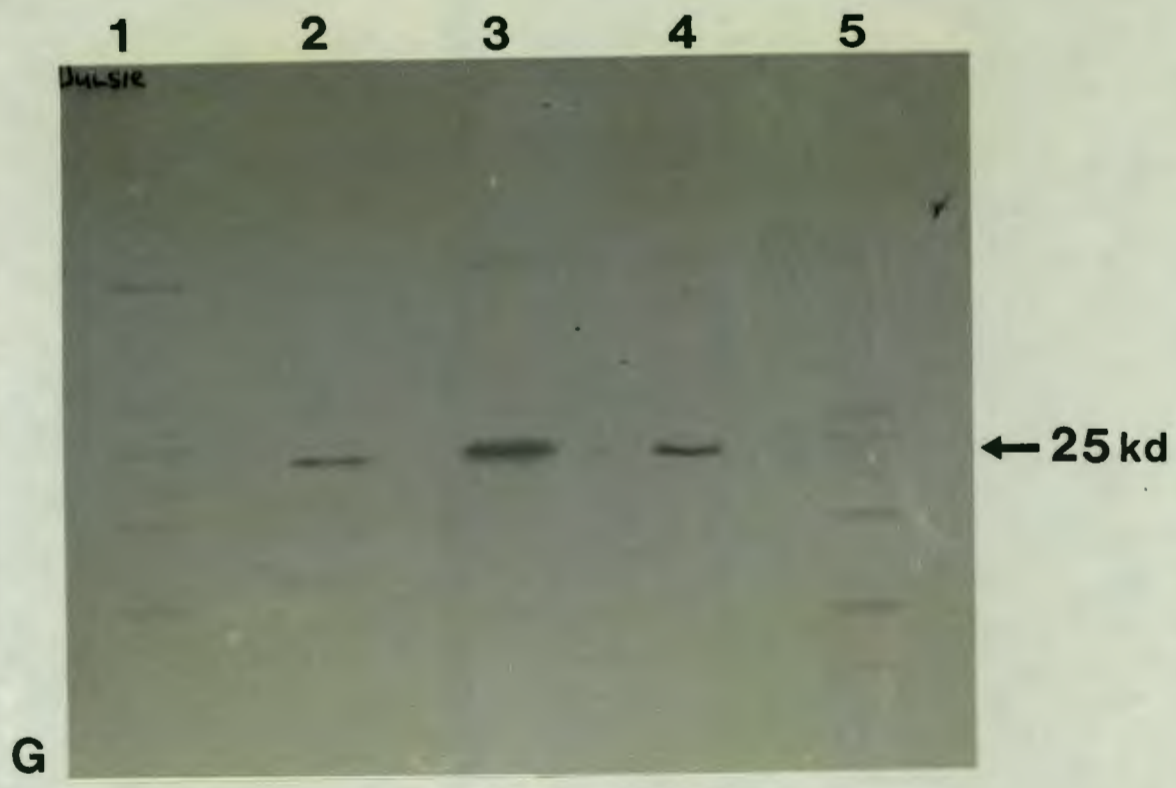


FIGURE 17 E & F : Blots of CMV strains and isolates probed with:
E. Anti-CMV-Is antiserum; F. Anti CMV-Imp antiserum
Track 1 : CMV-K
Track 2 : CMV-Q
Track 3 : CMV-Imp
Track 4 : CMV-Glad-SA



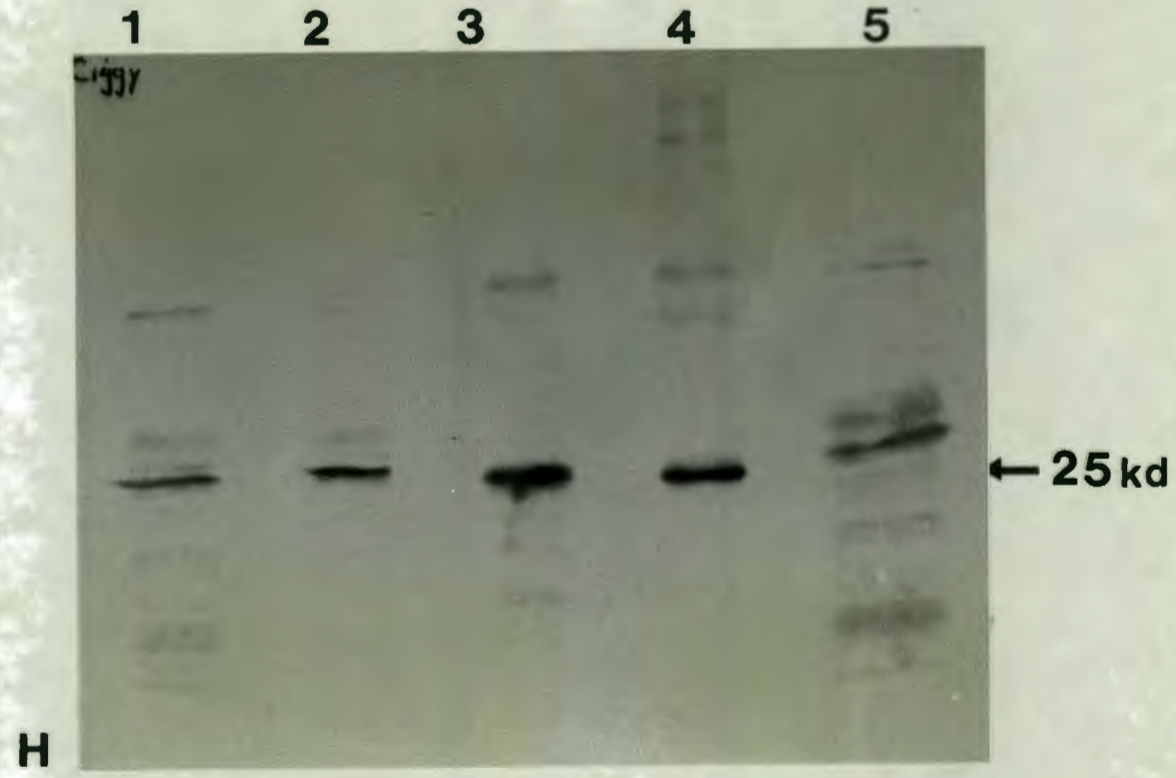


FIGURE 17 G & H : Blots of CMV strains and isolates probed with:
G. Anti-CMV-S antiserum; H. Anti-CMV-Y antiserum

Track 1 : CMV-K
Track 2 : CMV-Q
Track 3 : CMV-Tob
Track 4 : CMV-Nel
Track 5 : CMV-Is

TABLE 12 : Reaction Intensities between CMV Isolates/Strains and Anti-Cucumovirus Antisera in Enzyme-Immuno-Electroblots

