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COMPARATIVE STUDIES ON TOMATO ASPERMY AND CUCUMBER MOSAIC VIRUSES

by

Nooredin Habili B.Sc. (Jundi Shapur)

Department of Plant Pathology,
Waite Agricultural Research Institute,
The University of Adelaide,
South Australia.

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SUMMARY

Some properties of the V strain of tomato aspermy virus (TAV) and the Q strain of cucumber mosaic virus (CMV) have been compared. The size, morphology, sedimentation rate, RNA base ratio, and buoyant density of the two viruses are indistinguishable. The capsid structures of both viruses depend on RNA-protein interactions as both are dissociated in low concentrations of sodium-dodecyl sulphate (SDS) and stabilized with formaldehyde. However, the conditions required for their stability differ considerably. TAV is more resistant than CMV to degradation by pancreatic RNase and to precipitation by NaCl. Whereas TAV is stabilized by Mg^{2+}, CMV precipitates in the presence of the cation. CMV is stabilized but TAV is degraded by EDTA.

The requirement for a successful reconstitution of the viruses *in vitro* varies considerably. Mg^{2+} is required for the reconstitution of TAV and is deleterious for CMV. The reconstitution of CMV is not affected by EDTA, while TAV does not reconstitute in the presence of the chelating agent.

TAV and CMV are serologically distinct. Both viruses are poor immunogens in mice and in toads. The titre of antisera did not differ significantly when various doses of antigens were administered into mice. However, when the viral antigens were fixed with formaldehyde they were significantly more immunogenic.

Protein subunits of TAV were more soluble than CMV protein in low molarity salts. Whereas TAV coat protein was slightly immunogenic in mice, no detectable response was obtained with that of CMV. Analysis of SDS-treated viral proteins by polyacrylamide-gel electrophoresis showed that both viruses have protein subunits of molecular weight 24,500 daltons. The amino acid compositions of proteins from the two viruses, although similar, were distinguishable, and the calculated molecular weight of protein subunits were 26,100 and 26,300 daltons for TAV and CMV, respectively. The isoelectric point of TAV was about 5.7, and that of CMV, 4.7.

Molecular hybridization showed that there was no nucleotide base sequence homology between the RNA of the two viruses. TAV-RNA preparations contained species with molecular weights of T_1, 1.26×10^6, T_2, 1.10×10^6, T_3, 0.90×10^6, and T_4, 0.43×10^6, and CMV-RNA species of C_1, 1.26×10^6, C_2, 1.10×10^6, C_3, 0.77×10^6, and C_4, 0.34×10^6 daltons. These molecular weights of TAV-RNA and CMV-RNA species were estimated by polyacrylamide-gel electrophoresis in aqueous solution, but the results were not significantly different with electrophoresis in formamide. Isolated $T_1 + T_2$ or $C_1 + C_2$ had very low infectivity. However, the infectivity of $T_1 + T_2$ was greatly enhanced by the addition of either T_3 or C_3. The pseudorecombinant virus (PRV) resulting from infection by $T_1 + T_2 + C_3$ was shown to possess antigenic properties of CMV. Its behaviour to EDTA and Mg^{2+} was likewise similar to CMV. It induced host reactions indistinguishable from those of TAV. The

results suggest that C_3 contains a cistron for virus coat protein, and $T_1 + T_2$ are responsible for host reactions. RNA-RNA competition hybridization experiments showed that the PRV-RNA possesses more base sequences in common with TAV-RNA than with CMV-RNA.

Based on the behaviour of TAV and CMV in isopycnic density-gradient tubes, and comparison of RNAs extracted from the light and heavy fractions on polyacrylamide-gel electrophoresis, it was concluded that three types of particles were probably present in preparations of each virus.

The comparative studies carried out suggest that TAV and CMV are sufficiently similar to be included in the same taxonomic group. Nevertheless, the two viruses are distinct and the present nomenclature should be retained.

STATEMENT

This thesis has not previously been submitted for an academic award at this or any other University, and is the original work of the author, except where due reference is made in the text.

N. Habili

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CHAPTER 1

GENERAL INTRODUCTION

I. Cucumovirus Group

Harrison *et al.* (1971) classified plant viruses into sixteen groups. The name 'cucumovirus' was given to the sixth group, of which cucumber mosaic virus (CMV) is the type member (cryptogram R/1 : 1/18 : S/S : S/Ap). Other members include tomato aspermy virus (TAV) and peanut stunt virus. These viruses are polyhedral with a diameter of about 28 nm and a particle weight of 5.3 - 5.8 x 10^6 daltons, containing 17.7 to 18.5% RNA. The RNA is single-stranded with a molecular weight of approximately one million daltons per particle. Various publications on the properties of the type member, CMV, have appeared (Gibbs and Harrison, 1970). However, those of TAV have been confined to studies on the relationship with CMV using limited criteria such as cross-protection tests and serology. In this thesis experiments on the physical, chemical and biological properties of TAV and CMV are described in order to obtain more insight into their relatedness and to try to solve the old conflict as to whether they are strains of the same virus or are two distinct viruses.

1. Controversy on the relationship between TAV and CMV

Blencowe and Caldwell (1949) used the name 'aspermy' to describe a virus disease of tomato which caused severe mottling of leaves and

produced small fruits with reduced seeds. As the virus inhibited meiosis and stopped seed formation, they called the agent 'the virus of aspermy disease of tomato' or '*Lycopersicon* virus no. 7'. The name was modified to 'tomato aspermy virus' (TAV) by Brierley and Smith (1951). Early experiments carried out by Blencowe and Caldwell (1949) on the properties of this virus led them to conclude that although some of its properties were similar to those of CMV, TAV was not a strain of CMV. Their evidence was based on the fact that the virus did not protect the host against a challenge infection by CMV, and it could not develop symptoms on cucumber, a host that CMV infects systemically. Noordam (1952) investigated some properties of TAV such as dilution end point, longevity *in vitro*, electron microscopy, aphid transmission, serology and cross-protection and concluded that the virus was a strain of CMV and called it 'cucumis virus 1 strain chrysanthemum'. On the other hand, Brierley *et al*. (1955) concluded that differences between TAV and CMV are greater than those between CMV strains themselves. They found differences in aphid transmissibility, as TAV failed to be transmitted by *Aphis gossypii* and *Macrosiphum solanifolii*, two common vectors of CMV. They also found differences in some host reactions and that *Zinnia* plants infected with TAV were not protected against post-infection with CMV. Based on these experiments they suggested that the name of TAV should be retained.

Holmes (1956) observed a synergistic effect when tomato plants were infected simultaneously with TAV and CMV, which led him to conclude

that the two viruses were unrelated. However, Govier (1957) showed a positive cross-protection between three distinct strains of TAV and one of CMV and claimed that TAV was a strain of CMV. Graham (1957), using cross-protection tests, came to the same conclusion. The value of cross-protection as a criterion of strain relationship between TAV and CMV was questioned by Hollings (1955), who found a non-specific protection by Price's yellow strain of CMV (CMV-PY) against unrelated viruses such as potato virus Y and henbane mosaic virus. The same worker later concluded that the strain of virus, host plant, and the time of year influenced greatly the results obtained from cross-protection tests with these viruses (Hollings *et al.*, 1968).

Van Slogteren (1958) found a serological relationship between the Blencowe isolate of TAV and three CMV isolates. Grogan *et al.* (1963) found no serological relationship between CMV-Imperial 78 and some isolates of TAV in immunodiffusion tests. However, van Regenmortel (1966a) obtained a positive serological reaction between an antiserum against TAV obtained from Blencowe and four strains of CMV isolated in South Africa. Lawson (1967) studied the relationship between several isolates of a virus of chrysanthemum (CV) and TAV and CMV, using serology and host reactions. Taking into account the serological properties alone, it was concluded that TAV was a strain of CMV and distinct from CV. However, host reactions suggested that TAV and CV isolates were more closely related to each other than to CMV.

Stace-Smith and Tremaine (1973), working on an isolate of chrysanthemum virus obtained from Lawson (CV-L), concluded that its serological properties and amino acid compositions were indistinguishable from those of TAV isolated in Canada. Hollings *et al.* (1968) divided the CMV group into three distinct subgroups a) TAV isolates, b) ordinary CMV, and c) CMV-PY and related isolates. Strains in each subgroup showed very similar serological and host reactions. However, demonstrating a serological cross-reaction between the members of two subgroups required a 'broad-spectrum' antiserum.

Tomaru and Udagawa (1971) observed that phenol extracts of leaf tissues infected with TAV or CMV were more infectious than the corresponding buffer extracts. They regarded this as another property indicating that the two viruses were related. Alfalfa mosaic virus failed to behave in this way. However, Schlegel (1960) showed that two unrelated viruses, i.e. pea enation mosaic virus and tobacco necrosis virus, retained a relatively high infectivity after extracting the infected leaves with phenol. Recently Stace-Smith and Tremaine (1973) have shown that TAV and CMV share many physical and chemical properties as well as having a distinct serological relationship.

