A SEROLOGICAL STUDY OF SOME CAULIFLOWER MOSAIC VIRUS ISOLATES

BY

DION HENRI DU PLESSIS

SUBMITTED IN FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY,
IN THE
FACULTY OF SCIENCE,
UNIVERSITY OF CAPE TOWN.

DECEMBER, 1980

ABSTRACT

Enzyme-linked immunosorbent assay (ELISA) was used successfully to detect cauliflower mosaic virus (CaMV) in crude leaf extracts.

Small serological differences between CaMV isolates could be shown by ELISA and serum cross-absorption. Serological reactivity of CaMV was found to depend on the proteolytic degradation state of the virus coat protein so making it impossible to establish definite serological relationships among the virus isolates tested. Proteolysis during purification of CaMV could not be entirely eliminated.

The coat protein of CaMV was shown to be glycosylated by the specific binding of labelled Concanavalin A. The role of carbohydrate residues in CaMV serological reactivity was evaluated.

ADDENDUM

Publications in which portions of the work presented in this thesis have been reported:

DU PLESSIS, D.H. and VON WECHMAR, M.B. (1980). The effect of limited proteolysis by plant proteases on the antigenic stability of cauliflower mosaic virus. Virology 107, 298-301.

DU PLESSIS, D.H. and VON WECHMAR, M.B. (198). Detection of cauliflower mosaic virus in leaf extracts, protoplasts and aphids by enzyme-linked immunosorbent assay (ELISA). Phytopath.Z. 102, 270-278

DU PLESSIS, D.H. and SMITH, P. (1980). Glycosylation of the cauliflower mosaic virus capsid polypeptide. Virology 109, 403-408

ACKNOWLEDGEMENTS

I am deeply indebted to Dr. M. Barbara von Wechmar for assuming responsibility for the supervision of this project. Her assistance, guidance and encouragement at all times are sincerely appreciated. Thanks are also due to Prof. M.H.V. van Regenmortel for introducing me to the problems of CaMV serology, providing virus isolates, and for critically reading this manuscript.

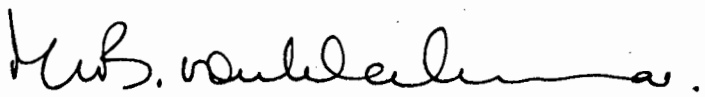
I also wish to express my appreciation to Prof. C. von Holt for his fruitful suggestions, for making available the facilities of the Department of Biochemistry, and for enabling me to continue with this project.

Sincere thanks are also due to the following:
Prof. D.R. Woods, who made the completion of this study possible;
Dr. A. Polson, for his many suggestions and novel ideas;
Mr. P. Buckton, who helped with the virus purification;
The Council for Scientific and Industrial Research and the University of Cape Town for the financial support which made this work possible.

Finally, I would like to thank my parents for encouragement and financial assistance.

CERTIFICATION OF SUPERVISOR

In terms of paragraph 9 of "Regulations for the Degree of PhD", I, as supervisor of the candidate D.H. du Plessis, certify that I approve of the incorporation in this thesis of material that has already been published or submitted for publication.



M.B. VON WECHMAR
SENIOR LECTURER
DEPARTMENT OF MICROBIOLOGY

ABBREVIATIONS

A_{260}, A_{280} etc	Absorbance at wavelength in nm. (eg. 260 nm).
BMV	Bromegrass mosaic virus.
BSA	Bovine serum albumin.
BSMV	Barley stripe mosaic virus.
Cabb-S	Strasbourg isolate of cauliflower mosaic virus.
CaMV	Cauliflower mosaic virus.
CGMMV	Cucumber green mottle mosaic virus (strain of tobacco mosaic virus).
Con A.	Concanavalin A.
DEAE	Diethylaminoethyl.
DMAP	Dimethylaminopropionitrile.
ELISA	Enzyme-linked immunosorbent assay.
FITC	Fluoroscein isothiocyanate.
NY 8153	New York isolate of cauliflower mosaic virus.
P1-8	Capsid polypeptides of CaMV.
PBS	Phosphate buffered saline.
PMSF	Phenylmethylsulphonylfluoride.
RØ	Distance migrated by virus in zone electrophoresis relative to phenol red.
SDS	Sodium dodecyl sulphate.
TEMED	N N N' N' tetramethylethylemediamine.
TMV	Tobacco mosaic virus.
U2	U2 strain of TMV.

CONTENTS

CHAPTER I

INTRODUCTION

The caulimoviruses (Harrison et al., 1971) are a group of spherical plant viruses which contain double-stranded DNA as their genetic material. The group is of particular interest since its genome is the smallest independently replicating DNA known in plant cells. It is likely that these viruses will one day play an important part in advanced molecular biological investigations.

Cauliflower mosaic virus (CaMV) naturally infects cruciferous hosts. It is the most extensively studied and characterized member of the group (Shepherd, 1976; 1979), but to date relatively little is known of its structure and replication. The virus particle is extremely stable and is able to withstand most of the usual methods which have been employed to disaggregate virus capsids. Disaggregated protein is also insoluble under physiological conditions. This phenomenon has been responsible to a large extent for the lack of knowledge regarding the intrinsic properties of the capsid proteins. Furthermore, in the past it was not possible to obtain reasonable quantities of purified CaMV. This is due to the fact that the virus particles of caulimoviruses are embedded within the matrix of inclusion bodies within the cytoplasm of infected plant cells (Fujisawa et al., 1967). It was necessary to develop a procedure to release the virus particles from the inclusion bodies in order to obtain increased yields of virus (Gomec 1973; Hull et al., 1976).

The presence of CaMV particles within inclusion bodies has also been responsible for difficulties which have been encountered in using standard serological techniques like precipitin tests to detect the virus in crude extracts of infected leaf tissue. Instead, owing to the low concentration of free virus, it has been necessary to utilize specialized and sensitive techniques like immune adherence (Nelson and Day, 1964) or serologically specific electron microscopy (Beier and Shepherd, 1978) for this purpose. In this thesis the applicability of the now widely used (Clark and Adams, 1977) enzyme-linked immunosorbent assay (ELISA) is investigated. The double antibody assay was found to be an effective means of detecting CaMV in crude extracts of infected leaves. However, like serologically specific electron microscopy, (Beier and Shepherd, 1978) the enzyme immunoassay showed limitations regarding the quantitation of CaMV in crude extracts.

It is well known that serological relationships exist among some members of the caulimovirus group (Shepherd 1970, Brunt 1971 ab) but is has not been established whether firm serological relationships exist among different CaMV isolates. This thesis reports investigations using immunodiffusion tests, serum cross-absorption, and ELISA. It could be shown that small serological differences between isolates existed. These differences were not always consistently repeatable, and with the publication of the paper in 1979 by Al Ani et al. (Al Ani et al., 1979b) which showed that the CaMV coat protein was very susceptible to proteolysis, it became necessary to re-examine CaMV

serological characteristics in the light of this finding.

It was found that serological differences between different degradation states of the same virus preparation may be of a similar order of magnitude as the differences between the various isolates. The effect of proteolytic degradation on electrophoretic mobility and homogeneity was also investigated.

Several workers have attempted to ascertain whether the CaMV capsid polypeptide is glycosylated (Kelly et al., 1974; Hull and Shepherd, 1976). Owing to the problems experienced in interpreting the polypeptide profiles and the limitations of the Schiff stain for carbohydrates, this question had not been resolved. This work reports the use of fluorescence and radiolabelled Concanavalin A to show glycosylation of the major CaMV polypeptide. The location of, and role played by carbohydrate residues in CaMV antigenicity is also evaluated.

ooo 000 ooo

CHAPTER II

REVIEW OF LITERATURE

A. PERSPECTIVE

The existence of mechanically transmissible viruses infecting cruciferous plants has long been known (Gardner and Kendrick, 1921; Schulz, 1921) and by 1945 the occurrence and properties of the virus now known as cauliflower mosaic (CaMV) had been reported by several workers (Tompkins, 1937; Tompkins and Thomas, 1938; Caldwell and Prentice, 1942; Walker et al., 1945).

In spite of the time CaMV has been known, purification difficulties and problems experienced in disrupting the capsid, have prevented the details of CaMV particle morphology and other properties from being adequately explored. In the early 1960's improved purification techniques made it possible to raise antisera to CaMV (Day and Venables, 1960; Pirone et al., 1961), but the serological properties of the virus have so far received little attention (Shepherd, 1970; 1976). It was only in 1979 that some indications of the location of the nucleic acid and the nature of the viral capsid have been obtained (Al Ani et al., 1979a and b; Chauvin et al., 1979), which is in sharp contrast to viruses like tobacco mosaic and the bromoviruses which have been extensively characterised. The DNA-containing plant viruses have been recently reviewed by Shepherd (1976, 1979).

B. SYMPTOMS AND HOST RANGE

Cauliflower mosaic virus is distributed world-wide causing mosaic type diseases of cruciferous plants which may result in losses of economically important crops (Shepherd, 1970, 1976; Tomlinson and Shepherd, 1978). Infected plants exhibit mottles and mosaics which have been described as "nondescript" (Shepherd, 1976), and for this reason, symptomatology has not proven a reliable diagnostic tool (Shepherd, 1976). Chronically infected plants may show changes in leaf pigmentation, and interveinal chlorosis becomes apparent with retention of chlorophyll along the major veins of the leaf (Shepherd, 1976). The symptoms produced depend on the strain of CaMV and the type of host. Brassica campestris, B. oleracea and Matthiola incana have been reported as useful diagnostic hosts (Shepherd, 1970).

At the microscopic level, a symptom which can be useful for diagnosis of the caulimoviruses, the taxonomic group of spherical double-stranded DNA plant viruses of which CaMV is a member, (Robb, 1964; Edwardson and Christie, 1978) is the production of characteristic inclusion bodies, or viroplasms, in the cytoplasm of infected leaf tissue (Fujisawa et al., 1967; Brunt and Kitajima (1973); Martelli and Castellano, 1971; Fenner, 1976).

These irregularly lobed spherical or elliptical bodies, containing many virus particles embedded in a proteinaceous matrix, can be stained with phloxine (Fujisawa et al., 1967) or are visible without staining under interference optics Martelli and Castellano, 1971). However, some isolates of

CaMV have been reported to produce small inclusion bodies which are not visible under the light microscope (Shepherd, 1976).

CaMV is confined mainly to the Cruciferae (Broadbent, 1957), but some strains have been reported capable of infecting Nicotiana clevelandii (Hills and Campbell, 1968) and Datura stramonium, causing local lesions on the latter host (Lung and Pirone, 1972). As propagation hosts, various cultivars of Brassica spp have been used by most workers.

C. PURIFICATION

Purified CaMV has, until recently, not been easy to obtain in reasonable quantities, since the particles are present mainly in inclusion bodies (Fujisawa et al., 1967) and may not be released after disintegration of the leaf tissue (Shepherd, 1976). In addition, the virions show a tendency to aggregate and loose solubility during the purification procedure (Shepherd, 1970). Most of the purification methods reported prior to 1976 have involved the use of butanol for initial sap clarification (Day and Venables, 1960; Pirone et al., 1961; Itoh et al., 1969; Shepherd et al., 1970), although Martini (1957) in an early attempt at CaMV purification, used a heat clarification step. Final purification of CaMV was achieved by sucrose density gradient centrifugation of the concentrated clarified extract (Pirone et al., 1960; 1961).

In 1973, Gomec developed a procedure for the purification of dahlia mosaic (DaMV) another caulimovirus. His method was

adapted for CaMV by Hull et al., (1976) who utilised the addition of 2,5% Triton X-100, a non-ionic detergent, and 1 M urea to the crude sap in order to disrupt the inclusion bodies and release the virus particles. Final purification was achieved by sucrose density gradient centrifugation after concentration of the extract by high speed centrifugation.

Hull and his coworkers found that, under their growth conditions, little virus could be extracted from the plants after eleven days and that maximum virus yield was obtained after 24 days and maintained until at least 35 days after infection.

Virus yield also depended upon the buffer used for extraction: if the sap was extracted in water, 0,1 or 1 M phosphate buffer pH 7,2, 0,2 M borate pH 8,2 or at room temperature, instead of in 0,5 M phosphate at 4°C, the virus yield was reduced. Despite Hull et al. finding that n-butanol was the major factor causing virus loss and variable yields in earlier procedures, Civerolo and Lawson (1978) combined a butanol clarification step with the Triton X-100/urea method in their purification of CaMV. The same authors used a similar procedure to purify carnation etched ring (CERV), a related caulimovirus. They obtained a greater yield of virus compared with the butanol procedure alone (Lawson and Civerolo, 1978).

In order to avoid high speed centrifugation of large volumes of leaf extract, Hull and coworkers as discussed in their 1976 paper, utilised the fact that inclusion bodies can be sedimented by low speed centrifugation in an alternate procedure.

They subjected extracted sap to centrifugation at 10 000 rpm for 15 minutes and resuspended the pellet in 1/10th of the original volume before treatment with Triton X-100 and urea. With this method they obtained yields of up to 70% of those given by their original technique. It was possible to precipitate CaMV with 40% saturated $(NH_4)_2SO_4$, Mg^{++}, ethanol, acidification to pH 3,5 and 5% PEG, but precipitates did not resuspend easily and Hull et al. considered that this problem precluded the use of these treatments during purification. They found that differences in yield obtained with some isolates were not fully explained by differences in the abundance of inclusion bodies, and the amount of virus they contained, but they felt that the stability of the viral inclusion bodies may be a factor affecting virus yield.

Several workers (Brunt et al., 1975; Hull and Shepherd, 1976) have noted that the treatment to which the virus preparation had been subjected affected the polypeptide profile obtained when CaMV was analysed by SDS electrophoresis. Storage at room temperature tended to result in the conversion of some capsid protein components to lower molecular weight species. The lighter ones were considered to be degradation products (Hull and Shepherd, 1976). Al Ani et al., (1979b) found that CaMV is very susceptible to proteolysis and reported that if frozen leaves were used for virus extraction, better preserved CaMV was obtained, presumably due to contaminating plant proteases being rendered inactive by a freeze-thaw cycle. No difference in the degree of proteolysis was detectable, however, between virus purified by the butanol or Triton X-100/urea methods. The capsid polypeptides are discussed in detail in subsequent sections of this review.

D. PHYSICAL AND CHEMICAL PROPERTIES

1. Biophysical Properties

Table 1 contains a list of important biophysical properties determined for CaMV.

Various workers have determined the sedimentation coefficient of the virus. Values ranging from 200 S (Al Ani et al., 1979a) to 222 S (Pirone et al., 1961) have been reported. Al Ani et al., (1979a) found a slight decrease in S values with increase in pH from pH 6 to pH 9. Recently, however, it was found that CaMV is very susceptible to proteolysis and that particles sedimented in sucrose density-gradients at different rates, depending upon their degradation state (Al Ani et al., 1979b), although no authors have mentioned such heterogeneity in analytical studies. Potato virus Y has also been found to sediment at different rates when its capsid protein has been degraded to different degrees (Hiebert and McDonald, 1976). Whether the relatively large disparity between some of the reported results for CaMV is also due to a similar phenomenon has not been investigated.

Hull et al., (1976), in their determination of the sedimentation and diffusion coefficients, found that interparticle interaction significantly affected both sedimentation and diffusion coefficients.

2. Nucleic Acid

CaMV was the first plant virus found to contain DNA, rather than RNA as its genomic material. The first indication that the nucleic acid of CaMV may be DNA was given by its

TABLE 1

BIOPHYSICAL PROPERTIES OF CaMV

Biophysical Property	Value	Reference
Sedimentation coefficient determined in 0,01 M phosphate buffer at 25°C.	214 S	Day and Venables (1960)
Sedimentation coefficient determined in cold water.	222 S	Pirone et al. (1961)
Sedimentation coefficient determined in 5 - 30% sucrose density gradients in 0,01 M phosphate buffer pH 7,5	206,5 S	Itoh et al. (1969)
Sedimentation coefficient determined in 0,1 M NaCl; 0,01 M phosphate buffer pH 7,2 at infinite dilution, corrected to $S_{20,w}$	208 S	Hull et al. (1976)
$S_{20,w}$ determined at pH 6 - 9 : Values showed a small increase with increasing pH	200 S	Al Ani et al. (1979a)
Partial specific volume	0,704 g/ml	Hull et al. (1976)
Diffusion coefficient $D_{20,w}$	0,753 x 10^{-10} cm^2/s	Hull et al. (1976)
Hydrodynamic radius	28,4 nm	Hull et al. (1976)
Buoyant density in CsCl	1,37 g/ml	Shepherd (1970)
Molecular weight of particle	22,8 x 10^6 daltons	Hull et al. (1976)
DNA molecular weight	3,9 x 10^6 daltons	Hull et al. (1976)
Percentage DNA	17%	Hull et al. (1976)
$E^{0,1\%}_{260}$ uncorrected for light scattering	about 7	Shepherd (1970)
$E^{0,1\%}_{260}$ corrected for light scattering	4,36	Shepherd (1970)

resistance to hydrolysis by acid and alkali and by the diphenylamine test (Shepherd et al., 1968). The buoyant density of the nucleic acid (Shepherd et al., 1970), as well as the fact that CaMV replication is affected by Actinomycin D (Tezuka et al., 1971) confirmed that the nucleic acid was in fact DNA. All members of the caulimovirus group (Shepherd, 1976, 1979), as well as the recently discovered gemini viruses (Harrison et al., 1977) possess DNA as genetic material.

The CaMV DNA is double stranded (Shepherd et al., 1969; 1970; Shepherd and Wakeman, 1971; Russell et al., 1971) and it is probable that its natural form is nicked circular (Shepherd, 1976; Hull and Shepherd, 1977), and twisted (Civerolo and Lawson, 1978), but not in the supercoiled form (Shepherd et al., 1970). There are at least three single-stranded gaps in the molecule as shown by electrophoresis in alkaline gels (Volvovitch et al., 1977, 1978). Another feature of CaMV-DNA is the presence of some covalently linked RNA (Shepherd, 1976; Hull and Shepherd, 1977), the function of which is not known. No unusual bases have been found in the DNA (Shepherd et al., 1970). Because of its DNA content, CaMV has been viewed as a potential candidate for recombinant DNA experiments (Howell and Hull, 1978; Shepherd, 1979). The role of restriction endonuclease mapping in the study of CaMV is discussed in a later section of this chapter.

3. Disaggregation

CaMV is characterised by its remarkable stability under protein-denaturing conditions. Shepherd (1976) observed that the exterior of the virus particle is very hydrophobic,

an attribute which he felt may account for its extreme resistance to degradation. He found that CaMV was resistant to most of the conditions usually used to denature proteins, for instance treatment with 5 - 6 M guanidine hydrochloride, phenol, phenol and cresol and/or dodecyl sulphate. However, in his 1979 review, Shepherd reported that boiling in 6 M guanidine hydrochloride was an effective means of dissociating the capsid. Salts like lithium iodide and sodium perchlorate did not disrupt the CaMV capsid. The virion can, however, be destroyed by proteolysis together with 0,25% SDS or by boiling in 1% SDS (Shepherd, 1976) or by high pH (Al Ani et al., 1979a). In 1972, Tezuka and Taniguchi found that when CaMV was digested stepwise with pronase a discrete component of 110 S appeared just before the DNA was released. They postulated that this was the inner core of a two-layer capsid (Tezuka and Taniguchi, 1972a).

Most workers who wished to disrupt the capsid to obtain the nucleic acid used proteolysis in the presence of SDS. Pronase (Shepherd et al., 1970; Shepherd and Wakeman, 1971; Civerolo and Lawson, 1978; Hull and Howell, 1978) or proteinase K (Lebeurier et al., 1978) have been used.

For studies on the protein of CaMV in polyacrylamide gels, workers have dissociated the virus capsid with SDS and 2-mercaptoethanol together with boiling, either in the presence (Tezuka and Taniguchi, 1972b; Kelly et al., 1974; Brunt et al., 1975; Hull and Shepherd, 1976) or absence of urea (Tezuka and Taniguchi, 1972b; Al Ani et al., 1979b). By 1976, no work had been reported on the free protein of CaMV

mainly because of the inability to solubilize the protein in the absence of SDS, (Shepherd, 1976). At present, the situation remains essentially the same.

4. Structural Changes

In an early study, Itoh et al., (1969) compared the S values of CaMV in sucrose gradients after treatment with SDS at 50°, 60° and 70° C. These treatments had the effect of converting 206 S particles to 190 S, 150 S and 110 S particles respectively. The slower sedimenting particles could be reverted to their original S values by dialysis against phosphate buffer pH 7,5 or could be fixed in their slow sedimenting forms by formaldehyde treatment. Electron microscopy showed that this change in sedimentation coefficient was accompanied by a change in conformation of the virus particles. Itoh et al. considered that this was due to a "loosening " of the structure, but they felt it unreasonable to compare this process with the pH-induced structural changes exhibited by the bromoviruses (Incardona and Kaesberg, 1964; Bancroft et al., 1967), as CaMV contains DNA and not RNA.

More recently, Al Ani et al. (1979a) also found that pH changes induced changes in conformation. The virus began to swell markedly at pH 10, and at pH 11,25 two components were detected with sedimentation coefficients of 110 S and 65 S. Electron microscopy showed that both components were swollen virus particles, and that the slower component had some of its DNA protruding. This structural transition could be reversed by back-dialysis to pH 7,3, with the DNA re-entering

the capsid. Above pH 12, the particles "collapsed" (Al Ani et al., 1979a). High salt concentration, which can stabilise some viruses (Kaper, 1975) did not stabilise CaMV, and the same "loosening" process was observed at high pH in 1,0 M and 0,1 M NaCl (Al Ani et al., 1979a). These authors noted that even in strongly alkaline medium, when the virus had dissociated completely, DNA strands with subunits still attached to them could be seen under the microscope. This indicated that the DNA-protein interactions in CaMV were able to withstand high pH conditions.Al Ani et al. (1979a) found no evidence for a "core" of subunits as postulated by earlier workers (Tezuka and Taniguchi, 1972 a and b ; Hull and Shepherd, 1976). They also pointed out that the easy exit and entry of the DNA suggests that the CaMV virion is assembled via a procapsid intermediate into which the nucleic acid is sucked. They postulated that a single protein, acting as a stopper, may be covalently linked to the DNA.

It is clear that although CaMV can undergo structural transitions, the conditions required to effect any detectable change are much more rigorous than those required for the bromoviruses. The work described above thus demonstrates the extreme stability of the CaMV capsid.

E. STRUCTURE OF THE VIRUS CAPSID

1. Electron Microscopy

Under the electron microscope, the CaMV capsid appears as an almost featureless sphere with no definite subunits (Day and Venables, 1960; Pirone et al., 1961). The diameter and appearance of the capsid is dependent upon the staining procedure used. When stained with phosphotungstate the particles appear "empty" with a diameter of 50 nm and a hollow "core" of 20 nm diameter. In tissue sections, staining with lead and osmium salts shows virions of 45 - 50 nm in diameter, with both "empty" and "full" capsids. Uranyl acetate, on the other hand, does not penetrate the virus (Hills and Campbell, 1968).

In their examination of CaMV structural transitions, Al Ani et al., (1979a) found no evidence for a core in CaMV particles. However, little information regarding CaMV structure has been obtained from electron microscope studies of the intact virus, and SDS polyacrylamide gel electrophoresis has proven more useful as a tool to gain information on the nature of the viral proteins.

2. Polyacrylamide Gel Electrophoresis

The proteins comprising the CaMV capsid have been extensively studied by polyacrylamide gel electrophoresis, but this technique has not yielded a final answer regarding the location of the viral polypeptides. For electrophoretic examination of the CaMV capsid protein, the virus was dissociated by SDS and 2-mercaptoethanol, or with urea, and electrophoresed in gels containing SDS or SDS and urea.

In 1972 (b) Tezuka and Taniguchi reported that two proteins of molecular weights 33 000 and 68 000 daltons probably formed the capsid of CaMV. They also considered the possibility that the 68 000 dalton species was a dimer of the lower molecular weight protein, but found that even if 6 M urea was present in the gel, the band produced by this protein was still present. They concluded that the virus probably contained two types of subunit, possibly forming more than one protein shell layer. In a similar study, Kelly et al. (1974) found two major components of 32 000 and 67 000 dalton molecular weight, as well as other bands representing protein species of 27 000, 40 000, 92 000 and 100 000 daltons. The two highest molecular weight components gave a positive Schiff reaction, indicating that they were glycoproteins. Kelly et al. suggested that these were precursors of the lower molecular weight species. They found, however, that the polypeptide profiles varied with the treatment of the SDS-disrupted particles before electrophoresis. If disrupted particles were stored for 80 days at room temperature and then treated again with SDS and 2-mercaptoethanol and urea, they found less of the 67 000 dalton species and more of the 27 000 dalton component upon electrophoresis. This they cautiously attributed to spontaneous cleavage of the 67 000 dalton component.

In 1975 Brunt et al. suggested that 2 major polypeptides with molecular weights of 67 000 and 42 000 were involved in the structure of CaMV. Other minor bands representing protein species of molecular weights between 100 000 and 15 000 were also apparent on their gels.

Brunt et al., by treating the virus preparation in various ways, concluded that some of the bands were due to degradation products, since their presence depended upon the particular treatment. Storage at 18°C for up to eight weeks, boiling with disrupting fluid for 15 minutes, or repeated freezing and thawing followed by boiling caused partial loss of the 68 000 dalton and the 42 000 dalton components, while other bands of 65 000 and 39 000 daltons respectively appeared. The high molecular weight species were thought to be aggregated polypeptides, and since the Schiff test proved negative, they concluded that these were not glycopeptides.

Brunt et al. also determined the amino acid composition of the whole virus. They found a large amount of basic amino acids, arginine and lysine making up 23% of the total. This suggested to them that at least one of the polypeptides was an internal protein with a strong affinity for DNA.

The next investigation was by Hull and Shepherd, who in 1976 found 96 000, 88 000, 64 000 and 37 000 dalton polypeptides. The 37 000 dalton polypeptide was found to break down to a 33 000 dalton component if the virus was stored for 2 weeks at room temperature. The Schiff test did not yield any positive proof of glycosylation of any of the polypeptides. Hull and Shepherd suggested, on the basis of previous studies with pronase degradation (Tezuka and Taniguchi, 1972(a)) and the molar ratios of the protein species they obtained, that the CaMV capsid may have two layers of subunits. They postulated that the 64 000 dalton component represented a

core protein, surrounded by an outer capsid composed of 33 000 dalton subunits.

A major step closer to resolving the location and identity of the CaMV capsid polypeptides was made by Al Ani and his Strasbourg coworkers in 1979, who used a discontinuous buffer system (Laemmli, 1970) in their gel electrophoresis experiments, in contrast to previous workers who had used continuous buffer systems. Al Ani et al. (1979b) investigated the effect of partial enzymatic digestion in vitro upon the CaMV capsid. They found that the variability in polypeptide profiles obtained by earlier workers could be explained by enzymatic degradation in situ or during purification. By means of one-dimensional peptide mapping (Cleveland et al., 1977) they established that sequence homologies existed between 42 000, 39 000, 37 000 and 33 000 dalton polypeptides. They concluded that the 42 000 dalton species was most likely the actual capsid subunit, but that it was a very labile polypeptide giving rise to a multiplicity of bands in the gel. The higher molecular weight species (70 - 85 000 daltons) they observed were believed to be heterologous and homologous polymers, probably formed by disulfide bridging, of the 42 000 dalton and lower molecular weight species. They also pointed out that polymers of this type can be present in some viruses, for example, southern bean mosaic virus (Seghal and Hsu, 1977), cowpea chlorotic mottle virus and tobacco necrosis virus (Rice, 1974).

In their discussion, Al Ani and coworkers reviewed the findings of earlier workers in the light of their results

and concluded that CaMV is actually a much simpler virus than had previously been supposed, and that it has a single protein species as its main structural protein. Two other virus polypeptides of molecular weight 55 000 and 49 000 daltons which were found not to be dimers of any lower molecular weight polypeptides, had sequence homologies with the 42 000 dalton polypeptide. Al Ani et al. suggested that these homologies were due to CaMV having overlapping genes, similar to SV40 (Contreras et al., 1977). Their data also ruled out any structural similarities with papovaviruses. Figure 1 illustrates the polyacrylamide gel profiles obtained by the various workers as summarised by Al Ani et al. (1979b).

Variable polypeptide profiles are not unique to CaMV. For example, SDS-polyacrylamide gel electrophoresis has shown that members of the potyvirus group (Hiebert and McDonald, 1973, 1976; Papa et al., 1973; Huttinga and Mosch, 1974; McDonald and Hiebert, 1975; Purcifull and Hiebert, 1979) and the potex virus group (Koenig et al., 1970; Lesnaw and Reichmann, 1970; Shepard and Secor, 1972; Tremaine and Agrawal, 1972; Tung and Knight, 1972; Koenig et al., 1978) exhibit capsid protein heterogeneity. In the case of the potex group this heterogeneity has also been accounted for by proteolysis of the capsid protein (Koenig et al., 1970; Shepard and Secor, 1972; Tremaine and Agrawal, 1972; Tung and Knight, 1972; Koenig et al., 1978).

3. Neutron Diffraction Studies

With the background of the data obtained by electron microscopy and polyacrylamide gel electrophoresis, Chauvin and

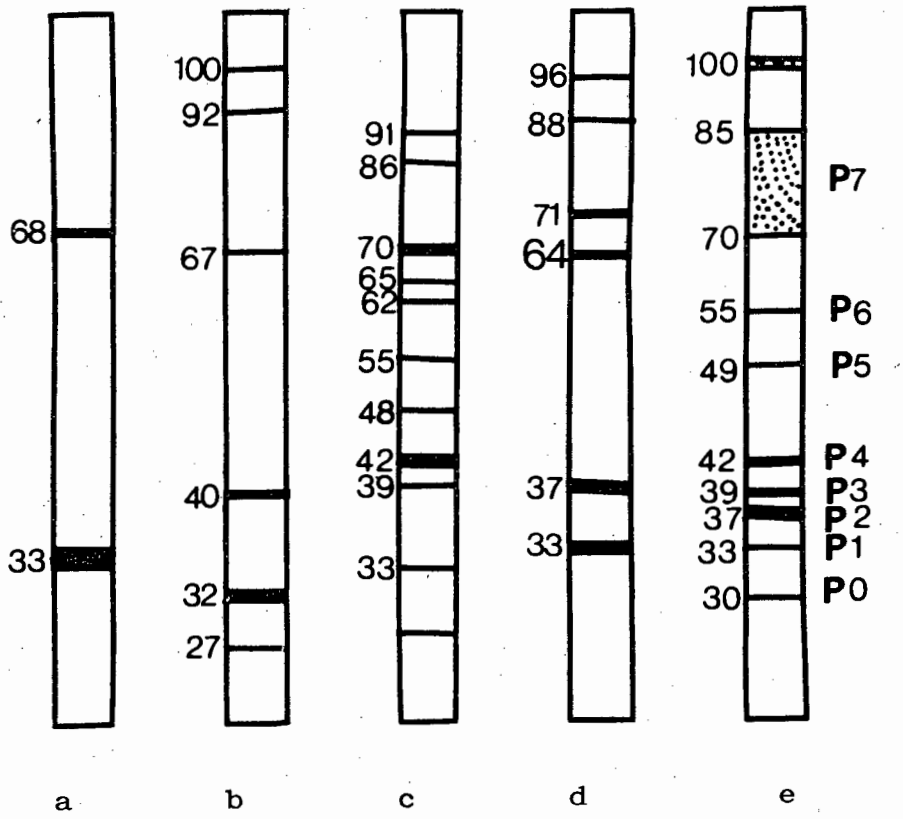


FIGURE 1

Comparison of CaMV polypeptide profiles obtained in polyacrylamide gel electrophoresis by various workers: (a) Tezuka and Taniguchi, (1972b); (b) Kelly et al., (1974); (c) Brunt et al., (1975); (d) Hull and Shepherd, (1976); (e) Al Ani et al., (1979b). (Adapted from Al Ani et al., 1979b). Molecular weights are given in thousands of daltons.

coworkers (1979) used the technique of neutron diffraction to determine the relative location of DNA and capsid protein in the intact CaMV virion. Neutron diffraction had previously yielded much valuable information in the case of spherical RNA viruses (Jacrot et al., 1977; Chauvin et al., 1978).

The respective contributions of the DNA and protein to the scattering of neutrons in heavy water was measured and the best approximation of the data to a spherical shell was ascertained.

The model presented by Chauvin et al. was a hollow sphere of protein with a double or single layer of DNA arranged on its inner surface, possibly penetrating the protein layer. Very little protein appeared to be found within the DNA layer, certainly not enough to form an inner shell as postulated by Tezuka and Taniguchi (1972 a and b) and Hull and Shepherd (1976). Chauvin et al. suggested that this internal protein may correspond to the minor polypeptides (P5 and P6) found in gels by Al Ani et al. (1979b). However, since these protein species are very susceptible to proteolysis, Al Ani et al. (1979b) suggested that they may be located on the capsid surface.

F. ISOLATES AND STRAINS

1. Biological Properties

Although Broadbent and Tinsely (1953) noted that minor variants can be distinguished by their virulence in cauliflower or turnip plants, Shepherd (1970, 1976) pointed out that few CaMV isolates are in fact pure strains. He noted that some isolates can become systemic in B. campestris, while others produce local lesions (Shepherd, 1970). However, he did not specify which isolate produced which symptoms. More recently, Tomlinson and Shepherd (1978) have been able to distinguish three strains by their virulence in Brussels sprouts and turnips.

In studies on various aspects of CaMV, a wide variety of isolates have been used. Table 2 contains a summary of the various isolates that have been studied. It should be noted that during serial transfer, some new isolates have arisen (Hull and Howell, 1978; Lebeurier, et al 1978) and that Tomlinson and Shepherd (1978), using nitrous acid and local lesion transfer, have isolated variants from a single strain.

2. Aphid Transmissibility

An important biological characteristic that distinguishes some CaMV isolates is their ability to be transmitted by aphids. Lung and Pirone (1973) investigated this aspect and found that the Cabbage B and NY8153 isolates were efficiently transmitted by Myzus persicae. Their KK Cabbage isolate was less efficiently transferred, but the Campbell

TABLE 2

REPORTED ISOLATES OF CAULIFLOWER MOSAIC VIRUS

ISOLATE	REPORTED IN STUDIES ON:	AUTHORS
Australian	Restriction endonuclease mapping	Hull and Howell (1978), Hull (1980)
	Inclusion bodies	Shepherd et al., (1980)
BARI (4 isolates)	Restriction endonuclease mapping	Hull (1980)
	Cytological comparison (Bari 1)	Shalla et al., (1980)
BKMV	Restriction endonuclease mapping	Hull (1980)
Brass 6	Restriction endonuclease mapping	Hull (1980)
	Inclusion bodies	Shepherd et al., (1980)
BSE	Restriction endonuclease mapping	Hull (1980)
Cabb B (-D)	Inclusion bodies	Shepherd et al., (1980)
	Restriction endonuclease mapping	Meagher et al., (1977); Hull and Howell (1978); Hull (1980)
	Cytological comparison	Shalla et al., (1980)
	Aphid transmissibility	Lung and Pirone (1973, 1974)
	Purification	Hull et al., (1976)
	Serologically specific electron microscopy	Beier and Shepherd (1978)
Cabb B (J-I) (arose from Cabb B)	Restriction endonuclease mapping	Hull and Howell (1978); Hull (1980)
	Multiplication in protoplasts	Howell and Hull (1978)
	Identification of gene products	Odell and Howell (1980)
(Cabb-S) (arose from isolate of Dr Conti)	Restriction endonuclease mapping	Lebeurier et al., (1978)
	Conformational changes	Al Ani et al., (1979a)
	Capsid polypeptides	Al Ani et al., (1979b)
Calif	Restriction endonuclease mapping	Hull (1980)

.../cont.