	ANTIGENS						
ANTISERA***	CMV-Tob	CMV-Is	CMV-Nel	CMV-Imp	CMV-Glad-SA	CMV-Q	CMV-K
Anti-CMV-Tob-2	*+++	+	++	++	++	++	+
Anti-CMV-Is	++	+++	+	+++	++	+	++
Anti-CMV-Imp-1	+	+	-	+++	++	-	++
Anti-CMV-Glad-SA (EB)**	-	+	-	+	++	-	-
Anti-CMV-S***	++	-	+	+	-	+	-
Anti-CMV-Y***	+++	+	++	++	+	+	+
Anti-TAV***	+++	++	+++	++	+	++	++

*Rating: +++ = Very strong (Relative reaction intensities were assessed by visual inspection of bands on blots).
++ = Strong
+ = Weak
- = Nil

**EB = Early bleeding

***For details of antisera, see Table 6.

The molecular weight of the coat proteins could be determined with the immunoelectroblotting technique. A potentially important observation was that antiserum dilutions that had been used to probe one or more blots occasionally, failed to react with certain of the CMV isolates. For example, anti-CMV-Is that had been used once, failed to react with CMV-Q protein in a second experiment (see Figures 17E & F). This phenomenon was probably due to the removal, on first probing, of a set of antibodies that recognized a particular determinant on CMV-Q protein. It could be surmised that such absorption rendered the antiserum non-reactive with CMV-Q due to the presence of perhaps only one cross-reactive epitope on the protein.

Anti-TAV antiserum, it was observed, strongly reacted with most CMV antigens as shown in Figures 17B & F. This confirms the serological relationship of CMV and TAV strains first described by Devergne and Cardin (1975) and Rao et al. (1982).

Anti-CMV-S, anti-CMV-Y, and anti-CMV-Glad-SA reacted relatively weakly with most antigens (see Figures 17G & H). Blots with anti-CMV-Glad-SA are not shown. The reason for this weak reaction maybe that early bleedings which were still weak were used. Another reason may be that the antiserum used was too dilute and therefore the concentration of the antibodies was too low. (Anti-CMV-S and anti-CMV-Y were gifts and the limited quantity allowed a minimum of tests only.)

Anti-CMV-Tob, anti-CMV-Is antisera and an antiserum to an Australian CMV*strain (gift of Dr. R.I.B. Francki) were compared by electroblotting CMV-Is, CMV-Tob, CMV-Nel, and healthy tobacco leaf extract, as illustrated in Figure 18. Anti-CMV-Tob-2 (see Table 4) reacted weakly with CMV-Is and strongly with both the homologous antigen and CMV-Nel. No band was observed in the healthy leaf extract (extracted as for CMV) track at the same position as for CMV. Anti-CMV-Is reacted with all three antigens. None of the sera reacted significantly with healthy tobacco extract.

(*The identity of this strain was not known).

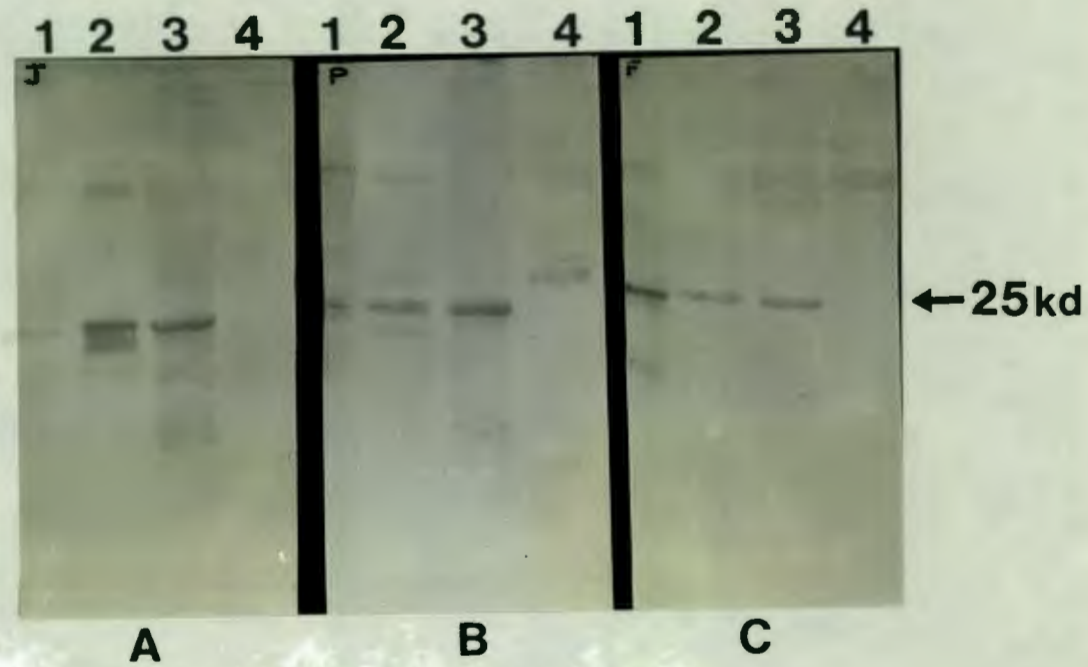


FIGURE 18 A, B & C : Blots of 3 CMV isolates probed with:
A. Anti-CMV-Tob antiserum B. Anti-CMV-Is antiseru
C. Antiserum to an Australian CMV strain.

Track 1 : CMV-Is
Track 2 : CMV-Tob
Track 3 : CMV-Nel
Track 4 : Healthy tobacco extract

L. ENZYME IMMUNOELECTROBLOTTING OF CMV-EXTRACTED FROM MAIZE

In the past year, the group of Professor M.B. Von Wechmar had started on a project to identify virus diseases of maize. Anti-CMV-Tob and anti-CMV-Is were included in serological tests. In several instances positive reactions were obtained with plants showing some abnormal symptoms like dwarfing, yellowing and leaf necrosis. This finding led to the reciprocal approach where maize seedlings were inoculated with CMV-Y and CMV-Lupin K5. Small necrotic lesions developed on inoculated leaves, soon disappearing as these leaves dried off. The later leaves of the same plants showed no apparent symptoms. To ascertain whether the absence of symptoms was indicative of the absence of virus, the inoculated plants were harvested after fourteen days and processed for CMV.

The virus extracted from the maize plants was electrophoresed on SDS-PAGE gels together with CMV-Lup-K5 and CMV-Y extracted from *N. glutinosa* plants. The gels were subsequently electroblotted with anti-CMV-Tob-2, anti-CMV-Is, anti-CMV-Imp-1 and anti-TAV. All four antisera reacted strongly with CMV-Lup-K5 extracted from both tobacco and maize. Anti-CMV-Tob and anti-TAV gave a strong reaction. Anti-CMV-Is and anti-CMV-Imp reacted weakly.

For all four antisera, two protein bands were observed around the 25 Kd position, with one band a little below and the other just above as if the virus coat protein had undergone proteolysis as illustrated in Figures 19A & B.

The positive result of the IEB indicated that CMV could infect maize when sap-inoculated with several different strains, although symptoms were not always associated with the infection.

The aspect of CMV being seed transmitted was not investigated in this project. However, having shown that certain CMV strains could infect maize, it seems feasible that CMV strains could exist that have adapted to maize specifically. This will have to be researched further to establish whether CMV is an important disease of maize.