2. Stability and immunogenicity of TAV and CMV

The instability and tendency of CMV particles to aggregate led to difficulties in purification of the virus, resulting in low yields (Scott, 1963). Failures to establish a serological relationship

between TAV and CMV led Grogan *et al.* (1963) to conclude that high titred antisera were required to prove such a claim. However, serological work on these viruses indicated that the viruses were poorly immunogenic (Francki *et al.*, 1966; Scott, 1968; Tomlinson *et al.*, 1973). Although it has been claimed by Hollings and Stone (1971) that TAV is a good immunogen, Stace-Smith and Tremaine (1973) argued against it. Francki *et al.* (1966) observed that CMV precipitated in 0.14 M NaCl and the antisera produced in rabbits had a titre of only 1/2 in immunodiffusion tests. Using tobacco ringspot virus and the same method of immunization a titre of 1/64 was readily obtained (Randles and Francki, 1965). Scott (1968) also concluded that the Y strain of CMV was a poor immunogen and that the virus dissociated in high ionic strength salts.

The conditions under which serological tests are carried out affect the antigenicity of the virus (Francki *et al.*, 1966; Tomlinson *et al.*, 1973). A serum titre of 1/32 was obtained in immunodiffusion tests when CMV-W was reacted in salt-free buffers. However, its titre decreased to 1/4 in the presence of 0.14 M NaCl (Tomlinson *et al.*, 1973).

Plant viruses capable of inducing antisera of high titres (Agrawal and Maat, 1964) probably have their capsids stabilized in a different way from those viruses with poor immunogenicity (Stace-Smith and Tremaine, 1973). It has been pointed out by Kaper (1972) that

in viruses with properties like those of turnip yellow mosaic virus, hydrophobic interactions between the adjacent protein subunits render the viral capsids very stable, even in high molarity salt solutions. However, in the case of CMV, the main contribution to the stability of the capsids is electrostatic interaction between protein and RNA. These interactions depend on the ionic strength of the medium, and are weakened by high salt concentrations. With CMV-like antigens the body of the animal provides an environment in which the weakening of these interactions, and hence degradation of the capsids, will take place. To minimize the deleterious effect of salt and high temperature on unstable viruses injected into animals for the production of antibodies one of the following two treatments or a combination of them could be applied:

a) Treatment of viral capsids with low concentrations of formaldehyde will make the virus structure less dependent on protein-RNA interactions. Formaldehyde reacts with the amino groups present on the surface of protein subunits (Kabat and Mayer, 1961) and provides linkage between them. Formaldehyde treatment has improved the immunogenicity of some plant viruses (Hollings and Stone, 1962). However, Scott (1968) failed to achieve such an effect on the Y strain of CMV.

b) Cold-blooded animals proved to be useful for obtaining relatively high-titred antisera against some unstable viruses (Langenberg and Middleton, 1969; Tolin *et al.*, 1972). Incubating the immunized animals

at a temperature lower than that of mammals (ca. 28°) probably prolonged the longevity of the antigens in the animal tissue resulting in a more efficient immune response.

3. Viral genomes

A large number of plant viruses have been found to have their functional genomes divided into two or more physically different capsids (for recent reviews see: Van Kammen, 1972; Brown and Hull, 1973). In the case of cucumoviruses no apparent physical heterogeneity of particles has been observed; purified preparations of these viruses sediment as a single homogeneous boundary. CMV has been classified together with the bromovirus group by Kaper *et al.* (1965) as 'one million RNA class' viruses, based on the assumption that a single RNA molecule with a molecular weight about 1.0 x 10^6 daltons contains all the information required for infectivity and maturation of the virus. Although the physical heterogeneity of CMV-RNA was observed by Diener *et al.* (1964), it was considered that the slower sedimenting RNA molecules were most probably degradation products of the larger molecule resulting from a single cleavage of the nucleotide chain by contaminating nucleases (Kaper *et al.*, 1965). Similar results were obtained with brome mosaic virus (BMV) by Bockstahler and Kaesberg (1965). However, more recently Lane and Kaesberg (1971) have provided evidence that BMV-RNA has a functionally divided genome. The purified

preparations of BMV contain three types of nucleoprotein particles which can be resolved by isopycnic density gradient centrifugation. It was also shown that preparations of BMV contain four species of RNA which are called RNA 1 to 4 in order of decreasing molecular weight. RNA 3 and 4 are enclosed in the same capsid, and the larger pieces, each in a separate capsid. They also found that the three largest RNAs were required for infectivity. It was shown that the nucleotide base sequence of RNA 4 occurs in RNA 3 (Shih *et al.*, 1972), and that both RNA species are capable of programming the synthesis of coat protein in a cell free protein synthesizing system derived from wheat embryo (Shih and Kaesberg, 1973). Nevertheless, RNA 4 is not required for infectivity (Lane and Kaesberg, 1971).

Similar studies have been carried out on CMV. Kaper and West (1972) showed by electrophoresis in polyacrylamide-gels (PAGs) that in preparations of CMV-RNA at least four major RNA species were present. They suggested the presence of three types of particles in purified preparations of CMV, similar to those observed with BMV. Peden and Symons (1973) showed that the three largest RNA species of CMV were needed for infectivity in the same way as with BMV-RNA. Schwinghamer and Symons (1974), using the technique of Shih and Kaesberg (1973) showed that CMV species 3 and 4 can function as messengers for the synthesis of coat protein. Lot *et al.* (1974) worked on the genome of some strains of CMV and confirmed that the three largest RNA species

were needed for infectivity. They also included in their work one of the two TAV strains described by Hollings *et al.* (1968). They called this virus a strain of CMV without providing any evidence, and referred to it as CMV-TAV-898. However, this strain was grouped separately from other CMV strains by Hollings *et al.* (1968). Although Lot *et al.* (1974) noted some physical differences between CMV-TAV-898 and the CMV isolates they used, these differences were attributed to the estimates being made in different laboratories.

II. Viruses Similar to the Cucumovirus Group

1. Bromovirus group

Members of the bromovirus group, i.e. brome mosaic virus (BMV: type member, cryptogram R/1 : 1/22 : S/S : S/(Ne)), cowpea chlorotic mottle virus (CCMV) and broad bean mottle virus (BBMV) share many properties with CMV (van Kammen, 1972; Schwinghamer and Symons, 1974). The similarity between the genomes of CMV and BMV has been discussed above. The three largest RNAs are required for infectivity of CCMV (Bancroft and Flack, 1972). The members of this group vary in their host specificity. For example, BMV is confined mainly to Gramineae, whereas CCMV is confined to Leguminosae. Nevertheless, a serological relationship has been found between the two viruses (Scott and Slack, 1971).

The bromoviruses are smaller in size than CMV with an average diameter of 25.5 nm. Their sedimentation rates vary between 78 to 87 S, depending on the pH of the suspending buffer, and the virus. The size of the protein subunit is also smaller with a molecular weight of 20,000 daltons (Agrawal and Tremaine, 1972). Although the cucumoviruses are transmitted by aphids, the method of transmission of the bromoviruses is not yet well understood. Based on the stability of particles in preparations, Kaper (1972) has classified the bromoviruses with CMV. However, unlike CMV, protein shells of BMV can be formed from subunits below pH 6.0 (Bancroft, 1970). Similar structures have not been formed with protein subunits of CMV at any pH tested (Kaper and Geelen, 1971). Studying the properties of cucumoviruses, Stace-Smith and Tremaine (1973) supported the proposal by Kaper (1968) that CMV should be included in the bromovirus group. However, referring to unpublished data, they observed that the amino acid compositions of coat protein of cucumo-viruses differed from those of bromoviruses. At present these groups are considered to be distinct (Harrison *et al.*, 1971).

2. Alfalfa mosaic virus

(cryptogram R/1 ; 1.3 + 1.1 + 0.9/18 ; U/U ; S/Ap)

Although alfalfa mosaic virus (AMV) is structurally different from the cucumo- and bromoviruses, they have some properties in common (Hull, 1969; Jaspars and van Kammen, 1972). AMV, like TAV and CMV,

is transmitted by aphids in a non-persistent manner and induces some host reactions indistinguishable from those produced by CMV (Gibbs and Harrison, 1970; Bos and Jaspars, 1971). Like CMV, AMV is precipitated with low concentrations of Mg^{2+}, dissociated in high ionic strength salts, and degraded with ribonuclease (RNase) (Hull, 1969; Bol and Veldstra, 1969). AMV, bromoviruses, and TAV are digested with trypsin (Hull, 1969; Stace-Smith and Tremaine, 1973), but CMV is not (Bol *et al.*, 1974). The viruses of these groups whose serological properties are adequately studied are poorly to moderately immunogenic and are associated with soluble antigens (van Regenmortel, 1966a; Hull, 1969). The protein subunit of AMV has a molecular weight of 24,500 daltons (Kruseman *et al.*, 1971) which is similar to the values of 24,200 and 25,200 obtained for CMV protein by Hill and Shepherd (1972) and van Regenmortel *et al.* (1972), respectively. AMV-RNA preparations like those of CMV and BMV resolve into four distinct species by electrophoresis in PAGs. However, unlike the bromoviruses and cucumoviruses, each RNA species of AMV is enclosed in a separate capsid, forming four physically heterogeneous nucleoprotein particles, the three largest being required for infectivity. Bol *et al.* (1971) demonstrated that protein subunits have a biological role in initiating infection. It was observed that the three largest RNA species are not infective unless either coat protein or the smallest RNA is added. Lot *et al.* (1974) failed to detect a similar reaction between CMV-protein and its RNA.