ISOLATE	REPORTED IN STUDIES ON:	AUTHORS
Campbell	Restriction endonuclease mapping	Hull (1980); Hull & Howell (1978)
	Local lesion formation	Lung and Pirone (1972)
	Aphid transmissibility	Lung and Pirone (1973)
	Purification	Hull et al., (1967)
	Cytological comparison	Shalla et al., (1980)
	Inclusion bodies	Shepherd et al., (1980)
CM 1841	Restriction endonuclease mapping	Hull and Howell (1978); Hull (1980)
	Identification of gene products	Odell and Howell (1980)
CM4-184	Restriction endonuclease mapping	Hull and Howell (1978); Hull (1980)
	Multiplication in protoplasts	Howell and Hull (1978)
	Purification	Hull et al., (1976)
	Cross protection and mutagenesis	Tomlinson and Shepherd (1978)
	Serologically specific electron microscopy	Beier and Shepherd (1978)
CM-78 (3 isolates)	Restriction endonuclease mapping	Hull (1980)
Datura	Cytological comparison	Shalla et al., (1980)
	Inclusion bodies	Shepherd et al., (1980)
GCRI (Glasshouse crops Research Institute)	Restriction endonuclease mapping	Hull and Howell (1978)
Horseradish	Cytological comparison	Shalla et al., (1980)
	Inclusion bodies	Shepherd et al., (1980)
Hungary	Restriction endonuclease mapping	Hull (1980)
Invergowrie	Purification	Hull et al., (1976)
Jap (2 isolates)	Restriction endonuclease mapping	Hull (1980)
Japanese (Chiba)	Conformational changes	Itoh et al., (1969)

.../cont.

ISOLATE	REPORTED IN STUDIES ON:	AUTHORS
KK-Cabbage	Local lesion formation	Lung and Pirone (1972)
	Aphid transmission	Lung and Pirone (1973, 1974)
Milan	Restriction endonuclease mapping	Hull (1980)
Nome	Purification	Hull et al., (1976)
	Restriction endonuclease mapping	Hull (1980)
	Cytological comparison	Shalla et al., (1980)
	Inclusion bodies	Shepherd et al., (1980)
NVRS (2 isolates) (National vegetable Research Station)	Restriction endonuclease mapping	Hull and Howell (1978); Hull (1980)
	Capsid polypeptides	Kelly et al., (1974)
	Cross protection and mutagenesis	Tomlinson and Shepherd (1978)
NY-8153 (New York)	Restriction endonuclease mapping	Hull and Howell (1978); Hull (1980)
	Local lesion formation	Lung and Pirone (1972)
	Aphid transmissibility	Lung and Pirone (1973, 1974)
	Purification	Hull et al., (1976)
	Serologically specific electron microscopy	Beier and Shepherd (1978)
NZ (New Zealand) (4 isolates)	Restriction endonuclease mapping	Hull (1980)
Phatak (PK)	Restriction endonuclease mapping	Hull (1980)
	Inclusion bodies	Shepherd (1976)
	Cross protection and mutagenesis	Tomlinson and Shepherd (1978)
	Capsid polypeptides	Kelly et al., (1974)
	Serologically specific electron microscopy	Beier and Shepherd (1978)
	Cytological comparison	Shalla et al., (1980)
	Inclusion bodies	Shepherd et al., (1980)
PV-45 (American type culture)	Nucleic acid conformation	Civerolo and Lawson (1978)
PV-147	Restriction endonuclease mapping	Volvovitch et al., (1979)
	Structure of nucleic acid	Volvovitch et al., (1978)

.../cont.

ISOLATE	REPORTED IN STUDIES ON:	AUTHORS
SHRI (Scottish Horticultural Research Institute)	Restriction endonculease mapping	Hull and Howell (1978)
V7 (M7)	Cross-protection and mutagenesis Cytological comparison	Tomlinson and Shepherd (1978) Shalla *et al*., (1980)
Wellesbourne	Purification	Hull *et al*., (1976)
Yug (2 isolates)	Restriction endonculease mapping	Hull (1980)

and CM4-184 isolates were not transmitted at all. Although they investigated the form and location of the virus in the plant cells, they found no evidence that location, concentration or distribution of the Cabbage B, NY 8153 or the Campbell isolates in the leaf tissue differed in any way. In 1974 they showed the existence of a 'transmission factor' which would allow non aphid-transmitted variants to be transmitted if aphids are first allowed to feed on plants infected with a transmissible strain. It appears that the virus specifies the vector transmission factor in the infected plant, but it is not known how this 'factor' functions (Lung and Pirone, 1973; 1974).

3. Local Lesion Formation

Lung and Pirone (1972) reported that the Campbell, CM4-184, KK Cabbage and New York 8153 isolates formed local lesions on Datura stramonium, but not the Cabbage B isolate. Local lesion production can thus be a further differentiating property of CaMV isolates. However, Shepherd (1976) had little success with Datura as a local lesion host, but he did not specify the light and other growth conditions to which his plants were subjected. Such environmental factors affect local lesion development by turnip yellow mosaic virus (Pleij et al., 1977) and may also influence symptom development in the case of CaMV. Local lesions are formed reproducibly on the F2 turnip hybrid "Just Right" by the NVRS, PK and CM4-184 isolates and this system has been found to be a suitable assay for CaMV by Tomlinson and Shepherd (1978). They also demonstrated that the time of inoculation, and whether the

plants received a darkening period before being inoculated, affected the development of local lesions by CaMV.

4. Biophysical and Biochemical Differences

a) Electrophoretic Mobility

Lung and Pirone (1972), in their investigation on the reason for differential aphid transmissibility subjected three CaMV isolates to electrophoresis in acrylamide/agarose gels. Small but distinct differences in electrophoretic mobility between the KK Cabbage, Campbell and NY8153 isolates were evident. There is no report of other physical differences between particles of different strains, although Day and Venables (1960) detected three electrophoretic components in the isolate they studied.

b) Restriction Endonuclease Mapping

The presence of DNA in CaMV (Shepherd et al., 1978) makes the virus amenable to study by the technique of restriction endonuclease mapping. This technique allows very fine differentiation between the nucleic acids of related DNA viruses (Studier, 1979), and in the absence of serological data, it was found useful for identifying and characterising different strains of CaMV. Using three restriction endonucleases, a physical map of the genome of CaMV was constructed by cleaving the DNA of the Cabb B isolate (Meagher et al., 1977). A feature of these results was the apparent heterogeneity in the native DNA population in the form of uncommon cleavage sites in a small proportion of the DNA. With the

help of fragments of CaMV-DNA cloned in E. coli, Meagher et al. found no extensive deletions, insertions or inversions, and suggested that minor modification of the sequence may occur. They proposed that such divergent molecules could be fixed in the population as a result of serial passage at high multiplicity of infection. A similar heterogeneity in restriction patterns of some isolates was also observed by Hull and Howell (1978), Lebeurier et al. (1978) and Hull (1980). An analogy to plant mitochondrial DNA (Quetier and Vedal, 1977) has been hinted at (Shepherd, 1979).

Hull and Howell (1978) compared the DNA genomes of several CaMV isolates and found 4 groups of isolates that showed differences in sequence by restriction enzyme analysis. They found that one region of the CaMV genome is subject to considerable change, while the rest is more highly conserved. Lebeurier et al. (1978) reported that an isolate which arose in their laboratory, Cabb-S, differed in its restriction endonuclease map from the isolates previously described by Volvovitch et al. (1977) and Hull and Howell (1978).

A comprehensive study entailing the restriction endonuclease mapping of thirty-three CaMV isolates was made by Hull (1980). He found considerable variation, both in numbers and map positions of the sites susceptible to the restriction enzymes. There appeared to be no correlation between the restriction maps and the biological characteristics or geographical origins of the various isolates. Hull suggested that new isolates of CaMV which are not suppressed by dominant isolates are arising continuously.

Thus although it has not yet been possible to assign definite gene functions to any of the CaMV-DNA restriction endonuclease fragments, the mapping technique has proven to be a reliable means of determining the heterogeneity of CaMV isolates, and of differentiating between them.

5. Inclusion Bodies

Isolated CaMV inclusion bodies have been found to consist of a single protein of 55 000 daltons (Shepherd and Wakeman, 1977). As inclusion bodies of different strains have been found to have different properties (Shepherd, 1976, 1979; Shalla *et al.*, 1980) it has been suggested (Shepherd, 1979; Shalla *et al.*, 1980) that the matrix protein is a virus-coded product. Shepherd (1979) also suggested that there may be characteristic chemical or immunological differences in the matrix proteins of different strains. To date this aspect has not been investigated in detail, but Hull *et al.* (1976), in their paper on the Triton X100/urea method of CaMV purification, suggested, on the basis of the virus yields of several isolates, that the stability of inclusion bodies produced by the isolates may be different.

Further evidence of differences came from Shalla *et al.* (1980) in their study of the comparative cytology of nine CaMV isolates. They found that inclusion bodies induced by the isolates could differ in the following characteristics: size, ratio of virions to amount of matrix protein, frequency of free virions in the cytoplasm. One isolate (CM4-184) formed inclusions in the leaf cell chloroplasts. Since these variances occurred in the same host, they suggested that

these effects were directed by the viral genome.

Shepherd et al., (1980) found that most strains of CaMV had two forms of inclusion bodies, one of which contained virus particles and was vacuolated, while the other was devoid of both virus and vacuoles. Difficulties were experienced in obtaining the matrix protein in a soluble state under physiological conditions, but the use of Triton X-100 enabled serological comparisons of this protein with intact CaMV to be made. No cross-reactivity was observed in immunodiffusion tests. Shepherd et al., (1980) stressed that further chemical or serological work is required to confirm that the CaMV inclusions are virus coded.

Edwardson and Christie, in their 1978 review on virus-induced inclusions, pointed out that caulimovirus inclusion bodies, or viroplasms, may be used as a diagnostic characteristic for CaMV and related viruses. Caulimovirus inclusion bodies are different in appearance under the light microscope from those induced by other virus groups, so making them suitable for this purpose.

G. SEROLOGY

The first successful production of an antiserum to CaMV was reported by Pirone et al. (1960). Several earlier workers (Larson et al., 1950; Martini, 1957) had failed to prepare a specific antiserum, but the introduction of butanol during purification as a sap clarification agent allowed the production of virus sufficiently pure and in sufficient quantities to be used to raise an antiserum (Pirone et al., 1960, 1961). Using semi-pure CaMV both for immunization and as the antigen in the ring interface test, Pirone et al. (1961) obtained a serum end-point titre of 1/1024. Suprisingly, their antiserum did not react with material from healthy plants. The microprecipitin test, however, showed that antibodies to plant components were present in this antiserum.

In an attempt to produce a more specific antiserum, they used virus purified by sucrose density-gradient centrifugation for antiserum production. The serum had a microprecipitin titre of 1/1280 and did not react with material from healthy plants. Pirone et al. attempted to use this antiserum for the detection of CaMV in clarified extracts by means of the microprecipitin assay. Their sap extracts were clarified either with butanol, or by heat treatment. Butanol-clarified extracts from healthy or infected plants showed non-specific precipitation with CaMV antiserum and with normal serum. Pirone and coworkers found that they could distinguish this non-specific precipitate from that produced by virus-containing extracts. They concluded, however, that the precipitin test was not specific or intense enough for the positive detection and identification of CaMV in clarified extracts.

In 1964 Nelson and Day published an account of the use of immune adherence to detect CaMV in both purified preparations and crude turnip sap. Their assay utilised the fact that CaMV reacted with rabbit antibody and human complement to form a complex which adhered to the surface of human erythrocytes causing haemagglutination. They found that CaMV could be identified in high dilutions of pure virus or with clarified sap of infected plants. More recently, Beier and Shepherd (1978) found that CaMV could be detected, but not quantitated by the technique of serologically specific electron microscopy (Derrick, 1973). This was attributed to the fact that the virions are occluded within viroplasms in the infected leaf cytoplasm.

CaMV was serologically compared with dahlia mosaic virus (DaMV) by Brunt (1966). By immunodiffusion tests, he showed that CaMV and DaMV reacted reciprocally with antisera to each other. Subsequently it has been reported that CaMV, DaMV and carnation etched ring virus (CERV) show close relationships in gel precipitin tests (Brunt, 1971a,b; Lawson et al., 1977). The serological relatedness of CaMV and DaMV was confirmed by Gomec (1973). He showed that they were not identical as indicated by spur formation in serological cross reactions in gel. There is no report of serological relationships within the CaMV group itself, and the various strains or isolates of CaMV have not been serologically characterised. CaMV is not serologically related to Mirabilis mosaic virus, another caulimovirus (Brunt and Kitajima, 1973).

CaMV has, however, been shown to exhibit heterogeneity of its capsid protein due to proteolysis (Al Ani et al., 1979b) and as mentioned earlier, the potex- and potyviruses are subject to a similar phenomenon. The effects of such heterogeneity on some properties of turnip mosaic virus (TuMV), a potyvirus, were investigated by Hiebert and McDonald in 1976. They found that TuMV which possessed a 28 000 dalton capsid protein, sedimented slower, had a slightly higher bouyant density, and could be serologically distinguished from virus containing a 34 000 dalton capsid protein.

Koenig (1978), in her study of the enzyme-linked immunosorbent assay (ELISA), found that the test could distinguish between potato virus X comprised of protein in the "slow" 29 000 dalton or "fast" 24 000 dalton form.

Antigenic and other differences may thus exist between different preparations of the same virus. The importance of this factor when making serological comparisons of the potyvirus group was pointed out by Purcifull and Batchelor (1977). The effect of the recently discovered proteolytic degradation of CaMV (Al Ani et al., 1979b) on the serological properties of this virus has not been ascertained.

In this thesis, this aspect is investigated in detail using a variety of serological procedures.

H. REPLICATION

The DNA of CaMV has a molecular weight of about $4,4 \times 10^6$ (Shepherd and Wakeman, 1971), placing it amongst the simplest of DNA viruses. Although the knowledge of CaMV replication is still at the "educated guess" stage, Shepherd, in his 1976 review suggests that the events are likely to be analogous to those occurring with other DNA viruses with a similar genome size. The findings of Al Ani and coworkers in 1979, which indicated that CaMV has at most three structural proteins, and that these have extensive sequence homologies, mean that less DNA is required to code for structural protein than had previously been supposed (Shepherd, 1976) on the basis of CaMV having four polypeptides (Hull and Shepherd, 1976).

The extreme resistance of CaMV to chemical degradation poses the problem of how the virus disassembles to uncoat its DNA in the host cell. Shepherd (1976) suggested that proteolytic action or conformational changes at the cell membrane could result in the release of the DNA. Since no RNA polymerase activity could be shown in CaMV, he suggested that host RNA polymerase probably participates in the initial transcription of the genome. Shepherd also suggested that like SV40 DNA, both DNA strands may have a messenger function, the strands being transcribed at different times in the infective cycle. It appears that intracytoplasmic inclusion bodies are the main sites of DNA replication as shown by ^{3}H-Thymidine uptake (Kamei *et al.*, 1969; Favali *et al.*, 1973). Caulimovirus assembly also appears to occur in these inclusion

bodies and intensive protein synthesis is suggested by the presence of electron dense material surrounded by numerous ribosomes. Virions can be seen in the matrix as the inclusion body develops (Kitajima et al., 1969; Martelli and Castellano, 1971; Lawson and Hearon, 1973), suggesting that they are virus assembly sites.

Although little is known of CaMV replication at present, the advantages of the caulimoviruses as a model system for studies on transcription, translation and DNA replication in plant cells is well understood. It can thus be anticipated that the use of experimental systems like protoplasts (Howell and Hull, 1978) and the application of modern biochemical techniques will ensure that the functions of the genome and details of CaMV structure and replication will be further elucidated. For instance, Odell and Howell (1980) have used an in vitro protein synthesizing system and hybrid-arrested translation to show that a 66000 dalton protein found in CaMV-infected leaves is coded for by a specific Eco-R1 fragment of the CaMV genome. Serological data could conceivably assist in the identification of the coat protein gene.

ooo 000 ooo

CHAPTER III

MATERIALS AND METHODS

A. VIRUS SOURCE

Five isolates of CaMV were used in this study. The Australian and New York 8153 isolates were obtained originally from Dr. R. Hull of the John Innes Institute, Norwich, England *via* Professor M.H.V. van Regenmortel of the Institut de Biologie Moleculaire et Cellulaire, Strasbourg, who also provided the Cabb-S isolate. These virus samples were received as infected desiccated leaf material. Two other isolates, Oudtshoorn and Banhoek, were obtained from Dr. M.B. von Wechmar of the University of Cape Town in dried and fresh leaf samples respectively. Tobacco mosaic virus (TMV) strains, Vulgare, U2 and CGMMV (Cucumber green mottle mosaic virus) purified by the method of von Wechmar and van Regenmortel (1970), after butanol clarification in the case of CGMMV were obtained from the stocks of the Department of Microbiology. Purified barley stripe mosaic virus was provided by Dr. M.B. von Wechmar.

B. VIRUS PROPAGATION

CaMV was routinely propagated on turnip *cv* Maizura (W. Atlee Burpee, U S A) or mustard *cv* Spahili (Kirchoff's, South Africa) in the controlled environment of plant growth rooms. White London mustard was used in some experiments. Temperature was 24°C with 16 hours of light per day. Soil used for plant growth was heat-sterilised, and the plants

were inoculated about 12 days after germination, at which stage there were usually 4 or 5 leaves present.

For inoculum, dried, frozen, or fresh leaf samples containing CaMV were ground up with 0,05 M phosphate buffer pH 7,5 and then strained through cheesecloth. A small quantity of celite was added and the inoculum was rubbed onto all the leaves of the young plants using a cottonwool pad. For work on the comparison of isolates, virus used for inoculation had undergone only one propagation cycle from the original stock. The type and speed of development of symptom expression depended upon the isolate used, but the virus was usually systemic by 15 days and the leaves were harvested after 21 to 24 days. Leaves were placed into large polythene bags and either used immediately for purification, or stored at 4°C for up to 2½ weeks.

C. VIRUS PURIFICATION

1. Buffers : Buffers used in virus extraction were prepared as described by Chase (1968).

Stock Solutions:

Solution A	0,5 M	KH_2PO_4
Solution B	0,5 M	K_2HPO_4

0,5 M Potassium phosphate buffer pH 7,5: (Standard Buffer)

This buffer was prepared by mixing 320 ml of solution A and 1 680 ml of solution B. EDTA was added to a final concentration of 0,001 M. The buffer also contained 0,02% NaN_3 and 0,01 M sodium sulphite.

0,5 M Potassium phosphate buffer pH 7,0: (Density gradient)

This buffer was prepared by mixing 780 ml of solution A and 1220 ml of solution B. The buffer contained 0,02% NaN_3 and 0,001M EDTA.

2. Standard Method

The Triton X-100/urea method described by Hull et al. (1976) was used. Infected leaves (0,5 - 2,0 kg) were homogenised in a Waring blender with cold (8°C) 0,5 M potassium phosphate pH 7.5 buffer in a w/v ratio of 1:1. The homogenate was filtered through cheesecloth into a beaker or a flask standing in an ice-bath. To the filtrate was added Triton X-100 to a final concentration of 1,25% and urea to 1,0 M. The solution was then stirred in a coldroom at 4°C for 15 to 18 hours. This step was followed by low speed centrifugation in a Sorvall GSA or GS-3 rotor at 8 000 rpm for 15 min. The supernatant fluid was then subjected to centrifugation at 34 000 rpm for 60 min in a Beckman Type 35 rotor. The resulting pellets were resuspended in 0,5 M phosphate buffer pH 7,0 (about 2 ml/pellet). After resuspension of the pellet, the preparation was again subjected to a cycle of low and high speed centrifugation. The pellet from the final high speed centrifugation was resuspended in about 2 ml of phosphate buffer pH 7,0 and applied to a 10 - 40% sucrose (BDH AnalaR Grade) density-gradient layered in a Beckman 30 ml cellulose nitrate tube. The gradient was centrifuged at 24 000 rpm for 2 - 2½ hours in a SW 27 rotor by which time the virus band was visible as an opalescent zone about halfway down the tube. The virus sample was recovered either by displacement from the bottom of the tube in an ISCO density-gradient fractionator, or by viewing the tube with side-lighting and withdrawing the virus band with a bent pasteur pipette (Brakke, 1967). The virus-containing sucrose solution was diluted 1/4 with water

and centrifuged again at 30 000 rpm for 90 min in a Beckman 60Ti rotor. The resulting light brown virus pellet was immediately resuspended in distilled water. Virus not subjected to sucrose density-gradient centrifugation is referred to as "semi-pure", while sucrose density-gradient virus is called "pure" in subsequent sections of this thesis.

3. Variations

Several variations on the basic technique were used to determine their effect on proteolysis of CaMV (Al Ani et al., 1979b).

a. Fresh leaves

Leaves were processed immediately after harvesting or after storage at 4°C for less than 4 hours.

b. 4°C Storage of leaves

In most of the initial work, before it was realised that proteolysis was a factor to be considered, leaves were stored at 4°C for up to 2½ weeks before processing.

c. Frozen leaves (-20°C)

These leaves were frozen at -20°C immediately after harvest and were processed some weeks later. The leaves were thawed in buffer before homogenization.

d. Frozen leaves (liquid nitrogen)

Fresh leaves were packed in a plastic bag and frozen by placing them into a liquid nitrogen freezer immediately after harvest. The frozen leaves were crushed while still frozen, buffer was added, in an approximately 1:1 w/v ratio and the mixture was further homogenised in a Waring blendor.

e. Protease Inhibitors

In some experiments, the following protease inhibitors were added to the buffers throughout purification:

phenylmethylsulphonyl fluoride (PMSF) (Merck, Darmstadt) to 0,001 M

iodoacetic acid (Merck) to 0,02 M

1 - 10 phenanthroline (Merck) to 0,02 M

(Weber et al., 1972)

To ensure complete solubilisation it was necessary to dissolve the PMSF in a small quantity of ethanol before adding it to the buffer. When the inhibitors were used, the sucrose solutions used for density-gradient centrifugation were boiled for 20 minutes before addition of the inhibitors, to inactivate any contaminating enzymes.

f. Reducing Agents

To determine the effect of reducing agents in the extraction buffer, sodium sulphite was omitted from the 0,5 M phosphate buffer pH 7,5.

g. Storage

Semi-pure CaMV from fresh leaves was stored at $4^{o}C$ for 2 weeks and at room temperature for 14 days before sucrose density-gradient purification.

D. INFECTIVITY TESTS

The infectivity of CaMV in leaves was tested by homogenising leaf samples with 0,05 M phosphate buffer pH 7,5 in a mortar and pestle, straining the homogenate through cheesecloth and inoculating 10 fold dilutions, up to 10^{-4}, in phosphate buffer onto White London or Spahili mustards. Approximately 12 - 15 plants were inoculated per dilution. After about

18 days the fraction of plants showing characteristic symptoms was determined.

E. SPECTROPHOTOMETRIC DETERMINATIONS

Ultraviolet spectra and absorbance values of purified CaMV preparations were determined with a Unicam SP 1700 or a Beckman DB spectrophotometer. When determining absorbance at 260 nm, no correction was made for light-scattering. A value of 7,0 was used as extinction coefficient (Shepherd, 1970) in calculating virus concentration.

F. ISOPYCNIC CENTRIFUGATION IN CAESIUM CHLORIDE

Caesium chloride crystals (Merck, Suprapur) were added to a semi-pure, or sucrose density-gradient purified CaMV sample in 0,5 M phosphate buffer pH 7,5 until the density of the solution as determined by refractometry was about 1,37 g/ml. The CsCl/virus mixture was centrifuged overnight at 35 000 rpm in a cellulose nitrate tube in a Beckman SW50 rotor at 4°C. The virus sample in the CsCl gradient was visible as a sharp band of opalescence. The gradient was fractionated by puncturing the bottom of the transparent tube and collecting 4 or 5 drop fractions from the bottom. The refractive indices of the various fractions were determined by means of an Abbé refractometer and converted to density values by means of standard tables.

In order to better observe the behaviour of CaMV preparations during isopycnic centrifugation, a Beckman Model E analytical ultracentrifuge was used. A virus preparation in water was diluted into a CsCl solution so that the final concentration

of virus was approximately 0,2 mg/ml and the density of the solution as determined by refractometry was 1,38 g/ml. The solution was centrifuged at 52 000 rpm at 20°C. After about 7 hours centrifugation, a photograph was taken using schlieren optics.

G. DETERMINATION OF SEDIMENTATION COEFFICIENTS

The sedimentation coefficients of CaMV preparations were determined by sedimentation velocity experiments in a Beckman Model E analytical ultracentrifuge. An AnD rotor with a standard 12 mm single sector Kel F or aluminium centrepiece was used. The virus samples were centrifuged at 24 000 rpm at 20°C and photographs were taken at various intervals using the instrument's schlieren optical system. The logarithm of the distance of the schlieren peak from the axis of rotation was plotted versus time and the S value was calculated from the slope of the resulting graph (Chervenka, 1969).

H. ELECTROPHORETIC TECHNIQUES

1. Electrophoresis in Agarose/Acrylamide Gels

Agarose acrylamide gel electrophoresis of purified CaMV was performed as described by Lung and Pirone (1973).

a. Stock solutions

The following solutions were used:

i) 20% acrylamide monomer solution made up by adding 19 g of acrylamide and 1,0 g of N,N,-methylene-bisacrylamide to 100 ml of water.

ii) 6,4% dimethylaminopropionitrile in water (DMAP).

iii) 1,6% ammonium persulphate in water.

iv) 0,02 M potassium phosphate buffer pH 7,5.

b. Preparation of gels

The gel was prepared by adding 80 ml of distilled water to 0,8 g of agarose (Miles Laboratories). The mixture was stirred and brought to the boil to dissolve the agarose. The solution was then placed into a water-bath at 40°C. Buffer (11,3 ml), DMAP (7 ml) and acrylamide monomer (11,3 ml) were mixed and warmed to 40°C in a water-bath. The agarose and the acrylamide solutions were mixed thoroughly and 4,5 ml of 1,6% ammonium persulphate was added. The solutions were mixed and poured into 14 cm x 1 cm glass tubes. The parafilm used to seal the bottom of the tubes was punctured several times when the gels had set and they were pre-electrophoresed in a Shandon tube electrophoresis apparatus at 4 mA/tube for 30 min. The buffer compartments contained 0,02 M potassium phosphate buffer pH 7,5. After pre-electrophoresis, 25 - 50 µl virus samples containing about 10 - 30 µg of CaMV in 0,02 M potassium phosphate buffer + 5,0 % glycerol + 0,05 % bromophenol blue were layered on top of the gels. Electrophoresis was at 4mA/tube at room temperature with a cooling current of air until the marker dye reached about 1 cm from the bottom of the gel column. The gels were then removed from the tubes and stained with 0,5% Coomassie blue in methanol: acetic acid: water (5v/1v/5v) for about 30 min. Destaining was in methanol: acetic acid: water (5%/10%/85%) for about 12 - 18 hours. The gels were scanned at 580 nm with an ISCO gel scanner.

2. Electrophoresis in Agarose gels

Electrophoresis on agarose slab gels was used to compare relative mobilities of purified preparations of several isolates.

Method

Agarose was added to 0,01 M phosphate buffer at various pH values to a final concentration of 0,6% w/v. The agarose was dissolved completely by boiling and after it had been allowed to cool to 40°C, 10 ml of agarose was poured onto a 7,5 cm x 7,5 cm clean glass slide on a level surface forming a layer about 2 mm thick. For smaller slides, the amount of agarose was reduced proportionately. When the gel had set, small (1 - 2 mm diameter) wells were cut in the agarose. Virus at a concentration of about 0,5 mg/ml in the buffer in which electrophoresis was to take place, was pipetted into the well, filling it completely (about 10 µl). The slide was placed into a general purpose electrophoresis chamber and a wick of cotton lint saturated in buffer was placed onto each end of the agarose slab, letting it overlap by about 1 cm. The other end of the wick was immersed in the buffer of the electrode chamber. An empty well was filled with 0,05% bromophenol blue to serve as a marker. Electrophoresis was at 20°C at 5 V/cm until the bromophenol blue marker had reached the wick at the opposite side of the gel. At pH values from 6,0 to 8,0, migration of virus was from the cathode towards the anode. Upon completion of electrophoresis the agarose was dried under a current of hot air and stained and destained in the same solutions as described for agarose/acrylamide electrophoresis. The staining and destaining of the thin agarose layer could be accomplished in 10 - 15 minutes. Rf values were calculated as the ratio of the distance travelled by the virus to that travelled by the bromophenol blue marker.

3. Zone Electrophoresis

The technique and apparatus have been described in detail by van Regenmortel (1964a, 1972) and by Polson and Russel (1967). Zone electrophoresis was performed on semi-pure or density-gradient purified CaMV samples in a sucrose density-gradient prepared in a 0,05 M phosphate buffer pH 7,5, or alternately in 0,1 M borate buffer pH 8,6. Phenol red was used as a standard reference to indicate the progress of electrophoresis and to determine the RØ values of the various components (van Regenmortel, 1968).

4. Polyacrylamide Gel Electrophoresis

This system was originally described by Laemmli (1970) and used by Al Ani et al. (1979b) for their study on CaMV proteolysis. The gel is prepared from a 37,5 : 1 (2,6% crosslinkage) acrylamide/bis mixture.

a. Stock solutions

i)	Acrylamide monomer	Acrylamide	30 g
		N-N'-methylenebisacrylamide	0,8 g
		Water	to 100 ml
ii)	Ammonium persulphate	Ammonium persulphate	0,5 g
		Water	to 100 ml
iii)	Resolving gel buffer (pH 8,8)	1,0 M HCl	24 ml
		Trishydroxymethylaminomethane	18,15 g
		Water	to 100 ml

3. Zone Electrophoresis

The technique and apparatus have been described in detail by van Regenmortel (1964a, 1972) and by Polson and Russel (1967). Zone electrophoresis was performed on semi-pure or density-gradient purified CaMV samples in a sucrose density-gradient prepared in a 0,05 M phosphate buffer pH 7,5, or alternately in 0,1 M borate buffer pH 8,6. Phenol red was used as a standard reference to indicate the progress of electrophoresis and to determine the RØ values of the various components (van Regenmortel, 1968).

4. Polyacrylamide Gel Electrophoresis

This system was originally described by Laemmli (1970) and used by Al Ani et al. (1979b) for their study on CaMV proteolysis. The gel is prepared from a 37,5 : 1 (2,6% crosslinkage) acrylamide/bis mixture.

a. Stock solutions

i)	Acrylamide monomer	Acrylamide	30 g
		N-N'-methylenebisacrylamide	0,8 g
		Water	to 100 ml
ii)	Ammonium persulphate	Ammonium persulphate	0,5 g
		Water	to 100 ml
iii)	Resolving gel buffer (pH 8,8)	1,0 M HCl	24 ml
		Trishydroxymethylaminomethane	18,15 g
		Water	to 100 ml

iv) Stacking gel buffer (pH 6,8)

1,0 M HCl	24 ml
Trishydroxymethylaminomethane	3,0 g
Water	to 100 ml

v) Electrode buffer (pH 8,3)

This buffer was made up in water and contained:

Trishydroxymethylaminomethane	0,025 M
Glycine	0,192 M
SDS (Sodium dodecyl sulphate)	0,1 %

vi) Disrupting fluid:

This consisted of stacking buffer to which was added:

SDS	2,0 %
2-mercaptoethanol	2,0 %
Glycerol	15,0 %
Bromophenol blue	0,05 %

vii) TEMED (NNN'N' Tetramethylethylenediamine)

b. Resolving Gel

Acrylamide monomer, resolving gel buffer and ammonium persulphate solution were mixed in various proportions to give between 7,5 and 12% polyacrylamide gels. Most separations were performed on 10% gels. SDS was added to a concentration of 0,1 % and the solution was degassed for a few minutes with a vacuum pump. TEMED (Eastman chemicals) was added to a final concentration of 0,025 % and the gel solution was immediately poured into a slab gel mould to give a gel 1,5 mm thick and with 9 - 10 cm path length. Water was carefully layered onto the surface and the gel was allowed to set. This took about 15 minutes at room temperature. When the gel had set the water was poured off and the stacking gel was cast.

c. Stacking gel

The monomer and the stacking gel buffer were mixed in suitable proportions to yield a 4 or 5 % gel. SDS was added to a concentration of 0,1%, the solution was degassed and TEMED (0,025 %) was added. The mixture was poured on top of the solidified resolving gel and a perspex slot former was put in place. When the stacking gel had set, the slot former was slowly and carefully removed.

d. Treatment of protein samples

CaMV at a concentration of approximately 1 mg/ml in water was diluted 1:1 into disrupting fluid and heated for two minutes in a boiling water bath. The standards used for molecular weight determinations were treated in the same way. Bovine serum albumin (68 000 daltons), ovalbumin (42 000 daltons), turnip crinkle virus protein (38 000 daltons), lactate dehydrogenase (36 000 daltons), chymotrypsinogen (27 500 daltons) and soybean trypsin inhibitor (21 500 daltons) were used as standards. The bands obtained with preparations of CaMV were identified by their positions relative to these markers (Al Ani et al., 1979b).

e. Electrophoresis

Electrophoresis of 30 µl samples of disrupted virus (about 30 µg of protein) was performed in a vertical slab gel apparatus for about 4 hours at 125 V until the ion front reached about 1 cm from the bottom of the gel. The lower buffer reservoir contained the anode.

f. Staining and destaining

The gels were stained and destained as described previously for agarose/acrylamide gels. Stained gels were photographed using diffuse back illumination with Ilford FP4 photographic film.

I. SEROLOGICAL TECHNIQUES

1. Antiserum Production

Antisera were routinely prepared in rabbits by intramuscular injections of density-gradient purified virus preparations emulsified in Freund's incomplete adjuvant. Rabbits were injected weekly, receiving about 4 to 6 injections of 1 ml each containing 0,5 - 1,0 mg of CaMV. The animals were bled from the peripheral ear vein at approximately weekly intervals. After clotting of the blood, the serum was extracted by centrifugation at 10 000 rpm for 10 min. The antisera were preserved by storage at -20°C.

Goat anti-rabbit IgG serum was prepared by immunising a goat with 4,0 mg of rabbit IgG, prepared by absorption to DEAE cellulose (Reif, 1969), in 1,0 ml of sterile 0,15 M NaCl emulsified with 1,0 ml of Freund's incomplete adjuvant. The animal was bled from the jugular vein.

Antisera to TMV protein and Brassica fraction 1 protein were obtained from the stocks of the Department of Microbiology.

2. Absorption of Antisera with Plant Material

To remove any possible contaminating plant protein antibodies, CaMV antisera used in immunoassays were absorbed with plant

material as follows:-

Uninfected mustard and turnip leaves were homogenised with 0,5 M sodium phosphate buffer pH 7,5 in a 1:1 w/v ratio and strained through cheesecloth. The filtrate was centrifuged at 10 000 rpm for 10 min. The resulting supernatant fluid was centrifuged at 50 000 rpm in a Beckman 50Ti rotor for 3 hours to produce a pellet of leaf material. This pellet was resuspended in 3 ml of undiluted antiserum and left at 37°C for 14 to 18 hours. The serum was clarified by centrifugation at 10 000 rpm for 15 to 20 minutes and then absorbed for a second time. Finally the antiserum was subjected to a further cycle of high speed centrifugation. Antisera treated in this manner showed no bands with homogenates from uninfected plants in Ouchterlony gel diffusion tests.

3. Double Diffusion in Gel (Ouchterlony method)

The Ouchterlony agar gel technique was used to detect antibodies to plant material and to determine end-point titres of antisera with homologous and heterologous virus isolates. The tests were performed (van Regenmortel, 1966) in plastic petri dishes of 10 cm diameter containing 20 ml of 0,6% agar, 0,075M NaCl buffered with 0,05 M borate pH 8,0.
Immunodiffusion tests in the presence of SDS were carried out in the following medium: 0,8% Ionagar, 0,5% SDS and 1,0% sodium azide (Purcifull and Batchelor, 1977).
To determine serum titres, each of a series of two-fold dilutions of antiserum was tested against a similar dilution of virus. The end-point titre was determined as the highest serum dilution at which a precipitin reaction was still visible.

4. Crossed Immunoelectrophoresis (Laurell Technique)

This technique was useful in determining the homogeneity of purified CaMV preparations and antisera, and was carried out according to the general method of Laurell (1965). A 10 µl aliquot of CaMV at 1 mg/ml was placed into a 1 mm diameter well cut in a 0,6% agarose slab on a 7,5 cm x 5,0 cm glass slide and electrophoresed as described for electrophoresis in agarose gels. After electrophoresis for 1½ to 2 hours, a 1 cm agarose strip containing the virus was removed from the slide and placed transversely across another identical glass slide. Agarose (0,6%) was added to the portion of the slide not covered by the strip to make a gel layer of 2 mm thick containing antiserum and electrophoresis at 5 V/cm was continued for 6 hours in the second dimension. After completion of electrophoresis, the slide was covered with wet filter paper and pressed with a weight of about 1 kg for 20 min. After pressing, non-precipitated serum protein was removed from the agarose by soaking the slide overnight in 0,1 M NaCl. The agarose layer was then dried under a current of hot air. Staining and destaining was carried out as described previously.