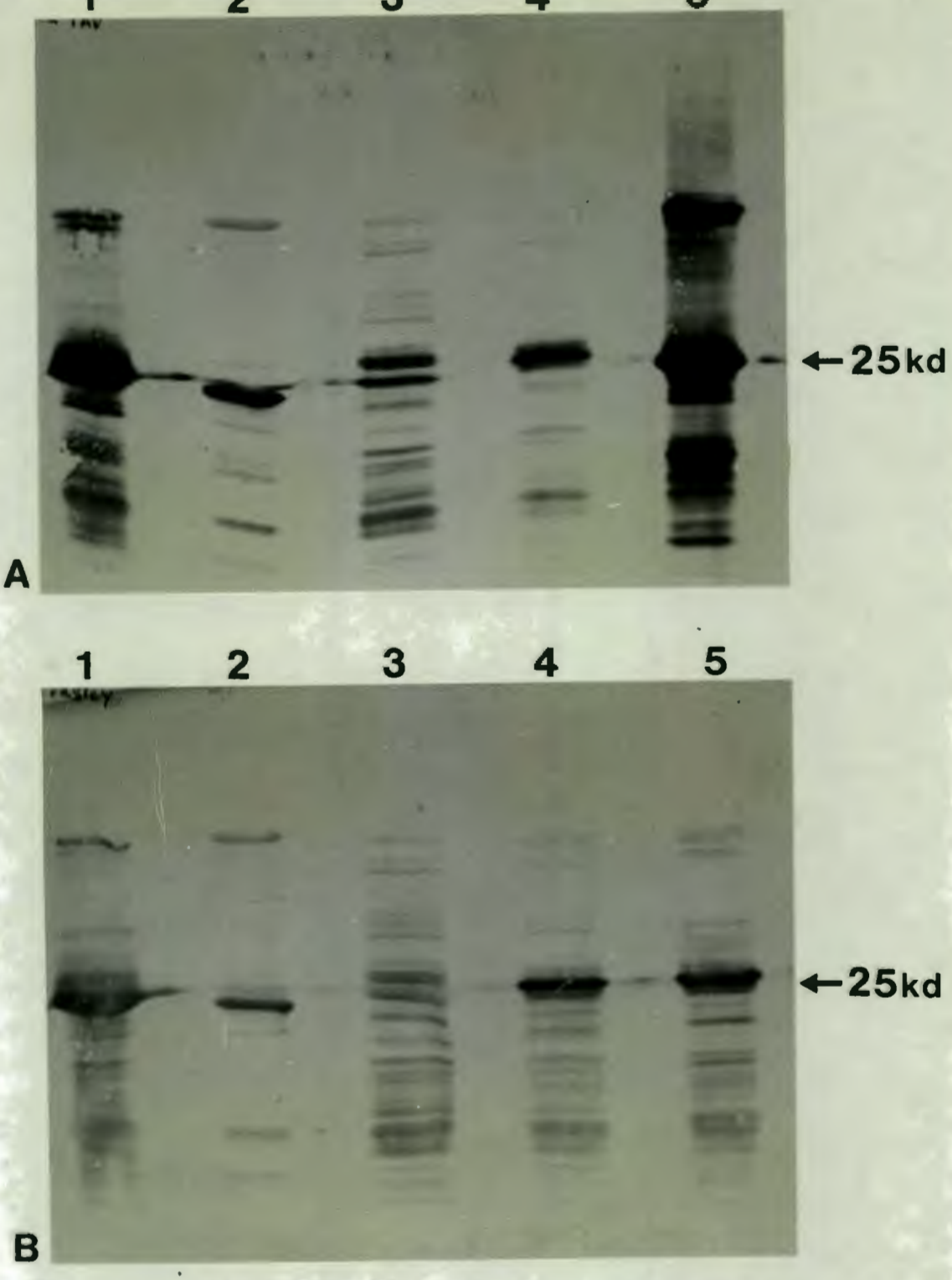


FIGURE 19 A & B : Blots of CMV-Lup-K5 and CMV-Y extracted from tobacco and maize plants probed with:

A. Anti-TAV antiserum; B. Anti-CMV-Imp antiserum

Track 1 : CMV-Lup-K5 extracted from tobacco

Track 2 : CMV-Lup-K5 extracted from maize

Track 3 : CMV-Y extracted from maize

Track 4 : CMV-Y extracted from tobacco

Track 5 : CMV-Imp extracted from tobacco

CHAPTER V

DETECTION OF CMV IN LUPINS

A. INTRODUCTION

Earlier work done by Van Regenmortel and his colleagues at Stellenbosch (Bekker, 1967; Hendry, 1969) showed that lupins were often infected with CMV. It is from this work that the isolate CMV-Lup-K5 that was also used in this project, originated. It is of interest that lyophilised plant sap sealed under vacuum in ampoules and stored at 4°C was infectious after 20 years when inoculated into *N. glutinosa* plants. (Personal communication, M.B. Von Wechmar.)

During the sixties, lupins were grown extensively in the Western Cape as a green manure and as a fodder crop, but became unpopular when severe mildew infections (*Erysiphe* sp) threatened the crop. Lupins made a comeback in recent years when mildew resistant cultivars became available.

Research on viruses of small-grains and maize, conducted in the plant virology laboratory at U.C.T. has shown that a virus, serologically related to CMV, can be detected in diseased barley and wheat (Von Wechmar et al., 1984; also personal communication) and in maize (Knox & Von Wechmar,1984). The antisera used in the tests were those prepared to CMV-Tob (anti-CMV-Tob-1) and CMV-Is (anti-CMV-Is) which were made for this project. The study of CMV-related viruses in small grains and maize is part of other departmental projects and will not be reported here.

My contribution to the larger departmental programme of detecting viruses in crop plants, was to investigate the presence of CMV in lupins.

As lupins are grown as alternate crops to wheat and barley, or in adjacent fields during the same season, it was considered important to look at the CMV infections present in lupins and, in particular, at transmission of this virus in lupin seeds, as this would be the primary source for field infections. If CMV could be detected in seeds of lupins grown locally, the virus could easily spread within the lupin crop by aphid transmission and also to adjacent crops, i.e. wheat and barley. CMV is transmitted non-persistently by a very wide range of aphid species (Francki et al., 1979). Even if the aphid species colonising lupins does not favour the alternate host of the virus, i.e. wheat or barley, it would not prevent the aphids transmitting CMV from lupins to these plants in the non-persistent fashion. The reverse may of course also occur

For the purpose of my project, I made a preliminary investigation of the presence of CMV in diseased field collected lupin plants and tested for the presence of CMV in young seedlings grown from commercial seeds.

B. DETECTION OF CMV IN DISEASED FIELD AND GREENHOUSE COLLECTED PLANTS

Lupin plants infected with CMV may exhibit different symptoms. The plants collected for this investigation were either dwarfed or yellow, had bent growth tips and shed their leaves prematurely. All plants tested were collected prior to flowering. The greenhouse plants showed typical mosaic symptoms and a backward curl of individual leaflets as illustrated in Figure 20. The greenhouse plants originated from Elsenburg and the field grown plants were collected at random in the Swartland by Professor Von Wechmar.