CHAPTER 2

GENERAL MATERIALS AND METHODS

I. Materials

1. Virus isolates

A strain of TAV originally isolated in Victoria, Australia, from chrysanthemum plants (strain V) and the Q strain of CMV (Francki *et al.*, 1966) were used throughout the course of these investigations. The U_1 strain of tobacco mosaic virus (TMV) (Siegel and Wildman, 1954) was also used in some experiments. A purified preparation of tobacco ringspot virus (TRSV) was obtained from Dr. R.I.B. Francki.

2. Chemicals

The name and source of some of the chemicals and biochemicals used are listed in Table 2-1. Other chemicals were of laboratory or analytical reagent grade. *Escherichia coli* ribosomal RNA (rRNA) was kindly provided by Dr. R.H. Symons. Antiserum prepared against synthetic polyinosinic : polycytidylic double-stranded polynucleotide (poly (I) : poly (C)) (Francki and Jackson, 1972) in rabbits was provided by Dr. R.I.B. Francki. A purified preparation of TRSV-RNA_1 (Rezaian and Francki, 1974) was a gift from Dr. Francki.

Table 2-1. The commercial sources of some chemicals and biochemicals used in the text.

Chemical	Source
Acrylamide	BDH Chemicals, England
Adjuvant, Freund's complete	Becton, Dickinson & Co., U.S.A.
Ampholine, pH 3-10, 40% (w/v)	LKB, Sweden
^{14}C bicarbonate (sodium salt)	The Radiochemical Centre, Amersham
Bovine serum albumin	Difco Laboratories, U.S.A.
Cytochrome *c* (type IV)	Sigma Chemical Co., U.S.A.
Dithioerythritol (Cleland reagent)	Sigma Chemical Co., U.S.A.
Dowex 50W-X8 (20-50 mesh), hydrogen form	BHD Chemicals, England
Formaldehyde, 40% (w/v)	May & Baker Ltd, England
Formamide	BDH Chemicals, England
N,N' Methylene-bis-acrylamide	Fluka, Buchs
Ovalbumin	Sigma Chemical Co., U.S.A.
Pepsin	Sigma Chemical Co., U.S.A.
Polyethylene glycol (PEG 6000)	Union Carbide, U.S.A.
Pronase (type VI, fungal protease)	Sigma Chemical Co., U.S.A.
RNase A (pancreatic)	Sigma Chemical Co., U.S.A.
RNase T_1	Sigma Chemical Co., U.S.A.
Spermidine trihydrochloride	Calbiochem, U.S.A.
N,N,N',N"-tetramethyl-ethylenediamine (TEMED)	Sigma Chemical Co., U.S.A.
Triton X100	Shell Chemical Co.
Trypsin	Sigma Chemical Co., U.S.A.

3. Instruments

High-speed centrifugations were carried out in either Beckman Model L or Model L2-65 ultracentrifuges. Isopycnic centrifugations were also done in a Beckman Model E analytical ultracentrifuge equipped with an AnF rotor. MSE Magnum with a swing-out rotor, MSE-18, Sorvall RC2-B and RC3 centrifuges were used for low speed centrifugations. Ultraviolet absorption spectra were measured with a Unicam SP1800 spectrophotometer. Sucrose density-gradients in centrifuge tubes were analysed at 254 nm with an ISCO u.v. analyser coupled to an external recorder. The PAG electrophoresis columns were scanned with a Joyce-Loebl Chromoscan.

II. Methods

Some of the methods commonly employed in the succeeding Chapters are described below:

1. Plants, inoculations and growth conditions

All plants were raised in an insect-proof glasshouse with natural illumination and an average temperature of 25°. Plants were dusted lightly with 400 mesh carborundum and inoculated mechanically with virus or viral RNA suspensions using the forefinger covered in a rubber fingerstall. The excess inoculum was washed off in a jet of water. Unless otherwise stated, the plants were transferred and

maintained in a growth chamber at 22° with a photoperiod of 12 h at 14,000 lux.

2. Infectivity assay

Purified preparations of TAV and CMV were assayed on opposite half-leaves of *Vigna sinensis* (L.) Endl. cv. Blackeye, or on *Chenopodium amaranticolor* Coste & Reyn. The latter host was found to be more sensitive to both viruses.

3. Virus purification

The plants were harvested two weeks after inoculation with purified preparations of TAV and CMV (100 μg/ml). In early experiments the purification method of Scott (1963) was employed. The leaf tissue was homogenized in citrate buffer (0.5 M sodium citrate, 0.1% thioglycollic acid, pH 6.5) and chloroform in a ratio of 1 g : 1 ml : 1 ml. The slurry was centrifuged at 3,000 *g* for 10 min. The supernatant was passed through a Whatman No. 4 filter paper, and dialysed against 5 mM borate buffer, pH 9.0, for 20 h. The preparation was then subjected to three cycles of high-speed (220,000 *g* for 60 min) and low-speed (5,000 *g* for 10 min) centrifugations. It was further purified by sucrose density-gradient centrifugation using a SW 25.1 rotor at 25,000 rpm for 3 h. All the purification steps were carried out at 0° to 4°. The procedure was laborious and low virus yields were obtained (10-50 mg/Kg leaf material). Therefore, the purification

method used by Lot *et al.* (1972) was employed in later experiments. The leaves were homogenized and the supernatant was recovered as described above, except that the concentration of thioglycollic acid was increased to 0.5%, and in the case of CMV 5 mM EDTA was added to the extracting buffer. Polyethylene glycol (PEG) 6000 (40%) was added to the homogenate to a final concentration of 10% and stirred for 45 min. The virus was sedimentated at 6,000 *g* for 20 min. The pellet was resuspended in 2% Triton X100 by stirring for 30 min (50-60 ml per 100 g leaf material). The suspension was clarified by centrifugation at 15,000 *g* for 20 min, and then given three cycles of differential centrifugation as mentioned before. CMV was resuspended in 5 mM borate buffer with 1 mM EDTA (pH 9.0) and TAV in 20 mM sodium-potassium phosphate buffer (pH 7.6) unless otherwise stated. The yield of TAV was usually about 2.0 g and of CMV 300 mg per Kg of fresh plant material. Purified preparations of CMV from cucumber cotyledons did not react with antiserum produced against healthy plant antigens prepared as described in Section 8 of this Chapter.

TMV was purified from *N. tabacum* L. using the method described by Francki and McLean (1968). The leaf tissue was homogenized in 1.5 volumes of 0.2 M Na_2HPO_4 and strained through cheesecloth. Decolorizing charcoal (0.05 g/ml) was added, shaken and filtered through a pad of celite. DEAE-cellulose (0.01 g/ml) was added to the filtrate and the filtration repeated. The preparations were subjected to three cycles of

high- and low-speed centrifugations. The final pellet was resuspended in distilled water.

4. Storage of purified preparations of TAV and CMV

Purified preparations of TAV in 20 mM phosphate buffer, pH 7.6, became contaminated with fungi and bacteria within the first week of storage, at 4°, resulting in the formation of a white precipitate. To eliminate the growth of organisms, the preparations were stored in 50% glycerol at -15° (Stace-Smith and Tremaine, 1973) when they were to be used for chemical and serological studies. However, prolonged storage (over 6 months) resulted in the loss of infectivity, and was avoided. Preparations of CMV in 5 mM borate buffer, 1 mM EDTA, pH 9.0, were stored at 4° without apparent growth of the organisms or loss of infectivity.

5. Treatment of TAV and CMV with formaldehyde

Formaldehyde was added to freshly purified TAV and CMV to a concentration of 0.2% and the mixtures were dialysed against 0.2% formaldehyde in 20 mM phosphate buffer, pH 7.6, for TAV and in 5 mM borate buffer, pH 9.0, for CMV. After 24 h incubation at 4°, the excess formaldehyde was removed by dialysis against formaldehyde-free buffers. Untreated virus preparations used as controls were subjected to similar dialysis steps against buffers without formaldehyde.

6. Rate-zonal density-gradient centrifugation

Samples containing 30-50 μg of virus were layered on 5-25% (w/v) linear sucrose density-gradients containing 20 mM phosphate buffer, pH 7.5, and centrifuged for 35 min at 50,000 rpm at 4° in the Beckman SW 50.1 rotor.

7. Electron microscopy

Copper grids coated with carbon-colloidon were placed on a drop of purified virus preparation in water (0.2 - 1.0 mg/ml). The excess fluid was removed with filter paper. The grids were stained with 2% uranyl acetate for 1-2 min and examined in a Siemens Elmiskop I electron microscope.