5. Enzyme Immunoassays

Enzyme immunoassays were used to detect and compare virus antigens (Clark and Adams, 1977; Koenig, 1978) and for detecting CaMV-specific antibodies (Bidwell et al., 1977).

a. Buffers (Clark and Adams, 1977)

i) Phosphate buffered saline (PBS, pH 7,4)

This buffer contained per 1000 ml of water:

NaCl	8,0 g
Potassium dihydrogen phosphate	0,2 g
Disodium hydrogen phosphate (12 H_2O)	2,9 g
Potassium chloride	0,2 g
Sodium Azide	0,2 g

ii) PBS-Tween

This consisted of PBS and 0,5% Tween-20

iii) Coating buffer

This buffer contained per 1000 ml of water:

Disodium carbonate	1,5 g
Sodium hydrogen carbonate	2,93 g
Sodium azide	0,2 g

iv) Substrate buffer

This buffer contained:

Diethanolamine	97 ml
Water	800 ml
Sodium azide	0,2 g
Magnesium dichloride	1 ml

The pH was adjusted to 9,8 with HCl and the buffer is made up to a final volume of 1 000 ml with water.

b. Preparation of IgG from antisera

i) Preparation of IgG

The IgG fractions of antisera were prepared by ammonium sulphate precipitation followed by ion exchange chromatography on diethylaminoethyl (DEAE) cellulose (Whatman's DE52) (Clark and Adams, 1977).

CaMV antiserum was diluted 1/10 with distilled water and precipitated by the addition of an equal volume of saturated

ammonium sulphate. The mixture was left at room temperature for 60 min to allow complete precipitation. The precipitate was sedimented by centrifugation at 10 000 rpm in a Sorvall SS-34 rotor and resuspended in 2 ml of PBS diluted 1:1 with distilled water (half-strength PBS). Ammonium sulphate was removed by exhaustive dialysis against half-strength PBS at 4°C. The dialysed preparation was then passed through a 4 cm x 1 cm column of DEAE cellulose equilibrated with half-strength PBS. Fractions absorbing at 280 nm were collected and pooled. The IgG preparation was then concentrated or diluted to 1 mg/ml using an $E_{280}^{0,1\%}$ of 1,4 (Clark and Adams, 1977) to determine the concentration.

ii) Preparation of specific antibodies

Antibodies specific for CaMV were prepared by mixing 1,0 ml of purified CaMV at approximately 3 mg/ml in 0,05 M phosphate buffer pH 7,0 with 1,0 ml of specific antiserum. The mixture was allowed to stand at 37°C overnight before centrifugation for 40 minutes at 30 000 rpm in a Beckman 50Ti rotor to sediment the virus/antibody complexes. The pellet obtained was resuspended in water (Hardie and van Regenmortel, 1977) and the pH was carefully adjusted to 2,9 with HCl to release the bound antibodies. The preparation was then recentrifuged and the pH of the supernatant fluid, containing the antibodies, was immediately adjusted to 7 with 0.1N NaOH.

c. Labelling of IgG with alkaline phosphatase

The IgG fraction of an antiserum, or specific antibodies were used at a concentration of 1,0 mg/ml - prepared as described above. Alkaline phosphatase was supplied by Boehringer

(Mannheim) at a concentration of 5 mg/ml as a suspension in ammonium sulphate. The enzyme (2,5 mg) was pelleted by centrifugation at 3 000 rpm in a glass tube. The supernatant fluid was carefully removed and the pellet was dissolved in 1 ml of IgG. The mixture was dialysed against PBS. Glutaraldehyde was used as a bifunctional reagent for coupling the enzyme to the antibody.

The volume of the dialysed mixture was determined and glutaraldehyde was added to a final concentration of 0,06%. After thorough mixing, the solution was left at 20°C for 4 hours, at the end of which time it had acquired a light yellow-brown colour. Excess unreacted glutaraldehyde was removed by dialysis against PBS at 4°C. After dialysis bovine serum albumin (BSA) was added to a concentration of 5 mg/ml to the conjugate solution which was stored at 4°C in a siliconised (Desicote, Beckman product) glass bottle.

d. The double antibody "sandwich" ELISA

This technique for detecting plant viruses has been described by Voller et al., (1976) and by Clark and Adams (1977). ELISA tests were performed in Cooke M29AR plastic microtitre plates or in "Microelisa" plates (Dynatech). Optimum concentrations of reactants were determined for each assay by cross-titrations. The wells of the plates were coated with unlabelled IgG or specific antibody by allowing 0,2 ml of IgG freshly diluted into coating buffer to remain in the wells for 4 hours at 37°C. The plate was kept in a humid chamber (plastic freezer box containing moist filter paper) during this and subsequent incubation steps. The contents of the wells were then shaken out and the wells were washed

out three times with PBS-Tween and the plate was shaken dry. The virus antigen under study was next pipetted into the wells (0,2 ml) and incubated at 4°C for about 16 hours. After another washing step 0,2 ml of enzyme-labelled IgG diluted in PBS-Tween and 0,2% ovalbumin or sometimes BSA were pipetted into the wells.

The plate was left for a further 4 hours at 37°C and after another washing step, 0,3 ml of p-nitrophenyl phosphate at 1 mg/ml in substrate buffer was added to the wells. After allowing 60 minutes for hydrolysis of the substrate, to take place the reaction was stopped by the addition of 50 µl of 3 M NaOH to each well. The contents of the wells were withdrawn and diluted 1/3 in water. The absorbance at 405 nm was determined with a Zeiss PM2D spectrophotometer.

e. ELISA for detection of CaMV-specific antibodies

i) Indirect test

The use of this technique with viral antigens has been examined by Bidwell et al., (1977). The procedure followed was similar to that described above. The wells of a microtitre plate were coated with CaMV in coating buffer. After washing, dilutions of the test antiserum were added to the wells and incubated overnight at 4°C. After another washing step, the IgG fraction of a goat anti-rabbit IgG antiserum labelled with alkaline phosphatase and diluted in PBS-Tween + 0,2% BSA was pipetted into the wells and left for 4 hours at 37°C. Subsequent steps involving the addition of substrate and determining the absorbance were as described above. Incubation times could be varied by at least 20% without affecting the final result.

ii) Inhibition of ELISA

An alternate method used to detect and compare antibodies which react with CaMV was to inhibit the antibody binding in a "sandwich" double antibody ELISA. Inhibition was carried out by incubating 0,2 ml of CaMV diluted to give an absorbance value of about 1,5 in an ELISA, with an equal volume of test antiserum for 2 hours at 37°C in pH 7,4 PBS-Tween and 0,2% ovalbumin. The virus was then assayed in a CaMV-specific "sandwich" ELISA as described previously. Percentage inhibition was calculated from the ratio of absorbance obtained with the inhibiting serum to that obtained in the absence of inhibitory serum, according to the formula:-

$$\% \text{ Inhibition} = 100 - \left(\frac{\text{Absorbance in the presence of inhibiting serum}}{\text{Absorbance in the absence of inhibiting serum}} \times \frac{100}{1}\right)$$

J. LECTIN BINDING TECHNIQUES

1. Concanavalin A (Con A)

Con A, prepared by the method of Agrawal and Goldstein (1967), was received from P. Smith of the Biochemistry Department of the University of Cape Town. Covalent coupling of fluorescein isothiocyanate was carried out by the method described by Malluci (1976).

2. Gel Staining with Fluorescein-labelled Con A

Electrophoresis gels were fixed in 25% ethanol, 7% acetic acid, then equilibrated in standard salt solution and subsequently stained in a solution of fluorescein-Con A (FITC-Con A) (1 mg/ml) in the same solution containing 5 mM Ca^{++}

and Mn^{++} each, for 3 hours at room temperature. Non-specific binding was monitored by staining in the presence of 0,1 M α-D-methylglucopyranoside, a ligand specific for Con A.

Gels were destained by diffusion in standard salt solution until the background was free of fluorescence. Stained gels were photographed for fluorescence using a short wave (254 nm) transilluminator (U V Products) through a Wratten type 61 filter with Ilford HP5 film.

3. Inhibition of Antibody Binding

This technique was used to show binding of Con A to the surface of the CaMV capsid.

a. Antibody binding

A dilution series from 10 to 0,1 µg/ml of CaMV in ELISA coating buffer pH 9,6 was made and 200 µl aliquots of each dilution were pipetted into the wells of a Cooke M29AR polystyrene microtitre plate. After 3 hours at 37°C the wells were washed with PBS-Tween. Alkaline phosphatase-conjugated antibodies specific for CaMV (Australia isolate) were added at different dilutions from 1/200 to 1/3 200 in PBS-Tween+0,2% ovalbumin. After overnight incubation at 4°C, the plate was washed with PBS-Tween and 300 µl of p-nitrophenyl phosphate at a concentration of 1 mg/ml in diethanolamine buffer pH 9,8 were added to each well. After 1 hour at room temperature (about 20°C) the hydrolysis was stopped by the addition of 50 µl of 3 M NaOH to each well. The contents of the wells were withdrawn and diluted 1/3 in distilled water and the absorbance at 405 nm was determined.

b. Inhibition with Con A

For the inhibition, concentrations of reactants were determined in a checkerboard titration as described above to yield an absorbance value of about 1,0. Using these conditions, a number of microtitre plate wells were coated with CaMV. Before the addition of labelled antibodies, dilutions of Con A in standard salt solution containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$ were added to the wells. For the control determinations, each dilution contained a 100-fold molar excess of methyl glucoside. After allowing 60 min at 20°C for Con A binding, the plate was washed and the labelled antibodies and substrate were added as described above.

Percent inhibition was calculated from the formula:

$$\% \text{ Inhibition} = 100 - \left(\frac{\text{Absorbance in presence of Con A}}{\text{Absorbance in absence of Con A}} \times \frac{100}{1}\right)$$

4. Iodination of Con A

Iodination of Con A was performed by the chloramine T method (Greenwood et al., 1963) using ^{125}I (The Radiochemical Centre, Amersham, U.K.). Unreacted iodine was removed by exhaustive dialysis against standard salt solution. The final preparation had a specific activity of approximately 17×10^7 cpm/mg of Con A.

K. BUFFERS

In addition to the specialised buffers and solutions described under virus extraction and polyacrylamide gel electrophoresis, the following buffers were used at different stages of this work:

1. Phosphate Buffers (Chase, 1968)

These buffers of different pH and ionic strength were prepared from the following stock solutions:

Solution A	NaH_2PO_4	0,2 M
Solution B	Na_2HPO_4	0,2 M

To make a buffer of 0,1 M of different pH values, the solutions were mixed as follows:

	ml/l Solution A	ml/l Solution B
pH 6,0	438,5	61,5
pH 7,0	195	305
pH 7,5	80	420
pH 8,0	26,5	473,5

Dilutions of these buffers to lower molarities were made with distilled water as required.

2. Phosphate Buffer 0,1 I, pH 7,5 (Miller and Golder, 1950)

This buffer was prepared from the following stock solutions:

0,5 M	Na_2HPO_4	24,3 ml
4,0 M	NaH_2PO_4	0,5 ml
5,0 M	NaCl	32,0 ml
	Distilled H_2O	to 2000 ml

3. Borate buffer pH 8,0 (Chase, 1968)

This buffer was prepared from the following stock solutions:

Solution A	Boric acid	0,2 M
Solution B	Sodium tetraborate	0,05 M

To 700 ml of solution A was added 300 ml of solution B.

4. Borate Buffer pH 8,6, 0,1 M (Polson and Russel, 1967)

This buffer contained the following:

Boric acid	0,035 M
NaOH	0,0175 M
HCl	0,0075 M
NaCl	0,073 M

5. Standard Salt Solution

This solution used in Con A binding studies was composed of 0,1 M NaCl buffered with 0,001 M sodium phosphate pH 7.0.

ooo 000 ooo

CHAPTER IV

RESULTS

A. PROPAGATION OF CaMV

Both turnip (cv Maizura) and Mustard (cv Spahili) plants were found to be suitable propagation hosts for CaMV. Depending upon the virus isolate in question, symptoms appeared 9 - 13 days after infection. Virus isolates produced mosaic and vein-clearing symptoms which could be classed as moderate (New York, Cabb-S, Oudtshoorn) or severe (Australia, Banhoek). Severe symptoms included dwarfing of the new leaves and leaf distortion particularly in the case of the Australia isolate.

In some experiments, the mustard cv White London was used. This host plant exhibited severe stunting after about 7 days, suggesting that it might prove useful as an indicator host. Datura stramonium was not suitable as a local lesion host for the isolates under study.

Leaves were used either immediately after harvesting for virus extraction or were stored for some time in plastic bags at $4^{o}C$.

B. PURIFICATION OF CaMV

1. Purification by the Triton X-100/Urea Method

CaMV was extracted from systemically infected leaves by the Triton X-100/urea method described by Hull et al. (1976). Final purification was by sucrose density-gradient centrifugation.

After centrifugation at 25 000 rpm on a 10 - 40% (w/v) sucrose density-gradient for 2 to 2½ hours, the virus band was removed from the gradient by means of a bent pasteur pipette or by displacement from the bottom of the tube with a 60% (w/v) sucrose solution. Virus after density-gradient centrifugation is referred to as "pure" virus. The absorbance at a wavelength of 260 nm was monitored and fractions were collected using an ISCO density-gradient fractionator. Fig. 2 shows the density-gradient profile from a typical centrifugation of CaMV prepared from fresh leaf material. The virus peak shows a small faster sedimenting shoulder. Such shoulders or asymmetric peaks were also observed by Al Ani et al. (1979b) and were found by them to represent different states of proteolysis of the virus capsid. For routine purification the entire virus-containing zone was removed from the gradient including the shoulder.

After a final high-speed centrifugation, the virus pellet was resuspended in water. Difficulty was experienced in resuspending the pellet completely and even after prolonged trituration, some large aggregates of virus were still present. An ultraviolet scan of a purified preparation of CaMV in water is shown in Fig. 3. An electron micrograph of density-gradient purified CaMV (taken by Dr R.G. Milne) showed no contaminating material. CaMV in water was stored at 4°C or at -20°C. Infectivity was not destroyed under these conditions. Virus yield depended upon the isolate, and varied from about 1 to 5 mg/kg of leaf material, but was up to 50% less if frozen leaves were

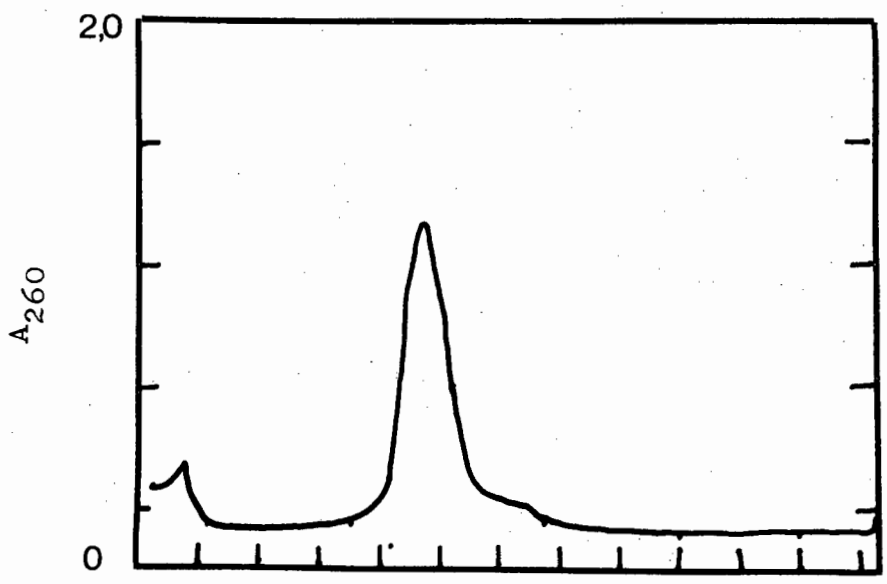


FIGURE 2

Sucrose density-gradient profile of a CaMV preparation prepared by the method of Hull et al. (1976) from freshly harvested virus-infected leaves. Sedimentation was from left to right. The entire region showing absorbance at 260 nm was removed from the gradient.

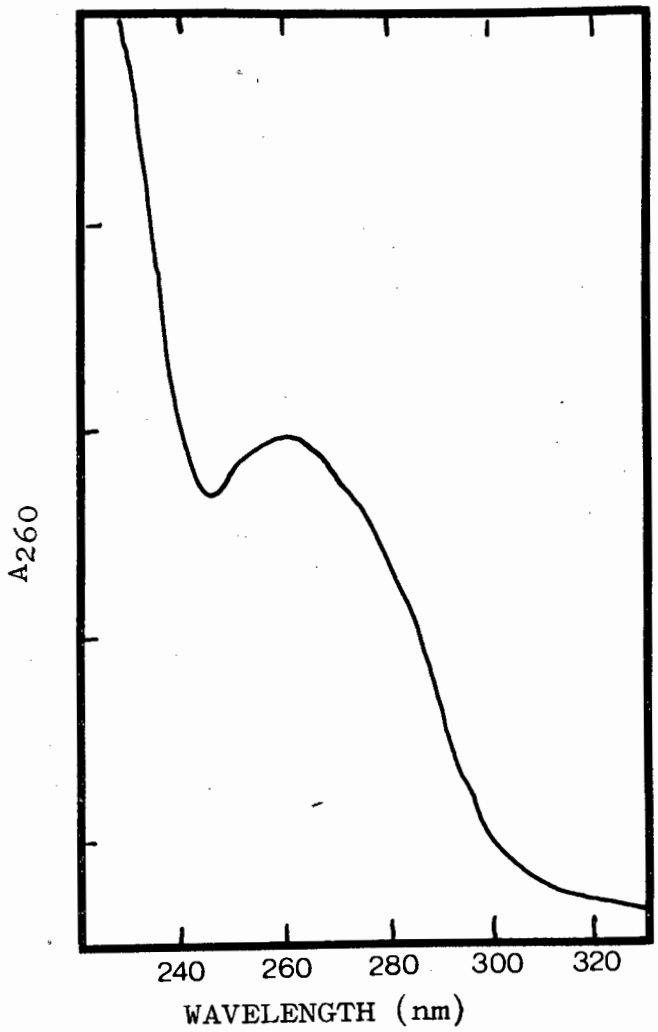


FIGURE 3

Ultraviolet absorbance profile of sucrose density-gradient purified CaMV (NY 8153) suspended in distilled water.

used. This could be attributed to difficulty experienced in resuspending the first high speed pellets obtained from frozen leaf material.

2. Extraction of CaMV using a Thin Layer Rotor

Because of the problems experienced in resuspending pelleted CaMV, the use of a rotor specially designed to avoid centrifuging virus to a pellet was investigated. The application of this thin layer rotor to the purification of filamentous viruses, has been described by Polson and Kiefer (1975). The rotor has a central cavity into which infected tissue is placed, and after centrifugation, extracted virus in solution is removed from a peripheral channel. The use of relatively low speeds and the fact that the virus is not pelleted, suggested its applicability in CaMV extraction. However the virus could not be extracted directly from frozen-thawed leaves, and it was necessary to use leaf homogenate which had been treated with Triton X-100 and urea.

CaMV infected leaves were homogenised and incubated with Triton X-100 and urea according to the method of Hull et al. (1976). After clarification by low speed centrifugation, the homogenate was placed into the central well of the thin layer rotor. A volume of about 120 ml of this preparation was centrifuged at 15 000 rpm for 45 min. After centrifugation, the fluid in the receiving cavity of the rotor was withdrawn using a pasteur pipette. This fluid (about 1,5 ml) was found by gel precipitin tests to contain concentrated CaMV, but its ultraviolet absorbance profile and its reaction with anti-fraction 1 plant protein antibodies (van Regenmortel,

1964) showed that further purification by sucrose density-gradient centrifugation was required. Practical considerations, such as the small capacity of this rotor precluded its use for routine purification. However, since the virus was not centrifuged to a pellet, the difficulty of resuspending aggregated CaMV particles was obviated.

3. Effect of the Purification Method on the State of the Virus Capsid

Although CaMV is considered to be an extremely stable virus particle (Shepherd, 1976), its capsid protein is very susceptible to proteolysis, especially during the purification process (Al Ani et al., 1979b). Several modifications of the standard purification procedure (eg. the use of protease inhibitors) were tested in an effort to prevent capsid degradation.

It should be noted that in the initial stages of this work, no special attempts were made to control proteolysis of the CaMV capsid. Leaf samples were used either fresh, or after storage for up to 2½ weeks at 4°C. In some cases, the virus preparations were stored at 4°C before final purification on a sucrose density-gradient. As will become apparent, such treatments can affect the degree of proteolysis of CaMV. Unless otherwise mentioned, the degree of proteolytic degradation of the CaMV preparations used in this thesis is unknown.

The state of proteolysis of the CaMV capsid protein purified by several different procedures was monitored on 10% polyacrylamide slab gels (Laemmli, 1970). In these

experiments all sucrose solutions used for centrifugation were boiled, and once purification was started it was continued with as little delay as possible. All buffers were used at a temperature of 4°C to 8°C and the virus sample (Oudtshoorn isolate) was kept as close to 4°C as possible at all times in an attempt to retard the action of any proteolytic enzymes. The nomenclature of the CaMV polypeptides (P1 to P7) used throughout this thesis is the same as that used by Al Ani et al. (1979b) but the highest molecular weight polymers (>85 000 daltons) have been labelled "P8".

a. Purification of CaMV from fresh leaves

Virus was extracted from host plant leaves immediately after harvesting. The time between harvest and commencement of extraction was usually less than 20 minutes. Leaves were not used later than 4 hours after harvesting. During this period the infected leaf sample was stored at 4°C. The polyacrylamide gel profile of virus prepared in this way is illustrated in Fig. 4 (Track 1). This gel profile shows the 33 000 dalton (P1), 37 000 dalton (P2), 39 000 dalton P3), 42 000 dalton (P4) and the 55 000 dalton (P6) bands, as well as the higher molecular weight polymers (P7 and P8).

According to Al Ani et al. (1979b), the 42 000 dalton P4 component is the major structural polypeptide of CaMV and all lower molecular weight components represent degradation products, formed by the action of proteolytic enzymes. The "P7" polypeptides represent stable polymers of P4 and its degradation products, while P5 (not present in this

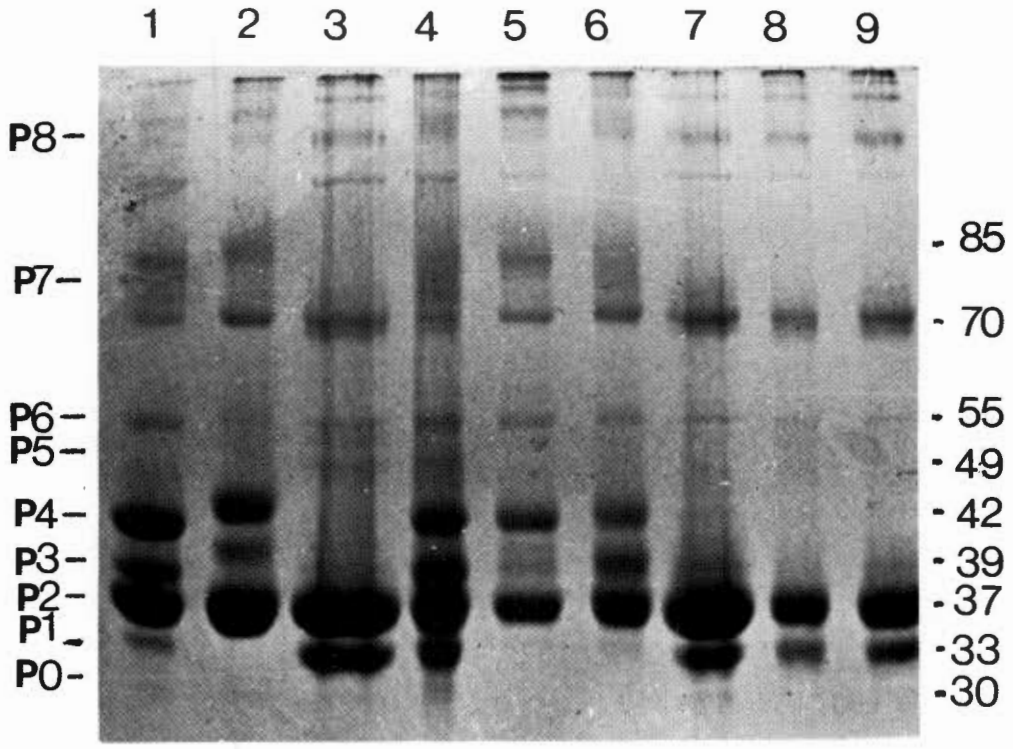


FIGURE 4

Polypeptide patterns of CaMV samples of various origins -

Track 1 : CaMV purified from freshly harvested leaves.

Track 2 : CaMV purified from freshly harvested leaves in the presence of protease inhibitors.

Track 3 : CaMV purified from freshly harvested leaves in the absence of sodium sulphite.

Track 4 : CaMV purified from liquid nitrogen-frozen leaves.

Track 5 : CaMV purified from liquid nitrogen-frozen leaves in the presence of protease inhibitors.

Track 6 : CaMV purified from -20°C frozen leaves in the presence of protease inhibitors.

Track 7 : CaMV purified from leaves after storage at 4°C for 14 days.

Track 8 : CaMV purified from fresh leaves and stored for 14 days in a semi-pure condition.

Track 9 : CaMV purified from leaves stored at 4°C for 7 days followed by storage at room temperature for 14 days in the semi-pure condition.

Molecular weights are indicated as multiples of 1 000 daltons.

preparation) and P6 show sequence homologies with P4, but are not polymers of any other polypeptides. They may form polymers in the "P8" region.

The presence in this preparation of components smaller than 42 000 daltons, and the absence of the 49 000 dalton P5, therefore indicates that this sample had undergone some proteolysis before or during purification.

b. Purification of CaMV from fresh leaves with the addition of protease inhibitors to the extraction buffers

Virus was extracted from fresh infected leaves as described above, but 1 mM phenylmethylsulphonylfluoride, 0,02 M iodoacetate and 0,02 M phenanthroline was added to all extraction buffers used in the purification. These substances are known to inhibit serine-, divalent cation- and cysteine-type proteases respectively (Weber et al., 1972).

The gel profile of CaMV purified in the presence of these inhibitors is illustrated in Fig. 4 (Track 2). There is no P1 band and P3 and P4 show slightly less migration than in the absence of inhibitors, (Track 1). The calculated molecular weights for the "P4" and "P3" bands in this preparation are 45 000 and 41 000 daltons respectively, and the P5 band is absent. It may be concluded that the addition of inhibitors had little influence on the proteolysis of the capsid protein.

c. Purification of CaMV in the Absence of Reducing Agents

In 1978 Koenig et al. found that proteolysis of potato virus X capsid protein was due to reducing agent-dependent proteases. To determine whether this was also the case with

CaMV, virus from fresh CaMV - infected leaves was extracted with potassium phosphate buffer pH 7,5 without the addition of sodium sulphite. The profile yielded by virus prepared in such a way is illustrated in Fig. 4 (Track 3). Both the minor proteins P5 and P6 are present, but the major protein, P4, appears to have been extensively degraded to P2 and P1 components.

As the absence of reducing agent had such a marked effect on degradation, virus from fresh infected leaves was extracted and purified with Na_2SO_3 in both the purification buffers, and not just in the extraction buffer as was usual (Hull et al., 1976). A gel profile similar to that of Track 1 was obtained, indicating that any proteolysis which is affected by the absence of reducing agents occurs in the early stages of the purification procedure.

d. Purification of CaMV from liquid nitrogen-frozen leaves

Al Ani et al. (1979b) found that freezing infected leaves prior to virus extraction had the effect of yielding better preserved CaMV. To determine the effect of freezing at very low temperatures, fresh leaves were frozen in liquid nitrogen immediately after harvest. The frozen leaves were then crushed and thawed in the presence of the standard buffer.

Fig. 4 (Track 4) shows the polypeptide profile of virus prepared in this way. Bands P0 (30 000 daltons), P1, P2, P3 and P4 as well as the minor bands P5 and P6 are apparent. This indicates that the major capsid protein (P4) had been fairly extensively degraded but that P5 and P6 were still intact. The relative amounts of P3 and P1 are greater

than in the case of virus prepared from fresh leaves, indicating more extensive degradation of P4.

e. CaMV prepared from liquid nitrogen-frozen leaves in the presence of protease inhibitors

As it seemed likely that proteases are liberated when the plant cells are disrupted during tissue homogenisation, an attempt was made to minimise their action in the early stages of extraction.

Infected leaves frozen in liquid nitrogen were crushed while still frozen and were then thawed and further homogenised in a Waring Blendor in buffer containing the protease inhibitors described in 3b. Inhibitors were also present in subsequent purification buffers, while boiled sucrose solutions were used for density-gradient centrifugation. The resulting virus exhibited the gel profile illustrated in Fig. 4 (Track 5). The polypeptides P4 and P2 are predominant, P5 is absent and P3 is visible as two very faint bands of molecular weights 41 000 and 39 000 daltons.

The treatment described above, prevented the degradation to P1 and lower molecular weight species, but did not prevent the disappearance of P5. The "P3" as seen in this preparation suggests that P4 has been cleaved yielding 2 degradation products of different molecular weights. It also suggests the possibility that degradation of P4 to P2 does not proceed via P3, since although P2 is present, a typical P3 is not.

f. The effect of freezing to -20°C and extraction in the presence of protease inhibitors

CaMV was extracted from leaves which had been frozen to -20°C. The leaves were thawed in buffer containing the protease inhibitors described above. The resulting gel profile of virus prepared in this way is illustrated in Fig 4 (Track 6). The polypeptide P5 is absent and there is little P1 and no P0, indicating that although some proteolysis had occurred, the virus capsid was less extensively degraded than that shown in Tracks 1, 3 or 4. The profile is similar to that found by Al Ani et al. (1979b) with frozen leaves and is identical to that obtained with frozen leaves extracted in the standard buffer. This finding suggests that some proteolysis of the CaMV capsid protein occurs very early in the purification procedure, or in situ before extraction.

g. The effect of storage of CaMV in infected leaf samples and as a semi-pure preparation

Before it became known that CaMV was susceptible to proteolysis (Al Ani et al., 1979b) the virus was sometimes stored in leaf samples at 4°C for up to 2½ weeks before extraction. It occasionally also stood for some time in a semi-pure condition at 4°C before being applied to sucrose density-gradients for final purification. The gel tracks 7, 8 and 9 (Fig. 4) show the condition of the capsid protein after 14 days storage at 4°C in the infected leaf, after 14 days at 4°C in the semi-pure condition, and after 7 days in the leaf followed by 14 days semi-pure at room temperature (about 20°C) respectively.

As the gel profiles show, these samples had undergone severe proteolytic degradation with almost all the capsid proteins being in the P2 and P1 form. These results suggest that some degradation occurs *in situ* in the infected leaf tissue during storage or purification. This is shown by comparing the polypeptide profile of virus prepared from stored leaves with that of virus extracted from fresh leaves (Tracks 1 and 7 in Fig. 4). Both samples had undergone identical extraction procedures.

The origin of the P7 band is also illustrated by the gel profile resulting from the above series of experiments. The bands comprising "P7" almost exactly mirror the P4 and lower molecular weight bands seen lower in the gel, which agrees with the conclusion that P7 consists of dimers of P4 and its degradation products which are stable under the conditions used for viral dissociation. In the gel very low molecular weight species which migrate with the ion fronts were also visible. These were not reported by Al Ani *et al*. (1979b), and it was thought possible that they were contaminating plant components. To test this possibility, a sample of CaMV which had not been treated with SDS and mercaptoethanol, was subjected to polyacrylamide gel electrophoresis. The stained gel showed that the virus did not penetrate the stacking gel, and no low molecular weight components were seen at the ion front, therefore showing the absence of contaminants.

None of the attempts to control proteolysis yielded CaMV with the major capsid protein entirely in the P4 state. Some degradation products were always present. This meant that CaMV which was used in the studies described later in

this thesis was not in the native state. Attempts to use glutaraldehyde-treated gelatin to remove proteolytic enzymes (Polson, 1977) resulted in a loss of almost all the virus.

In the course of the experiments described above it was found that purified CaMV stored at -20°C could be repeatedly thawed and refrozen without any change in its polypeptide pattern. Preparations of the virus in a known state of proteolysis were therefore stored at -20°C.

4. Isopycnic centrifugation in caesium chloride density-gradients

Attempts were made to use equilibrium centrifugation in a CsCl density-gradient for separating different degradation states of the virus and for obtaining relatively undegraded, or possibly even native virus. This approach was based on the finding that CaMV in different states of proteolysis could be separated from a preparation of relatively undegraded CaMV by rate zonal centrifugation in sucrose density-gradients (Al Ani et al., 1979b).

It seemed plausible that equilibrium centrifugation might effect a separation into discrete components on the basis of buoyant density differences originating in different states of proteolysis. Such centrifugation to equilibrium was found by Polson (1971) to allow a clear separation of a natural mixture of Nudaurelia viruses.

Some preliminary experiments using a Beckman SW 50 rotor had shown that purified CaMV condensed as a

single opalescent band in a CsCl density-gradient. The buoyant density of all preparations tested (the state of proteolysis of which was not known), as determined by refractometry varied between 1,361 and 1,378, which is close to the reported figure of 1,37 g/ml (Shepherd, 1970). To enable better observation of the behaviour of CaMV in density-gradients, virus preparations were centrifuged to equilibrium in a Beckman Model E analytical ultracentrifuge. A standard cell with a Kel F centrepiece and an An D rotor were used for these studies.

Purified CaMV was mixed with CsCl so that the initial density of the solution as determined by refractometry was about 1,38 g/ml. The virus concentration in the final solution was adjusted to be approximately 0,2 mg/ml. The solution was then centrifuged at 52 000 rpm at 20°C for up to 7½ hours. By this time a CsCl density-gradient was formed in which the CaMV had condensed at its isopycnic density level. A photograph was taken with schlieren optics.

Fig. 5 illustrates an isopycnic centrifugation diagram obtained with a preparation of CaMV (Oudtshoorn isolate). This virus preparation was extracted from fresh leaves in the presence of protease inhibitors (See Section 3e of this Chapter) and exhibited the polypeptide profile shown in Track 2 of Fig. 4. The same preparation had been shown to be electrophoretically heterogeneous (Fig. 26). However, as is apparent from the schlieren photograph any heterogeneity in buoyant density was not sufficient to allow separation into different entities which would be seen as more than one "zig-zag" pattern. As further proof

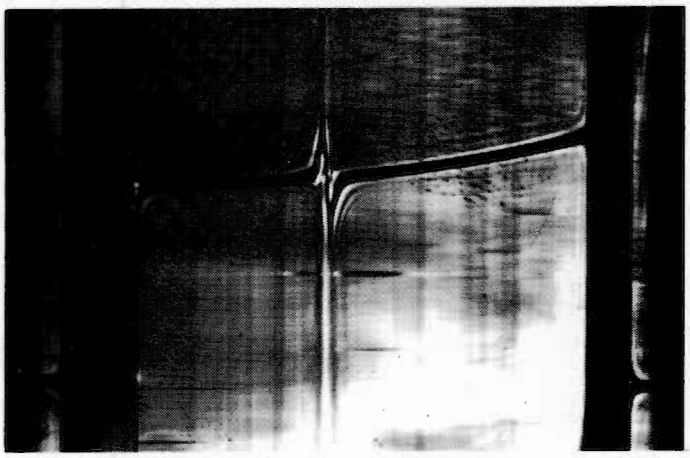


FIGURE 5

Schlieren photograph of CaMV after centrifugation to equilibrium in a CsCl density-gradient. The starting density of the CsCl solution was 1,38 g/ml and the concentration of the virus was 0,2 mg/ml. The preparation was centrifuged at 52 000 rpm for 7½ hours. Note the single zig-zag pattern which represents the differential of a Gaussian distribution, showing that the preparation was homogenous with respect to buoyant density. This virus preparation was however electrophoretically heterogeneous.

that particle buoyant density is not appreciably altered by natural proteolytic degradation, a mixture of partially degraded (Fig. 4, Track 2) and extensively degraded (Fig. 4, Track 9) CaMV was similarly subjected to isopycnic centrifugation. No heterogeneity was apparent in the schlieren pattern. Furthermore, when the preparation shown in Fig. 5 was centrifuged at a 10 times higher concentration, no other components became apparent.