The DAS-ELISA technique was used with antisera to CMV-Tob, CMV-Is and CMV-Imp. Healthy tobacco sap was used as a negative control and CMV-Is infected tobacco leaf sap as a positive control.

FIGURE 20 : A greenhouse lupin seedling showing mosaic symptoms and the curling back of leaves.

Note also the chlorosis.

Three of the four greenhouse lupin samples collected at Stellenbosch gave a strongly positive reaction, while one out of the three field samples from the Swartland gave a weakly positive result. The results are listed in Table 13.

The absorbances were read at 405 nm (A_{405}) in a Titertek Multiscan plate reader (Flow Laboratories). The results shown in Table 13 show that the greenhouse grown lupins were infected with CMV. The results for the fieldgrown plants were only marginally positive with anti-CMV-Is antiserum.

The Elsenburg CMV was infectious when inoculated onto Soulouk and *N. glutinosa* tobacco. The virus was further propagated, purified and used to immunize rabbits. Unfortunately, this antiserum was not ready for further testing for purposes of this project. It should, however, be useful for further studies on lupin CMV infections by new incumbents on the project.

TABLE 13: DAS-ELISA Results of Lupin Plants (ex Elsenburg and Malmesbury) with Antisera against Different Isolates of Cucumber Mosaic Virus

Plant No.	A_{405}			**Rating
Random Greenhouse collected samples ex Elsenburg	Anti CMV-Tob	Anti CMV-Is	Anti CMV-Q	
1	1,404	1,163	0,622	++
2	0,140	0,297	0,019	+
3	0,991	0,902	0,503	++
4	1,496	1,244	0,613	++
Random field collected samples ex Swartland( )				
5	0,087	0,149	0,023	-
6	0,064	0,381	0,029	-
7	0,045	0,274	0,036	-
*Healthy Tobacco sap				
8	0,042	0,068	0,012	-
9	0,039	0,072	0,027	-
10	0,049	0,077	0,028	-
Lupin seedling with no symptoms				
11	0,101	0,353	0,022	
CMV-Is infected tobacco sap				
	0,694	0,617	0,440	+

*FOOTNOTE: Values twice the average value of the healthy tobacco sap for this antisera were regarded as positive.

** + = infected
++ = strong infection
- = not infected

C. DETECTION OF CMV IN LUPIN SEEDLINGS USING DAS-ELISA

(i) Commercial Fodder Lupin Seed

One hundred seeds taken randomly from a batch of commercial Steb lupin seeds (*Lupinus angustifolius*) were planted. The seeds were first germinated by incubating in moist vermiculite in an incubator at a temperature of 28-30°C. At about 48 hours after incubation the germinated seeds were planted in pots containing sterile soil. The plants were grown in plant growth rooms under the same conditions as those used for CMV propagation plants.

After fourteen days the lupin seedlings, most of which showed disease symptoms were harvested and divided into two groups according to the following symptoms:

A. Seedlings with no detectable symptoms
B. Seedlings with necrosis on the youngest leaves

The seedlings were crushed in PBS-Tween BSA with pestle and mortar and the sap expressed through cheesecloth before loading into the wells of the microtitre tray.

As negative control, healthy tobacco leaf sap was used and as positive control, CMV-Is infected tobacco leaf sap was used.

Anti CMV-Tob, anti-CMV-Is and anti-CMV-Q were used for the assay. Results are listed in Table 14.

The choice of antiserum was a practical one as enzyme conjugates of the above three antisera were available. A more obvious choice would have been antiserum to the lupin isolate (CMV-Lup-K5), but only early bleedings of this immunized rabbit were avialable at the time.

TABLE 14 : DAS-ELISA Results of 'Fodder' Lupin Seedlings with Anti-sera against Different Isolates of Cucumber Mosaic Virus

Plants were divided into two categories viz: plants with no detectable symptoms and plants with necrotic symptoms.

Plant No.	A_{405}			Rating*
A. Plants with no detectable symptoms	Anti CMV-Tob	Anti CMV-Is	Anti CMV-Q	
1	0,081	0,089	0,042	-
2	0,054	0,071	0,031	-
3	0,072	0,189	0,059	-
4	0,054	0,077	0,055	-
5	0,069	0,091	0,039	-
6	0,089	0,109	0,069	-
7	0,264	0,314	0,099	+
8	0,019	0,046	0,007	-
9	0,076	0,099	0,061	-
10	0,114	0,232	0,082	+
11	0,034	0,062	0,017	-
12	0,029	0,059	0,013	-
13	0,093	0,135	0,073	+
14	0,081	0,107	0,062	-
15	0,077	0,094	0,061	-
16	0,014	0,032	0,008	-
17	0,109	0,192	0,083	+
18	0,090	0,132	0,077	-
19	0,051	0,075	0,039	-
20	0,048	0,068	0,024	-
21	0,082	0,091	0,066	-
22	0,116	0,139	0,085	+
23	0,009	0,027	0,002	-
24	0,010	0,034	0,006	-
25	0,089	0,108	0,060	-
26	0,042	0,076	0,029	-
27	0,051	0,081	0,033	-
28	0,074	0,097	0,041	-
29	0,039	0,063	0,026	-
30	0,067	0,084	0,041	-
B. Plants with necrotic symptoms				
31	0,142	0,342	0,103	+
32	0,088	0,127	0,072	-
33	0,096	0,182	0,081	+
34	0,047	0,093	0,032	-
35	0,031	0,079	0,014	-
36	0,039	0,082	0,019	-
37	0,055	0,098	0,023	-
38	0,269	0,361	0,191	+

TABLE 14 continued:

Plant No.	A405			Rating
B. Plants with necrotic symptoms cont.	Anti CMV-Tob	Anti CMV-Is	Anti CMV-Q	
39	0,050	0,081	0,037	-
40	0,581	0,983	0,414	+
41	0,062	0,081	0,039	-
42	0,051	0,072	0,041	-
43	0,322	0,544	0,266	+
44	0,091	0,126	0,071	-
45	0,014	0,039	0,009	-
46	0,022	0,041	0,011	-
47	0,290	0,361	0,201	+
48	0,071	0,219	0,054	-
49	0,049	0,071	0,031	-
50	0,050	0,086	0,034	-
51	0,164	0,224	0,109	+
52	0,056	0,084	0,034	-
53	0,154	0,199	0,121	+
54	0,061	0,130	0,033	-
55	0,059	0,082	0,041	-
56	0,068	0,097	0,031	-
57	0,047	0,073	0,026	-
58	0,061	0,104	0,037	-
59	0,049	0,068	0,021	-
60	0,135	0,242	0,112	+
61	0,009	0,024	0,012	-
62	0,147	0,182	0,126	+
63	0,085	0,108	0,077	-
64	0,026	0,044	0,019	-
65	0,077	0,093	0,067	-
66	0,080	0,112	0,059	-
67	0,114	0,128	0,087	+
68	0,017	0,036	0,013	-
69	0,062	0,086	0,054	-
70	0,050	0,094	0,029	-
71	0,033	0,065	0,017	-
72	0,091	0,120	0,056	-
73	0,067	0,071	0,044	-
74	0,254	0,318	0,177	+
75	0,044	0,068	0,032	-
76	0,032	0,052	0,026	-
77	0,066	0,088	0,049	-
78	0,022	0,040	0,011	-
79	0,014	0,027	0,004	-
80	0,020	0,038	0,012	-
81	0,040	0,061	0,027	-
82	0,061	0,074	0,040	-
83	0,042	0,083	0,032	-
84	0,004	0,022	0,012	-