8. Serological techniques

Double immunodiffusion tests were employed in all experiments so that: (1) small volumes of serum could be used; (2) the non-specific precipitation of CMV in precipitin tube tests could be avoided (Francki *et al.*, 1966); (3) the same serum sample could be concurrently titrated against more than one antigen. The tests were carried out in glass petri dishes 10 cm in diameter containing 12 ml of 0.75% agar in 10 mM phosphate buffer, pH 7.6, with 0.02% sodium azide added as preservative. Holes, 3 mm in diameter and 3.5 mm apart, were cut, and each was charged with 10 μl of serum or antigen. The plates were observed for precipitin

lines after 4 to 7 days' incubation at 25° in a moist atmosphere.

An antiserum to healthy cucumber antigens was produced in one rabbit. The antigens were obtained by the clarification method described for virus infected tissues except that the pellet from a single high-speed centrifugation of 1.5 h was used as the antigen after suspension in adjuvant. Antisera to viral antigens were prepared as described in Chapter 6.

9. Isolation of viral RNA

For most experiments the RNA was isolated from TAV and CMV using the procedure described by Peden and Symons (1973). Virus preparations were emulsified in a mixture of 78% phenol containing 0.1% 8-hydroxyquinoline, and a buffer consisting of 0.6% SDS, 0.6 M sodium acetate and 20 mM EDTA (1 : 1 : 1). The suspension was shaken vigorously for 10 min and the aqueous phase was recovered after centrifugation at 5,000 *g* for 5 min. A volume of phenol equal to that originally used was added and the extraction repeated. The traces of phenol were removed by washing the aqueous phase with 2½ volumes of ether. The excess ether was evaporated under vacuum. The RNA was precipitated with two volumes of re-distilled ethanol and stored at -15° overnight.

To obtain maximum RNA recovery from dilute virus preparations and from preparations treated with formaldehyde, the Pronase extraction

method described by Murant *et al.* (1972) was used. Virus preparations were mixed with an equal volume of SSC buffer (0.15 M NaCl, 15 mM sodium citrate) containing 0.2% Pronase and 1% SDS, which was pre-incubated at 37° for 30 min to eliminate nuclease contamination. The mixture was incubated at 37° overnight and the RNA was precipitated as described above. The precipitated RNA was sedimented by centrifugation at 5,000 *g* for 10 min and dissolved in TNE buffer (0.1 M NaCl, 20 mM Tris HCl, 1 mM EDTA, pH 8.5) containing 10% sucrose and stored at -15° for PAG electrophoresis.

TMV-RNA was isolated using the phenol extraction method, except that 3 to 4 cycles of extraction were carried out. All the glassware and solutions coming in contact with RNA were washed with 0.1 N NaOH or autoclaved as a precaution against contaminating RNase.

10. PAG electrophoresis of viral RNA

a. Recrystallization of acrylamide and bis-acrylamide

Acrylamide and bis-acrylamide were recrystallized by the method of Loening (1967). Acrylamide was dissolved in hot chloroform (50°), at a concentration of 7% (w/v), and filtered. Crystals were obtained after storing at -15° and were separated by filtration. Bis-acrylamide was dissolved in hot acetone (45°), at a concentration of 1% (w/v), and crystals were obtained as described for acrylamide. The reagents were stored at -15° until required.

b. PAG electrophoresis in aqueous solution

The RNA preparations were analysed in 2.6% PAGs using the technique described by Loening (1967). One hundred ml of stock electrophoresis buffer (pH 7.5) was prepared by mixing 40 ml of 1 M Tris, 10 ml of 2 M sodium acetate, 10 ml of 0.2 M EDTA, 2 ml glacial acetic acid and 38 ml water. A solution of acrylamide monomer containing 20% acrylamide and 1% bis-acrylamide in water was stored in the dark at 4°. To make up 30 ml of 2.6% acrylamide, sufficient for 12 tubes (9.0 x 0.6 cm), 3.9 ml of the acrylamide solution, 3.0 ml of the stock buffer, and 22.8 ml of deionized water were mixed and degassed under vacuum. Freshly prepared ammonium persulfate solution (10% w/v, 0.25 ml) and TEMED (0.025 ml) were added, mixed, and the mixture poured into the tubes to a depth of 7.5 cm. The tubes had been closed at the bottom with dialysis membrane. Water was layered on the gels to obtain a flat surface. The tubes were transferred to a 4° room for at least 1 h where acrylamide was allowed to polymerize. The stock buffer was diluted one in ten and each vessel of the electrophoresis apparatus (Davis, 1964) was filled with 200 ml of the buffer. The dialysis membrane was pierced and gels were pre-run at 80 volts (5 mA/tube) for 1 h, before layering the RNA samples (10-20 µg/gel). Bromophenol blue (0.2%, 5 µl) was added to one tube to serve as a tracking dye. The electrophoresis was continued at 4° for 3-4 h. The gels were then stained for 20 min with 0.05% toluidine blue 0 in 50 mM sodium acetate, 5 mM acetic acid, and 0.1 mM EDTA, pH 5.5 (Peden and Symons, 1973).

They were destained in water and scanned at 620 nm.

c. PAG electrophoresis in formamide

A modification of the procedure of Staynov *et al.* (1972) as described by Duesberg and Vogt (1973) was used. 'Dowex' resin was washed, successively, in 1 N NaOH (200 g/l), H_2O, 1 N HCl, and H_2O and dried thoroughly before mixing with the formamide. Formamide was deionized by stirring in a 3% suspension of the resin at room temperature for 1 h and was filtered through filter paper. Phosphate-buffered formamide (pH 7.0) was prepared for electrophoresis by dissolving 142 mg of Na_2HPO_4 and 156 mg of $NaH_2PO_4.2H_2O$ in 100 ml of deionized formamide. The acrylamide and bis-acrylamide monomers in the ratio used for the electrophoresis in aqueous solutions were dissolved in the buffered formamide. The gels (3.5%) were prepared in the same way and the tubes (12 x 0.6 cm) were filled to a depth of 7 cm. Polymerization of the gels was at room temperature. RNA samples (0.5 mg/ml) in the formamide containing 20 mM NaCl and 10% sucrose were incubated at 37° for 15 min (Peden and Symons, 1973) and layered on the gels (10-20 µg/gel) under a 5 cm column of the buffered formamide. The electrode buffer was 40 mM sodium phosphate, pH 7.0. Electrophoresis was carried out at room temperature and the bands of various RNA species were detected by staining as described above.

11. Spectrophotometry

Virus and RNA concentrations were determined from their absorbance at 260 nm using $E_{260}^{0.1\%}$ = 5.0 for CMV and TAV (Francki *et al.*, 1966; Stace-Smith and Tremaine, 1973), 3.3 for TMV, and 25.0 for RNA.

CHAPTER 3

PHYSICAL AND CHEMICAL PROPERTIES

Introduction

Although the physical and chemical properties of CMV have been investigated in some detail (Kaper *et al.*, 1965; Francki *et al.*, 1966; van Regenmortel, 1967; van Regenmortel *et al.*, 1972; Kaper and West, 1972), those of TAV are not well known (Hollings and Stone, 1971; Stace-Smith and Tremaine, 1973). This Chapter deals with a comparative study on the physical and chemical properties of the two viruses.

Methods

1. Isolation of viral RNA

For base ratio analyses the RNA was isolated by the LiCl precipitation method (Francki *et al.*, 1966). One volume of 4 M LiCl in 20 mM Tris.HCl, pH 7.2, was added to the virus preparation and frozen at -15° for at least 3 h. The mixture was then thawed slowly and the RNA was collected by centrifugation at 3,000 *g* for 10 min. For other studies, the RNA was isolated as described in Chapter 2.

2. Isolation of viral protein

Protein was recovered from the supernatant after separation of RNA with 2 M LiCl (see above). The solution was dialysed exhaustively at 4° against 10 mM Tris.HCl (pH 7.2) containing 1 mM Cleland reagent. The protein precipitated and was recovered by centrifugation at 3,000 *g* for 10 min. Then it was lyophilised and stored desiccated at 4° until required.

3. Determination of protein solubility

Aliquots of 0.1 ml (0.15 mg) of isolated protein in 2 M LiCl were added to 0.9 ml of various concentrations of LiCl and the solubility was measured in two ways: (a) the samples were incubated at room temperature for 10 min and the amount of light-scattering was measured at 300 nm; (b) 1 ml of each sample (0.15 mg protein per ml) was dialysed against various concentrations of LiCl at 4° for 20 h, centrifuged at low speed and the optical density of the supernatant was measured at 280 nm.

4. Rate-zonal density-gradient centrifugation of viral RNA

RNA preparations suspended in TNE buffer (20 mM Tris.HCl, 0.1 M NaCl, 1 mM EDTA, pH 8.5) were subjected to centrifugation for 4.5 h in linear density-gradients containing 10-40% (w/v) sucrose in 20 mM Tris.HCl, pH 7.2, at 4°, as described in Chapter 2. Before layering the RNA samples on the gradients, 0.1 ml of 2% (w/v) EDTA-

treated bentonite (Fraenkel-Conrat *et al.*, 1961) was layered on each gradient to serve as RNase inhibitor.