C. ANTISERA

Antisera specific for CaMV were raised in rabbits by intramuscular injection of preparations purified on sucrose density-gradients. The resulting antisera did not react with homogenates of healthy or infected host plant leaves in gel precipitin tests, but gave a single precipitin band near to the antigen well with density-gradient purified CaMV (Fig. 9). With the homologous antigen, titres of up to 1/256 were obtained in immunodiffusion tests.

Since antisera were raised in the early stages of this work, before it became apparent that proteolysis was a factor, it should be noted that the CaMV preparations used for antiserum production were not monitored to ascertain the condition of their capsid protein, and it is likely that virus in different states of proteolysis was used for antiserum production.

For use in the enzyme-linked immunosorbent assay (ELISA), which is much more sensitive than precipitin tests, the antisera were exhaustively absorbed as a precautionary measure

with a healthy host extract to ensure that they contained a minimum of antibodies to host proteins. In subsequent ELISA tests with homogenates of healthy plant material at a 1/10 dilution, absorbance values of less than 0,03 absorbance units above background were obtained, showing that assays prepared with such antisera were highly specific for CaMV.

D. DETECTION OF CaMV IN CRUDE EXTRACTS

1. Immunodiffusion Tests in the Presence of SDS.

It is not possible to detect CaMV in crude leaf extracts by normal serological means, due probably to the low concentration of free virus in the leaves. The use of immunodiffusion tests in the presence of SDS for this purpose (Purcifull & Batchelor, 1977) was investigated since it was envisaged that sufficient virus may be liberated by SDS treatment from the inclusion body matrix to form a visible precipitate.

Infected leaves were homogenized in a mortar and pestle in the presence of 3% SDS in water (1 g leaf for every 1 ml of SDS solution). The homogenate was either first heated at 100°C in a boiling water bath for 2 min. or placed immediately into wells cut into an agar plate containing 1% SDS. CaMV antisera were used undiluted. Control antigens were uninfected leaf homogenate and purified CaMV at a concentration of 1 mg/ml. A diffuse band formed near the antigen well with pure CaMV, but no virus-specific precipitates were visible in the case of the infected leaf material.

2. Enzyme-linked Immunosorbent Assay (ELISA)

a. Detection of CaMV in leaf extracts

Because the majority of CaMV particles in infected leaves are embedded in the matrix of intracytoplasmic inclusion bodies (Fujisawa et al., 1967), the widely used technique of gel diffusion has in the past proven ineffective for the detection of virus in crude homogenates of infected leaf material. Immune adherence was successfully used with crude leaf extracts in 1964 by Nelson and Day, indicating that a technique of high sensitivity is required to detect the small amount of free non-included CaMV contained in infected leaves. The applicability of the enzyme-linked immunosorbent assay (ELISA) was investigated in this study because of its many advantages, including sensitivity.

In order to produce visible precipitin lines with its homologous antiserum in agar gel diffusion tests, purified CaMV was used at concentrations exceeding 0,5 mg/ml. By contrast, a double antibody "sandwich" ELISA prepared with a hyperimmune serum directed against the Cabb-S isolate made it possible to detect as little as 20 ng/ml of purified CaMV (Fig. 6).

The same high sensitivity of this assay for detecting pure CaMV was not obtained when an early antiserum was used for ELISA.

ELISA was also found to be effective for detecting CaMV in crude leaf extracts. Systemically infected leaves were ground up in a mortar and pestle with PBS-Tween in a 1:1

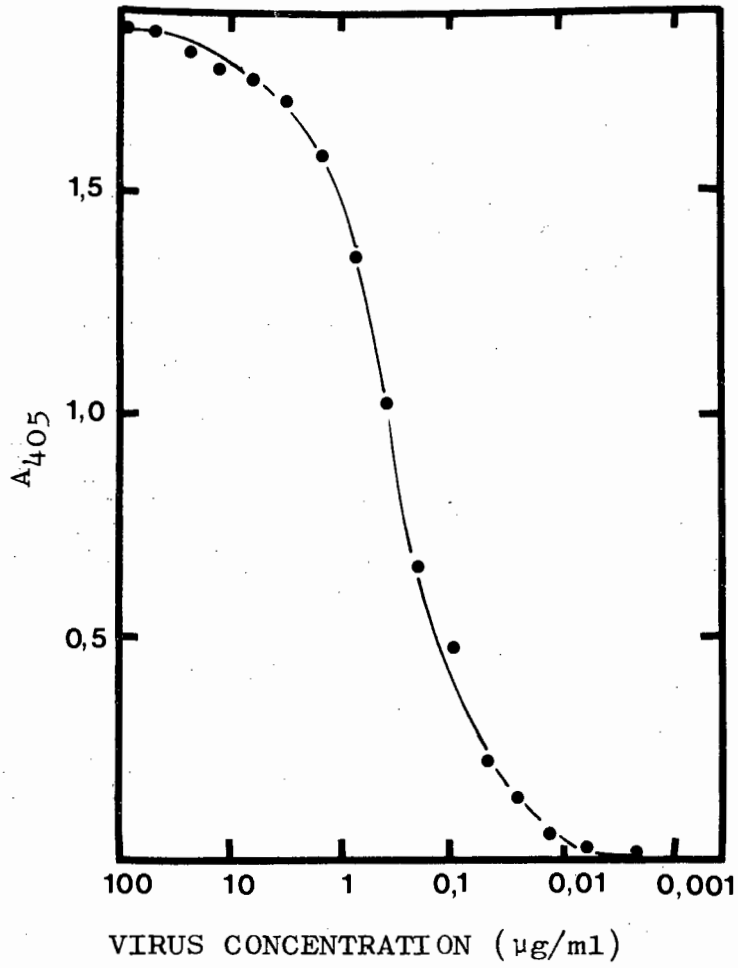


FIGURE 6

Absorbance values at 405 nm obtained in ELISA with purified CaMV (Cabb-S) and its homologous antiserum. For this assay the microtitre plate was coated with IgG at a concentration of 1,0 µg/ml and enzyme-labelled antibody was added at a dilution of 1/800.

w/v ratio and filtered through cheesecloth. Dilutions of the homogenate were then tested in ELISA. Fig. 7 shows the results of a test made with mustard cv Spahili leaves 9 days after infection. Although the CaMV isolate (Cabb-S) used for infection had not yet begun to show its characteristic symptoms, virus was detectable by ELISA. There was a slight non-specific reaction with healthy plant extract, indicating that some host plant component antibodies were still present in this particular antiserum. This non-specific colour could be reduced by about 50% by the addition of antibodies to host plant Fraction I protein (Van Regenmortel, 1964b) at the same time as the conjugate was added (Koenig, 1978), but this was not routinely done as it was only at high concentrations of sap (1/10) that non-specific reactions were significant.

ELISA was also used to screen a number of potential CaMV-infected cruciferous plants from a local farm. CaMV was positively identified in mustards, swedes, kale and cabbage samples with absorbance values at 405 nm of about 0,3 being obtained with 1/50 dilutions of leaf homogenates. The kale leaves were used as a source of virus for further propagation (Banhoek isolate). An advantage of the immunological assay was its ability to detect virus in both frozen and dried leaf samples. Dried samples were ground up in PBS-Tween, diluted in the same buffer and assayed as usual. Another South African isolate (Oudtshoorn) was detected in this way in dried leaf tissue which had been stored for 10 years at 4°C in the presence of self-indicating silica-gel.

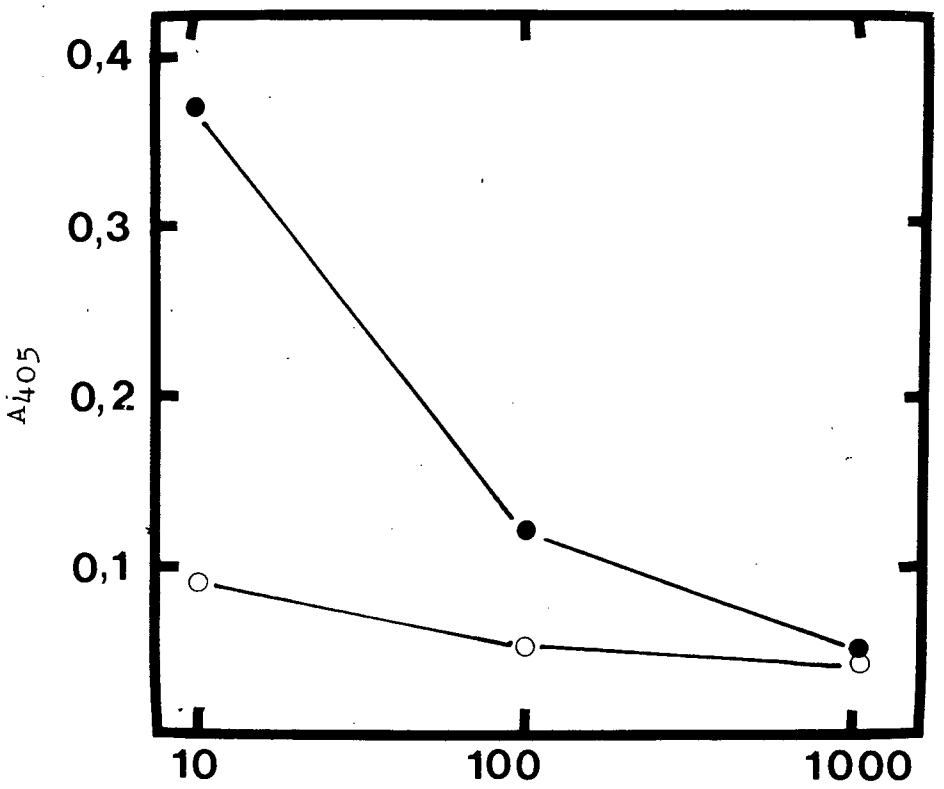


FIGURE 7

ELISA absorbance values obtained with extracts of healthy (○) and infected (●) turnip leaves 9 days after infection with CaMV (Cabb-S). Concentrations of reactants for ELISA are identical to those described in Figure 6.

No attempt was made in the foregoing tests to relate the colour development obtained in assays with crude leaf extracts to the calibration curve for pure CaMV (Fig. 6). This is because it is not certain whether only free CaMV, or CaMV embedded in inclusion bodies reacts in ELISA, as a single inclusion body may contain many virus particles inaccessible to virus antibodies. This aspect was studied in subsequent experiments.

b. Development of CaMV in host plant leaves

In an attempt to follow the progress of the development of CaMV in systemically infected leaves, symptom expression, ELISA values and infectivity of homogenates of foliage from 3 different CaMV-infected host cultivars were monitored at intervals after infection.

Maizura turnip, and Spahili and White London mustard plants were inoculated with CaMV (Cabb-S) about 7 - 10 days after germination. Times of inoculation were arranged so that the plants, all harvested on the same day, had been infected for different lengths of time. About 16 - 18 plants were used for each time interval. The foliage of the plants was homogenised with PBS-Tween in a 1:1 w/v ratio and tested for the presence of CaMV by ELISA. Fig. 8 shows the ELISA values obtained at different times after infection. Infectivity tests performed with representative leaf samples showed a concomitant rise in infectivity to a maximum at about 21 days, followed by a decrease as the plants aged (Table 3). Severity of symptom expression closely followed a similar pattern to the maximum at about 21 days. It is evident from Fig. 8, that development in White London

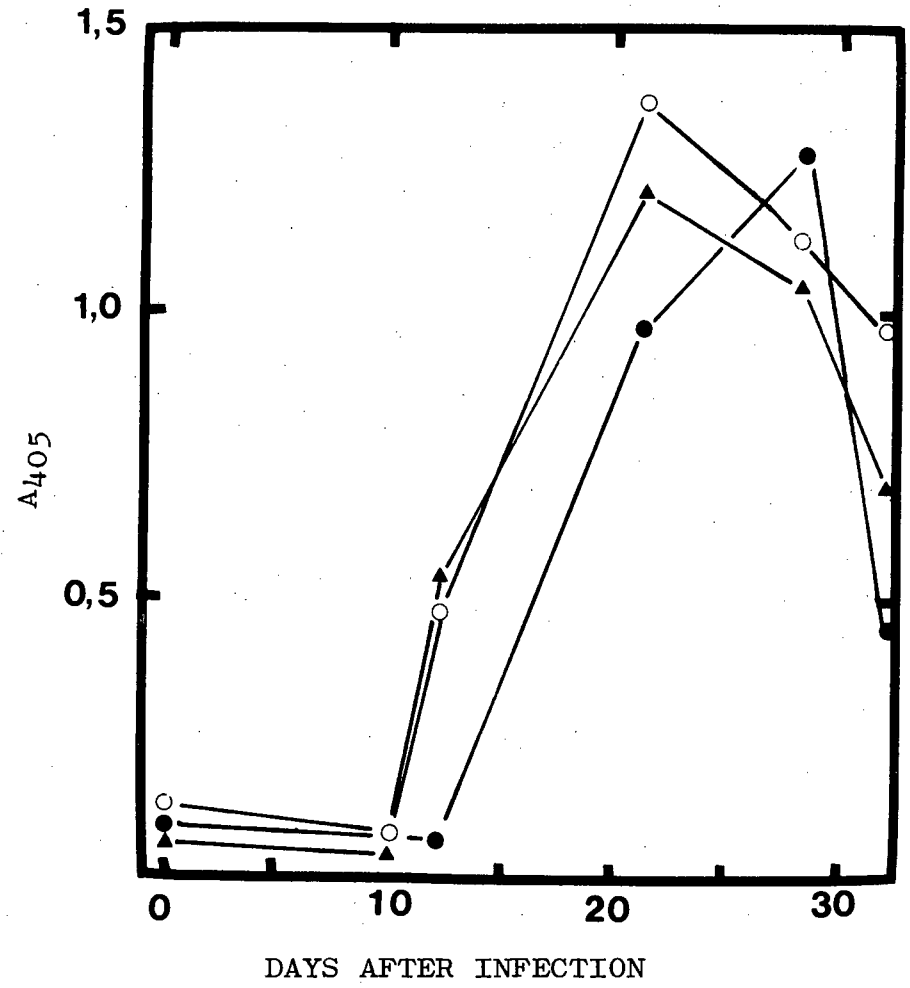


FIGURE 8

ELISA absorbance values obtained with extracts of Maizura (○), Spahili (▲) and White London (●) host plants at different times after inoculation with CaMV (Cabb-S). The microtitre plate wells were coated with IgG at a concentration of 1,0 µg/ml and enzyme-antibody conjugate was added at a dilution of 1/800.

TABLE 3

Results of infectivity tests with systemically infected turnip leaves at various times after inoculation with CaMV (Cabb-S). The host plants used in this test were Maizura turnip and Spahili mustards. The fraction of plants showing CaMV-type symptoms was determined 19 days after inoculation. These results should be compared with the ELISA values presented in Figure 8.

Time after infection (days)	Dilution of inoculum	Fraction of infected plants
10	10^{-1}	12/12
	10^{-2}	12/12
	10^{-3}	1/15
	10^{-4}	0/13
14	10^{-1}	8/8
	10^{-2}	8/10
	10^{-3}	5/12
	10^{-4}	0/11
21	10^{-1}	11/11
	10^{-2}	11/11
	10^{-3}	7/11
	10^{-4}	5/11
27	10^{-1}	10/10
	10^{-2}	9/10
	10^{-3}	1/11
	10^{-4}	0/11
32	Insufficient material	

mustards was slower. However, even though ELISA values and infectivity declined after reaching a maximum, the amount of virus, as determined by the ultraviolet absorption of pure CaMV, extracted from Maizura and Spahili plants 21, 24 or 27 days post infection was essentially similar (about 2,5 mg/kg). This is in accordance with the findings of Hull et al. (1976) who found that a constant amount of virus was extracted from their leaves from 24 - 35 days after infection with CaMV.

c. Detection of CaMV multiplication in protoplasts

Leaf mesophyll protoplasts were prepared from Wong Bok Chinese cabbage by Mr. W.S. Nowakowski of the Department of Microbiology, University of Cape Town, according to the method of Howell and Hull (1978). These protoplasts were mixed in the presence of 0,1% poly-l-ornithine with CaMV (Australia isolate) so that the final concentration of virus in the mixture gave an ELISA absorbance value of about 0,3 which is equivalent to a final concentration of about 0,1 μg/ml of CaMV. The protoplast/virus mixture was kept under conditions of high light intensity in a plant growth room for 72 hours in simplified Takebe medium (Howell and Hull, 1978). At the end of this time the protoplasts were ruptured by a freeze-thaw cycle and dialysed from the mannitol protoplast medium into PBS-Tween and diluted in 10-fold steps. A 0,2 ml aliquot of each dilution of control non-infected and infected protoplasts was placed into a microtitre plate well for assay. It was found that the ELISA value had increased compared with that obtained with the concentrations of CaMV added before incubation: the undiluted protoplast dialysate yielded an ELISA absorbance

value of 1,0 compared with 0,1 before incubation, while a 1/10 dilution showed an increase from 0 to 0,35. This experiment illustrates that ELISA can be a simple and effective means of assaying virus or virus protein multiplication in a protoplast system.

d. Detection of CaMV in aphids

It was reported in 1978 by Gera et al. that cucumber mosaic virus in viruliferous aphids was detectable by ELISA. As several aspects of aphid transmissibility of CaMV are not resolved (Lung and Pirone, 1973; 1974; Shepherd, 1976), the applicability of ELISA to the detection of CaMV in aphids was investigated.

Aphids (Brevicoryne brassicae) which had been found on CaMV field-infected cabbage leaves were homogenised in a mortar and pestle in PBS-Tween. About 0,3 g of aphids were homogenised with 0,3 ml of buffer and tested undiluted and at a 1/10 dilution in an ELISA. A negligible background absorbance value of 0,02 was obtained with an undiluted sample of "clean" aphids, while the aphids which had been feeding on the CaMV-infected plants yielded ELISA absorbance values of about 0,1.

E. SEROLOGICAL DIFFERENCES BETWEEN CaMV ISOLATES

1. Gel Diffusion Tests

Attempts were made to differentiate the Cabb-S, NY8153, Australia and Banhoek isolates of CaMV by precipitin tests in agar gel. The precipitin end-point titres of several antisera with homologous and heterologous CaMV isolates were

determined using purified CaMV at about 0,5 mg/ml. The results of these determinations are shown in Table 4. It is evident that any serological differences between the isolates tested could not be detected by differences in homologous and heterologous end-point titres in gel - a single dilution step difference was not considered significant.

When homologous and heterologous CaMV isolates were placed in adjacent wells surrounding the antiserum well in an Ouchterlony test, no crossing or spur formation of the precipitin lines was apparent (Fig. 9).

The presence of SDS in the immunodiffusion medium (Purcifull and Batchelor, 1977) resulted in less sharp precipitin bands, but no difference regarding spurring was observed.

2. Enzyme-linked Immunosorbent Assays (ELISA)

a. Double-antibody "Sandwich" ELISA

Koenig (1978), in an investigation into homologous and heterologous reactions of plant viruses in ELISA, found that the double-antibody or sandwich assay was an effective means of discriminating between closely related virus strains. As the precipitin tests described above were not sufficiently sensitive to discriminate between the CaMV isolates, the double-antibody sandwich ELISA was tested for its ability to distinguish between three CaMV isolates. It is thought that the high strain selectivity of this system may be due to lowered affinity of the conjugated antibody (Koenig, 1978).

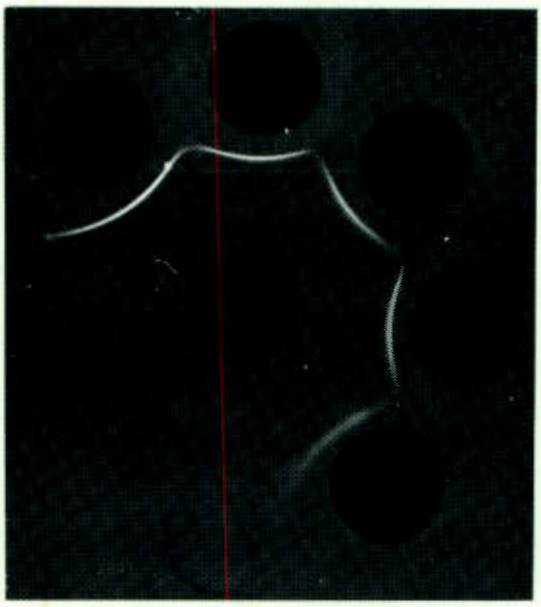


FIGURE 9

Precipitin bands formed by purified preparations of CaMV. The central well contained an antiserum directed against the Cabb-S isolate of CaMV at a dilution of 1/4 in saline. The peripheral wells contained the following isolates in clockwise order: Australia, NY 8153, Cabb-S, Oudtshoorn and Banhoek. Virus concentration was approximately 0,5 mg/ml.

TABLE 4

Precipitin end-point titres of several CaMV antisera with sucrose density-gradient purified CaMV preparations. Note the similarities between titres with homologous and heterologous antigens. A single dilution step difference was not considered significant. Figures in brackets refer to the number of days after the first immunization when the serum was collected.

Antiserum directed against	Antigen	End-point titre
Cabb-S (84)	Cabb-S	1/256
	NY 8153	1/256
	Australia	1/256
	Banhoek	1/256
Australia (14)	Australia	1/32
	Cabb-S	1/32
(98)	Australia	1/256
	Cabb-S	1/128
NY 8153 (60)	NY 8153	1/128
	Cabb-S	1/128
Banhoek (20)	Banhoek	1/64
	Cabb-S	1/64
	Australia	1/64

For this study, a hyperimmune serum directed against the Cabb-S isolate of CaMV was used in ELISA. The CaMV isolates (Cabb-S, Australia, NY-8153) were prepared from fresh and/or stored leaves without any knowledge of their state of proteolysis. The pure virus preparations were diluted in PBS-Tween to an absorbance value at 260 nm of 0,713 - equivalent to approximately 100 µg/ml of pure virus, using an extinction coefficient of 7,0 for all the isolates. Serial dilutions in PBS-Tween of each of the three isolates were pipetted into the wells of the IgG-coated microtitre plate, and the ELISA was carried out as described (Chapter 3, Section I). Fig. 10 illustrates the results of one such determination. Each of the isolates reacted to a very similar degree in the assay, and differences in the amount of colour development were only apparent at low dilutions of antigen (below 1/512). Differences in the absolute absorbance values were obtained in other determinations using the same virus preparations and were probably due to variations in the microtitre plate manufacture, but the results were essentially the same, with the homologous Cabb-S showing most colour development, followed by the NY-8153 and Australia isolates. On a single assay plate, duplicate determinations at identical antigen dilution were usually within 0,05 absorbance units of each other. Wherever ELISA was used for comparing antigens, preliminary tests with different concentrations of coating IgG and conjugate were carried out to ascertain the optimal conditions of the test (Koenig, 1978).

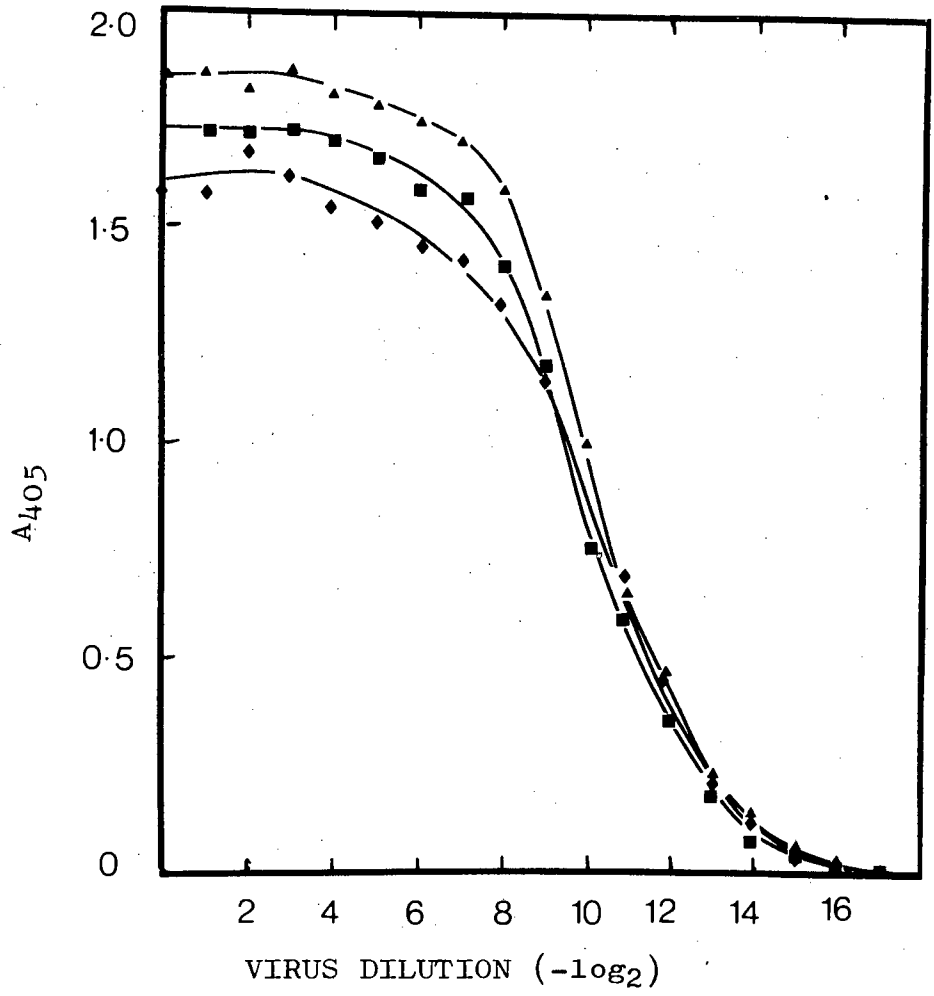


FIGURE 10

Comparison of the serological reactivities of 3 CaMV isolates in ELISA with an antiserum to CaMV (Cabb-S). Starting concentrations for the dilution series were 100 µg/ml. For the assay, the wells of the microtitre plate were coated with IgG at 1,0 µg/ml and enzyme IgG conjugate was added at a dilution of 1/800. Note the difference in maximum colour development at low antigen dilutions.

▲ Cabb-S ■ NY 8153 ◆ Australia

In order to rule out the possibility that the observed differences in the titration curves were due to small differences in virus concentration and not to serological differences, dilutions of the same preparations of Cabb-S and Australia isolates used in the above experiment were compared at different starting concentrations. The Australia isolate was diluted to 125 µg/ml and the Cabb-S to 80 µg/ml. Dilutions in PBS-Tween were made and serological reactivities were compared by ELISA as described above.

The results shown in Fig. 11 indicate that although the Cabb-S isolate was used throughout at a lower concentration than the Australia isolate, the maximum colour development by the homologous antigen exceeded that of the heterologous isolate at low dilution. This result indicates that small concentration differences do not affect the relative maximum colour development by homologous and heterologous antigens in this ELISA system, and that the differences in maximum colour development observed are likely to be due to differences in serological reactivity of the 3 isolates tested. Possible reasons for these serological differences are examined in Section F of this chapter.

The serological reactivities of the Cabb-S and Australia isolates were also compared in ELISA using antibodies specific for the Australia isolate. The antibodies used in this assay were obtained by absorption of a hyperimmune antiserum directed against CaMV Australia, at pH 7,0, followed by release at pH 2,9 (Hardie and van Regenmortel, 1977). When

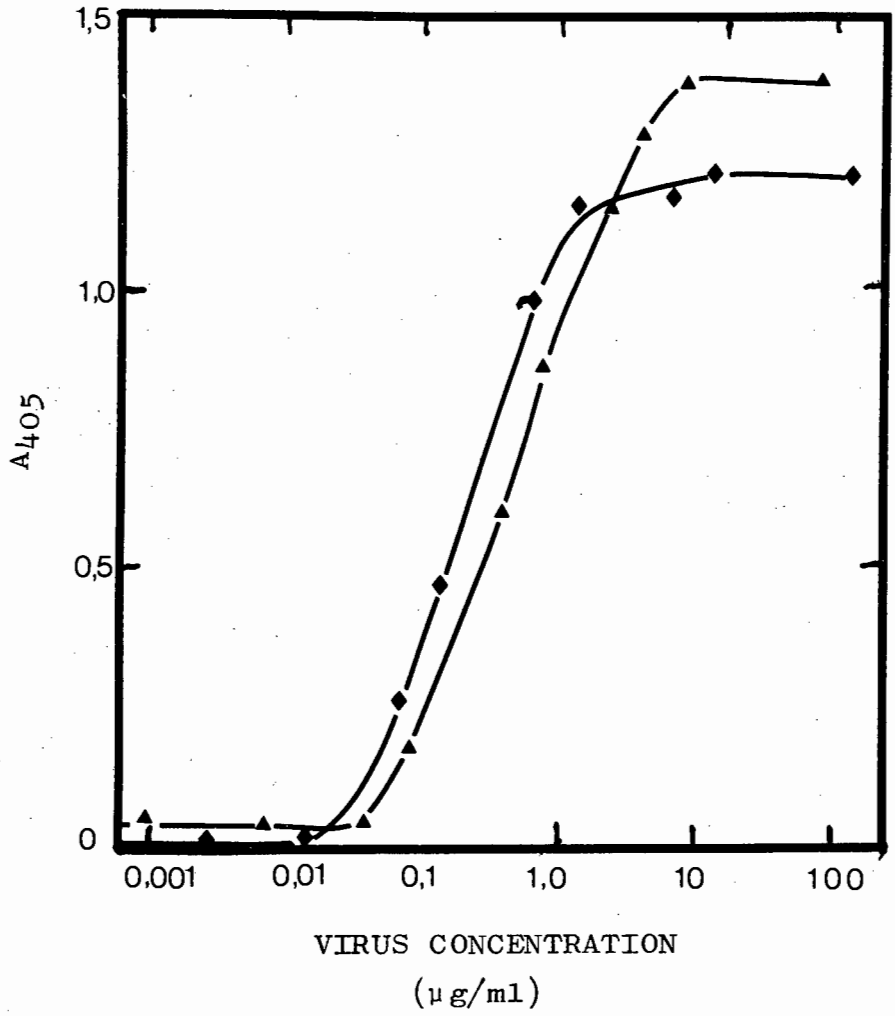


FIGURE 11

Comparison of serological reactivities of two CaMV isolates in ELISA with antiserum directed against CaMV (Cabb-S). The microtitre plate wells were coated with IgG at a concentration of 1.0 µg/ml and enzyme-conjugated antibody was added at a dilution of 1/800. Note that while the position of the linear portion of the curve is affected by virus concentration, the maximum colour development at high antigen concentrations by the homologous antigen (Cabb-S) is higher than that in the case of the heterologous (Australian isolate) antigen.

▲ Cabb-S ◆ Australia

purified antibodies were used in ELISA it was not possible to discriminate between the homologous and heterologous CaMV isolates (Fig. 12)

b. Inhibition of ELISA with IgG directed against homologous and heterologous isolates

In order to discriminate between the three CaMV isolates, an attempt was made to utilise a serum-blocking assay, a system which was used to identify rotaviruses from different animal species (Yolken et al., 1978).

Three IgG preparations, directed against the Australia, Cabb-S and New York 8153 isolates respectively, were titrated in Ouchterlony tests. All were then diluted to yield identical end-points (1/32) with their homologous antigens. A two-fold dilution series of each of the preparations was made in PBS-Tween. A volume of 0,2 ml of each dilution was mixed with an equal volume of CaMV (Australia isolate), the concentration of which had previously been adjusted to give an absorbance at 405 nm of 1,5 in a double antibody "sandwich" ELISA, at a point in the antigen titration curve near the end of the plateau region. After 2 hours of incubation at 37°C, 0,2 ml of the IgG/virus mixture was pipetted into wells which had been coated with specific antibodies directed against the Australia isolate. The assay was then performed as usual.

The inhibition of antibody binding in ELISA is illustrated in Fig. 13. All preparations were capable of 100% inhibition with slightly more inhibitory power at high dilution being shown by the homologous preparation. However,

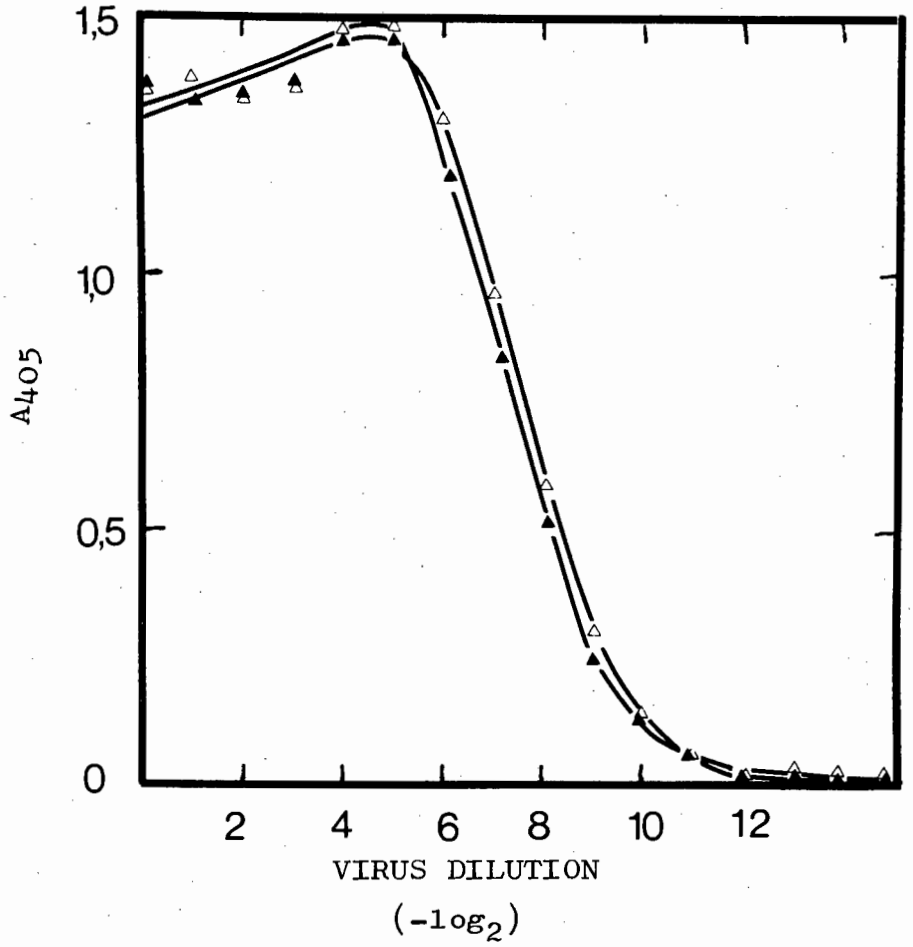


FIGURE 12

Comparisons of serological reactivities of the Cabb-S and Australia isolates in ELISA prepared with antibodies directed against the Australia isolate. These specific antibodies were obtained by binding at pH 7 and dissociation at pH 3. The wells of the assay plate were coated with the IgG at 1,0 µg/ml and conjugated antibody was added at a dilution of 1/1 000. The starting concentration of both antigens was about 80 µg/ml. This assay fails to show any differences in serological reactivity.