TABLE 14 continued:

Plant No.	A_{405}			Rating
C. Controls	Anti CMV-Tob	Anti CMV-Is	Anti CMV-Q	
Healthy tobacco sap	0,051	0,064	0,044	-
Tobacco sap infected with CMV-Is	0,576	1,014	0,442	+

*FOOTNOTE: Plants with values twice the average value of the negative controls for two antisera were regarded as infected.

One hundred seeds were selected randomly for germination. Ninety eight seeds germinated and on planting, eighty-four grew.

- = not infected
+ = infected

Summary of Table 14

A. Plants with No Detectable Symptoms

-	+	Total
25	5	30

B. Plants with Chlorosis

-	+	Total
42	12	54

Percentage of infected seedlings = 20% (based on relatively small sample)

If a less stringent rating is applied, where positive ratings for only one antiserum are included, the percentage of infected seedlings would be 26%, 36% and 20% for the three different antisera, respectively.

C(ii) Detection of CMV in Lupin Seeds obtained from Elsenburg

By kind cooperation of Mr. Loots from Elsenburg, Winter-rainfall Region, Department of Agriculture and Water Supply, we obtained batches of lupin seeds from propagation stocks. Three of these were planted as in VB. The cultivars were Steb, Illyarie and Jak.

The seedlings were examined regularly. Steb seedlings showed strong yellowing after three weeks. Plants with yellowing later developed necrosis. Other plants, not yellowed, remained dwarfed and the small leaves rolled backwards, giving the leaves a 'clenched fist' appearance. Some plants not exhibiting yellowing or leaf curling, showed mosaic symptoms.

To determine the percentage seed infection, one hundred seedlings of the cultivar Steb, were tested in DAS-ELISA, using antisera to CMV-Imp, CMV-Is and CMV-Tob.

The results are listed in Table 15. The A_{405} readings are given to illustrate the distribution of strongly infected seedlings in the various symptoms groups present in 100 seedlings. It is noticeable that symptom expression does relate to the presence of virus in seedlings as the majority of symptomless plants reacted negatively and plants with curled leaves had the highest concentration of virus per plant.

TABLE 15: DAS-ELISA Results of Lupin (*L. angustifolius* cv. Steb) Seedlings with Antisera against Different Isolates of Cucumber Mosaic Virus

Plants were divided into four categories according to symptom expression.

	A_{405}[1]			
Plant No.	Anti CMV-Tob[2]	Anti CMV-Is[2]	Anti CMV-Imp[2]	Rating[4]
A. No detectable symptoms				
1	0,200	0,196	0,193	-
2	0,229	0,218	0,263	-
3	0,249	0,206	0,233	-
4	0,207	0,211	0,206	-
5	0,217	0,209	0,206	-
6	0,270	0,220	0,258	-
7	0,310	0,232	0,301	+
8	0,236	0,218	0,193	-
9	0,183	0,224	0,202	-
10	0,231	0,214	0,211	-
11	0,153	0,184	0,166	-
12	0,181	0,189	0,177	-
13	0,174	0,174	0,176	-
14	0,156	0,183	0,156	-
15	0,178	0,181	0,152	-
16	0,150	0,188	0,146	-
17	0,164	0,190	0,158	-
18	0,165	0,192	0,137	-
19	0,153	0,289	0,173	-
20	0,178	0,205	0,164	-
21	0,157	0,309	0,148	-
22	0,188	0,295	0,176	-
23	0,155	0,275	0,173	-
24	0,174	0,322	0,150	-
25	0,172	0,286	0,147	-
26	0,145	0,314	0,161	-
27	0,234	0,309	0,168	-
28	0,143	0,308	0,173	-
29	0,161	0,314	0,159	-
30	0,147	0,297	0,154	-
B. Chlorosis and necrosis in youngest leaves				
31	0,192	0,179	0,149	-
32	0,138	0,266	0,192	-
33	0,345	0,268	0,232	-
34	1,306	0,658	0,617	+

TABLE 15 continued

Plant No.	A_{405}[1] Anti CMV-Tob[2]	Anti CMV-Is[2]	Anti CMV-Imp[2]	Rating[4]
B. Chlorosis & necrosis in youngest leaves cont.				
35	0,408	0,763	0,226	+
36	0,342	0,215	0,272	+
37	0,340	0,300	0,317	+
38	1,042	1,113	0,534	+
39	0,366	0,261	0,232	-
40	0,634	1,228	0,697	+
41	0,144	0,238	0,144	-
42	0,144	0,233	0,134	-
43	0,133	0,264	0,128	-
44	0,147	0,248	0,195	-
45	0,134	0,298	0,169	-
46	0,264	0,354	0,181	-
47	1,873	1,929	1,395	++
48	0,423	0,276	0,343	+
49	0,130	0,183	0,190	-
50	0,175	0,194	0,248	-
51	0,138	0,219	0,286	-
52	0,135	0,346	0,131	-
53	0,351	0,383	0,128	+
54	1,772	1,844	1,315	++
55	1,684	1,719	1,213	+
56	0,181	0,244	0,259	-
57	0,252	0,186	0,319	-
58	0,134	0,193	0,121	-
59	0,144	0,182	0,131	-
C. Mosaic Symptoms				
60	1,570	1,802	1,191	++
61	1,522	1,937	1,192	++
62	0,142	0,325	0,335	-
63	1,336	1,668	0,823	++
64	0,230	0,355	0,301	-
65	0,176	0,273	0,293	-
66	0,145	0,224	0,220	-
67	0,153	0,286	0,171	-
68	0,149	0,223	0,182	-
69	0,150	0,195	0,252	-
70	0,148	0,507	0,276	-
71	0,279	0,492	0,277	+
72	0,304	0,398	0,345	+
73	0,313	0,361	0,360	+
74	0,175	0,415	0,266	+

TABLE 15 continued

Plant No.	A_{405} Anti CMV-Tob	Anti CMV-Is	Anti CMV-Imp	Rating
D. Leaf Curling[3]				
75	0,143	0,274	0,224	–
76	1,427	1,698	1,029	++
77	0,179	0,401	0,255	–
78	0,263	0,334	0,373	–
79	1,876	1,828	1,384	++
80	0,213	0,325	0,263	–
81	1,437	1,667	0,953	++
82	1,688	1,698	1,086	++
83	0,264	0,336	0,303	–
84	0,172	0,285	0,501	–
85	1,696	1,874	1,054	++
86	0,143	0,440	0,189	–
87	1,109	1,636	0,746	++
88	0,163	0,350	0,228	–
89	1,527	1,718	1,102	++
90	0,152	0,535	0,233	–
91	0,166	0,597	0,246	–
92	1,614	1,606	0,969	++
93	0,154	0,367	0,266	+
94	1,614	1,921	1,421	++
95	0,154	0,234	0,179	–
96	0,204	0,263	0,210	–
97	0,222	0,281	0,197	–
98	0,177	0,283	0,175	–
99	0,162	0,248	0,205	–
100	0,171	0,259	0,184	–
E. Controls				
Healthy tobacco leaf sap (average)	0,141	0,192	0,133	–
	0,148	0,201	0,126	–
	0,150	0,187	0,131	–
Tobacco leaf infected with CMV-Is	0,855	1,554	1,429	++

FOOTNOTE: Values that were twice the average value of the negative control for at least two antisera were regarded as positive (+). Values that were equal to or more than A_{405} of 1 000 for two antisera were regarded as strongly positive.