5. Isopycnic density-gradient centrifugation

The viruses were fixed with 0.2% formaldehyde as described in Chapter 2, and mixed with an equal volume of CsCl of density 1.72 g/cm^3, in 5 mM sodium borate buffer, pH 9.0. The mixtures were centrifuged at 38,000 rpm at 4°, for 20-40 h in the Beckman SW 50 rotor, or at 35,600 rpm in a Beckman Model E ultracentrifuge for 17 h, using the AnF rotor. After centrifugation in the preparative rotor, fractions were collected from the bottom of the tube. The density of each fraction was obtained gravimetrically as described by Szybalski (1968). A 0.1 ml-micropipette was first filled with water, weighed and dried with acetone under vacuum. It was then refilled with the various fractions and weighed at 25°. The densities were calculated using the following equation:

$$\rho_o = \frac{\text{net weight of each fraction}}{\text{net weight of water}} \times 0.99704$$

The fractions were diluted before measuring optical densities at 260 nm.

6. RNA base ratio analyses

RNA samples were hydrolysed in 1 N HCl at 100° for 1 h, the products were separated by paper chromatography in isopropanol-HCl and the base ratios determined as described by Markham (1955).

7. Amino acid analyses

Each sample of lyophilized viral protein (1-2 mg) was dissolved in 1 ml of 6 N HCl with a drop of 5% aqueous phenol added to prevent extensive degradation of tyrosine (DeLange *et al.*, 1969). The mixture was hydrolysed at 110° for 24 and 72 h. The resulting amino acids were evaporated to dryness, resuspended in 12.5% sucrose in 0.1 N HCl and applied to a Beckman 120C Amino Acid Analyser. Tryptophan was determined spectrophotometrically as described by Spies and Chambers (1949).

8. PAG electrophoresis of viral proteins

Protein analyses were carried out in 10% PAGs in SDS-phosphate as described by Weber and Osborn (1969). To make up 30 ml of gel solution, 10 ml of stock acrylamide solution (30 g acrylamide, 0.8 g bis-acrylamide, and water to 100 ml), 3 ml of stock buffer (1 M sodium phosphate, pH 7.2, containing 1% SDS) and 17 ml of water were mixed. The mixture was degassed for 1 min, and 0.15 ml freshly dissolved ammonium persulphate (10%) and 0.015 ml of TEMED were added. It was

then swirled and poured into tubes (10 x 0.6 cm) to a depth of 8 cm. The gels were allowed to polymerize at room temperature. The stock buffer was diluted ten times, 2-mercaptoethanol was added to 0.1% and used as the electrophoresis buffer. The protein samples were electrophoresed for 10 h at 8 mA/gel (60-70 volts). The gels were stained overnight in a mixture containing 0.2% Coomassie Brilliant Blue R 250, 50% methanol and 7% acetic acid at 37°, followed by destaining in 7% acetic acid (Maizel, 1971).

Both viral and marker proteins were prepared as described by Agrawal and Tremaine (1972). The viral and marker proteins (1 mg/ml) in a mixture of 4 M urea, 1% SDS, 1% 2-mercaptoethanol, 0.1 M sodium phosphate buffer, pH 7.2, were heated at 100° for 1 min. Glycerol was then added to 50% and kept frozen at -15°. Each time before application to the gel, the samples were heated as described above. The protein markers used for the estimation of molecular weights of TAV and CMV protein subunits were: bovine serum albumin (mol. wt. 68,000), ovalbumin (mol. wt. 43,000), pepsin (mol. wt. 35,500), and trypsin (mol. wt. 23,300). The viral proteins were coelectrophoresed with two to three markers in each run. Cytochrome *c* (mol. wt. 12,400) was present in all tubes to calculate the relative mobility of the other proteins.

9. Determination of isoelectric point

In early experiments the isoelectric points of TAV and CMV

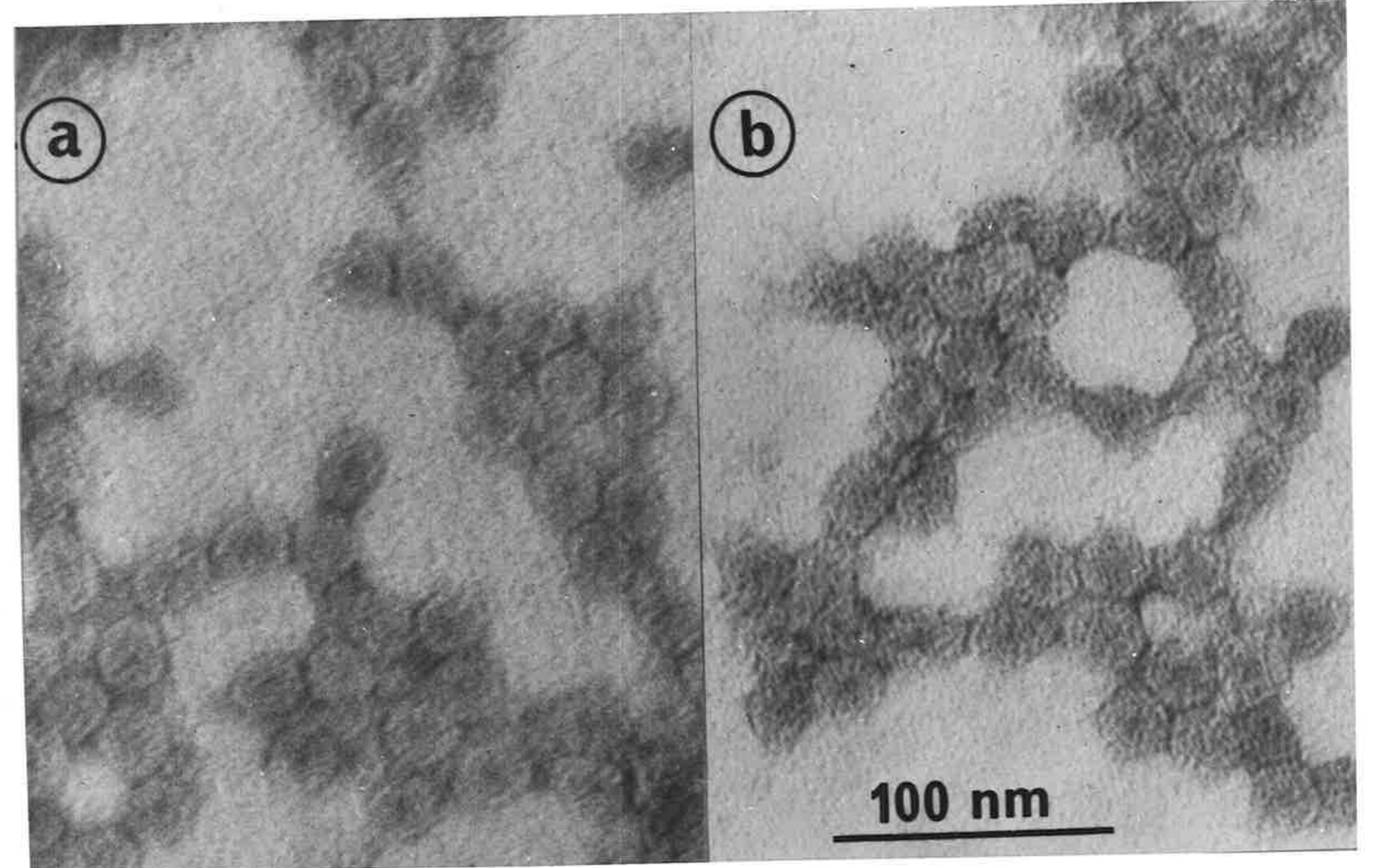
preparations were determined by a pH-titration method. The viruses at 100 µg/ml were incubated at 4° for 20 h in 20 mM sodium acetate buffer (pH 3.8-6.8), 20 mM sodium phosphate buffer (pH 6.5-8.5) or 5 mM sodium borate buffer (pH 8.0-9.5). The samples were centrifuged at 3,000 *g* for 15 min, and the solubility of the virus was detected by measuring the optical density of the supernatant at 260 nm. In later experiments the isoelectric point was determined by the electrofocusing technique in a LKB 110 ml-column. A pH-gradient was established using 1% carrier ampholine (pH 3-10) stabilized by sucrose density-gradients as described in the LKB manual. The dense solution was 50 ml of 47% (w/v) sucrose in distilled water containing 2/3 of the carrier ampholine. The light solution was 50 ml distilled water containing the remaining (1/3) ampholine and 2 to 5 mg of each virus. The sucrose gradients were made using the gradient-making device supplied with the column. The anodic solution was 24 ml 50% (w/v) sucrose acidified with 0.2 ml phosphoric acid and the cathodic solution was 10 ml of 1% NaOH. The pH gradient was allowed to stabilize at 400 volts and 4° for 48 h. Two-ml fractions were collected for pH and optical density measurements.