▲ Cabb-S

△ Australia

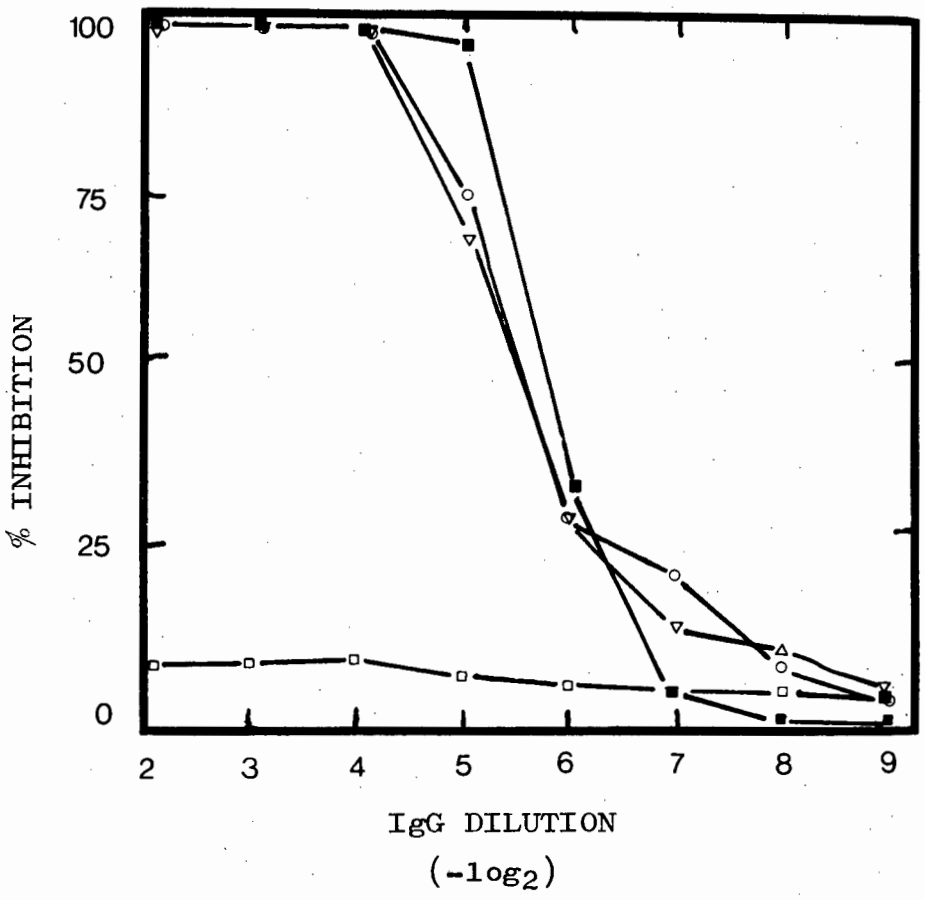


FIGURE 13

Inhibition of a double antibody "sandwich" ELISA by IgG preparations directed against homologous and heterologous CaMV isolates. The assay was prepared with specific anti-Australia isolate antibodies. A value of 0% inhibition represents an ELISA absorbance value of 1,5. The micro-titre plate was coated with antibodies to the Australia isolate at 1,0 µg/ml and enzyme antibody conjugate was added at a dilution of 1/1 000. The inhibition was performed as described in the text.

■ Australia isolate
▽ Cabb-S isolate
○ NY 8153 isolate
□ Normal rabbit serum.

the method was clearly not successful in effectively distinguishing homologous and heterologous reactions. Moreover, the technique of comparing antigens through a study of the differences between their respective antisera is subject to many shortcomings. For this reason this approach was discontinued. Nevertheless the experiments described above establish the existence of a close serological relationship between the three isolates under study.

c. Binding of enzyme-labelled IgG to CaMV isolates.

In this series of experiments, alkaline phosphatase-labelled IgG (diluted 1/5 000) was absorbed with equal amounts of homologous and heterologous CaMV isolates respectively. After centrifugation of the virus/antibody complexes, an attempt was made to measure the amounts of bound antibody brought down. It was envisaged that in such a direct binding system, the homologous CaMV isolate would absorb more labelled antibody from the antiserum than the heterologous isolates. Volumes of 0,2 ml each of CaMV at 1 mg/ml (Cabb-S and Banhoek isolates), alkaline phosphatase-conjugated IgG directed against CaMV (Cabb-S) diluted 1/1 000 in PBS-Tween + 0,2% ovalbumin and centrifuged at 30 000 rpm for 90 min to sediment the virus/antibody complexes. The pellet was then resuspended in 1 ml of 1 mg/ml p-nitrophenylphosphate in diethanolamine substrate buffer pH 9,8. After 60 min at room temperature, the reaction was stopped by the addition of 500 µl 3 M NaOH. The absorbance at 405 nm was then determined.

Differences in absorbance at 405 nm produced by the conjugate

brought down by the homologous and heterologous CaMV isolates at various dilutions ranged from very small to indistinguishable. TMV was used as a non-specific control, but some non-specific absorption was apparent. This approach was abandoned as a means of discriminating between CaMV isolates.

d. Serological differentiation of CaMV isolates by serum cross-absorption

As the serological differences between isolates were shown tobe very small by the previously described enzyme immuno-assays, an attempt was made to develop an assay system capable of clearly differentiating such closely related virus antigens. The following experiments were designed to exploit the discriminatory powers of serum cross-absorption (van Regenmortel, 1967), combined with the potential for sensitivity and quantitation offered by ELISA. An anti-serum to an isolate of CaMV was exhaustively cross-absorbed with preparations of homologous and heterologous CaMV isolates. Any residual non-crossreacting antibodies were then detected in ELISA. It was anticipated that the homologous antigen would absorb all antibodies from the serum, but heterologous antigens would be incapable of removing certain classes of antibody. The closer the serological relationships of the heterologous strains to the antigen used for serum production, the less antibody would be detectable after absorption.

With a sensitive technique like ELISA, it is possible that even small quantities of contaminating anti-host antibodies

could affect the experimental results. To obviate this, the antiserum used for cross-absorption was raised against sucrose density-gradient purified CaMV and then absorbed twice with healthy host plant extract obtained after centrifugation of homogenised leaf material at 50 000 rpm for 3 hours, as a precautionary measure. Sera absorbed in this way and used for a "sandwich" ELISA did not react significantly with healthy plant extract diluted 1/100 (See section D of this chapter), showing that no detectable host antibodies were present in this antiserum when used at high dilution. To determine whether some low molecular weight plant components, ie. those which did not sediment after high speed centrifugation, were able to interfere with the test, an antiserum against these soluble host components was prepared and tested for its ability to react with CaMV. The variation of the ELISA used to detect antibodies capable of reacting with CaMV ("Indirect" ELISA) relies upon the sensitization of microtitre plate wells with purified antigen. The test serum is added and any antibodies in this serum which absorb specifically to the virus are shown by the addition of enzyme-labelled anti-globulin antibody.

A homogenate of plant leaves in 0,5 M phosphate buffer pH 7,5 was centrifuged at 50 000 rpm for 3 hours, and the resulting supernatant fluid was used to immunise a rabbit. The antiserum produced contained specific anti-host antibodies as was shown by precipitation in gel with healthy leaf homogenate. It did not react with sucrose density-gradient purified CaMV in gel precipitin tests nor in indirect ELISA. No antibodies reacting with CaMV preparations were detectable

at a 1/10 dilution of this anti-serum in ELISA. Host/anti-host reactions of this nature with purified CaMV can thus be ruled out. With an antiserum free of all detectable anti-host antibodies, it was possible to use CaMV purified by differential centrifugation for the cross-absorption experiments.

Such an antiserum directed against CaMV (NY-8153) was diluted 1/2 000 in PBS-Tween containing 0,2% bovine serum albumin. At this dilution, the serum gave an ELISA absorbance value of approximately 1,0 at 405 nm in indirect ELISA (Fig. 14). For this assay the wells of a microtitre plate were coated with CaMV (NY-8153) at 0,5 µg/ml in carbonate buffer pH 9,6 and alkaline phosphatase-conjugated goat anti-rabbit IgG was added at a dilution of 1/1 000.

The cross-absorption with CaMV was done as follows:- to 4,0 ml aliquots of the diluted antiserum, was added 1,0 ml of each of the isolates of CaMV at a concentration of about 0,5 mg/ml. The virus and antiserum were thoroughly mixed and incubated for 2 hours at 37°C. The virus/antibody complexes were sedimented by centrifugation in a Beckman SW 50 rotor using polyallomer tubes for 60 min at 35 000 rpm. To 4,5 ml of the resulting supernatant fluid was added a further volume of 0,5 ml of each of the same virus isolates. This virus/antiserum mixture was incubated overnight at 37°C and again centrifuged as above. The resulting supernatant fluid, containing any antibodies which may not have been absorbed out, was then assayed in an ELISA.

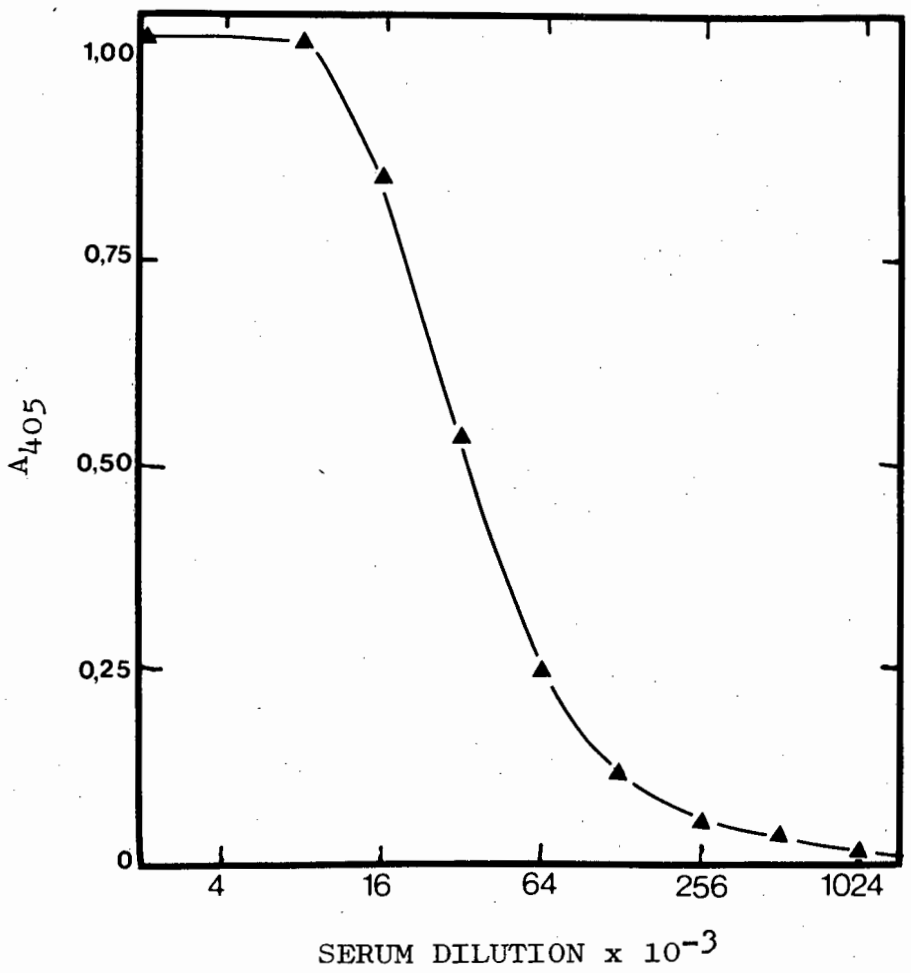


FIGURE 14

ELISA titration curve of anti-NY 8153 serum used for the cross-absorption experiments. The microtitre plate was coated with pure CaMV NY 8153 at 0,5 µg/ml. Alkaline phosphatase-labelled goat anti-rabbit IgG was used at a dilution of 1/1000.

A microtitre plate was coated with CaMV (NY-8153) at pH 9,6. The serum samples were pipetted into the wells and after overnight incubation alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies were added to the wells. After incubation with the substrate, the ELISA absorbance values shown in Table 5a were obtained. It can be seen that absorption with the homologous (NY-8153) isolate removed all detectable antibody from the serum. Tobacco mosaic virus (TMV), used as a non-specific control, did not change the ELISA value of the absorbed antiserum significantly. The heterologous isolates removed most of the antibody from the antiserum, which confirms the close relationships of the isolates as shown by the Ouchterlony and ELISA experiments described previously. For example, the Banhoek isolate caused a decrease in ELISA absorbance values from about 1,0 at 1/2 000 to 0,032 which, from Fig. 14 is equivalent to a serum dilution of 1/512 000.

The cross-absorbed antiserum was also tested after concentrating it tenfold in a Minicon serum concentrator (Amicon Corp., U.S.A.). Even after concentration, no antibody activity could be detected in the homologously absorbed antiserum, indicating that the absorption procedure was sufficiently exhaustive. After concentrating the residual non-absorbed antibodies, their ELISA values were shifted to the central position of the calibration curve (Fig. 14), thus making the relative differences in the amounts of residual antibody more apparent. The cross-absorption was repeated on a small scale using 0,2 ml aliquots of diluted antiserum. Similar results were obtained.

TABLE 5

ELISA absorbance values obtained after absorption of (a) an anti-NY 8153 and (b) an anti-Banhoek serum with homologous and heterologous CaMV isolates. The absorbed antiserum was assayed for residual antibody at its original concentration (x1) and after a ten-fold concentration step (x10).

	Virus isolate used for absorption	Absorbance at 405 nm x1	x10
a)	CaMV NY-8153	0,000	0,000
	CaMV Australia	0,010	0,046
	CaMV Cabb-S	0,029	0,168
	CaMV Oudtshoorn	0,031	0,294
	CaMV Banhoek	0,032	0,336
	TMV vulgare	1,007	
	Unabsorbed antiserum	1,025	
b)			
	CaMV NY-8153	0,006	0,014
	CaMV Australia	0,008	0,017
	CaMV Cabb-S	0,139	0,689
	CaMV Oudtshoorn	0,225	0,711
	CaMV Banhoek	0,010	0,020
	TMV vulgare	1,311	
	Unabsorbed antiserum	1,341	

In order to ascertain whether the observed relationships were reciprocal and quantitative if an antiserum against a different isolate was used, the experiment was repeated with an antiserum directed against the Banhoek isolate of CaMV. This isolate was apparently most distantly related to the NY-8153 isolate (Table 5a). An ELISA calibration curve using CaMV Banhoek and homologous antiserum was constructed as described above. Using this curve, it was established that the antiserum at a dilution of 1/1000 gave an ELISA absorbance value of about 1,3. The CaMV isolates used in the cross-absorption were propagated from desiccated leaf samples obtained from the first propagation from the original stock.

The results of the reciprocal cross-absorption are listed in Table 5 (b). It is apparent that there was a marked lack of reciprocity in serological relatedness. The Banhoek (homologous) and the NY-8153 and Australia isolates removed almost all detectable antibody from the antiserum, indicating that they had many determinants in common with the immunogen. The Oudtshoorn and Cabb-S isolates, which in the first cross-absorption were apparently closely related to Banhoek, were unable to remove appreciable amounts of antibody from the Banhoek antiserum in the reciprocal test. It is possible that this lack of reciprocal serological relatedness in the different experiments was due to the fact that the isolates are in different states of proteolysis since they were all propogated from the same stock material.

The influence of proteolysis on the serological reactivity of CaMV is investigated in the next section of this chapter.

F. EFFECT OF THE PROTEOLYTIC DEGRADATION STATE OF CaMV ON ITS SEROLOGICAL REACTIVITY

In the foregoing section of this chapter, it was shown that serological differences between CaMV isolates could be detected by techniques of high discriminatory power like the "sandwich" ELISA, and cross-absorption of antisera.

When it became evident (Al Ani et al., 1979b), that many of the CaMV polypeptides seen on polyacrylamide gels may be proteolytic degradation products of a 42 000 dalton component, the question arose as to whether this proteolytic process could lead to any serological changes in the capsid protein. If this were the case some of the serological differences described above may be due to the presence of different degradation states in preparations of the same strain, rather than to true strain differences originating as differences in DNA sequence. In the following experiments the effect of proteolysis on serological activity of a single CaMV isolate was investigated. The "sandwich" technique of ELISA was used as a discriminatory method, with antisera to intact CaMV.

1. Influence of Degradation on the Serological Reactivity of an Isolate.

An ELISA was prepared with an early antiserum (30 days post injection) from a rabbit which had been injected with CaMV (Oudtshoorn)

in an unknown state of degradation. Dilutions of density-gradient purified CaMV from fresh leaves and CaMV which had been stored for two weeks in a semi-pure condition, were made, both starting at 100 µg/ml. Fig. 4 shows the polypeptide profiles of these preparations (Tracks 1 and 8). The dilutions were pipetted into microtitre plate wells which had been coated at a concentration of 0,5 µg/ml with anti-CaMV (Oudtshoorn) IgG. The absorbance values obtained with these preparations in the immunoassay are presented graphically in Fig. 15. The CaMV from fresh leaves with polypeptides P4 and P3, as well as P2 and P1 visible on gel gave higher absorbance values at low dilutions than the stored preparation showing only the P2 and P1 polypeptides. This suggests that the antiserum had some antibodies to determinants on P4 and P3, possibly produced by the injection of relatively well-preserved virus, and shows that distinct serological differences between degraded and undegraded CaMV could be detected by this particular antiserum. A similar experiment was performed with CaMV (Cabb-S) preparation which had been prepared from fresh leaves. On a sucrose density-gradient it was possible to separate two populations of virus (Al Ani et al., 1979), the polypeptide profiles of which are shown in Fig. 16. The "fast" component showed an enriched "P4" band compared with the "slow" component. The reactivity of each preparation in ELISA using an antiserum directed against the Cabb-S isolate was tested. As can be seen in Fig. 17, the amount of maximum colour development differed. The faster sedimenting, less degraded fraction showed higher absorbance values at low dilutions than the degraded one,

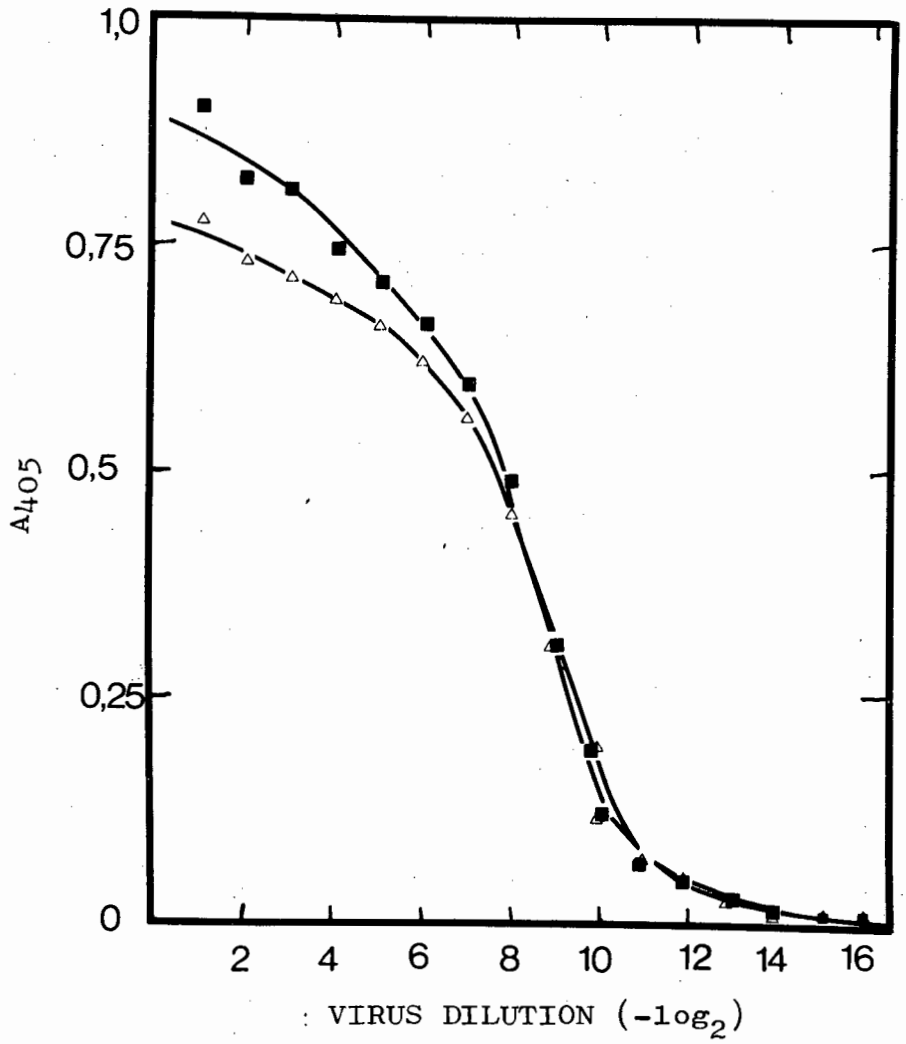


FIGURE 15

ELISA absorbance values obtained with CaMV (Oudtshoorn isolate) from -

■ Fresh leaves

△ Fresh leaves, after storage for 2 weeks at 4°C as a semi-pure preparation.

The antiserum (30 days) was directed against CaMV (Oudtshoorn) and the plate was coated with IgG at 0,5 µg/ml. Conjugate was added at a dilution of 1/500.

See Figure 4, Tracks 1 and 8 for polypeptide profiles of these preparations.

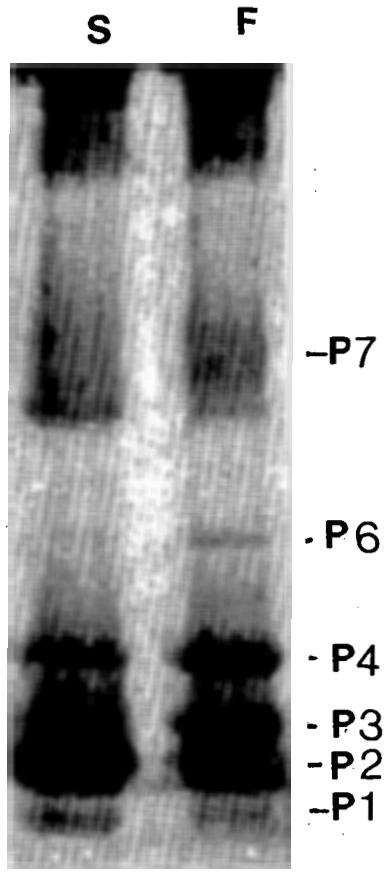


FIGURE 16

Polypeptide profiles of CaMV (Cabb-S) components which had been separated on 10 - 40% sucrose density-gradients.

F - fast sedimenting component
S - slow sedimenting component

The difference in serological reactivity of these preparations is illustrated in Figure 17.

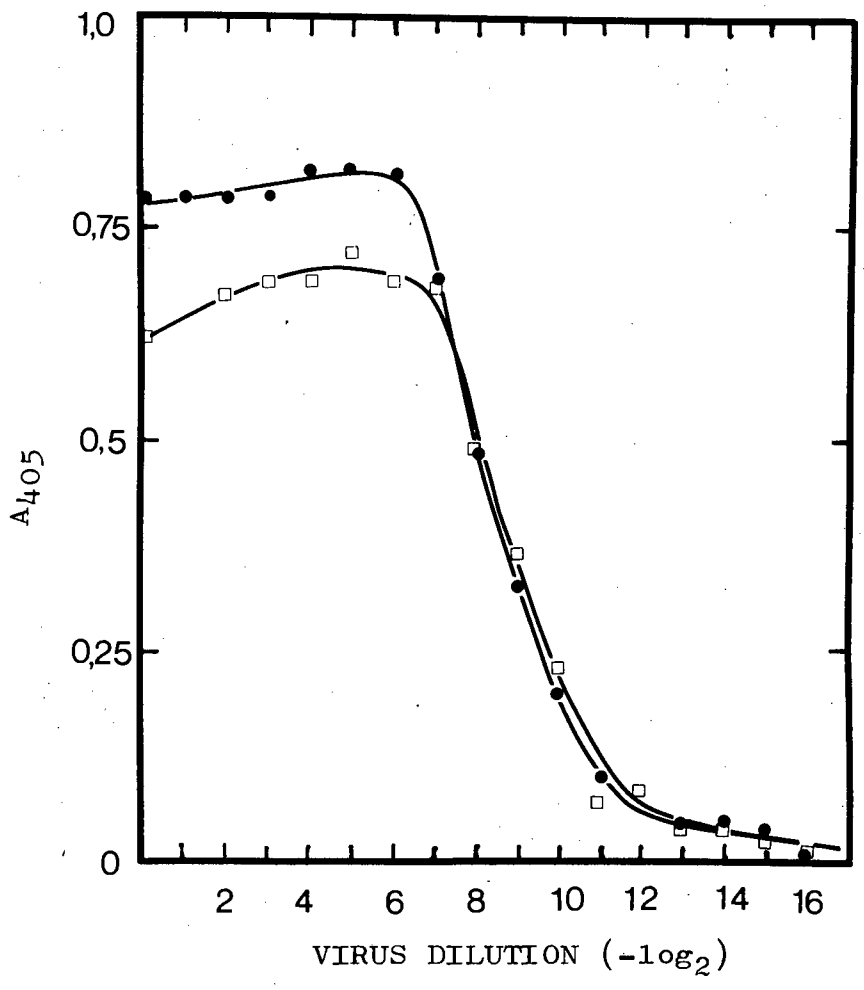


FIGURE 17

ELISA absorbance values obtained with CaMV (Cabb-S) extracted from fresh leaves. The preparation was separated on a 10 - 40% sucrose density-gradient into two components, the profiles of which are illustrated in Figure 16.

● fast sedimenting component.
□ slow sedimenting component.

For the ELISA, the microtitre plate was coated with IgG directed against CaMV (Cabb-S) at a concentration of 1,0 µg/ml. Labelled IgG was added at a dilution of 1/800.

confirming that ELISA can detect differences in capsid degradation state. As these differences were of a similar order of magnitude to those found between different isolates, the validity of the previously established differences is doubtful (Fig. 10).

In order to establish whether there was a direct relationship between the proteolytic degradation state of a CaMV preparation and its serological reactivity in ELISA, a semi-pure preparation of Australia isolate was incubated in 0,5 M potassium phosphate buffer + 0,02%NaN_3 at 20°C and aliquots were purified at different time intervals by sucrose density-gradient centrifugation. The state of proteolysis of these aliquots was monitored on polyacrylamide gels (Fig. 18). A reduction in the amount of P3 and P4 as incubation proceeded is clearly apparent, along with a concomitant increase in the proportion of P1. However, as can be seen from Fig. 19 when the ELISA absorbance values of the different proteolytic states of the virus were compared, no direct correlation between the degree of serological reactivity and the preparation's proteolytic state could be made. Furthermore, not all antisera were equally suitable for distinguishing antigenic differences resulting from proteolysis (Fig. 20).

2. Effect of Proteolytic State on Apparent Serological Relationships

It was noted in the previous section of this chapter that an antiserum directed against CaMV was able to distinguish between different degradation states of the same isolate. These differences were of a similar magnitude to the apparent

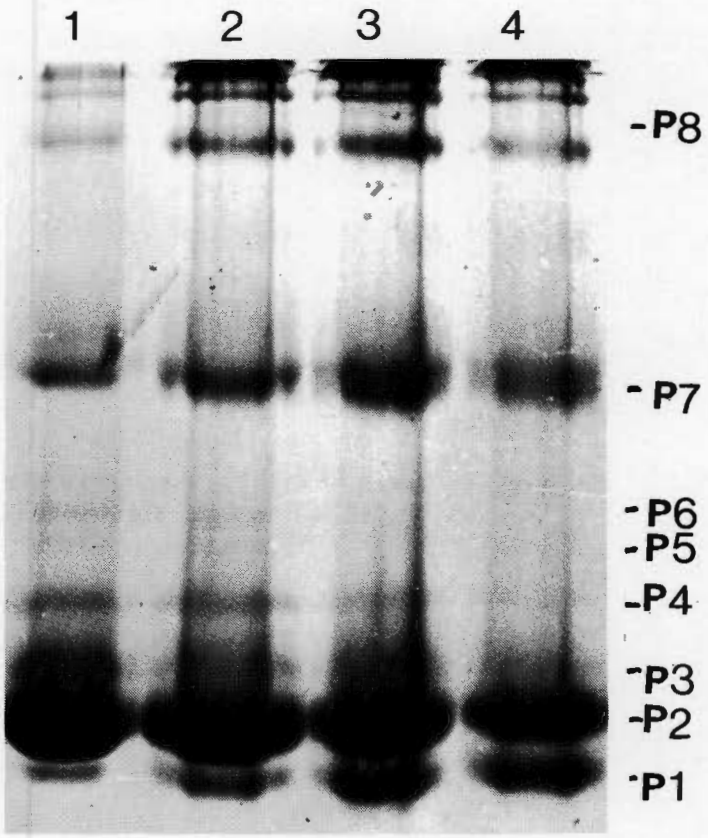


FIGURE 18

Polypeptide profiles of CaMV (Australia isolate) after storage as a semi-pure preparation for various lengths of time at 20°C prior to sucrose density-gradient purifications.

Track	Days storage as a semi-pure preparation
1	0
2	4
3	14
4	20

The serological reactivities of these preparations are compared in Figure 19.

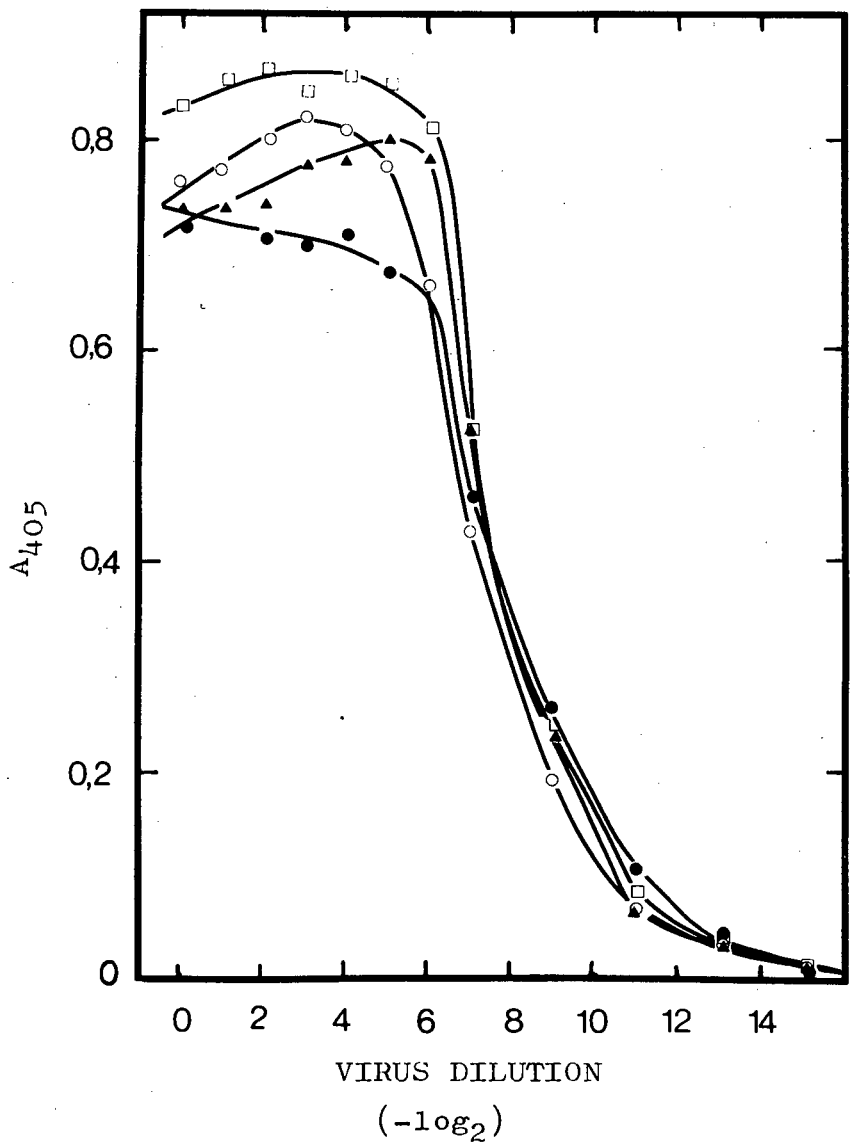


FIGURE 19

ELISA absorbance values obtained with CaMV (Australia isolate) after storage for various lengths of time as semi-pure preparations followed by purification on 10% - 40% sucrose density-gradients. The polypeptide profiles of these preparations are illustrated in Fig. 18. For ELISA, the microtitre plate wells were coated with IgG directed against the Cabb-S isolate of CaMV at a concentration of 1,0 µg/ml. Alkaline phosphatase labelled IgG was added at a dilution of 1/800.

Absorbance after storage for (○) 0 days (●) 4 days (▲) 14 days (□) 20 days

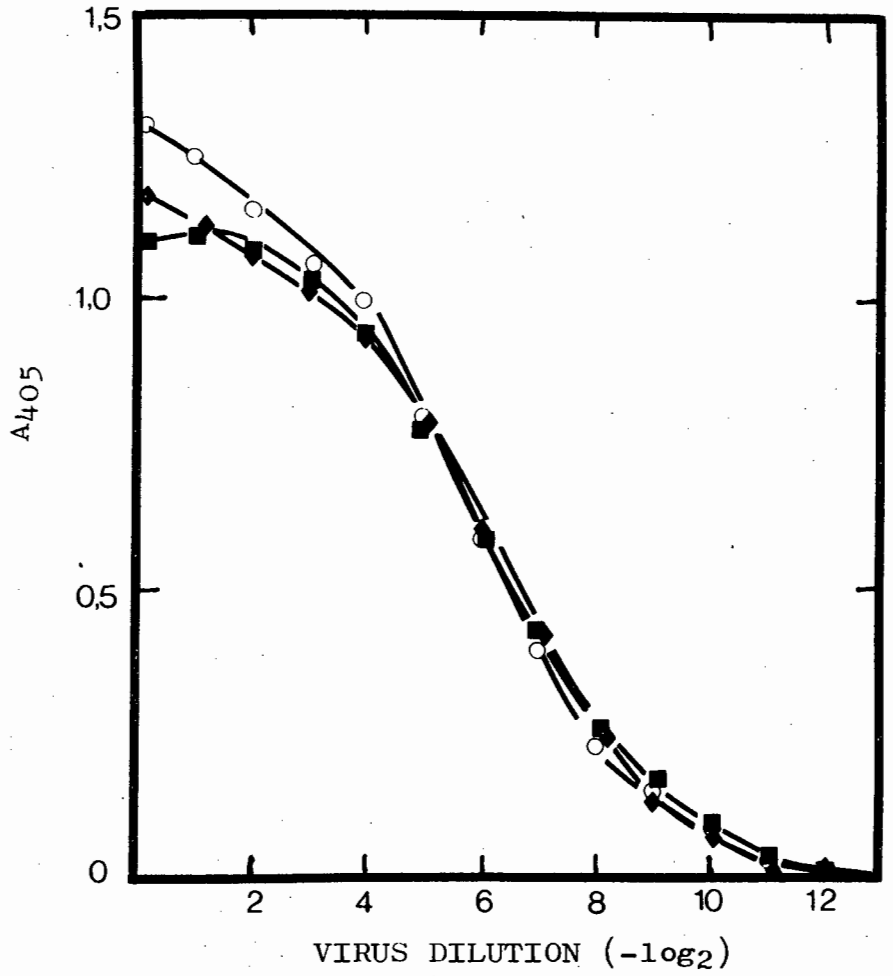


FIGURE 20

ELISA absorbance values obtained with CaMV (Oudtshoorn) prepared from -

- ○ CaMV extracted from fresh leaves.
- ■ CaMV extracted from fresh leaves after storage for 2 weeks at 4°C as a semi-pure preparation.
- ◆ CaMV prepared from frozen leaves in the presence of protease inhibitors.

The antiserum was raised against a CaMV preparation received from M.H.V. van Regenmortel and was reported to have a high proportion of P4. (van Regenmortel, personal communication) (pooled bleedings). The microtitre plate was coated with IgG at 0,1 µg/ml. Conjugated antibody was added at a dilution of 1/800.

See Figure 4, Tracks 1, 8 and 6 for polypeptide profiles.

serological differences between isolates (Fig. 10). The experiments which follow show that a spurious order of serological relationships can be deduced from ELISA results as a result of proteolytic degradation of the viral capsid.