1. Absorbance at 405 nm
2. Antisera to CMV-Tob, CMV-Is and CMV-Imp
3. Leaves curled backwards giving a "clenched fist" appearance (see Fig.20).
4. See p.90.

4. Rating

- = not infected
+ = infected
++ = strongly infected

Summary of Table 13

A. Plants with No Detectable Symptoms

-	+	++	Total
29	1	0	30

B. Plants with Necrosis

-	+	++	Total
18	9	2	29

C. Plants with Mosaic Symptoms

-	+	++	Total
8	4	3	15

D. Plants with Leaf Curling Symptoms

-	+	++	Total
16	1	9	26

Percentage of infected seedlings = 29%

D. DISCUSSION ON CMV IN LUPINS

As I have no agricultural experience, I discussed the problem of seedborne CMV in lupins with Professor Von Wechmar. Points raised in this section crystalised from this discussion and are not my own.

Two viruses have, in the past, been recognised to infect lupins, i.e. CMV and bean yellow mosaic virus (Wood & McLean, 1975). As both of these viruses are sap, aphid and seed-transmissible (Bos, 1970; Francki et al., 1979) it is not surprising that they are often encountered as dual infections.

In this study no attempt was made to investigate the presence of bean yellow mosaic virus, so that it is not possible to state whether this virus was present or not.

Looking at the data presented in Tables 14 and 15, it is obvious that the observed symptoms in seedlings did not correspond closely with the presence of CMV detected in such plants. It will be necessary to determine whether the presence of bean yellow mosaic virus could influence symptom expression and to what degree the observed symptoms are attributable to bean yellow mosaic virus only or a combination of both viruses. The fact that a relatively high percentage of seedborne CMV was determined in small samples of commercial seedlots is disturbing.

It is feasible to imply that seedlings that are primarily stressed by seedborne CMV may be infected more readily by secondary fungal pathogens. Although direct evidence is lacking, this may be an interesting aspect to follow up, particularly in view of the attention that mildew and root-infecting fungi attract in lupins.

Another aspect that would need following up is to establish what factors determine CMV to become seedborne : is it the weakly infected plant that gives rise to seedborne CMV (the strongly

infected ones normally do not produce normal flowers), or is it secondary aphid transmitted CMV that gives rise to viruses entering the embryo?

Within the limits of this project it was not possible to touch on these questions. The availability of antisera to several different CMV strains and isolates and procedures for testing for CMV should, however, facilitate such a study in the future.

Another question of interest for the farmer and seed producers, is whether seedborne CMV in lupins can go over to maize and small grains and if so, by what mechanism, as Von Wechmar et al. (1984) and Knox and Von Wechmar (1984) have already shown that CMV occurs in small grains and maize.

As lupins are used as an alternate crop in small grain production, it appears probable that CMV may survive in such crops. This may be another interesting aspect to investigate.

CHAPTER VI

DISCUSSION

A. IDENTIFICATION AND CHARACTERIZATION

1. Separation of viruses (IV.A.2.a)

The initial separation of CMV-Tob from the presumptive filamentous virus was the start of this project. The methods of identification and characterization adopted were those advocated by Hamilton et al. (1981). Host range studies, on selected plant species proved to be useful in the characterization of CMV. The selectivity of certain plant families (such as the *Cucurbitacea* in this case) made it possible to separate CMV from the other unidentified virus. In spite of its important role in the identification and characterization, symptomology could only play a secondary role to other properties such as physical, chemical and antigenic properties, because of the complex variables affecting symptom expression (Francki & Hatta, 1980; Kaper & Waterworth, 1981). CMV induces a variety of symptoms depending on virus strain and host cultivar (Francki et al., 1979).

Separation of the viruses using the dilution effect and the rate of multiplication was based on the difference in the properties of the two viruses. CMV-Tob apparently multiplied faster and out-competed the flexuous virus. By using the new growth of tobacco plants which only exhibited the mosaic symptoms, it was possible to multiply the CMV-Tob isolate in the absence of the second virus.

2. Identification (IV.F.G & J)

The purified virus particles had a spherical shape as shown by electron microscopy. The R_{ϕ} value in zone electrophoresis of the particles was 0,55 and 0,54 for CMV-Tob and CMV-Is, respectively; these values are close to those obtained by van Regenmortel (1968) for CMV-S and CMV-Y. The molecular weights of the coat proteins in SDS-polyacrylamide gels ranged from 24,5 - 26,0 Kd. CMV-Tob had a sedimentation coefficient of 98S. The physico-chemical properties were thus in good agreement with those documented in literature for CMV (Francki et al., 1979; Francki & Hatta, 1980; Kaper & Waterworth, 1981; Matthews, 1982).

Although symptomatology was of secondary importance in identification, it was useful in virus separation and thus contributed in the identification of CMV.

B. SYMPTOMATOLOGY

Symptomology did not play a major role in the characterization of CMV isolates. This is due to the variability of symptoms caused by different strains of CMV on certain plant species (Francki et al., 1979). Symptoms, however, were useful in, for example, the isolation of CMV-Tob and the selection of a suitable propagation host. *N. glutinosa* was selected as the major maintenance host because the virus-infected *N. glutinosa* plants could stay for months under the tables in the plant rooms, mature slowly, and are unattractive to aphids. A further advantage is that tobacco mosaic virus (TMV) does not infect this host systematically. TMV is always a contamination risk in a teaching laboratory. Symptoms of CMV-Tob and CMV-Nel and also CMV-Is and CMV-Imp, respectively, were so similar on most host plants, that it was difficult to tell them apart (see Table 5). On the other hand, CMV-Glad-SA appeared to be different from any of the other isolates.

This similarity in symptoms could imply a close relationship between these CMV isolates. This closeness in symptomology is consistent with the results obtained in DAS-ELISA (see IV I(i) 2), in which the CMV isolates causing similar symptoms, have a close serological relationship.