Results

I. Morphology of Virus Particles

Electron micrographs of TAV and CMV stained with uranyl acetate revealed that the particles were similar (Fig. 3-1). The mean diameters

Fig. 3-1. Electron micrographs of TAV (a) and CMV (b) stained in 2% uranyl acetate.


a
b
100 nm

of the particles based on samples of 100 particles, were 29.0 ± 0.28 nm for TAV, and 28.8 ± 0.22 nm for CMV. When the two viruses were mixed and examined in the electron microscope, particles could not be distinguished into two different populations and their mean diameter was 28.4 ± 0.20 nm.

II. Sedimentation Properties of the Viruses

The sedimentation rates in sucrose density-gradients of TAV and CMV were indistinguishable and, when the two viruses were mixed, a single homogeneous peak was observed.

III. Properties of Viral RNA

The molar base ratios of TAV-RNA and CMV-RNA were similar (Table 3-1) and could not be distinguished by the techniques used. The values were also indistinguishable from those obtained by other workers (Table 3-1).

When TAV-RNA and CMV-RNA preparations were centrifuged in sucrose density-gradients, most of the material sedimented as a peak with an approximate sedimentation coefficient of 22 S (peak b, Fig. 3-2). However, in the sedimentation profile of CMV a minor distinct peak (20 S) was observed which corresponded to the shoulder of peak b of TAV (Fig. 3-2).

Because of the relatively poor resolution of the RNA species of

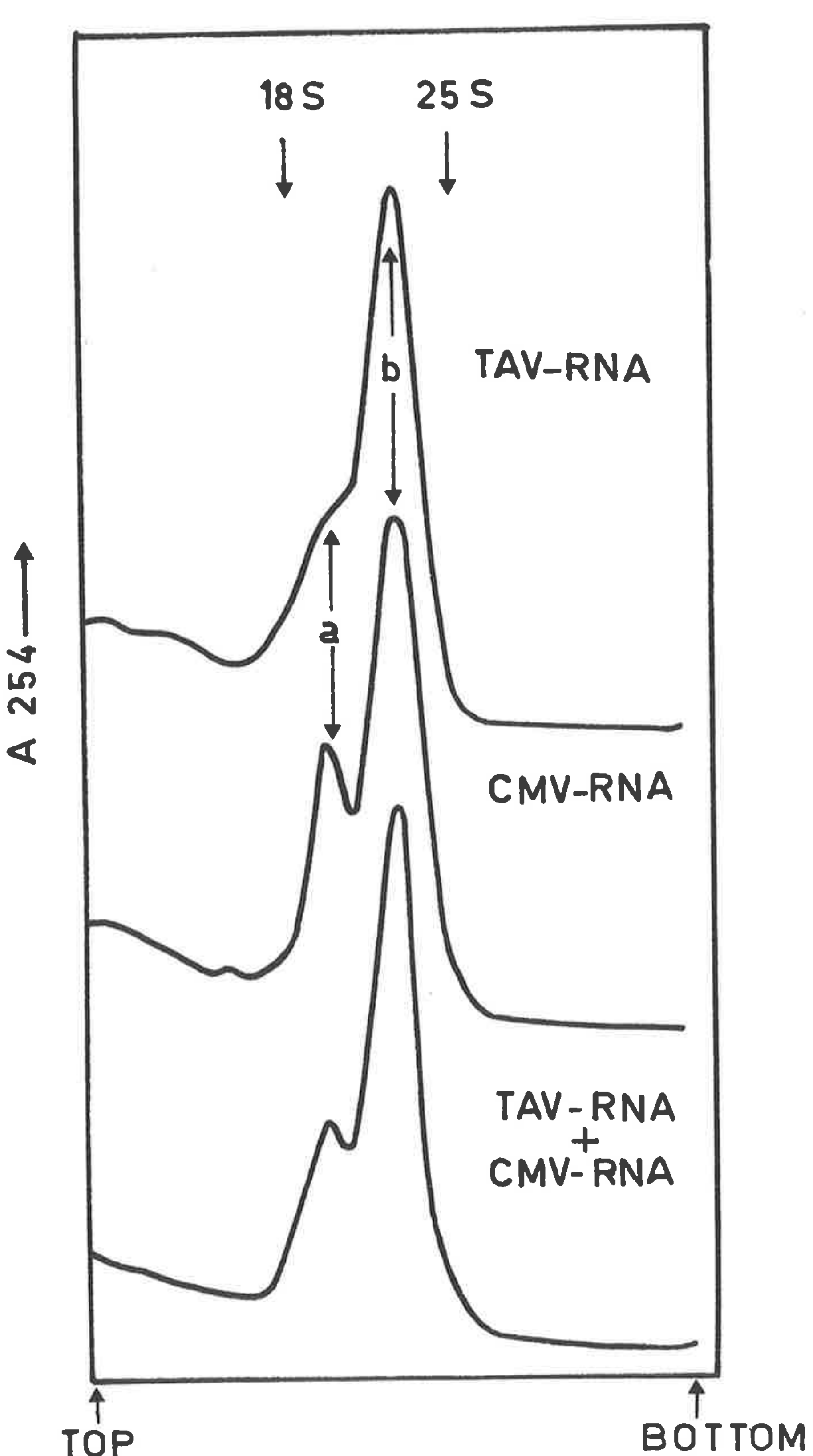
Table 3-1. Molar base ratios of TAV and CMV

Virus	Reference	Mole %			
		G	A	C	U
TAV (Canadian)	Stace-Smith and Tremaine (1972)	23.7	26.4	21.2	28.7
TAV (Canadian)	Stace-Smith and Tremaine (1973)	23.1	26.2	21.6	29.0
TAV (V strain)	This work	23.9[a] ± 0.48	24.6 ± 0.47	21.4 ± 0.29	30.1 ± 0.39
CMV (Y strain)	Kaper *et al.* (1965)	23.4 ± 0.4	24.3 ± 0.5	23.2 ± 0.7	29.0 ± 0.7
CMV (Q strain)	Francki *et al.* (1966)	24.7 ± 0.33	22.4 ± 0.22	22.8 ± 0.45	30.1 ± 0.50
CMV (Q strain)	This work	24.0[b] ± 0.50	23.5 ± 0.39	21.8 ± 0.29	30.7 ± 0.39

a Means of ten determinations from three separate RNA preparations.

b Means of seven determinations from three separate RNA preparations.

Fig. 3-2. Sedimentation profile of TAV and CMV-RNA preparations in sucrose density-gradients. Of each viral RNA, 40 µg was layered on 10 to 40% linear sucrose density gradients in 0.02 M Tris HCl buffer, pH 7.5, and centrifuged in the Beckman SW 50.1 rotor at 50,000 rpm for 4.5 h at 4°.


18 S
25 S
TAV-RNA
b
a
A 254
CMV-RNA
TAV-RNA
+
CMV-RNA
TOP
BOTTOM

both TAV and CMV in sucrose density-gradients, RNA preparations of both viruses were analysed by PAG electrophoresis. Results of these experiments are summarised in Figs. 3-3 and 3-4 and it appears that RNA of both viruses can be resolved into 4 species (Fig. 3-3). The two slowest migrating RNA species of TAV (T_1 and T_2) appear to be indistinguishable from those of CMV (C_1 and C_2). However, the other two components of TAV (T_3 and T_4) migrate at different rates from those of CMV (C_3 and C_4). By using *E. coli* rRNA as a marker, the molecular weights of the various RNA species of TAV and CMV have been determined (Fig. 3-4). T_1 and C_1 have molecular weights of 1.26 $\pm$ 0.08; T_2 and C_2, 1.10 $\pm$ 0.07; T_3, 0.90 $\pm$ 0.06; C_3, 0.77 $\pm$ 0.05; T_4, 0.43 $\pm$ 0.03, and C_4, 0.34 $\pm$ 0.02 x 10^6 daltons. Two minor RNA species, migrating ahead of RNA 4 were sometimes present in RNA preparations of each virus. The origin of these RNA species is not known (Lot *et al.*, 1974).

In all preparations of TAV-RNA, the proportion of T_1 was highest and T_4 was not always detected (Fig. 3-3). In preparations of CMV-RNA, the species C_2 was highest and C_3 and C_4 were always present in approximately equimolar proportions.

To demonstrate whether the differences in the molecular weights of T_3, C_3 and T_4, C_4 are likely to be due to differences in their secondary structures or not, the RNAs of both viruses were treated with formamide as described by Peden and Symons (1973) and electrophoresed in PAG in the presence of 98% formamide (see Chapter 2 for methods). Using *E. coli* r RNA and TMV-RNA as markers in the same tube, the molecular

Fig. 3-3. Analysis of TAV-RNA and CMV-RNA preparations by PAG electrophoresis. RNA samples (20 µg) were subjected to electrophoresis in 2.6% polyacrylamide gels for 3 h at 5 mA/gel. The gels were stained in toluidine blue as described in Chapter 2, and scanned at 620 nm with a Joyce-Loebl Chromoscan.