The Australian and NY-8153 isolates were purified from a semi-pure preparation of CaMV by means of sucrose density-gradient centrifugation and subjected to SDS-polyacrylamide gel electrophoresis. The polypeptide profile of these preparations is shown in Fig. 21a. The Australia isolate showed P4, P3, P2 and P1 on gel while the NY-8153 isolate capsid polypeptide was degraded to almost entirely P2 and P1. It seemed likely that a heterologous antiserum might possibly lead to better differentiation. For this reason these two isolates were compared in a "sandwich" ELISA as described previously using a heterologous (Cabb-S) antiserum. The Australia isolate showed a higher serological reactivity than the NY-8153 isolate (Fig. 22a). This is in contrast to a previous determination (Fig. 10). The same semi-pure preparations were then kept at 20°C for 18 days. The virus was purified by sucrose density-gradient centrifugation, and the virus polypeptide profiles were monitored on gels; the serological reactivities were compared with the same antiserum as described.

The polypeptide profile of the NY-8153 isolate still showed mainly P2 and P1 whereas the P4 in the case of the Australia isolate had disappeared (Fig. 21b). When serological reactivities were compared by ELISA the same two isolates showed a slight but detectable difference in the order of serological

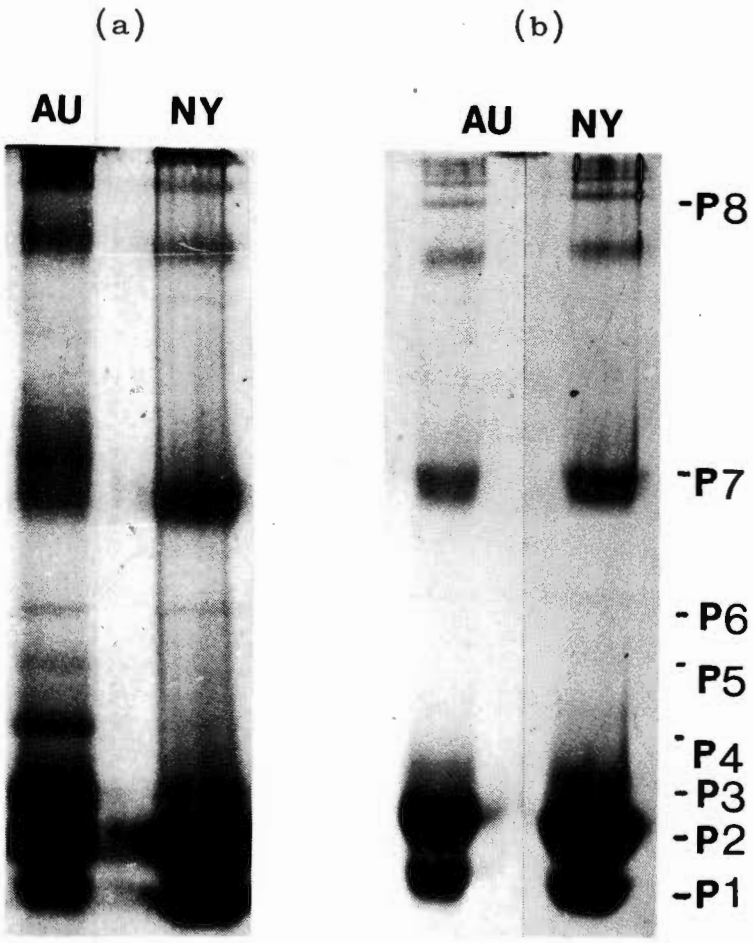


FIGURE 21

a) Polypeptide profiles of CaMV preparations used in the serological comparisons described in the text and illustrated in Figure 22.

AU - Australia isolate
NY - NY 8153 isolate

b) Polypeptide profiles of the same preparations of CaMV after 18 days storage at 20°C as semi-purified preparations.
The virus was purified by sucrose density gradient centrifugation prior to gel electrophoresis.

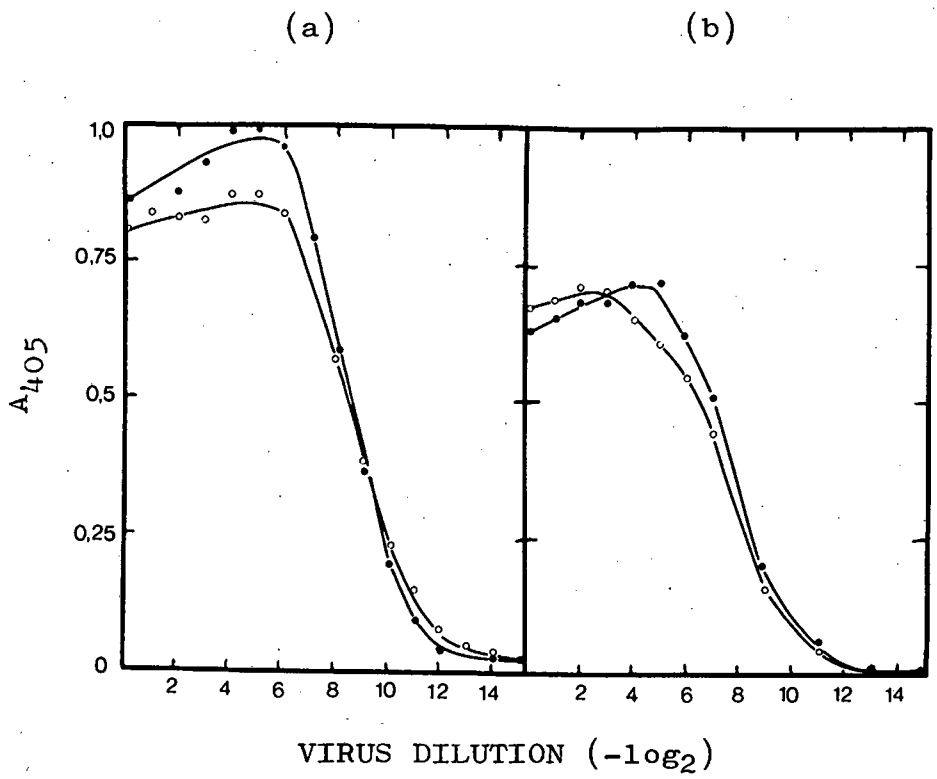


FIGURE 22

a) ELISA absorbance values obtained with CaMV preparations which exhibited the polypeptide profiles shown in Figure 21a.

● - Australia isolate
○ - NY-8153

b) ELISA absorbance values of the same preparations after storage for 18 days at 20°C. The polypeptide profiles of these samples are illustrated in Figure 21b.

For the ELISA, the microtitre plates were coated with IgG directed against the Cabb-S isolate at a concentration of 1,0 µg/ml. Alkaline phosphatase labelled IgG was added at a dilution of 1/800.

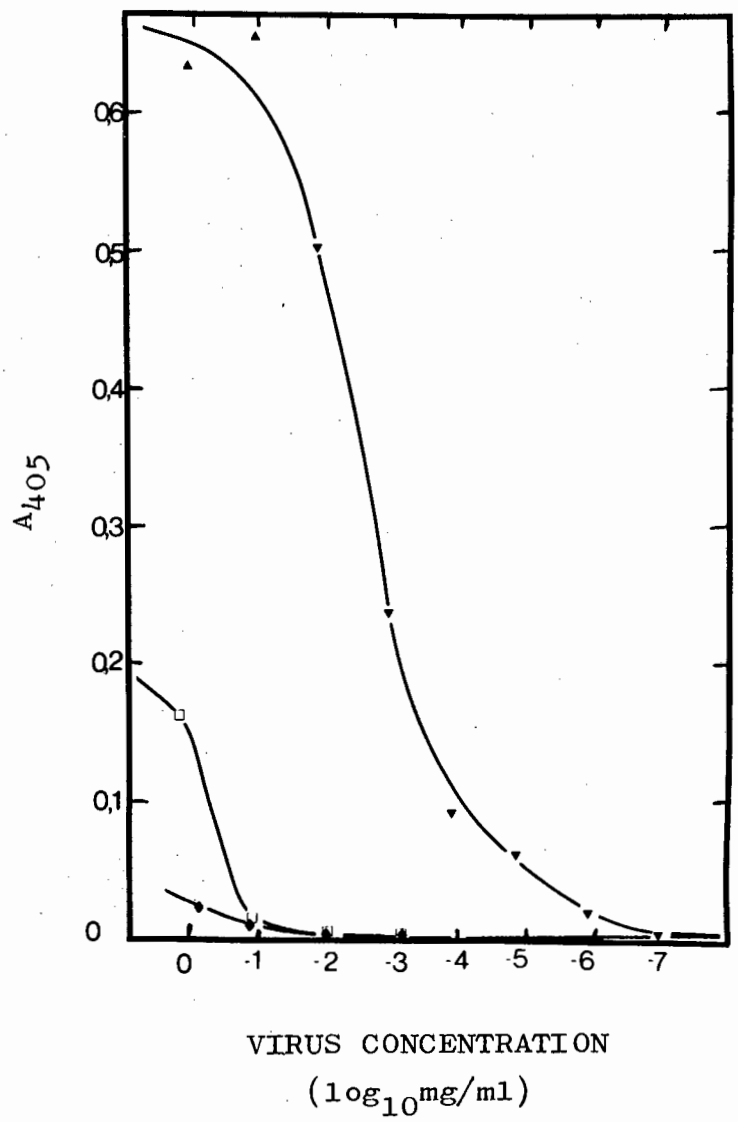


FIGURE 23

Comparison of serological reactivity of 3 strains of Tobacco mosaic virus (TMV) in an ELISA prepared with an antiserum directed against the protein subunits of TMV vulgare. The wells of the microtitre plate were coated with IgG at a concentration of 10 µg/ml, while antibody-enzyme conjugate was added at a dilution of 1/800. Note the clear serological distinction between strains achieved in this assay.

▼ vulgare □ U2 ◆ CGMMV

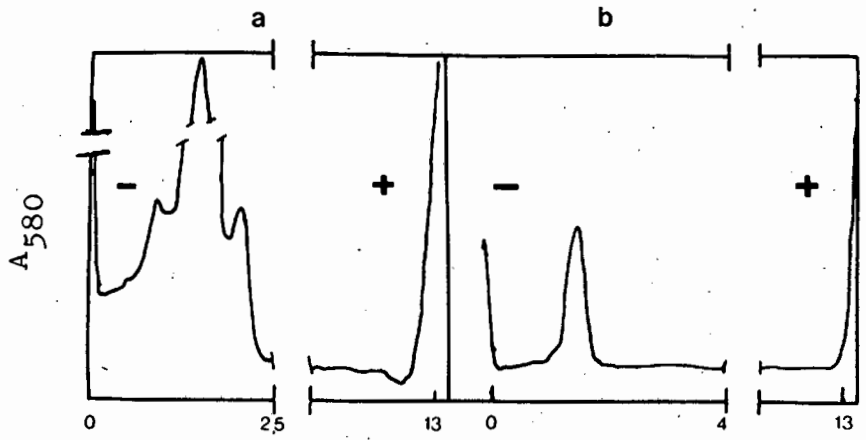


FIGURE 24

Absorbance (580 nm) profile of CaMV after electrophoresis in agarose/acrylamide gels stained with Coomassie blue.

a) Cabb-S isolate

b) NY-8153

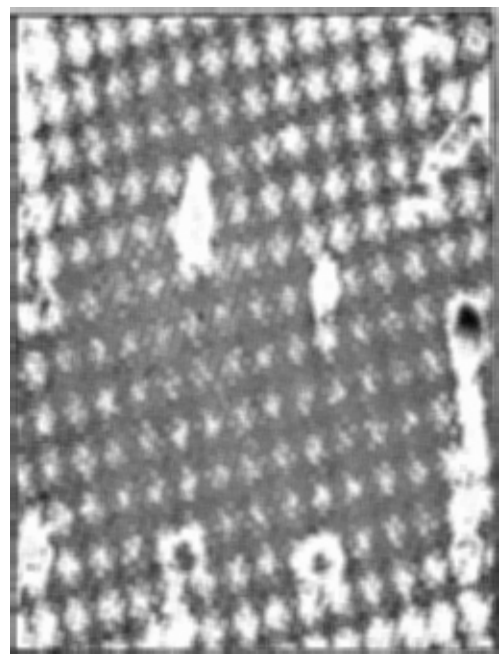
Electrophoresis was at a potential difference of 190 V for 100 minutes.

TABLE 6

Ratios of the distance moved by 4 CaMV isolates to that migrated by the marker dye (Rf's) in an agarose/acrylamide composite gel electrophoresis experiment. Electrophoresis was in 0,02 M phosphate buffer pH 7,5 for 100 min at 4 mA/tube.

CaMV isolate	Rf
Cabb-S	0,088
Australia	0,099
Oudtshoorn	0,135
New York 8153	0,139

ANODE



1 2 3 4

CATHODE

FIGURE 25

Electrophoresis of 4 CaMV isolates in agar slab gel showing relative differences in electrophoretic mobility. Electrophoresis was for 50 minutes at 5 V/cm potential.

1) Australia isolate
2) NY 8153 isolate
3) Oudtshoorn isolate
4) Cabb-S isolate.

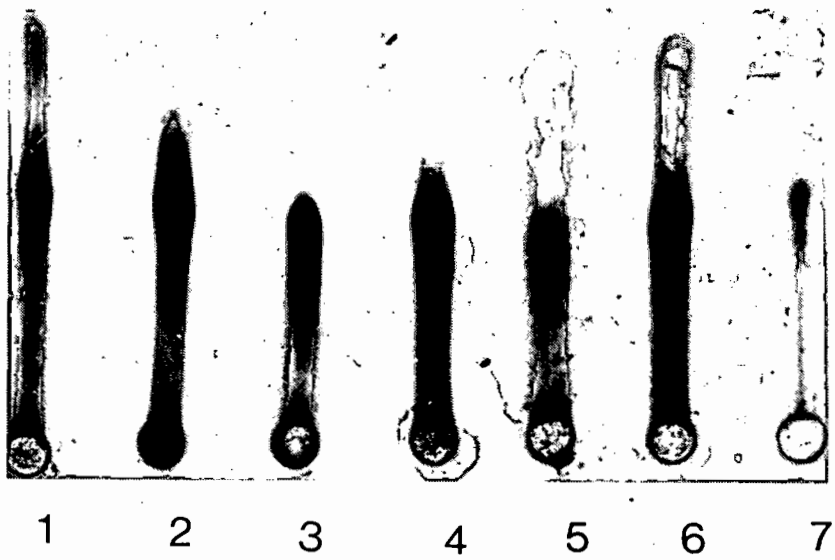
shown in Fig. 4 (Tracks 1 - 7).

The relative positions migrated by the CaMV samples showing different polypeptide profiles is illustrated in Fig. 26. The electrophoretic mobility of the major virus component, as well as the presence or absence of a faster migrating component was affected by the capsid degradation states. The samples containing predominantly the P2 and P1 polypeptides (Fig. 4 Track 3) show no second component and reduced mobility in agarose gel when compared with CaMV preparations containing appreciable quantities of P4 and P3. (Tracks 1,2,4 and 6). The sample represented by Track 5, containing 2 bands in place of P3 also shows reduced mobility. The above results suggest that some charged groups on the CaMV capsid are located on regions of the viral subunit which are altered by proteolytic degradation, and as a result, the virus shows differing electrophoretic mobility and homogeneity, depending upon the relative proportions of native P4 and its degradation products present. This finding casts doubt on the validity of using electrophoretic mobilities of CaMV strains or isolates for characterisation and identification purposes, unless the virus preparation can be obtained in an homogeneous and defined state of proteolysis. The origin of CaMV electrophoretic heterogeneity is examined further in a subsequent section of this chapter.

3. Electrophoresis at pH Values Near to the Isoelectric Point of CaMV

An attempt was made to determine whether any differences in isoelectric points between the Cabb-S, NY-8153, Australia

ANODE



CATHODE

FIGURE 26

Agarose gel electrophoresis of CaMV (Oudtshoorn isolate). The numbers of the sample refer to the preparation method used. (See section B2, Figure 4).

1) Fresh leaves
2) Fresh leaves and protease inhibitors.
3) Fresh leaves, no Na_2SO_3 in extraction buffer.
4) Liquid nitrogen-frozen leaves.
5) Liquid nitrogen-frozen leaves and protease inhibitors in buffer.
6) Leaves frozen to -20°C and protease inhibitors in buffer.
7) Leaves stored for 14 days at 4°C.

The polypeptide profiles of the various samples are shown in Figure 4 (Tracks 1 - 7).

and Oudtshoorn isolates could be detected by differences in rate or direction of migration at pH values around 4,0 - 5,0. The isolates were electrophoresed at pH 4,0, 4,5 and 5,0 in thin agarose slabs using an acetate buffer system. Precipitation of the virus in the wells at the origin at these pH values prevented any differences in mobility from being distinguished.

4. Origin of CaMV Electrophoretic Heterogeneity

Some preparations of CaMV were found to have more than one electrophoretic component in agarose/acrylamide gels (Fig. 24a). The existence of a faster migrating component appeared to depend upon the polypeptide profile of the preparation in question. As can be seen by comparing Figs. 4 and 26, virus preparations showing relatively large quantities of P4 and P3 in acrylamide gels showed a second faster migrating component.

This observation was verified by electrophoresing virus (Oudtshoorn isolate) prepared from fresh leaves and leaves which had been stored at +4°C for 16 days. In agarose electrophoresis the fresh leaf preparation showed a second component, while the stored leaf preparation did not. It was not possible, due to streaking, to recognise any components slower than the major one using this technique. The polypeptide profiles in acrylamide gels were found to be similar to track 1 (fresh preparation) and track 6 (stored preparation) in Fig. 4.

As the polypeptide profile appeared to influence the electrophoretic homogeneity of intact CaMV, it appeared likely that

the different electrophoretic components represented CaMV particles in different degradation states present in a single preparation of the virus. This possibility was investigated using zone electrophoresis (Polson and Russell, 1967).

Preparations of CaMV were electrophoresed in 0.1M borate buffer pH 8,6 (Polson and Russel, 1967), or in 0,05 M phosphate buffer pH 7,5 (Miller and Golder, 1950). Pure or semi-pure virus samples of about 1,0 ml each containing up to 5 mg of CaMV were subjected to overnight electrophoresis at 20 mA. The apparatus was cooled by a current of air to help reduce convection. Phenol red was incorporated in the sample in some runs as a marker. To obtain absorbance profiles of the column, the column was pumped out via the capillary. Fractions of 2 ml were collected, the absorbance values of which were monitored at 260 nm. To avoid mixing, and contamination from the capillary, the faster migrating fraction used for polyacrylamide gel electrophoretic analysis was removed from the top of the column using a long pasteur pipette with a bent tip.

As is apparent from the photographs and absorbance profiles presented in Fig. 27 and 28, CaMV preparations in a relatively undegraded state prepared from fresh leaves migrated as a diffuse zone with an RØ of about 1, followed by a slower component with an RØ of about 0,7. After removal from the column, these zones were initially shown to contain CaMV by their ultraviolet absorbance profiles. The virus-containing samples were concentrated

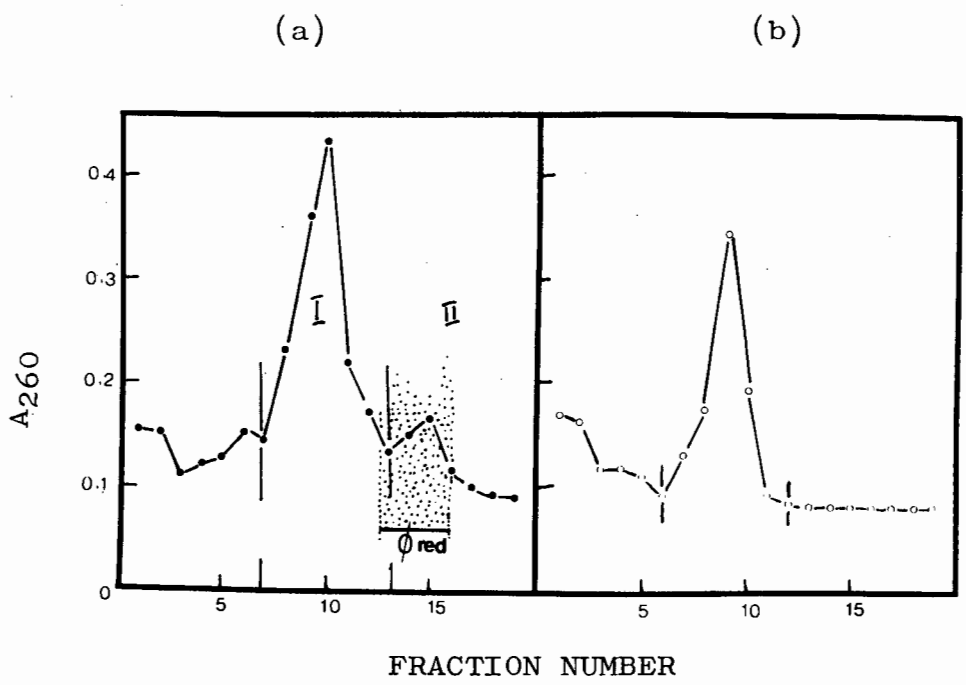


FIGURE 27

a) Absorbance profile of CaMV (Oudtshoorn isolate) prepared from fresh leaves after electrophoresis in a sodium phosphate (pH 7,5) zone electrophoresis column.

b) Absorbance profile after electrophoresis under identical conditions of CaMV (Oudtshoorn) prepared from leaves which had been stored at 4°C for 16 days.

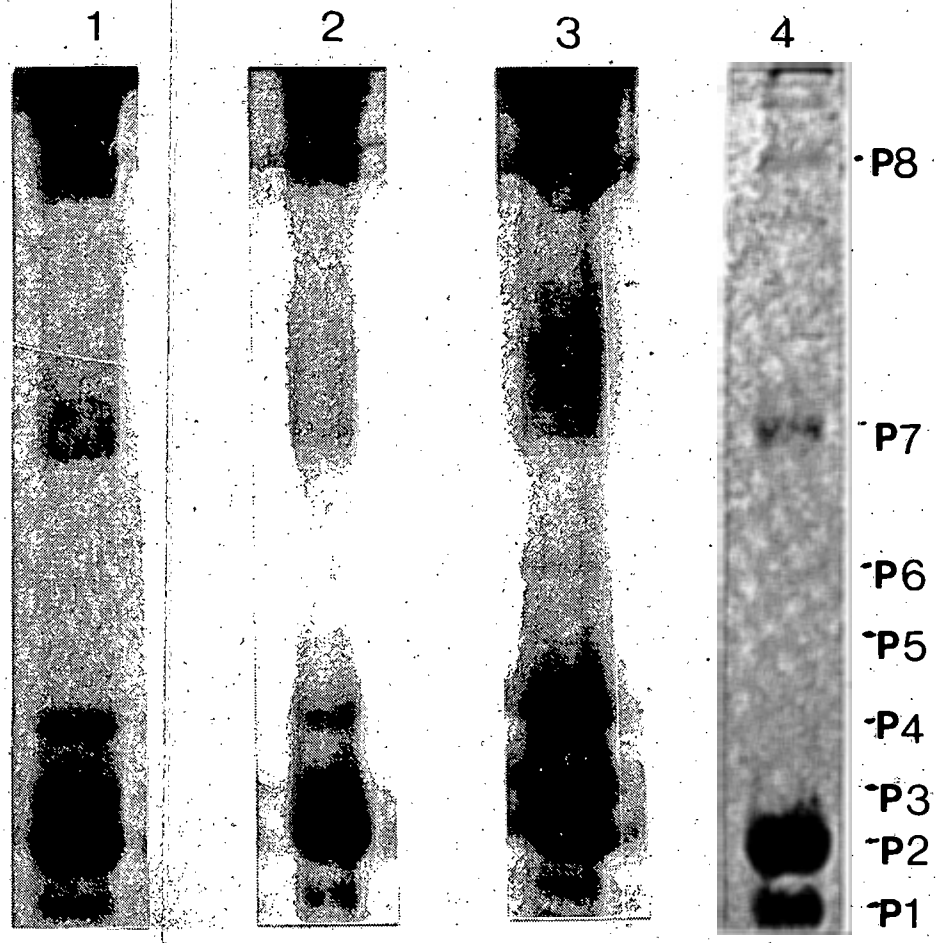


FIGURE 28

Polypeptide profiles of CaMV removed from the zone electrophoresis experiments illustrated in Figure 27 (a) and Figure 27 (b).

1 Profile of the virus sample applied to the column in (a).

2 Profile of the virus sample removed from virus containing zone I in (a).

3 Profile of the virus sample removed from virus containing zone II in (a).

4 Profile of the virus sample applied to the column in (b).

by centrifugation at 30 000 rpm for 90 minutes. In polyacrylamide gels, the virus removed from different zones showed differences in the proportion of the various polypeptides present. The faster component (RØ = 1) had an enriched P4 band compared to the slower one. In different determinations, the proportions and amounts of the polypeptides were not always identical, due probably to variations in sampling and the degree of degradation of the sample. However, the fast moving virus component invariably showed the greater proportion of P4 and P3 on polyacrylamide gel.

When CaMV with its capsid protein degraded to P2 and P1 by storage was electrophoresed, the virus sample migrated as a single band with an RØ of approximately 0,7 (Fig. 27b). These findings are in accordance with the results obtained in agarose gels (Section G.1.).

It can be concluded from the above results that the nett charge of the CaMV virus particle is subject to alteration as the result of enzymatic degradation, and this in turn can result in a single preparation showing more than one electrophoretic component. It also means that in a single preparation of CaMV one may find populations of virus particles in different states of degradation. In a relatively well-preserved preparation, eg, from fresh leaves, the P4, P3, P2 and P1 capsid polypeptide species seen in gel need not all exist simultaneously in a single virion. This confirms the analogous findings of Al Ani et al. (1979b), using sucrose density-gradients.

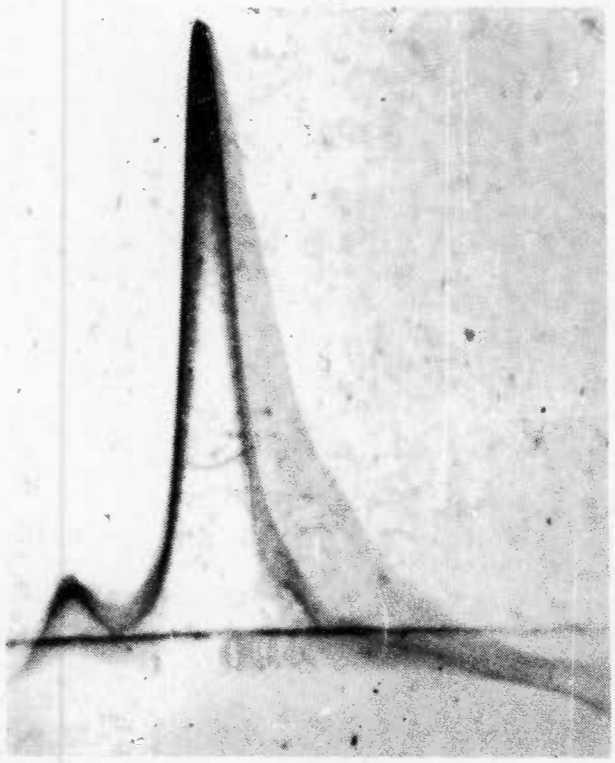
H. TWO-DIMENSIONAL IMMUNOELECTROPHORESIS

Two-dimensional immunoelectrophoresis (Laurell) was used as a means of ascertaining the homogeneity of CaMV preparations and antisera. In this technique, the virus preparation is first electrophoresed in a thin agarose layer at pH 7,0. A second electrophoretic run at right angles to the first is made into another agarose gel in which CaMV antibodies are evenly distributed. The antigen and antibody both migrate, the electrophoretic movement of the antibody being counteracted to some extent by electroendosmosis. Precipitin zones form for each antigen/antibody system. For a given antibody component, the height of the peak formed is roughly proportional to the concentration of the antigen if the run is sufficiently long.

The antisera used in this series of experiments were raised against virus which had been purified without knowledge of its proteolytic state. CaMV preparations (various isolates) were tested against several antisera. When CaMV (Cabb-S) prepared from fresh leaves was tested, two clear precipitin peaks were apparent, with a third smaller peak also evident in some runs (Fig. 29). These peaks probably represent the components seen in the agarose/acrylamide and agarose gel electrophoresis (Fig. 24). There was no clear serological distinction between the peaks as would be shown by a crossing-over or spurring of the precipitin lines of the respective peaks. However, the precipitin profile of preparations of all CaMV isolates tested showed a "shadow" and evidence of blurred or superimposed peaks. This heterogeneity was also seen with CaMV showing only a single peak.

ANODE IN SECOND ELECTROPHORESIS

+



+

ANODE
IN FIRST ELECTROPHORESIS

FIGURE 29

Precipitin pattern formed by CaMV (Cabb-S) after two-dimensional (Laurell) immunoelectrophoresis with a homologous antiserum. The experiment was performed in 0,01M sodium phosphate buffer pH 7 in 0.6% agarose. The antiserum dilution was 1/400.

These results suggest that the CaMV antisera used in these tests displayed more than one specificity, due probably to a heterogeneity of the immunogen. The experiments described in section G of this chapter, as well as the findings of Al Ani et al. (1979b), indicate that such heterogeneity may be due to a single virus preparation containing particles in more than one enzymatic degradation state. It seems likely that faster or slower migrating components are due to electrophoretic heterogeneity of the preparation as seen in agarose and zone electrophoresis. Antigenic heterogeneity may be responsible for "shadows" and blurred boundaries of the precipitin peaks.

I. TREATMENT OF CaMV WITH 2-CHLOROETHANOL

In order to obtain CaMV protein subunits which could be used in serological studies, an attempt was made to disrupt the virus using the organic solvent 2-chloroethanol. This solvent is thought to act on viruses in a similar fashion to acetic acid, breaking intersubunit hydrophobic bonds (Schubert and Frank, 1970).

About 3 mg of CaMV (Cabb-S) were centrifuged to a pellet and resuspended in 0,2 ml of distilled water. A volume of 9,0 ml of 2-chloroethanol (Merck) was then added. This mixture was left at room temperature (about 20°C) and then subjected to centrifugation to remove any intact CaMV. The supernatant fluid was withdrawn and the CaMV-like pellet which was found after this treatment was resuspended in water. Chloroethanol shows minimal absorbance in the ultraviolet, and the ultraviolet absorbance of the supernatant was determined. A scan

showed an absorption maximum at about 270 nm. The resuspended pellet yielded a typical CaMV ultraviolet scan with a maximum at 260 nm. Using an $E_{260}^{0,1\%}$ of 7,0, the amount of virus in the pellet was found to be about 96% of that initially treated.

For further examination, the supernatant fluid was evaporated to dryness by a current of nitrogen at room temperature. A small quantity of light brown residue was obtained. This residue was suspended in 0,3 ml of distilled water yielding a cloudy solution. When tested in a precipitin test in agar gel with a CaMV antiserum, no precipitin lines were obtained. The remainder of the solution was treated at 100°C with SDS, urea and 2-mercaptoethanol and electrophoresed on a 10% polyacrylamide gel using a continuous tris-boric acid buffer system (Hull and Shepherd, 1976). No bands were obtained with the supernatant, but the virus pellet treated in the same way showed a polypeptide profile typical of partially proteolysed CaMV (P2 and P1). In addition, the 2-chloroethanol-treated virus was serologically undistinguishable from untreated virus in a "sandwich" ELISA (Fig. 30).

These experiments show that although 2-chloroethanol is unable to degrade CaMV, a substance with a characteristic protein-like ultraviolet absorption profile was separated. It was not possible to identify the component removed, and no serological change in the treated virus was detectable.

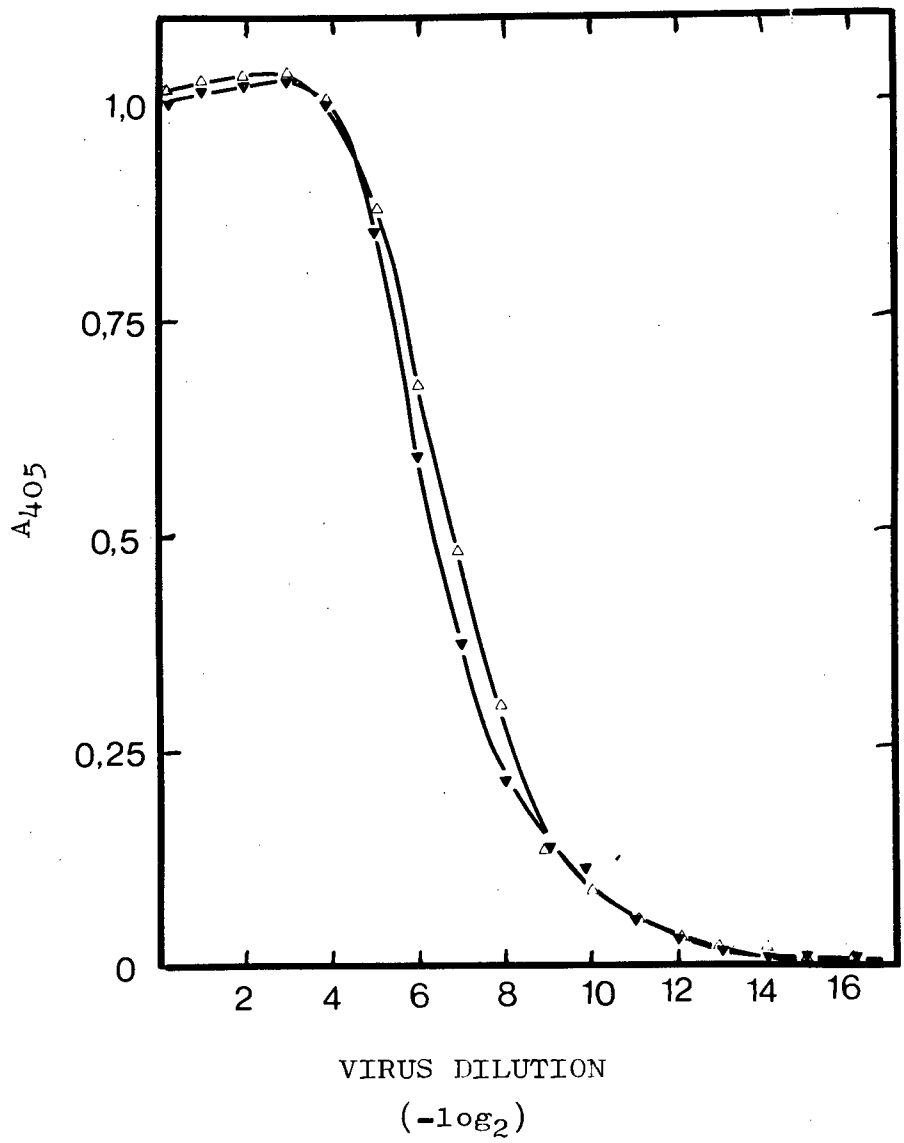


FIGURE 30

ELISA absorbance values obtained with CaMV (Cabb-S isolate) after treatment with 2-chloroethanol (△) compared with values obtained with untreated virus (▼). For ELISA the microtitre plate was coated with IgG directed against the Cabb-S isolate at a concentration of 1,0 µg/ml. Labelled IgG was added at a dilution of 1/800.

J. LECTIN BINDING STUDIES

1. CaMV Polypeptides

Several workers have attempted to determine which, if any, of the CaMV polypeptides observed in gels were glycopeptides, using Schiff's reagent (Kelly et al., 1974; Hull and Shepherd, 1976). However, no conclusive results were obtained, due mainly to variable polypeptide patterns, the origin of which was not understood, and to the unreliability of the Schiff test at low levels of glycosylation (Hull and Shepherd, 1976). In 1979 the problem of CaMV polypeptide variability was resolved by Al Ani et al. (1979b), who showed that enzymatic degradation and polymerisation was responsible for the multiplicity of bands observed on gel with disrupted CaMV.

The plant lectin Concanavalin A (Con A) is a globular protein composed of identical 25 500 dalton subunits arranged in dimeric, tetrameric and higher order forms. Con A generally binds to saccharides containing α-D-mannose or α-D-glucose residues. The lectin appears to recognise terminal as well as internal saccharide residues (Nicholson, 1976).

In this work, instead of the Schiff test, the binding of a fluorescent derivative of Con A to CaMV polypeptides which had been electrophoresed in gels was used to detect possible glycosyl residues attached to the CaMV polypeptides. The preparation of fluorescent Con A derivatives and binding studies were performed with the help of P. Smith of the University of Cape Town Biochemistry Department. Fluorescein isothiocyanate-conjugated Con A (FITC-Con A) was prepared by the method described by Malluci (1976).