C. PROPAGATION AND ISOLATION

There were certain initial difficulties experienced in the propagation and isolation of CMV. During studies on symptomatology certain CMV isolates seemed to be losing infectivity and were not infective when inoculated on certain plant species. Various inoculation buffers were subsequently used to preserve infectivity. Gibbs and Harrison (1976) have reported that the ionic content of the inoculum can greatly affect its infectivity. They also report that it is not easy or not possible to transmit viruses from some plant species to certain other species by mechanical inoculation of the sap (such as *Chenopodium* spp and *Phytolacca* spp). The most common cause of this phenomenon is the presence of inhibitors of infection in the sap of the source plant. The effect of these inhibitors could be minimised by diluting the inoculum because the effect of the inhibitor decreases more quickly with dilution than does virus infectivity (Gibbs & Harrison, 1976).

Several methods of purification were tried in an attempt to select one that could be adopted for the South African CMV isolates but were found to be unsatisfactory for the CMV isolates under investigation (Scott, 1963; Murant, 1965). Most of the virus was lost during low speed centrifugation. It was felt that the use of organic solvents such as chloroform, butanol, and carbon tetrachloride, contributed to loss of virus during purification. Buffers of high molarity and pH around neutrality (pH 6,5-pH 8,0) were found to be satisfactory for virus extraction as was observed by other researchers who worked with CMV (Scott, 1963; Murant, 1965; Lot et al., 1972; Habili & Francki, 1974; Mossop et al., 1976). Buffers of low molarity and alkaline pH (8,0-9,0) were suitable for storage of viruses in the pure state in order to preserve infectivity.

D. VIRUS STABILIZATION

For the purpose of this study, formaldehyde (FA) was found to be suitable for the preservation of the integrity of the virus particles. Some isolates such as CMV-Is, CMV-Nel and CMV-Imp lasted longer in the formalinized state than others which were less stable such as CMV-Tob and CMV-Glad-SA. Virion stabilisation could be visualised in Ouchterlony tests in which the less stable isolates either gave protein bands which are products of degradation or no bands at all. Electron microscopy was also used to determine the extent to which particle structure was preserved.

As intact particle structure was not essential for the serological comparison of the viruses in DAS-ELISA and IEB, further conditions for stabilising the particles were not investigated. It is of interest that Francki and Hatta achieved satisfactory stabilisation by using a low percentage glutaraldehyde (GA) in addition to FA. The viruses worked with, differ sufficiently that it is feasible to anticipate that each strain/isolate would require its own specific optimal conditions for stabilisation, not only with FA and GA, but starting earlier with buffer conditions (ionic strength, buffer salt and pH).

E. ELECTRON MICROSCOPY

In this study, 2% Uranyl acetate pH 4,5 was the most suitable negative stain for CMV particles in electron microscopy. The particles were disrupted when stained in 1% phosphotungstic acid at pH 7,0. In 2% ammonium molybdate pH 5,0 the virus particles had hazy images and could not be viewed properly. The use of all three stains has been reported by many workers who had varying degrees of success with them.

Francki et al. (1966) successfully used phosphotungstic acid to stain CMV-Q particles. Similarly, several other researchers have used phosphotungstic acid and found it to be satisfactory for their CMV work (Francki & Habili, 1972; Bos & Maat, 1974). Others have reported the disruption of CMV particles in phosphotungstic acid and the suitability of ammonium molybdate (Bouwen et al., 1978). Uranyl acetate has been found to be satisfactory for CMV by other workers (Tolin, 1977; Francki & Hatta, 1980). Francki and Hatta (1980) reported that particles of numerous viruses are disrupted when stained with phosphotungstic acid, which is usually adjusted to near neutrality. However, particles of such viruses are usually stable and well stained in uranyl acetate. The disadvantage is that uranyl acetate must be used under acid conditions at pH$\sim$4,5. These authors state that the way to prevent precipitation of virus and host proteins under such conditions, is to first apply the sample preparations to the grids in order to get the particles attached to the membrane before they are stained. The problem of precipitation of virus particles under such conditions in this investigation was avoided by following the same procedure of staining.

The average radii of virus particles was 28,3 $\pm$ 0,5nm.

F. SEROLOGY

1. Ouchterlony Tests

The Ouchterlony technique was one of the methods used to study relationships among CMV isolates. The immunogenicity of the virus - which is generally regarded as being poor (Francki et al., 1979) - was enhanced by stabilisation with formaldehyde (Francki & Habili, 1972). The best conditions for the test were found to be low salt concentration, buffers of low molarity and pH at around neutrality. Several workers found similar conditions to be suitable for the strains they were working with (Francki et al., 1966; Scott, 1968; Musil et al., 1983). The virus used in the test was stabilized as it had a tendency to degrade in agar gels if unstabilized (Scott, 1968).

Except for CMV-Nel and CMV-Is, which formed a single spur, the individual CMV isolates could not be differentiated from each other using the Ouchterlony test. Although they were stabilized with formaldehyde, CMV strains K, Q and Y degraded in agar gels thus giving protein precipitin lines. Under the circumstances, it was not possible to study serological relationships between isolates and strains using this method.

2. Double-Antibody Sandwich ELISA (DAS-ELISA)

The DAS-ELISA technique was found to be the most satisfactory method of studying serological relationships amongst CMV isolates. Use of the method enabled the extent of relationships between isolates and strains to be determined.

The isolates CMV-Tob, CMV-Imp and CMV-Is appeared closely related, while CMV-Q was relatively distantly related to each of the three local isolates. Although closely related to one

another, the three isolates were distinctly different: each produced a strong homologous reaction in the respective ELISA tests, while heterologous reactions were markedly weaker. Anti-CMV-Q antiserum reacted only weakly with the isolates.

Relationships between CMV-Nel and CMV-Glad-SA and between these and other isolates, were difficult to determine because of the lack of antisera specific for these two isolates.

However, CMV-Nel seemed to be closer to CMV-Tob, CMV-Is and CMV-Imp than to CMV-Glad-SA.

The relationships shown by DAS-ELISA tests amongst local isolates and Australian strain Q are diagrammatically represented in Figure 21. (See Table 8 and Figs 11, 12, 13 and 14).

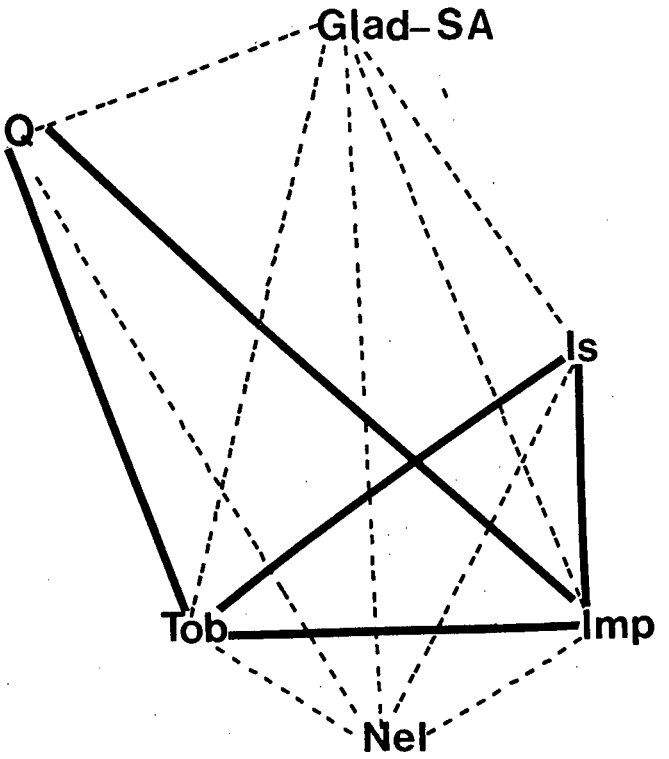


FIGURE 21: Diagrammatic representation of the serological relationships amongst CMV isolates and strains.