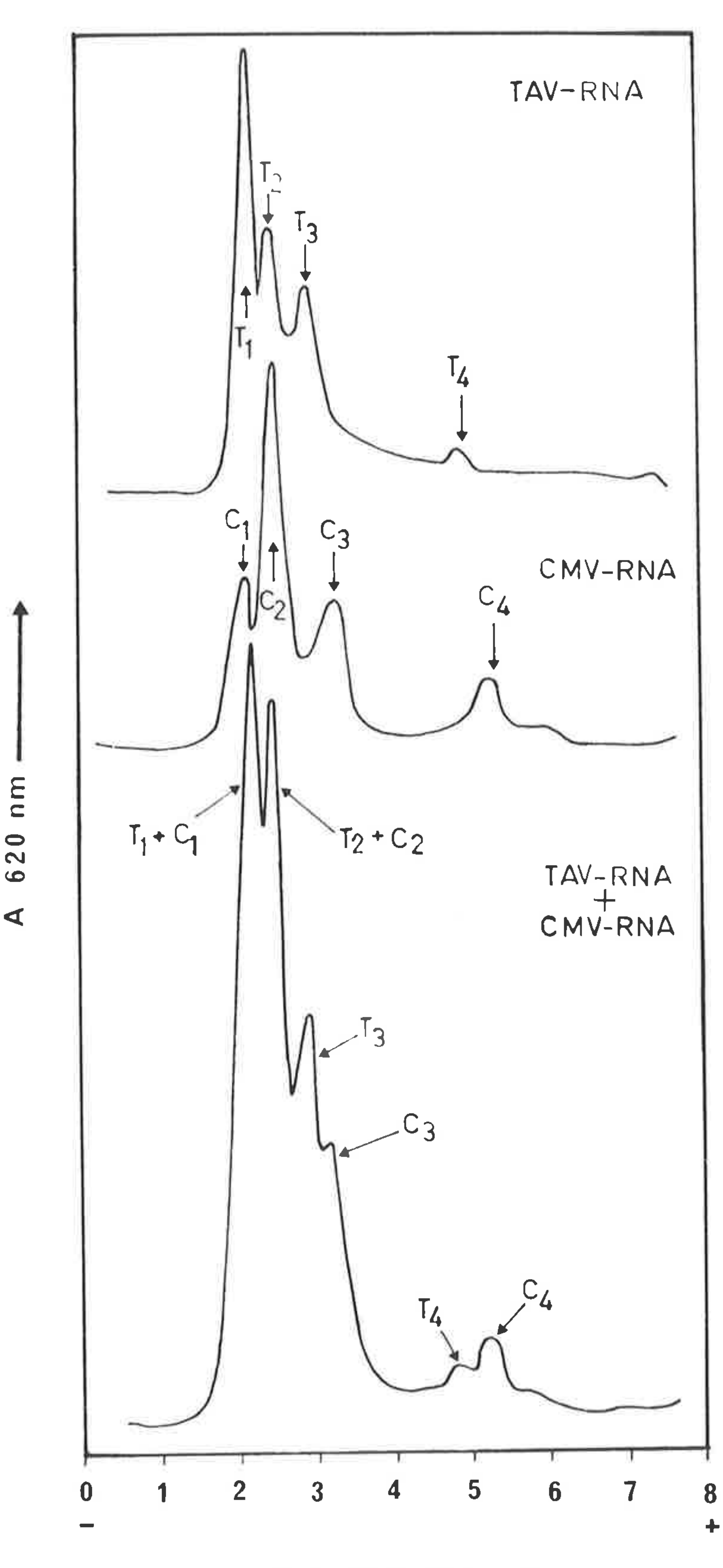
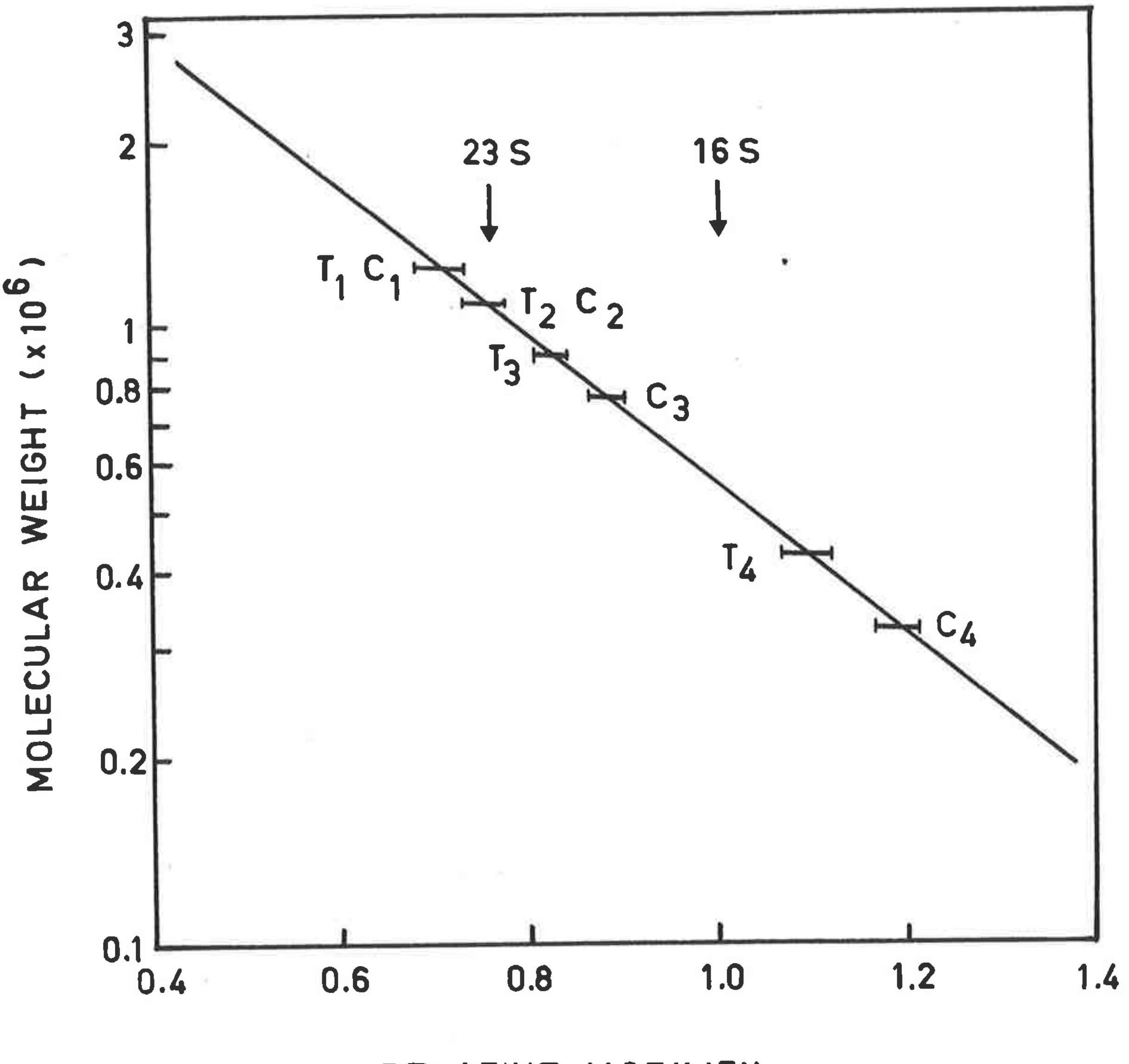

TAV-RNA
T1
T2
T3
T4
C1
C2
C3
C4
CMV-RNA
T1 + C1
T2 + C2
TAV-RNA
+
CMV-RNA
A 620 nm
0
1
2
3
4
5
6
7
8
-
+
MIGRATION (cm)

Fig. 3-4. Molecular weight determination of TAV and CMV-RNA species by PAG electrophoresis (methods as described in Fig. 3-3). *Escherichia coli* rRNA was used as a marker. In each experiment the mobility of each viral-RNA relative to that of 16 S *E. coli* rRNA was determined. Molecular weights of 23 S and 16 S *E. coli* rRNAs were taken as 1.07×10^6 and 0.55×10^6 daltons, respectively (Stanley and Bock, 1965). The values of this figure are means of 16 independent determinations from 10 different RNA preparations. T_4 was detected only in 7 of these preparations.



MOLECULAR WEIGHT (x10⁶)
3
2
1
0.8
0.6
0.4
0.2
0.1
23 S
16 S
T1 C1
T2 C2
T3
C3
T4
C4
0.4
0.6
0.8
1.0
1.2
1.4
RELATIVE MOBILITY

weights of various RNA species under the denaturing condition were determined and found to be not significantly different from the above values.

The estimates of molecular weights of the four RNA species of CMV and TAV agree reasonably well with the values reported by other workers (Table 3-2). Brown and Hull (1973) reported the molecular weights of RNA species 1 to 4 of CMV to be 1.25, 1.19, 0.96 and 0.5 x 10^6 daltons, respectively. The values of the RNA 3 and 4 are significantly higher than those reported by other workers for these species of CMV-RNA (Table 3-2). The molecular weight estimates of 0.96 x 10^6 and 0.5 x 10^6 daltons agree reasonably well with those of RNA 3 and 4 of TAV, respectively, as summarized in Table 3-2. Hull (personal communication) reported that Brown and Hull (1973) used the CMV-TAV-898 isolate described by Lot *et al.* (1974) for estimating molecular weights of the various species of CMV-RNA.