As little as 0,5 µg of carbohydrate can be detected by this method using horse-radish peroxidase as a standard glycoprotein. A CaMV preparation from fresh leaves was electrophoresed in several tracks on polyacrylamide slab gels. After electrophoresis the gel was cut vertically into two slabs, each containing CaMV polypeptides. One slab was stained with Coomassie blue in the usual way, while the other was fixed in ethanol/acetic acid/water in a 25%/7%/68% v/v/v ratio. After thorough soaking in standard salt solution, the fixed gel was then immersed in a solution of Con A, left for 3 hours at room temperature. Unbound FITC-Con A was removed by further soaking in standard salt solution. Under ultraviolet (254 nm) illumination, the regions of the gel corresponding to the CaMV polypeptides were found to fluoresce (Fig. 31). If methyl glucoside was present in the Con A solution, no fluorescence was apparent, thus establishing the specificity of the system.

Polypeptides P4, P3 and P2 were found to exhibit strong fluorescence, showing FITC-Con A binding while the minor polypeptides could not be positively identified as exhibiting fluorescence. Not only P4, the apparent main structural polypeptide of CaMV (Al Ani et al., 1979b), bound Con A, but also its degradation products. This finding suggests that at least some carbohydrate is not lost during enzymatic breakdown. It was not found possible to quantitate the carbohydrate as the carbohydrate moiety of a series of standard glycoproteins may differ in its specificity for Con A.

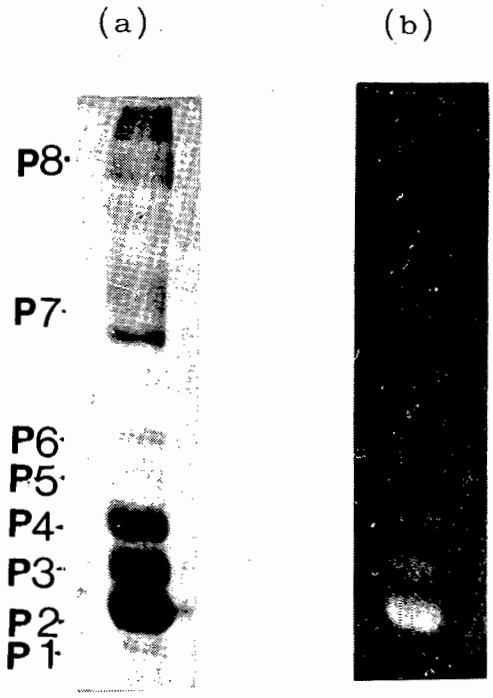


FIGURE 31

Polypeptide profile of CaMV (Cabb-S) after electrophoresis in 10% polyacrylamide gels.

a) Stained with Coomassie blue.

b) After treatment with fluorescent Con A. Fluorescence was visualised under ultraviolet illumination.

2. Binding of Con A to Intact CaMV

a. Agglutination with Con A

Attempts were made to agglutinate CaMV with Con A, both in liquid and gel (Goldstein, 1976). It was found that CaMV at a concentration of 1,0 mg/ml did not precipitate in liquid or in gel when tested with Con A at concentrations up to 5 mg/ml.

b. Inhibition of antibody binding by Con A

The following experiment was designed to show the specific adsorption of Con A to intact CaMV by inhibiting the attachment of labelled antibodies to virus which had previously been adsorbed to the surface of polystyrene microtitre plate wells.

CaMV was adsorbed to the microtitre plate in the same way as in an ELISA, and after a washing step, alkaline phosphatase conjugated specific antibody was added. The presence of bound antibody was shown by the hydrolysis of p-nitrophenol phosphate. A preliminary checkerboard titration was carried out in order to ascertain the optimal concentrations of CaMV and specific labelled antibody required to yield an absorbance value of approximately 1,0. This was achieved by coating the well with CaMV at a concentration of 10 µg/ml and adding specific labelled antibody at a dilution of 1/800 in PBS-Tween at 0,2% ovalbumin.

For the inhibition, dilutions of Con A in standard salt solution containing 1 mM $MnCl_2$ and 1 mM $CaCL_2$ was added to the virus-coated wells after the first washing step. After

60 min at 20°C the plate was washed again and conjugated antibody and substrate was added as described. Ovalbumin was present in the buffer in which the labelled antibody was suspended in order to prevent non-specific adhesion of antibodies to the plastic well. The ovalbumin would also conceivably occupy free binding sites on the Con A, which may have been available to bind IgG, itself a glycoprotein.

It was found that the Con A inhibited the attachment of conjugated antibody to the immobilised CaMV, so causing a reduction in absorbance values. If the sugar, methyl glucoside (methyl-α-D-glucopyranoside) was present in a 100-fold molar excess over the Con A, no such reduction in absorbance values was obtained. This establishes the specificity of the system. The inhibition of antibody binding is illustrated in Fig. 32. These findings imply that the Con A was bound to accessible carbohydrate residues on the surface of the CaMV capsid.

c. Direct binding of iodinated Con A

In order to confirm the foregoing results which suggested the presence of carbohydrate residues on the surface of CaMV, radiolabelled Con A was allowed to react with purified virus preparation, both in the absence and presence of methyl glucoside. After centrifugation to a pellet, the amount of radioactivity associated with the virus was then determined.

Volumes of 50 µl of purified preparations of CaMV (Australia isolate), bromegrass mosaic virus (BMV) and barley stripe mosaic virus (BSMV) at an approximate concentration of

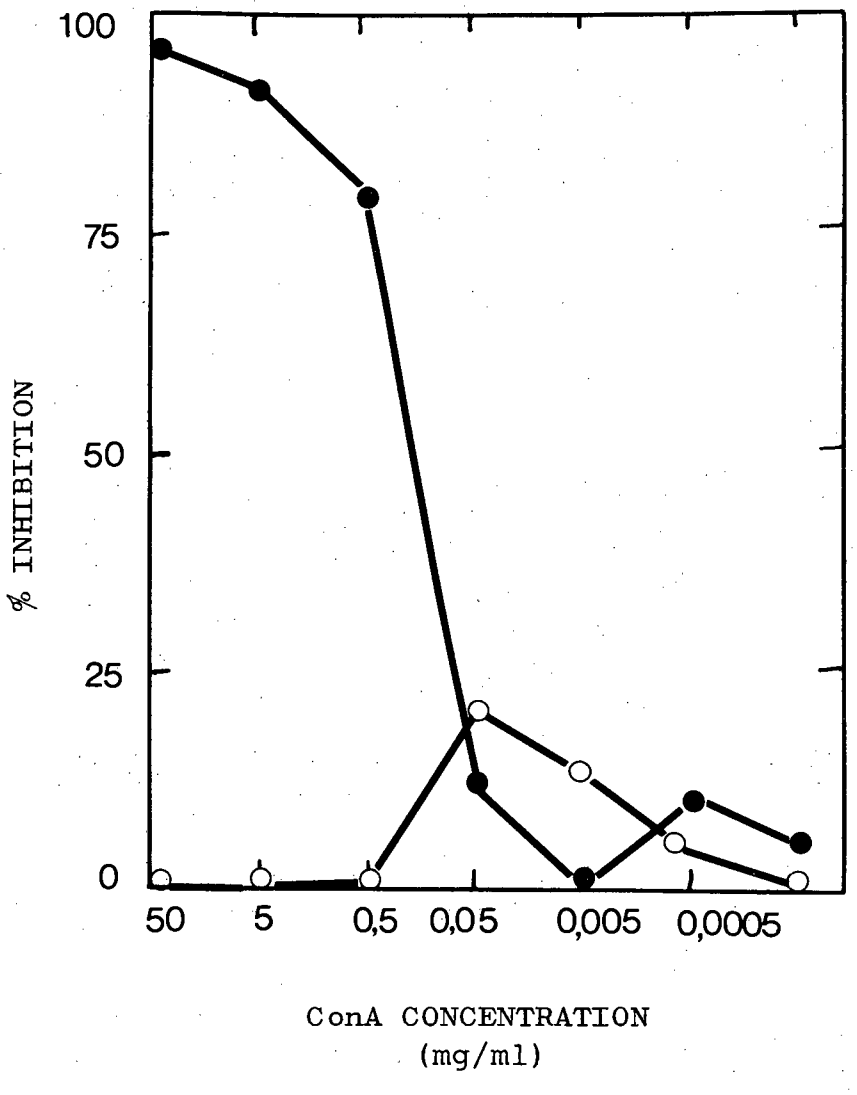


FIGURE 32

The inhibition of labelled antibody binding to immobilised CaMV by ConA. Percentage inhibition was calculated as described in the text.

Inhibition by

● ConA in standard salt solution.

○ ConA in standard salt solution containing a 100-fold molar excess of methyl glucoside.

1 mg/ml were each mixed with 10 µl of iodinated Con A in a Beckman polyallomer SW 50 centrifuge tube. To the test samples were added 10 µl of standard salt solution containing 0,2% BSA, while the control samples each received an identical aliquot containing methyl glucoside at a concentration of 50 mg/ml. The mixture was left at room temperature for 60 min, after which time 5 ml of standard salt solution containing 0,2% BSA was added in order to fill the centrifuge tubes. After centrifugation for 40 min at 40 000 rpm in a Beckman SW 50 swing-out rotor, the supernatant fluid was discarded and the tubes were allowed to drain thoroughly. A further 5 ml of standard salt solution and 0,2% BSA was added, the virus pellets were allowed to resuspend and the centrifugation was repeated and the supernatant fluid was discarded. Using the probe of a radiation monitor, it was established that all detectable radioactivity was located at the bottom of the tube. The entire tube was then placed into a scintillation vial and the associated radioactivity was measured with a Packard gamma counter.

It was found that the presence of methyl glucoside in the initial reaction mixture had the effect of reducing the amount of radioactivity associated with the virus pellets by 28,78%, 37,81% and 8,87% (table 7) in the case of CaMV, BSMV and BMV respectively compared with total radioactivity brought down in the absence of the ligand.

As the BSMV protein subunit is glycosylated (Partridge et al., 1974), while that of BMV is not, it can be inferred that the reduction in radioactivity in the case of CaMV was due to

TABLE 7

Radioactivity associated with different virus preparations after incubation with ^{125}I-labelled Con A.

Virus	cpm x 10^4 in pellet		% Reduction in radioactivity due to the presence of methyl glucoside
	(a)	(b)	
BMV	35,4	32,26	8,87.
BSMV	25,56	15,89	37,81
CaMV	27,9	19,92	28,78

(a) Counts/min (cpm) in abscence of ligand.

(b) Counts/min in presence of ligand.

reduced binding of the labelled Con A to the intact CaMV virion, as a result of competition by the ligand, methyl glucoside. These findings confirm that the protein subunit of CaMV is glycosylated and that some carbohydrate residues are accessible on the surface of the virion.

d. Inhibition of antibody binding by free saccharides

In the previous experiments, it was shown that antibody binding to CaMV could be inhibited by the attachment of Con A to the intact virus. It is possible that the inhibition was due to steric blocking of antibody attachment or alternatively, due to the carbohydrate residues of the capsid glycoprotein being implicated in the virus antigenic determinants. If the latter were the case, it should be possible to reduce antibody binding by competitive inhibition with sugars, as some antibodies would perhaps be carbohydrate-specific.

An attempt to show such inhibition was made using an indirect ELISA. Microtitre plate wells were coated with CaMV (Australia isolate) at a concentration of 5 µg/ml. After washing, antibodies specific for CaMV (prepared by binding at pH 7 and release at pH 2,9) were placed in the wells at sufficient concentration to give an ELISA absorbance value of about 0,5. This was on the descending portion of an antibody titration curve, and any reduction in antibody binding would be easily detectable. The antiserum was incubated with different concentrations of the following sugars: methyl-α-D-glucopyranoside, lactose, D-glucose and sucrose. The assay was then performed as usual.

No reduction of antibody binding due to the presence of any of the sugars was detectable, indicating that carbohydrate residues are not likely to be part of an antigenic determinant of the virus.

K. THE EFFECT OF CAPSID CONDITION ON SEDIMENTATION COEFFICIENT

The S values of some of the CaMV preparations described in section B (Oudtshoorn isolate) were determined in 0,1 M NaCl, buffered with 0,01 M sodium phosphate buffer, pH 7,2 at 20°C. As the object of these experiments was to compare sedimentation of virus in different states of proteolysis under identical conditions, the values quoted are uncorrected. The concentration of all preparations was adjusted photometrically to 0,5 mg/ml, by suitable dilution of purified CaMV, first into water, and then 1:1 into double strength buffered NaCl solution. Fig. 33 shows the schlieren pattern obtained after 32 min of centrifugation at 20 000 rpm. The preparation obtained from fresh leaves (Fig. 4, Track 1) had an uncorrected S value of 220, whereas the value obtained for the CaMV preparation shown in Fig. 4, Track 3 was 206S. Table 8 shows the S_{20} values obtained with some other CaMV preparations, the gel profile of whose capsid polypeptides is shown in Fig. 4. (None of these preparations showed any sign of heterogeneity in the model E, and in sucrose all were found to sediment as a single band.) It is apparent that there is no visible direct correlation between the S values obtained and the state of capsid proteolysis as shown in gel electrophoretic analysis.

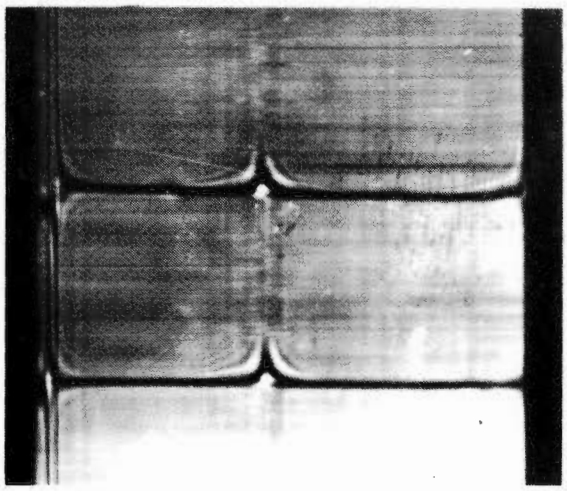


FIGURE 33

Schlieren diagram of CaMV preparations (Oudtshoorn isolate) after centrifugation at 20 000 rpm for 32 min. These preparations were the same as those showing the polypeptide profiles in Figure 4. The schlieren pattern from the plain cell represents Track 1 and the 1^{o} Wedge cell represents Track 3. S_{20} values were calculated to be 220 S and 207 S respectively.

TABLE 8

S values of CaMV (Oudtshoorn isolate) prepared under different conditions described in section 3 of this chapter. The polypeptide profiles of these preparations are shown in Fig. 4.

Track No (Fig. 4)	S value
1	220
2	201
3	207
4	200
5	203
7	208
9	208

However, there does appear to be some agreement with published S values for CaMV: For example, the values of 222 S (Pirone et al., 1961), 206,5 S (Itoh et al., 1969) 208 S (Hull et al., 1976) and 200 S (Al Ani et al., 1979a), are close to the 220 S (Track 1), about 208 S (Tracks 3, 7, 9) and about 200 S (Tracks 2, 4, 5) obtained in these experiments.

ooo 000 ooo

CHAPTER V

DISCUSSION

A. SEROLOGICAL DETECTION OF CaMV

In the past, the serological detection of CaMV proved to be problematic. Techniques which can readily be used to detect other plant viruses in crude leaf extracts, for example precipitin assays, have not been successful with CaMV (Pirone et al., 1961; Brunt, 1971). Furthermore, electron microscopy, particularly the quick dip method, has been found to be a relatively ineffective means of detecting CaMV particles (Shepherd, 1976). Improved results were obtained using serologically specific electron microscopy. This technique however, is unreliable for CaMV quantitation (Beier and Shepherd, 1978). These difficulties have been attributed to the fact that the CaMV virus particles are contained within inclusion bodies in the cytoplasm. As a result in crude homogenates, there are very few free virus particles available to react in serological assays, or be visualized on the electron microscope grid. Furthermore, the use of SDS in this study in immunodiffusion media apparently did not liberate sufficient virus particles to form a visible precipitate with specific antisera in Ouchterlony tests.

In 1964, the highly sensitive method of immune adherence was found to be suitable for detection of CaMV in clarified sap (Nelson and Day, 1964), but the technique did not gain acceptance. ELISA, however, a technique which is now

widely applied to plant virus detection appeared to have sufficient sensitivity, and its applicability to CaMV was investigated in this study.

With the "sandwich" double antibody variation of this technique (Clark and Adams, 1977) it was possible to detect CaMV antigen in crude leaf extracts even before symptoms became visible (Fig. 7), to show virus multiplication in isolated leaf mesophyll protoplasts, and to detect the presence of virus in aphids which had fed on CaMV-infected plants.

When purified virus was used in the assay, virus at a concentration of about 20 ng/ml was detectable (Fig. 6). This was about 50 times less than the concentration that could be detected by a local lesion assay for some strains of the virus (Tomlinson and Shepherd, 1978). As none of the attempts to obtain local lesions with isolates used in this study were successful, a reliable means of bioassay was not available.

The high strain specificity of ELISA (Koenig, 1978; Rochow and Carmichael, 1979), which may prevent detection of distantly related virus strains, did not prevent the detection in field samples of two local South African isolates of CaMV with a single antiserum. Subsequent work showed that these CaMV isolates were serologically very similar (Fig. 10, Tables 3 and 4). This suggested that in the case of CaMV, ELISA would be able to detect several strains with equal facility.

The antisera used for preparation of enzyme immunoassays were all absorbed routinely with healthy host plant material, so that non-specific reactions were negligible. As the virus preparations used as immunogens were purified on sucrose density-gradients, and the antisera were absorbed with plant material, the presence of appreciable amounts of antibodies to host components in the antisera which were used in immunoassays must be considered extremely unlikely. In some cases, non-specific colour development in the test at low sap dilutions was apparent. This colour could be reduced by about 50% by the addition of antibodies to host fraction 1 protein. On the other hand such low sap dilutions (1/10) are not used for routine detection and can therefore be ignored. It was noticed in tests where low sap dilutions were used, that it was difficult to flush the sap from the wells. This stickiness of sap components possibly contributes to the non-specific colour reaction. In no case did non-specific reactions mask virus-specific reactions in tests with field samples.

The experiments describing the detection of CaMV multiplication in protoplasts, while of a preliminary nature, indicate the potential of ELISA for this type of study. ELISA may have advantages over other methods which have been used such as the co-sedimentation with radioactive virus used by Howell and Hull (1978) or infectivity tests (Okuno et al., 1977).

The detection of virus in aphids is an indication that ELISA could be of use in studies on the behaviour of CaMV in its

insect vector, an aspect which is not entirely resolved (Shepherd, 1976). The assay may also be useful in epidemiological studies and in the screening of other suspected CaMV vectors.

Although a standard curve with pure CaMV was available (Fig. 6), no comparison with ELISA absorbance values obtained with crude extracts was made. This is because it is not clear whether the immunoassay is capable of detecting only free non-included virus, or whether the viral inclusion bodies which contain many virus particles can attach to virus-specific antibodies. In this regard it is of interest that Beier and Shepherd (1978) experienced difficulty in quantitating CaMV by immune electron microscopy. A calibration curve constructed using purified CaMV may thus be meaningless when related to ELISA values which have been obtained with crude extracts. The experiment designed to follow the development of CaMV in infected host plant leaves provides some confirmation of this. After infection, ELISA absorbance values and infectivity of leaf homogenates increased to a maximum and then showed a decline (Fig. 8, Table 3). On the other hand, after the maximum was reached, virus yield remained constant until 32 days after infection, at which time ELISA values had declined to about 50% of their maximum level. This can be explained by the fact that, in the later stages of infection, very few free virus particles are to be found in the cytoplasm (Fujisawa et al., 1967), in this way causing reduced ELISA and infectivity values. The process of purification by Triton X-100 and urea releases these particles.

It is possible that CaMV inclusion bodies have some virus particles accessible to antibodies on their surface. This is suggested by the findings of Lawson and Civerolo (1978) who showed that injection into a rabbit of the inclusion bodies of the related caulimovirus, CERV, resulted in the production of antibodies to purified virus. The possibility also exists, though, that the viroplasm matrix was degraded in the rabbit, so releasing virus particles.

It is thus not certain whether CaMV inclusion bodies possess accessible virus antigenic determinants. This point may be clarified by ferritin or fluorescent-labelled antibody studies in situ. However, even though it appears that ELISA cannot be used for the absolute quantitation of CaMV in crude leaf extracts, the experiments described in this study show that the assay can be of use to detect, and to determine relative quantities of CaMV in the early stages of infection. In the later stages, however, no direct correlation between ELISA value and the quantity of virus contained in an infected leaf as measured after disruption of the inclusion bodies by Triton X-100 and urea and extraction of the virus exists.

A further consideration is that ELISA, using an antiserum directed against intact virus, could also conceivably detect non-assembled virus protein in infected leaf or protoplast tissue. The assay may thus be of use in studies on CaMV morphogenesis.

B. DIFFERENTIATION OF ISOLATES

The results of restriction endonuclease analyses of the nucleic acid of many CaMV isolates have been reported (Meagher et al., 1977; Hull and Howell, 1978: Lebeurier et al., 1978; Volvovitch et al., 1977, 1979; Hull, 1980; Gardner et al., 1980) but so far, other than a proposal for the location of the viroplasm gene (Al Ani et al., 1980), it has not been possible to assign gene functions to any regions of the CaMV genome. Recently, a 42 000 dalton polypeptide "P4" was identified as the main structural component of the CaMV capsid (Al Ani et al., 1979b). Differences in amino acid sequence of this coat protein in different CaMV isolates could conceivably be correlated with changes in their DNA restriction patterns. It could be expected that such differences would be expressed antigenically and in this study an attempt was made to identify any such serological variation between several isolates.

1. Serology

All available serological data point to a close relationship between members of the caulimovirus group (Brunt 1966, 1971a and b; Gomec, 1973), but no information on the serological characteristics of CaMV strains has been reported. In this thesis, immunodiffusion tests and ELISA were used for examining antigenic relationships between some CaMV isolates. In addition, electrophoresis was studied as a possible way of distinguishing isolates. No

significant serological differences between the Cabb-S, NY-8153, Australian, Oudtshoorn and Banhoek isolates were discernible by comparison of homologous and heterologous serum end-point titres in gel (Table 4). Furthermore such immunodiffusion tests yielded reactions of serological identity with no spurring of precipitin lines being apparent (Fig. 9).

The double antibody "sandwich" variation of the ELISA technique has been reported to be a sensitive means of discriminating between closely related virus antigens (Koenig, 1978; Rochow and Carmichael, 1979). In the experiments described in this thesis, the "sandwich" ELISA assay showed up small differences in serological reactivity of the Cabb-S, NY8153 and Australia (Fig. 10), in contrast with the results of immunodiffusion tests. It was also established that minor variations in antigen concentration did not affect the relative maximum colour development in the ELISA due to homologous and heterologous reactions (Fig. 11). The high degree of specificity attainable in the "sandwich" ELISA has been attributed to lowered affinity of the enzyme-labelled antibody (Koenig, 1978).

When the Australian and Cabb-S isolates were compared in an ELISA prepared with specific anti-Australia isolates antibodies, no difference in homologous and heterologous ELISA values was found (Fig. 12). This may be a function of the particular antiserum, as antisera from different bleedings of different rabbits may differ in homologous and heterologous

reactivity (van Regenmortel and von Wechmar, 1970; Koenig and Givord, 1974, van Regenmortel, 1975). As will be discussed later, the possibility of different states of proteolytic degradation of the viral capsid leading to a fortuitous reaction of serological identity must also be considered.

The other techniques utilising enzyme labelled antibodies which were investigated, confirmed the close serological relationship of the isolates which were observed in the gel diffusion tests and sandwich immunoassays. The results of the experiments in which the inhibition of antibody binding by antisera directed against different isolates was tested, although suggesting serological differences, should be treated with caution (Fig. 13). This is because, different antisera are likely to vary in antibody affinity and avidity and the small differences in inhibition may be due to the part played by such variables. Direct binding of enzyme-labelled antibodies to CaMV, followed by centrifugation and quantitation of the antibodies by the addition of substrate, was not successful as a discriminatory technique for distinguishing CaMV isolates.

The cross-absorption of an antiserum with homologous and heterologous isolates, followed by the assay of residual antibody activity by indirect ELISA, was found to be a successful means of showing serological differences between five CaMV isolates. In the case of the cross-absorption of the NY-8153 antiserum, the homologous antigen removed all detectable antibodies from the antiserum. Different

amounts of antibodies which reacted with the NY-8153 isolate were detectable after absorption with the four heterologous isolates (Table 5). It could be expected that the more distant the serological relationship of these isolates to NY-8153, the less antibody would be removed by the cross-absorption. Hence more antibodies would be available to react with the NY-8153 isolate, since the heterologous isolates possess antigenic determinants which do not react with the NY-8153 paratopes. By this reasoning, Table 5 portrays an ostensible order of serological relationships. However, when the cross-absorption was repeated on the Banhoek antiserum and with new preparations of CaMV, a reciprocal and quantitative relationship was not obtained. Again, this may be due to affinity and avidity differences between the antisera, but some other possible reasons must be considered.

As the Australia and NY-8153 isolates were capable of removing almost all detectable antibodies from the Banhoek antiserum, the possibility of contamination of these isolates with Banhoek isolate is suggested. Care was taken to avoid this happening during virus propagation, but it is not impossible that the isolates used were not entirely pure strains. Restriction maps of CaMV-DNA have often revealed underlying patterns typical of other isolates (Meagher et al., 1977; Hull and Howell, 1978; Lebeurier et al., 1978; Hull 1980). In addition, new isolates have arisen during transfer, and on the basis of the wide variation in restriction patterns of CaMV isolates, it has been postulated that

this may occur continuously (Hull, 1980). If isolates are indeed cross-contaminated, the "sandwich" ELISA should yield more meaningful serological data. This is because dilution to the low concentrations (about 100 µg/ml starting concentration) used in this assay may effectively reduce the contribution of such contaminants below the threshold of ELISA sensitivity.

At the time when the initial serological work reported in this thesis was performed, little information on the identity and location of the CaMV capsid polypeptides was available. In the course of this work it became known however, that the CaMV coat protein was extremely sensitive to proteolysis (Al Ani et al., 1979b). The possibility that such enzymatic hydrolysis would affect CaMV serological reactivity was therefore investigated, and the results of earlier experiments were re-evaluated in the light of these findings.

It was found that in making serological comparisons of CaMV isolates, the proteolytic state of the capsid protein is an important factor. This was made clear by the experiments in which the serological reactivity of CaMV preparations was found to depend upon the degree to which the capsid protein had been degraded by plant proteases. Using several antisera, this phenomenon was shown by the "sandwich" double antibody ELISA in the case of several virus preparations which exhibited different polypeptide profiles on polyacrylamide gels. Antisera directed against intact CaMV (Fig. 15, 17 and 19) were capable of clear discrimination between preparations of the same isolate which differed

only in the proportions of native capsid polypeptide and its degradation products.

In the case of one particular antiserum, it was also established that no direct correlation existed between the degree of proteolysis and the serological reactivity of a CaMV preparation (Fig. 18 and Fig. 19), ie. there was no concomitant reduction or increase in ELISA reactivity as degradation proceeded. Possible reasons for this will become apparent when the specificity of CaMV antisera is discussed. Further, not all antisera showed equal discriminatory powers (Fig. 20).

When Fig. 10 and Fig. 17 are compared, it is clear that serological differences due to proteolysis may be of equal or greater magnitude than apparent serological differences between isolates. In order to confirm that these changes can lead to the appearance of spurious serological differences, two CaMV isolates were compared serologically by ELISA in two different states of degradation. As is evident from Fig. 21 and Fig. 22, the apparent degree of serological relatedness changed, depending upon the capsid protein's proteolytic state. From these results it may be concluded that serological reactivity is not an effective means of establishing relationships within the CaMV group. Taking these findings into account, it is apparent that the higher ELISA absorbance values obtained with homologous antigens in "sandwich" ELISA tests (Fig. 10) may have been fortuitous as may apparent reactions of identity (Fig. 12). It follows that it has not been possible to identify serological markers which could be correlated with DNA sequence differences.

Other workers have also noted that *in situ* degradation of potex and potyvirus capsid proteins, can affect virus serological reactivity. Such a serological change may be detectable in immunodiffusion tests (Hiebert and MacDonald, 1976; Purcifull and Batchelor, 1977), or it may require a sensitive technique like ELISA to be distinguished (Koenig, 1978). The importance of taking into account possible antigenic differences between preparations of the same virus when making serological comparisons has been pointed out by Purcifull and Batchelor (1977).

A critical factor in making serological comparisons of virus strains is the specificity of the antiserum used. It will thus be necessary to discuss CaMV antisera in the light of what is known of the immunogen. The antisera which were used throughout this study, having been raised early on in this study, were directed against CaMV preparations which were not monitored to ascertain their state of proteolysis. It was shown that storage of virus, both in the infected leaf and as a semi-pure preparation affected the polypeptide profile of CaMV (Fig. 4 and Fig. 18). It is highly likely, therefore, that CaMV in a variety of proteolytic states was used during the course of immunisation. Furthermore, it had been shown that a single preparation of CaMV may contain populations of virus particles in different states of degradation (Al Ani *et al.*, 1979b). Al Ani and coworkers were able to separate these virus populations on sucrose density-gradients. It was shown in this thesis that populations of CaMV exhibiting different polypeptide profiles

on polyacrylamide gels could also be separated by zone electrophoresis (Fig. 27 and 28). As the serological comparisons showed, proteolysis of the capsid resulted in an alteration of the virus antigenic structure. In none of the different preparation methods tested in this study was it possible to obtain virus showing native P4 capsid polypeptide alone. This means that during the course of immunisation, there is a likelihood that the animal was presented with a spectrum of antigenic determinants. It follows that a heterogenous antibody population must be produced. Since it is likely that CaMV purified from plant material does not possess a unique set of antigenic determinants it may be impossible at this stage to make valid serological comparisons between CaMV isolates.

Some evidence for the serological heterogeneity of CaMV preparations and their antisera may be seen in the results of two-dimensional (Laurell) immunoelectrophoresis experiments. Precipitin peaks showed "shadows" and poorly defined boundaries which may reflect immunological heterogeneity of antigen and antibody (Fig. 29). The smaller peaks probably represent different degradation states of the virus, as was shown in the zone electrophoresis experiments to be discussed below.

A comparison of the serological reactivities of TMV and two related strains (U2 and CGMMV) showed the degree of discrimination of which ELISA is capable (Fig. 23). The degradation of the P4 (42 000 dalton) capsid polypeptide to P2 (37 000 daltons) can be equated with the loss of about 50 amino acid residues. The difference in ELISA

reactivity of a preparation showing P4 and P3 and one with only P2 and P1 is shown in Fig. 15. However, by comparison, the U2 strain of TMV which has 41 amino acid residue exchanges, all of which are not located in immunologically active regions of the subunit (van Regenmortel, 1967; Milton and van Regenmortel, 1979) reacted to a far lesser extent in ELISA than did TMV vulgare.

A possible reason for the CaMV antisera showing relatively poor discriminatory power may be that the portion of the CaMV polypeptide cleaved by the plant proteases does not occupy an immunologically active region of the virus surface. As these regions are accessible to proteolytic enzymes and are thus most probably on the virus surface (Al Ani et al., 1979), the latter possibility seems unlikely.

Other factors which should be considered in the study of CaMV serology are whether capsid proteins in different states of proteolysis may exist simultaneously on a single CaMV virion, the immunological role of the minor capsid polypeptides P5 and P6, and the possibility that proteolytically cleaved peptides may adhere to the virus surface due to hydrophobic or other non-covalent bonding.

In none of the preparations used throughout this work was only the native P4 capsid polypeptide present in SDS-polyacrylamide gel electrophoresis analysis. Density-gradient centrifugation (Al Ani et al., 1979b) and zone electrophoresis could be used to prepare CaMV showing a greater proportion of P4, but lower molecular weight degradation products were always present (Fig. 16 and 28). This raises the possibility

that capsid polypeptides in different states of proteolysis are present on a single virion, although it is difficult to envisage why such differential degradation should occur. If this were the case, it would make any immunochemical study of CaMV extremely complex and ambiguous, until such time as methods for obtaining native, homogeneous, and undegraded CaMV have been developed.

The minor polypeptides P5 and P6 appear, on the basis of their lability, to be located on the surface of the intact CaMV capsid (Al Ani et al., 1979b), and they may thus play a role in the antigenic determinants of the virus. On the other hand, it has also been postulated on the basis of neutron diffraction experiments, that they may be located within the capsid (Chauvin et al., 1979). In view of the small quantity of P5 and P6 present, their contribution to CaMV antigenicity could be expected to be masked by that of P4 and its degradation products. At present it is not possible to speculate on the antigenic role of P5 and P6.

Also of interest is the fate of the polypeptides cleaved by the plant proteases. Are they removed entirely, or do they remain on the viral subunit, held perhaps by hydrophobic bonding, and if so, what contribution to viral antigenicity do they make? If the fragments do not remain upon the virus surface, the biophysical properties of the particle would depend upon the proteolytic state of the virus. It is known that the rate of sedimentation of CaMV in sucrose gradients depends upon its degradation state and in the analytical centrifugation studies reported in this

thesis, some variations in S value were noted (Table 8). It was not possible however, to directly correlate this phenomenon with degradation state and these variations are probably not significant. In buoyant density determinations in high CsCl concentrations, no heterogeneity was apparent. Since the removal of large amounts of protein from the surface of a virus particle could be expected to alter its buoyant density significantly, it seems likely that most of the enzymatic cleavage products are retained on the virus surface. If this is the case, the reason for sedimentation heterogeneity in sucrose density gradients may result in part from an alteration of the particles' hydrodynamic properties, due perhaps to a further "loosening" of the virus surface structure. This conformational change may then be antigenically detectable (Fig. 16 and 17).

The treatment of CaMV with 2-chloroethanol, a solvent which can break intersubunit bonds of some viruses (Schubert and Frank, 1970), resulted in the removal of a substance with an ultraviolet absorbance maximum at about 270 nm. It was not possible to identify the substance removed, and although it might have been contaminating plant protein, this seems unlikely as no evidence of such contamination of sucrose density-gradient preparations was found (Fig. 5). If cleaved proteolytic peptides were in fact removed by chloroethanol treatment no detectable effect on virus antigenicity was apparent. The question of what happens to the cleaved peptides is thus not conclusively answered.

To sum up, the experiments presented in this thesis have shown that the serological characterization of CaMV isolates is not practicable with virus preparations which have their capsid protein in a proteolysed condition. The difficulty of obtaining strains which are genotypically homogeneous is a further complicating factor.

2. Electrophoresis

It has been reported that CaMV is electrophoretically heterogenous (Day and Venables, 1960), and that different isolates may have different electrophoretic mobilities (Lung and Pirone, 1972). In this thesis a possible reason for this heterogeneity is presented.

By means of electrophoresis of intact CaMV in agarose/acrylamide composite gels, the electrophoretic mobility of four CaMV isolates was determined. There were found to be small differences in electrophoretic mobility between the isolates (Table 6), and in the case of some preparations, more than one component was visible (Fig. 24). In gels, a slower component may represent the dimers that are observed in analytical ultracentrifugation studies (Hull et al., 1976; Al Ani et al., 1979a). When the same preparations that were used in the agarose/acrylamide experiments were electrophoresed in thin agarose gels in glass slides, the same order of relative mobilities was obtained. However, when this experiment was repeated on new samples of each isolate, the relative mobilities were found to have changed. It became necessary therefore to determine the effect of the capsid proteolytic degradation state on virus electrophoretic

behaviour. Different preparations of CaMV which had undergone different treatment before and during preparation, and which showed different polypeptide profiles on acrylamide gels (Fig. 4) were subjected to electrophoresis in agarose slabs.

Differences in the mobility of these preparations were found (Fig. 26). As all the preparations originated from the same propagation stock of the same isolate, the differences in mobility can be attributed to differences in the condition of the capsid protein. A second faster migrating component was also apparent in these experiments. This component was present in preparations containing P4, the undegraded capsid polypeptide, while if the virus preparation had been extensively degraded, the second component was absent. When the nature of this electrophoretic heterogeneity was investigated by zone electrophoresis, it was found to represent different degradation states of the capsid protein. The faster migrating component in zone electrophoresis was found to correspond to the less degraded capsid (Fig. 27 and 28).