3. Immunoelectron Microscopy

Immunoelectron microscopy (IEM) was the least satisfactory method for studying strain relationships. The optimal proportions of virus to antibody were extremely difficult to obtain. No difference could be detected between a homologous and a heterologous reaction. The decorated particles were not clearly defined. Rao et al. (1982), when comparing the Ouchterlony test, DAS-ELISA and IEM found the latter technique to be the least satisfactory for studying serological relationships amongst Cucumoviruses. The method could probably better be used for detecting distantly serologically related viruses.

G. ELECTROPHORESIS

1. SDS-PAGE of Proteins

The molecular weights of the coat proteins of the CMV isolates were in good agreement with the values given in literature (Habili & Francki, 1974; Francki et al., 1979; Francki & Hatta, 1980; Matthews, 1982). The average molecular weight of the CMV isolates was 25,0 ± 0,5 Kd in six determinations. Only CMV-Nel, at 24,5 Kd, and CMV-Imp at 26,5 Kd, were slightly different. This difference could probably be attributed to a variation in the amino acid composition (Regenmortel et al., 1972; Habili and Francki, 1974).

2. RNA Electrophoresis

CMV RNA was studied very briefly in this investigation. Extreme care had to be exercised when handling RNA because of the ubiquitous RNases. The equipment and the reagents had to be sterilized in order to eliminate these.

CMV had four, five or six RNA species that could be detected on gels. Usually, four RNA species were detected when the virus was extracted from squash plants and five or six when extracted from tobacco. The fifth RNA species could be CARNA 5 and the sixth band could be degraded RNA. The presence of more than four RNA species has been reported by several workers (Kaper, Tousignaut & Lot, 1976; Kaper and Waterworth, 1977). Richards et al. (1978) were able to determine a sequence of 335 nucleotide residues for the CARNA 5. It was not possible to determine accurate molecular weights of the RNA species because of the difficulty of getting a good standard curve from the RNAs used as standards. However, no nucleic acid species migrating slower than the 'putative' CMV 'RNA 1' were detected. This was like BMV (Peden & Symons, 1973).

H. ENZYME IMMUNOELECTROBLOTTING OF PROTEINS

The technique was used by Rybicki and Von Wechmar (1982) in studying the serological relatedness of Bromoviruses.

In this study, the technique was found to be useful in detecting serologically related viruses. The difference in the molecular weights of CMV isolates and strains helped differentiate between them. However, the technique was not suitable for studying the serological differences. The intensity of the protein reactions was usually very similar. Differences could only be detected using 'early bleed' low-titre antisera.

What was also of interest was the strongly positive reaction between the anti-TAV antiserum and the CMV isolates and strains. This was interpreted as indicating a close serological relationship between the Italian TAV strain and the CMV isolates and strains used in this study. This result also re-affirms the serological relatedness of TAV and the CMV group (Devergne and Cardin, 1973; Rao et al., 1982 ; Rybicki & Von Wechmar, 1985).

However numerous reactions with host proteins were observed in all the electroblots. This was because semi-purified virus preparations were used.

I. APHID TRANSMISSION

As vector transmission is an important aspect in the identification of plant viruses (Hamilton et al., 1981), in particular CMV, as it is transmitted in a non-persistent fashion by a large number of aphid species (Francki et al., 1979), it could have been expected that this project would have dealt with that aspect.

However, aphid transmission of CMV was not performed in this project because this would have competed for space with the ongoing cereal aphid projects in the department which involve different aphid species. Introducing aphid species transmitting CMV freely, could also have endangered these programmes as little is known about the transmission of CMV to cereals generally.

The criteria presented were considered to characterise the CMV isolates sufficiently well to omit this biological aspect.

CONCLUSIONS

Isolating the CMV-Tob from infected ornamental plants was a difficult task mainly because it occurred as a mixed infection with a filamentous virus and the unfamiliarity on my part of working with an unstable virus.

Once antisera to CMV-Tob and CMV-Is were prepared, it was much easier to identify other isolates and monitor experimental data with serological techniques.

With regard to symptomatology, CMV-Is and the Pretoria CMV-Imp isolate and also the CMV-Nel isolate (from *Nicotiana glauca*) and the CMV-Tob isolates appeared to fall into two groups. The CMV-Glad isolated from imported gladiolus corms produced symptoms that differed from all others.

The Ouchterlony test was not found useful to determine relationships and differences. Apart from spur reactions between CMV-Is and CMV-Nel, all other isolates were indistinguishable under the experimental conditions employed. The test was useful for monitoring serum titres prior to enzyme labelling for ELISA and also for the crude detection of anti-host antibodies.

The ELISA was the most sensitive technique employed to show differences and similarities between the isolates and strains. This is not surprising as this technique utilises antibodies against both intact virions and structural subunits. Throughout the investigation, ELISA therefore, was the method of choice for identification and differentiation.

SDS-polyacrylamide gels were useful to determine biochemical differences between the strains. The molecular weight of the coat proteins of CMV-Imp and CMV-Nel differed sufficiently to separate them as distinct isolates from the other isolates and strains. This difference is also reflected in the serological relationship outlined in Fig. 21.

The multipartite nature of CMV-RNA was shown, although this aspect was not examined further.

The immunoelectroblotting method was used to investigate relationships of the South African isolates to some Australian. As this technique combines two parameters, i.e. protein molecular weight and antigenic specificity, it introduced an additional dimension to serological relationships. Both the foreign strains, CMV-K and CMV-Q were found to be strongly related to the South African isolates. Incorporating antiserum to an Italian tomato aspermy virus strain confirmed the relationship of CMV and TAV.

Of more applied interest was the test determining the presence of CMV in lupin seedlings. Finding a relatively high percentage of infection in seedlings grown from seed produced for commercial purposes, implies that losses due to virus infection could be expected either as early plant death or weakly infected plants serving as primary infection for aphids to disseminate it further.

Although maize plants appear not to develop strong visible symptoms when infected with CMV, it could be shown that maize tissue supports the replication of this virus to high concentrations. As virus replication strongly competes for metabolites in the cell, it can be expected to affect the performance of the plant generally. This will be investigated as part of another project.

To summarise : Various CMV isolates were collected and identified as being similar to CMV strains known to cause severe diseases in other parts of the world. With the availability of CMV antisera, the identification of CMV induced disease should, in future, be much faster.

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