The results of the electrophoresis experiments point to the loss of charged groups with proteolysis, or alternatively, to the exposure of new charged groups which may neutralise some available charges and so reduce the nett charge of the virus particle. However, the possibility that the presence of other electrophoretic components in a preparation of CaMV may be due to contamination by other isolates is not ruled out. As regards the use of electrophoretic mobility as a means of characterising CaMV isolates, the same limitations

applicable to CaMV serology regarding strain purity and native CaMV should be considered.

C. ATTEMPTS TO OBTAIN NATIVE CaMV

In an attempt to obtain virus with the capsid polypeptide in a native, undegraded state, variations on the standard CaMV extraction and purification method (Hull et al., 1976) were investigated. These methods involved freezing of the infected leaves and the use of protease inhibitors. This approach was suggested by the finding of Al Ani et al., (1979b) who showed that better preserved virus was extracted from frozen, rather than from fresh leaves. These workers also suggested the use of protease inhibitors during virus extraction.

It is evident from Fig. 4 that the use of protease inhibitors resulted in the absence of P1, but in no case, either with fresh or frozen leaves was it possible to obtain CaMV which showed the P4 peptide alone (Tracks 2, 5, 6). Liquid nitrogen freezing of infected leaves resulted in fairly extensively proteolysed CaMV (track 4), probably due to plant cell vesicular membranes being ruptured, releasing enzymes. An extensive degradation due to storage and to purification in the absence of reducing agents was also observed (Tracks 3, 7, 8, 9 in Fig. 4). Since degradation also occurred when the extraction was performed in the absence of reducing agents, it may be concluded that the proteases responsible for CaMV degradation are not analogous to those which are thought to be responsible for the proteolysis of the capsid protein of potato virus X (Koenig et al., 1978).

Caesium chloride density-gradient centrifugation (Fig. 5) and zone electrophoresis (Figs. 27 and 28) were equally unsuccessful as ways of separating native CaMV from a preparation of the virus. This may provide evidence for the existence of capsid polypeptides in different degradation states on a single virion, although buoyant density differences due to capsid protein degradation are not always apparent (Mayo and Cooper, 1973).

Although it was not possible to obtain native CaMV, the extraction of CaMV in the presence of protease inhibitors has confirmed that the variability in CaMV polypeptide profiles results from proteolysis and it highlights the difficulty of obtaining CaMV with its very labile capsid polypeptide in a native state.

D. GLYCOSYLATION OF THE CaMV CAPSID POLYPEPTIDE

Attempts to detect the presence of carbohydrate residues on the putative CaMV structural polypeptides by means of the Schiff test have met with little success (Kelly *et al.*, 1974; Hull and Shepherd, 1976). After it became known that the structural protein of CaMV was likely to be a 42 000 dalton polypeptide (Al Ani *et al.*, 1979), it was possible to re-examine the question of CaMV polypeptide glycosylation. For this study fluorescent-and ^{125}I-labelled derivatives of Con A were used, rather than the Schiff test. Attempts to precipitate the virus with Con A were not successful. This may be as a result of a low level of glycosylation (Gumpf and Shannon, 1978), a possibility which is supported by inconclusive staining of

protein or even assembled capsids can be obtained in this way. The use of monoclonal antibodies in plant virus serology can also be expected in the near future. However, this thesis has demonstrated the difficulty of interpreting serological data obtained so far with CaMV.

The significance of the finding that the CaMV coat protein is glycosylated is not clear. It is also not possible to postulate where or when this glycosylation occurs as little is known of CaMV replication. Whether the carbohydrate residues are perhaps implicated in the infective process as receptors, could be elucidated by treating the virus with glycosidases.

The biological significance of the coat protein's region of "loose structure" which may be responsible for the polypeptides ability to enzymatic hydrolysis (Al Ani et al., 1979b) is also not clear. It may result from redundant sequences which have not been exposed to selection processes, or on the other hand, it may be essential for the release of the DNA of this otherwise stable virus.

In conclusion it is clear that a serological evaluation of relationships amongst available isolates of CaMV can only be achieved once the proteolytic degradation of the capsid can be controlled. In addition any serological study of CaMV must take into account the possible genetic heterogeneity of isolates. Serological data on its own cannot yield unequivocal information.

ooo 000 ooo

CHAPTER VI

SUMMARY

1) ELISA was investigated as a means of detecting CaMV in crude extracts of infected leaf tissue. The assay was found to be suitable for this purpose, and its extreme sensitivity enabled the detection of virus in leaves before symptoms became visible. The serological assay was found to be unsuitable for quantitation of virus in infected leaves, particularly in the later stages of infection. With this assay it was also possible to show the presence of CaMV in viruliferous aphids and in infected leaf mesophyll protoplasts.

2) An attempt was made to show serological differences between several CaMV isolates. This was not possible using immunodiffusion tests, but small differences in serological reactivity were detectable by ELISA. Serum cross-absorption was used to help accentuate differences between CaMV isolates. Relationships established in this way were not reciprocal with different antisera.

3) The role of proteolytic degradation of the capsid polypeptide on the serological charactaristics of the virus was investigated. Differences in serological reactivity between virus preparations in different states of proteolysis were detectable. There was no direct correlation between the degree of degradation and serological reactivity. Relationships established between isolates were influenced by the degradation state of the capsid. Attempts to

obtain undegraded virus using a variety of extraction procedures were not successful.

4) Differences in electrophoretic mobility were investigated as ways of characterizing CaMV isolates. Electrophoretic mobility and homogeneity of CaMV preparations were found to be dependent upon the proteolytic state of the preparation.

5) CaMV antigens and antisera were examined by two-dimensional crossed immunoelectrophoresis. Evidence of more than one electrophoretic and antigenic component was found.

6) The glycosylation of the CaMV capsid polypeptide was re-examined using fluourescent and radio-labelled Con A. The lectin bound to the dissociated capsid polypeptides in gels as well as to intact CaMV. It also inhibited the attachment of virus-specific antibodies. A variety of saccharides did not inhibit CaMV-specific antibody binding to the intact virus.

REFERENCES

AGRAWAL, H.O. and GOLDSTEIN, I.J. (1967). Isolation of Concanavalin A by specific absorption on cross-linked dextran gels. Biochim. et Biophys. Acta 147, 262-271.

AL ANI, R., PFEIFFER, P., LEBEURIER, G. and HIRTH, L. (1979a) The structure of cauliflower mosaic virus. I. pH-Induced structural changes. Virology 93, 175-187.

AL ANI, R., PFEIFFER, P. and LEBEURIER, G. (1979b). The structure of cauliflower mosaic virus. II. Identity and location of the viral polypeptides. Virology 93, 188-197.

AL ANI, R., PFEIFFER, P., WHITECHURCH, O., LESOT, A., LEBEURIER, G. and HIRTH, L. (1980). A virus specified protein produced upon infection by cauliflower mosaic virus (CaMV). Ann. Virol. (Inst. Pasteur) 131E, 33-53.

BANCROFT, J.B., HILLS, G.J. and MARKHAM, R. (1967). A study of the self assembly process in a small spherical virus. Formation of organized structures from protein subunits in vitro. Virology 31, 354-379.

BAR-JOSEPH, M. and SALOMON, R. (1980). Heterologous reactivity of tobacco mosaic virus strains in enzyme-linked immunosorbent assays. J. Gen Virol. 47, 509-512.

BEIER, H. and SHEPHERD, R.J. (1978). Serologically specific electron microscopy in the quantitative measurement of two isometric viruses. Phytopathology 68, 533-538.

BERCKS, R., KOENIG, R. and QUERFURTH, G. (1972). Plant virus serology. In. Principles and Techniques in Plant Virology. (ed. C.I. Kado and H.O. Agrawal) pp. 466-472. Von Nostrand-Reinhold, N.Y.

BIDWELL, D.E., BARTLETT, A. and VOLLER, A. (1977). Enzyme immunoassays for viral diseases. J. Inf. Diseases 136 Supplement, 274-278.

BRAKKE, M.K. (1967). Density gradient centrifugation. In Methods in Virology. (ed. Maramarosch, K. and Koprowski, H.) Vol II. Academic Press.

BROADBENT, L. (1957). Investigations of Virus Diseases of Brassica Crops. 94 pp. Cambridge Univ. Press. London and New York.

BROADBENT, L. and TINSLEY, T.W. (1953). Symptoms of cauliflower mosaic and cabbage black ring spot in cauliflower. Plant Path. 2, 88-92.

BRUNT, A.A. (1966). Partial purification, morphology and serology of dahlia mosaic virus. Virology 28, 778-779.

BRUNT, A.A. (1971a). Some hosts and properties of dahlia mosaic virus. Ann. Appl. Biol. 67, 357-368.

BRUNT, A.A. (1971b). Dahlia mosaic virus. Commonw. Mycol. Inst. Descriptions of Plant Viruses, 51.

BRUNT, A.A. and KITAJIMA, E.W. (1973). Intracellular location and some properties of Mirabilis mosaic virus, a new member of the cauliflower mosaic group of viruses. Phytopath.Z. 76, 265-275.

BRUNT, A.A., BARTON, R.J., TREMAINE, J.H. and STACE-SMITH, R. (1975). The composition of cauliflower mosaic virus protein. J. Gen. Virol. 27, 101-106.

CALDWELL, J. and PRENTICE, I.W. (1942). A mosaic disease of broccoli. Ann. Appl. Biol. 29, 366-373.

CASPER, R. (1977). Testung von Prunus avium samen auf prune dwarf virus mit den ELISA-Verfaren. Phytopath.Z. 90 (1977) 91-94.

CHASE, M.W. (1968). Composition of selected buffers. In Methods in Immunology and Immunochemistry. (ed. C.A. Williams and M.W. Chase). Vol II Appendix II. Academic Press.

CHAUVIN, C., JACROT, B., LEBEURIER, G. and HIRTH,L. (1979). The structure of cauliflower mosaic virus: A neutron diffraction study. Virology 96, 640-641.

CHAUVIN, C., PFEIFFER, P., WITZ, J. and JACROT, B. (1978). Structural polymorphisms of bromegrass mosaic virus: A neutron small angle scattering investigation. Virology 88, 138-148.

CHERVENKA, C.H. (1969) A manual of methods for the analytical ultracentrifuge. Published by Spinco division of Beckman instruments.

CIVEROLO, E. L.and LAWSON, R.H. (1978). Topological forms of cauliflower mosaic virus nucleic acid. Phytopathology 68, 101-109.

CLARK, M.F. and ADAMS, A.N. (1977) Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34, 475-483.

CLEVELAND, W., STUART, G., FISCHER, G.S., KIRSCHNER, M.V. and LAEMMLI, U.K. (1977). Peptide mapping by limited proteolysis in sodium dodecyl sulphate and analysis by gel electrophoresis. J. Biol. Chem. 252, 1102-1106.

CONTRERAS, R., ROGIERS, R., van de VOORDE, A. and FIERS, W. (1977). Overlapping of the VP_2 - VP_3 gene and the SV40 genome. Cell 12, 529-538.

DAY, M.F. and VENABLES, D.G. (1960). Purification of cauliflower mosaic virus. Virology 11, 502-505.

DERRICK, K.S. (1973). Quantitative assay for plant viruses using serologically specific electron microscopy. Virology 56, 652-653.

EDWARDSON, J.R. and CHRISTIE, R.G. (1978). Use of virus-induced inclusions in classification and diagnosis. Ann. Rev. Phytopathology. 16, 31-56.

ENGVALL, E. and PERLMANN, P. (1971). Enzyme-linked immunosorbent assay (ELISA) Quantitative assay of immunoglobulin G. Immunochemistry 8, 871-874.

FAVALI, M.A., BASSI, M. and CONTI, G.G. (1973). A quantitative autoradiographic study of intracellular sites for replication of cauliflower mosaic virus. Virology 53, 115-119.

FENNER, F.F. (1976). Classification and nomenclature of viruses. Intervirology 7, 1-115.

FUJISAWA, I., RUBIO-HUERTOS, M. and MATSUI, C. (1971). Incorporation of thymidine ^{3}H into carnation etched ring virus. Phytopathology 61, 681-684.

FUJISAWA, I., RUBIO-HUERTOS, M., MATSUI, C. and YAMAGUCHI, A. (1967). Intracellular appearance of cauliflower mosaic virus particles. Phytopathology 57, 1130-1132.

GARDNER, M.W. and KENDRICK, J.B. (1921). Turnip mosaic. J. Agric. Res. 22, 123-124.

GARDNER, C.O., MELCHER, V., SHOCKEY, M.W. and ESSENBERG, R.C. (1980). Restriction enzyme cleavage maps of the DNA of two cauliflower mosaic virus isolates. Virology 103, 250-254.

GERA, A., LOEBENSTEIN, G. and RACCAH, B. (1978). Detection of cucumber mosaic virus in viruliferous aphids by enzyme-linked immunosorbent assay. Virology 86, 542-545.

GOLDSTEIN, I.J. (1976). Quantitation of the precipitin reaction between Concanavalin A and Carbohydrate polymers. In: "Concanavalin A as a Tool" pp 161-167. (Eds. H. Bittiger and H.P. Schnebli) Published by John Wiley and Sons.

GOMEC, B. (1973). Properties and biochemical characterization of dahlia mosaic virus. Ph.D. Thesis, Univ California, Davis, Calif.

GREENWOOD, F.C., HUNTER, W.M. and GLOVER, J.S. (1963). The preparation of ^{131}I-labelled human growth hormone of high specific activity. Biochem. J. 89, 114-123.

GUMPF, D.J. and SHANNON, L.M. (1978). The carbohydrate composition of barley stripe mosaic virus capsid protein, NSP strain. Abstracts: Third Int. Congr. Plant Path., München.

GUMPF, D.J., CUNNINGHAM, D.S., HEICK, J.S. and SHANNON, L.M. (1977). Amino acid sequence in the proteolytic glycopeptide of barley stripe mosaic virus. Virology 78, 328-330.

HARDIE, G. and VAN REGENMORTEL, M.H.V. (1977). Isolation of specific antibody under conditions of low ionic strength. J. Immunol. Meth. 15, 305-314.

HARRISON, B.D., BARKER, H., BOCK, K.R., GUTHRIE, E.J. and MEREDITH, G. (1977). Plant viruses with circular single-stranded DNA. Nature 270, 760-763.

HARRISON, B.D., FINCH, J.T., GIBBS, A.J., HOLLINGS, A.J., SHEPHERD, R.J., VALENTA, V. and WETTER, C. (1971). Sixteen Groups of Plant Viruses. Virology 45, 356-363

HENNIG, B. and WITTMANN, H.G. (1972). Tobacco mosaic virus; mutants and strains. In. Principles and Techniques in Plant Virology. (Ed. C.I. Kado and H.O. Agrawal.) Published by Van Nostrand Reinhold.

HIEBERT, E. and McDONALD, J.G. (1973). Characterization of some proteins associated with viruses in the potato Y group. Virology 56, 349-361.

HIEBERT, E. and McDONALD, J.G. (1976). Capsid protein heterogeneity in turnip mosaic virus. Virology 70, 144-150.

HILLS, G.J. and CAMPBELL, R.N. (1968). Morphology of broccoli necrotic yellows virus. J. Ultrastruct. Res. 24, 134-144.

HIRUKI, C., RAO, D.V., MATSUMOTO, T., CHEN, M.H. and BAINS, P. (1978). Clover yellow mosaic virus in cowpea mesophyll protoplasts: infection and multiplication studies. Abstracts: Fourth International Congress Virology, The Hague.

HOWELL, S.H. and HULL, R. (1978). Replication of cauliflower mosaic virus and transcription of its genome in turnip leaf protoplasts. Virology 86, 468-481.

HULL, R. (1980). Structure of the cauliflower mosaic virus genome. III Restriction endonuclease mapping of thirty-three isolates. Virology 100, 76-90.

HULL, R. and HOWELL, S.H. (1978). Structure of the cauliflower mosaic virus genome. II Variation in DNA structure and sequence between isolates. Virology 86, 482-493.

HULL, R. and SHEPHERD, R.J. (1976). The coat proteins of cauliflower mosaic virus. Virology 70, 217-220.

HULL, R. and SHEPHERD, R.J. (1977). The structure of cauliflower mosaic virus genome. Virology 79, 216-230.

HULL, R., SHEPHERD, R.J. and HARVEY, J.D. (1976). Cauliflower mosaic virus: An improved purification procedure and some properties of the virus particles. J. Gen. Virol. 31, 93-100.

HUTTINGA, H. and MOSCH, W.H.M. (1974). Properties of viruses of the potyvirus group. 2. Buoyant density, S value, particle morphology and molecular weight of the coat protein subunit of bean yellow mosaic virus and potato virus Y. Neth. J. Plant Pathol. 80, 19-27.

INCARDONA, N. and KAESBERG, P. (1964). A pH-induced structural change in bromegrass mosaic virus. Biophys. J. 4, 11-21.

ITOH, T., MATSUI, C. and HIRAI, T. (1969). Conformation changes in cauliflower mosaic virus. Virology 39, 367-372.

JACROT, B., CHAUVIN, C. and WITZ, J. (1977). Comparative neutron small-angle scattering study of small spherical RNA viruses. Nature 266, 417-421.

KAMEI, T., RUBIO-HUERTOS, M. and MATSUI, C. (1969). Thymidine-^{3}H uptake by X-bodies associated with cauliflower mosaic virus infection. Virology 37, 506-508.

KAPER, J.M. (1975). The chemical basis of virus structure, dissociation and reassembly. In Frontiers of Biology (ed. A. Neuberger and E.L. Tatum) Vol. 39. published by North Holland, Amsterdam.

KAPLAN, M. and SCHLAMOWITZ, M. (1972). Investigation of the antigenicity of the carbohydrate moiety of chicken ovalbumin. Immunochemistry 9, 737-747.

KELLY, D.C., COOPER, J. and WALKEY, D.G.A. (1974). Cauliflower mosaic virus structural proteins. Microbios 10, 239-245.

KIHO, Y., ABE, T. and OHASHI, Y. (1980).
Disassembly of tobacco mosaic virus by membrane-lipid isolated from tobacco leaves and polyornithine.
Microbiol and Immunol. 23, (In Press).

KITAJIMA, E.W., LAURITIS, J.A. and SWIFT, H. (1969).
Fine structure of zinnia leaf tissue infected with dahlia mosaic virus. Virology 39, 240-249.

KOENIG, R. (1978). ELISA in the study of homologous and heterologous reactions of plant viruses.
J. Gen. Virol. 40, 309-318.

KOENIG, R. and GIVORD, L. (1974). Serological inter-relationships in the turnip yellow mosaic virus group.
Virology 58, 119-125.

KOENIG, R., TREMAINE, J.H. and SHEPARD, J.F. (1978).
In situ degradation of the protein chain of potato virus X at the N- and C- termini.
J. Gen. Virol. 38, 329-337.

KOENIG, R., STEGEMANN, H., FRANCKSEN, H. and PAUL, H.L. (1970).
Protein subunits in the potato virus X group. Determination of the molecular weights by polyacrylamide electrophoresis. Biochim. et Biophys. Acta. 207, 184-189.

LAEMMLI, U.K. (1970).
Cleavage of structural proteins during the assembly of the head of Bacteriophage T4.
Nature 227, 680-685.

LARSON, R.H., MATTHEWS, R.E.F. and WALKER, J.C. (1950).
Relationships between certain viruses affecting the genus Brassica. Phytopathology 40, 955-962.

LAWSON, R.H. and CIVEROLO, E.L. (1978).
Formation of virus specific antibody in rabbits immunized with carnation etched ring virus inclusion bodies.
Abstracts: Third Int. Congr. Plant Path. München.

LAWSON, R.H., and HEARON, S.S. (1978).
Ultrastructure of carnation etched ring virus infected Saponaria vaccaria and Dianthus caryophyllus.
J. Ultrastruct. Res. 48, 201-215.

LAWSON, R.H., HEARON, S.S. and CIVEROLO, E.L. (1977). Carnation etched ring virus. Commonw. Mycol. Inst. Descriptions of Plant Viruses. 182.

LEBEURIER, G., WHITECHURCH, O., LESOT, A. and HIRTH, L. (1978). Physical map of DNA from a new cauliflower mosaic virus strain. Gene, 4, 213-226.

LESNAW, J.A. and REICHMANN, M.E. (1970). Determination of molecular weights of plant viral protein subunits by polyacrylamide gel electrophoresis. Virology 42, 724-731.

LODISH, H.F. and ROTHMAN, H.F. (1979). The assembly of cell membranes. Sci. American 240, 38-53.

LUNG, M.C.Y. and PIRONE, T.P. (1972). Datura stramonium, a local lesion host for certain isolates of cauliflower mosaic virus. Phytopathology 62, 1473-1474.

LUNG, M.C.Y. and PIRONE, T.P. (1973). Studies on the reason for differential transmissibility of cauliflower mosaic virus isolates by aphids. Phytopathology 63, 910-914.

LUNG, M.C.Y. and PIRONE, T.P. (1974). Acquisition factor required for aphid transmission of purified cauliflower mosaic virus. Virology 60, 260-264.

MALLUCI, L. (1976). Preparation and use of fluorescent Concanavalin A derivatives. In. "Concanavalin A as a Tool" (ed. H. Bittiger and H.P. Schnebli). John Wiley and Sons.

MARTELLI, G.P. and CASTELLANO, M.A. (1971). Light and electron microscopy of the intracellular inclusions of cauliflower mosaic virus. J. Gen. Virol. 13, 133-140.

MARTINI, C. (1957). Versuche zur Reinigung und Herstelling der Antiseren von Blumenkohlmosaik (Cauliflower mosaic virus) und Schwarzringfleckigkeit des Kohles (Cabbage black ring spot virus). Naturwissenschaften 44, 426-427.

MAYO, M.A. and COOPER, J.I. (1973).
Partial degradation of the protein of tobacco rattle virus during storage.
J. Gen. Virol. 18, 281-289.

McDONALD, J.G. and HIEBERT, E. (1975).
Characterization of the capsid and cylindrical inclusion proteins of three strains of turnip mosaic virus.
Virology 63, 295-303.

MEAGHER, R.B., SHEPHERD, R.J. and BOYER, H.W. (1977).
The structure of cauliflower mosaic virus.
1. A restriction endonuclease map of cauliflower mosaic virus DNA. Virology 80, 362-375.

MELCHERS, F. and SELA, M. (1970).
Search for antibodies against the carbohydrate portion of two mouse myeloma proteins.
Immunochemistry 7, 749-754.

MILLER, F. (1971). Glycopeptides of human immunoglobulins II. Contribution to the antigenicity of the heavy chain.
Immunochemistry 8, 99-111.

MILLER, G.L. and GOLDER, R.H. (1950). Buffers of pH 2 to 12 for use in electrophoresis.
Arch. Biochem 29, 420-423.

MILTON, R.C. de L. and VAN REGENMORTEL, M.H.V. (1979).
Immunochemical studies of tobacco mosaic virus
III. Demonstration of five antigenic regions in the protein sub-unit.
Mol. Immunol. 16, 179-184.

NELSON, D.S. and DAY, M.F. (1964). Detection of cauliflower mosaic virus by immune adherence.
Phytopathology 54, 395-398.

NIBLETT, C.L. and SEMANCIK, J.S. (1969).
Conversion of the electrophoretic forms of cowpea mosaic virus *in vivo* and *in vitro*. Virology 38, 685-693.

NICHOLSON, G.L. (1976). Concanavalin A: The tool, the techniques and the problems. In: "Concanavalin A as a Tool". (Ed. H. Bittiger and H.P. Schnebli). John Wiley and Sons.

OKUNO, T., FURUSAWA, I and HIRUKI, C. (1977). Infection of barley protoplasts with brome mosaic virus. Phytopathology 67, 610-615.

OTSUKI, Y., SHIMOMURA, T. and TAKEBE, I. (1972). Tobacco mosaic virus multiplication and expression of the N gene in necrotic responding tobacco varieties. Virology 50, 45-50.

PAPA, G., MICHELIN-LAUSAROT, P. and CASETTA, A. (1973). Purification and properties of alliaria mosaic virus. Phytopath. Z. 78, 344-345.

PARTRIDGE, J.E., SHANNON, L.M., GUMPF, D.J. and COLBAUGH, P. (1974). Glycoprotein in the capsid of plant viruses as a possible determinant of seed transmissibility. Nature 247, 391-392.

PEACOCK, A.C. and DINGMAN, C.W. (1968). Molecular weight estimation and separation of ribonucleic acid by electrophoresis in agarose-acrylamide composite gels. Biochemistry 7, 668-674.

PIAZZOLLA, P., DIAZ-RUIZ, J.R. and KAPER, J.M. (1979). Nucleic acid homologies of eighteen cucumber mosaic isolates determined by competition hybridization. J. Gen. Virol. 45, 361-369.

PIRONE, T.P., POUND, G.S. and SHEPHERD, R.J. (1960). Purification and properties of cauliflower mosaic virus. Nature 186, 656-657.

PIRONE, T.P., POUND, G.S. and SHEPHERD, R.J. (1961). Properties and serology of purified cauliflower mosaic virus. Phytopathology 51, 541-546.

PLEIJ, C.W.A., KOOT-GRONSWELD, E.A.M. and van VLOTEN-DOTING, L. (1977). A sensitive and easy local lesion assay for turnip yellow mosaic virus. J. Gen. Virol. 35, 593-595.

POLSON, A. (1971). New approaches to ultracentrifugation. In: Methods in Virology, Vol V (Eds. Maramarosch, K. and Koprowski, H.). Academic Press, New York and London.

POLSON, A. (1977). Removal of proteolytic enzymes with aldehyde treated gelatin. Prep. Biochem. 7, 257-259.

POLSON, A. and KIEFER, A. (1975).
Purification of filamentous plant viruses by thin layer centrifugation. Prep. Biochem. 5, 199-218.

POLSON, A. and RUSSEL, B. (1967).
Electrophoresis of viruses.
In: Methods in Virology (Eds. Maramorosch, K. and Koprowski, H.) Vol II pp 39-496.
Academic Press.

PURCIFULL, D.E. and BATCHELOR, D.L. (1977).
Immunodiffusion tests with sodium dodecyl sulphate (SDS) - treated plant viruses and plant viral inclusions.
Bulletin 788, Florida Agric. Exp. St. Gainesville, 39 pp.

PURCIFULL, D.E. and HIEBERT, E. (1979).
Serological distinction of watermelon mosaic virus isolates.
Phytopathology 69, 112-116.

QUETIER, F. and VEDAL, F. (1977).
Heterogeneous population of mitochondrial DNA molecules in higher plants. Nature 268, 365-368.

RAMANATHAN, L., GUYER, R.B., KARAKAWA, W., BUSS, E.G. and CLAGETT, C.O. (1979). Immunochemistry of the glycopeptide derived from avian riboflavin-binding protein. Mol. Immunol. 16, 935-942.

REIF, A.E. (1969). Batch preparation of rabbit γG globulin with DEAE cellulose.
Immunochemistry 6, 723-731.

RICE, R.H. (1974). Minor protein components in cowpea chlorotic mottle virus and satellite of tobacco necrosis virus. Virology 61, 249-255.

ROBB, S.M. (1964). Location, structure and cytochemical staining reactions of the inclusion bodies found in Dahlia variabilis infected with dahlia mosaic virus.
Virology 23, 141-144.

ROCHOW, W.F. and CARMICHAEL, E.L. (1979).
Specificity among barley yellow dwarf viruses in enzyme immunosorbent assays.
Virology 95, 415-420.

ROTHMAN, J.E. and LODISH, H.F. (1977).
Synchronized transmembrane insertion and glycosylation of a nascent membrane protein.
Nature 269, 775-780.

RUSSELL, G.J., FOLLET, E.A.C., SUBAK-SHARP, P.A. and HARRISON, B.D. The double-stranded DNA of cauliflower mosaic virus.
J. Gen. Virol. 11, 129-138.

SCHUBERT, D. and FRANK, H. (1970).
The use of 2-chloroethanol for reversible depolymerisation of the protein shell of bacteriophage Fr.
Z. Naturforsch. 25b, 711-713.

SCHULZ, E.S. (1921). A transmissible mosaic disease of chinese cabbage, mustard and turnip.
J. Agric. Res. 22, 173-177.

SEHGAL, O.P. and HSU, C.H. (1977).
Identity and location of a minor protein component in southern bean mosaic virus.
Virology 77, 1-11.

SHALLA, T.A. SHEPHERD, R.J. and PETERSEN, L.J. (1980).
Comparative cytology of nine isolates of cauliflower mosaic virus.
Virology 102, 381-388.

SHEPARD, J.F. and SECOR, G.A. (1972).
The effect of enzymatic digestion on the molecular weight and antigenic specificity of potato virus X protein.
Phytopathology 62, 1154-1160.

SHEPHERD, R.J. (1970).
Cauliflower mosaic virus.
Commonw. Mycol. Inst. Descriptions of plant viruses 24.

SHEPHERD, R.J. (1976).
DNA viruses of higher plants.
Advan. Virus Res. 20, 305-339.

SHEPHERD, R.J. (1977).
Intrinsic properties and taxonomy of aphid-borne viruses.
In: Aphids as Virus Vectors. p 121-136.
(Eds. Harris, K.F. and Maramarosch, K.)
Academic Press, New York and London.

SHEPHERD, R.J. (1979)
DNA plant viruses.
Ann. Rev. Plant Physiol. 30, 405-423.

SHEPHERD, R.J. and WAKEMAN, R.J. (1971).
Observation on the size and morphology of cauliflower mosaic acid deoxyribonucleic acid.
Phytopathology 61, 188-193.

SHEPHERD, R.J. and WAKEMAN, R.J. (1977).
Isolation and properties of the inclusion bodies of cauliflower mosaic virus.
Proc. Am. Phytopathol. Soc. 4, 145.

SHEPHERD, R.J., BRUENING, G.E. and WAKEMAN R.J. (1969).
The double-stranded nature of cauliflower mosaic virus DNA.
Phytopathology 59, 1049.

SHEPHERD, R.J., BRUENING, G.E. and WAKEMAN, R.J. (1970).
Double-stranded DNA from cauliflower mosaic virus.
Virology 41, 339-347.

SHEPHERD, R.J., RICHINS, R. and SHALLA, T.A. (1980).
Isolation and properties of the inclusion bodies of cauliflower mosaic virus.
Virology 102, 389-400.

SHEPHERD, R.J., WAKEMAN, R.J. and ROMANKO, R.R. (1968).
DNA in cauliflower mosaic virus.
Virology 36, 150-152.

STOLLAR, V., STOLLAR, B.D., KOD, R., HARRAP, K. and SCHLESINGER, R.W. (1976). Sialic acid contents of sindbis virus from vertebrate and mosquito cells. Equivalence of biological and immunological viral properties. Virology 69, 104-115.

STUDIER, W. (1979). Relationships among different strains of T7 and among T7 related bacteriophages.
Virology 95, 70-84.

TEZUKA, N. and TANIGUCHI, T. (1972a).
Stepwise degradation of cauliflower mosaic virus by Pronase.
Virology 47, 142-146.

TEZUKA, N. and TANIGUCHI, T. (1972b).
Structural protein of cauliflower mosaic virus.
Virology 48, 297-299.

TEZUKA, N., TANIGUCHI, T. and MATSUI, C. (1971).
Inhibition of Cauliflower mosaic virus multiplication by Actinomycin D.
Virology 43, 717-718.

THRESH, J.M., ADAMS, A.N., BARBARA, D.J. and CLARK, M.F. (1977). The detection of three viruses of hop (Humulus lupulus) by enzyme-linked immunosorbent assay (ELISA). Ann. Appl. Biol. 87, 57-65.

TOMLINSON, J.A. and SHEPHERD, R.J. (1978).
Studies on mutagenesis and cross-protection of cauliflower mosaic virus.
Ann. Appl. Biol. 90, 223-231.

TOMPKINS, C.M. (1937). A transmissible mosaic disease of cauliflower.
J. Agric. Res. 55, 33-46.

TOMPKINS, C.M. and THOMAS, H.R. (1938).
A mosaic disease of Chinese cabbage.
J. Agric. Res. 56, 541-551.

TREMAINE, J.H. and AGRAWAL, H.O. (1972).
Limited proteolysis of potato virus X by trypsin and plant proteases.
Virology 49, 735-744.

TUNG, J.S. and KNIGHT, C.A. (1972).
The coat protein subunits of potato virus X and white clover mosaic virus: a comparison of methods for determining their weights and some *in situ* degradation products of potato virus X protein.
Virology 49, 214-233.

VAN REGENMORTEL, M.H.V. (1964a)
Purification of plant viruses by zone electrophoresis.
Virology 23, 495-502.

VAN REGENMORTEL ,M.H.V. (1964b).
Separation of an antigenic plant protein from preparations of plant viruses. Phytopathology, 54, 282-289.

VAN REGENMORTEL, M.H.V. (1966).
Plant virus serology.
Advan. Virus. Res. 12, 207-271.

VAN REGENMORTEL, M.H.V. (1967).
Serological studies on naturally occurring strains and chemically induced mutants of tobacco mosaic virus.
Virology 31, 467-480.

VAN REGENMORTEL, M.H.V. (1968).
Zone electrophoretic R Ø values of plant viruses.
S. Afr. Med. J. 42, 118.

VAN REGENMORTEL, M.H.V. (1972).
Electrophoresis. In: Principles and Techniques in Plant Virology (Ed. C.I. Kado and H.O. Agrawal). Published by Van Nostrand Reinhold Co.

VAN REGENMORTEL, M.H.V. (1975).
Antigenic relationships between strains of tobacco mosaic virus.
Virology 64, 415-420.

VAN REGENMORTEL, M.H.V. (1978).
Applications of plant virus serology.
Ann. Rev. Phytopath. 16, 57-81.

VAN REGENMORTEL, M.H.V. and VON WECHMAR, M.B. (1970).
A reexamination of the serological relationship between tobacco mosaic virus and cucumber virus 4.
Virology 41, 330-338.

VON WECHMAR, M.B. and VAN REGENMORTEL, M.H.V. (1970).
A simple procedure for purifying tobacco mosaic virus strains.
S. Afr. Med. J. 44, 151.

VOLVOVITCH, M., DRUGEON, G., and YOT, P. (1978).
Studies on the single-stranded discontinuities of the cauliflower mosaic virus genome.
Nucleic Acid Res. 5, 2913-2925.

VOLVOVITCH, M., DUMAS, J.P., DRUGEON, G. and YOT, P. (1977).
Single-stranded interruptions in cauliflower mosaic virus DNA.
In: Acides Nucleiques et Synthese dez Proteines chez les Vegetraux. ed. by L. Bogorad and J.H. Weil. pp 635-641 Colloq. 261 CNRS Paris.

VOLVOVITCH, M., DRUGEON, G., DUMAS, J.P., HAENNI, A.C. and YOT, P. (1979).
A restriction map of cauliflower mosaic virus DNA (Strain PV 147).
Eur. J. Biochem. 100, 245-255.

VOLLER, A., BIDWELL, D.E. and BARTLETT, A. (1976).
Enzyme immunoassays in diagnostic medicine: theory and practice.
Bulletin of the World Health Organization 51, 209-211.

VOLLER, A., BARTLETT, A., BIDWELL, D.E., CLARK, M.F. and ADAMS, A.N. (1976).
The detection of viruses by enzyme-linked immunosorbent assay (ELISA).
J. Gen. Virol. 33, 165-167.

WALKER, J.C., LE BEAU, F.J. and POUND, G.S. (1945).
Viruses associated with cabbage mosiac.
J. Agric. Res. 70, 379-404.

WEBER, K., PRINGLE, J.R. and OSBORN, M. (1972).
Measurement of molecular weights by electrophoresis on SDS-acrylamide gel.
Methods in Enzymology XXVI, p 10 (Eds. L.H.W. Huis and S.N. Timasheff) Academic Press.

WEST, C.M., McMAHON, D. and MOLDAY, R.S. (1978).
Identification of glycoproteins, using lectins as probes, in plasma membranes from Dictostelium discoideum and human erythrocytes.
J. Biol. Chem. 253, 1716-1724.

YOLKEN, R.H., BARBOUR, B., WYATT, R.G., KALICA, A.R., KAPIKIAN, A.Z. and CHANOCK., R.M. (1978).
Enzyme-linked immunosorbent assay for identification of rotaviruses from different animal species.
Science 201, 259-262.