IV. Properties of Viral Proteins

Results of the amino acid analyses of TAV and CMV coat proteins are summarized in Table 3-3 and are compared with those of two strains of TAV (Canadian and CVL, Stace-Smith and Tremaine, 1973) and the S strain of CMV from South Africa as determined by van Regenmortel (1967) and recalculated by Stace-Smith and Tremaine (1973). It appears from the data that the amino acid composition of all the viruses compared,

Table 3-2. Molecular weights of various RNA species of TAV and CMV determined by PAG electrophoresis

Virus	Reference	Source of RNA marker	Mol. wt of RNA species (x10^6 daltons) 1[a]	2	3	4	5	6
TAV (Hollings strain)	Lot *et al.* (1974)	*E.coli* ribosomal	1.23	1.17	0.97	0.50	0.17	-[b]
TAV (Hollings strain)	"	*E.coli* ribosomal	1.36[c]	1.27	1.04	0.49	-	-
TAV (V strain)	This work	*E.coli* ribosomal	1.26	1.10	0.90	0.43	-	-
TAV (V strain)	"	" + TMV	1.30[d]	1.22	0.94	0.50	-	-
CMV (S strain)	Kaper and West (1972)	TYMV, MS2	1.01[e]	0.89	0.68	0.33	0.11	0.01
CMV (S strain)	Kaper and Waterworth (1973)	TYMV, MS2	1.07[e]	0.95	0.69	0.33	-	-
CMV (D strain)	Marchoux *et al.* (1973)	AMV	1.10	0.95	0.70	0.34	0.16	-
CMV (D strain)	"	BMV	1.26	1.12	0.76	0.38	-	-
CMV (D strain)	"	*E. coli* ribosomal	1.26	1.17	0.86	0.48	-	-
CMV (Q strain)	Peden and Symons (1973)	*E. coli* ribosomal	1.30	1.13	0.78	0.34	0.12	0.05
CMV (Q strain)	"	*E. coli* ribosomal	1.35[d]	1.16	0.85	0.35	-	-
CMV (Q strain)	This work	*E. coli* ribosomal " + TMV	1.30[d]	1.17	0.83	0.35	-	-
CMV (Q strain)	"	*E. coli* ribosomal	1.26	1.10	0.77	0.34	-	-

a RNA species number.
b Not determined.
c RNA treated with formaldehyde.
d Electrophoresis was carried out in PAG-formamide system (see Chapter 2).
e Determined by centrifugal method of Boedtker (1968) and electrophoresis of formaldehyde-treated RNA.

show similarities. However, it can also be seen that the TAV isolates form a group distinguishable from the two CMV strains.

The calculated molecular weights of TAV and CMV protein subunits from the amino acid analyses (Table 3-3) are 26,100 and 26,300 daltons, respectively. When the isolated viral proteins were subjected to electrophoresis in 10% PAGs (Weber and Osborn, 1969) single bands were detected with very similar electrophoretic mobilities (Fig. 3-5). On coelectrophoresing the TAV and CMV protein preparations, the two proteins migrated as a single band that could not be resolved into two components (Fig. 3-5). The molecular weight of the proteins was determined by co-electrophoresis with marker proteins and shown to be 24,500 $\pm$ 270 daltons (Fig. 3-6) which agrees reasonably well with values of 26,300 reported by Stace-Smith and Tremaine (1973), 25,200 by van Regenmortel *et al.* (1972) and 24,200 by Hill and Shepherd (1972).

The solubility of TAV and CMV proteins in various concentrations of LiCl is shown in Fig. 3-7, and it appears that both proteins are poorly soluble in the absence of salt. However, the solubility of TAV protein is much greater than that of CMV in LiCl below 0.5 M.

V. Isopycnic density-gradient centrifugation of the viruses

Both TAV and CMV were degraded in CsCl. Most of the degraded material produced a band at a density of 1.28 g/cm^3, showing a u.v. absorption spectrum characteristic for proteins. However, when the

Table 3-3. Amino acid composition of TAV and CMV.

Amino acid	TAV strains: VTAV Relative molar ratio[a]	TAV strains: VTAV Integer value	TAV strains: Canadian TAV[b]	TAV strains: CVL[b]	CMV strains: QCMV Relative molar ratio[a]	CMV strains: QCMV Integer value	CMV strains: S-CMV[c]
Lys	14.5	15	15	14	15.0	15	15
His	5.0	5	4	4	3.6	4	3
Arg	15.9	16	17	17	20.3	20	20
Asp	26.6	27	29	29	24.0	24	24
Thr[d]	19.4	19	17	17	13.9	14	14
Ser[d]	23.1	23	21	19	26.2	26	26
Glu	18.6	19	19	19	15.6	16	16
Pro	14.3	14	15	15	17.6	18	15
Gly	13.5	14	13	13	13.7	14	13
Ala	19.2	19	15	16	14.4	14	14
Cys	0.8	1	2	2	0	0	0
Val	16.6	17	16	17	17.6	18	18
Met	2.1	2	2	4	5.3	5	6
Ile	13.1	13	15	14	11.6	12	13
Leu	19.2	19	20	20	21.7	22	22
Tyr[d]	7.9	8	7	7	9.4	9	9
Phe	6.6	7	7	7	5.8	6	6
Trp[e]	1.4	1	1	1	1.2	1	1
Total	-	239	235	235	-	238	235

a Means of 24 and 72 h hydrolyses of three separate protein preparations.

b Data from Stace-Smith and Tremaine (1973).

c Data from van Regenmortel (1967).

d Extrapolated to zero hydrolysis time.

e Determined by colorimetric analysis as described by Spies and Chambers (1949).

Fig. 3-5. Electrophoresis of TAV protein (1), CMV protein (2), and a mixture of the two proteins (3) in 10% PAGs in the presence of SDS. The gels were run for 10 h at 8 mA/gel (70 volts), and stained with Coomassie blue. The protein markers used were bovine serum albumin (BSA), ovalbumin (OA), and cytochrome *c* (Cyt. c). Band (a) was a contaminant of the BSA preparation and was not detected in the gels run without BSA (results not shown).

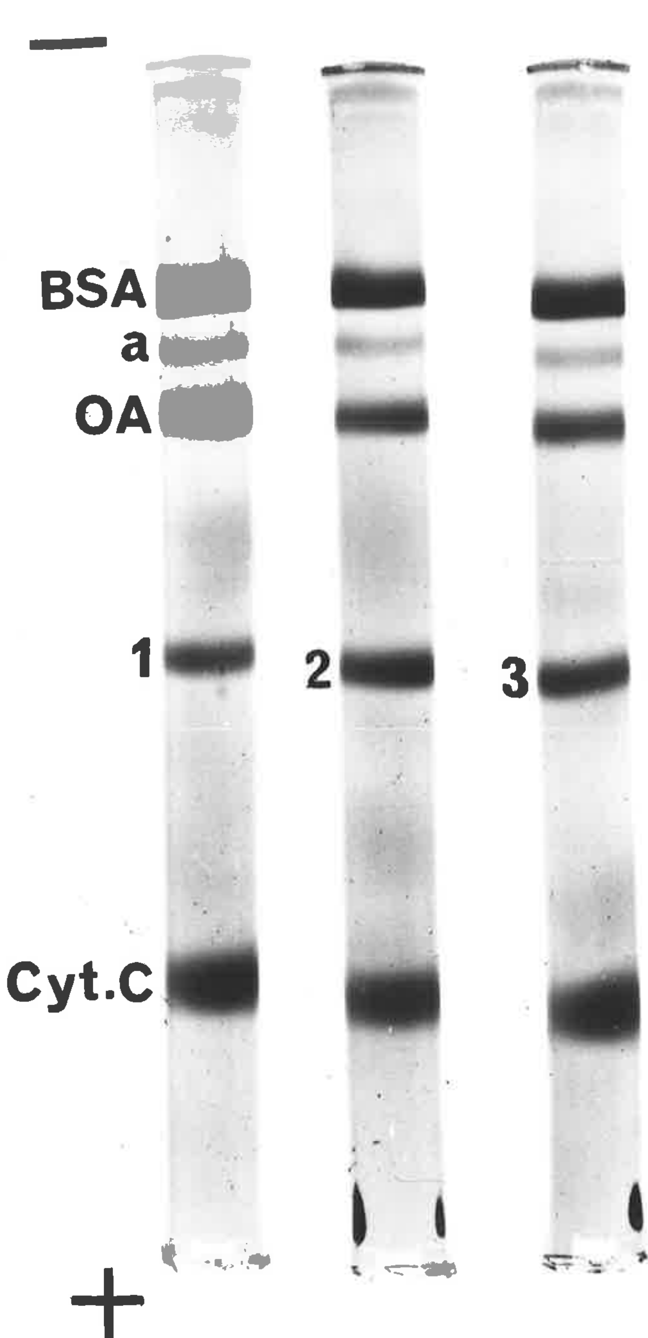
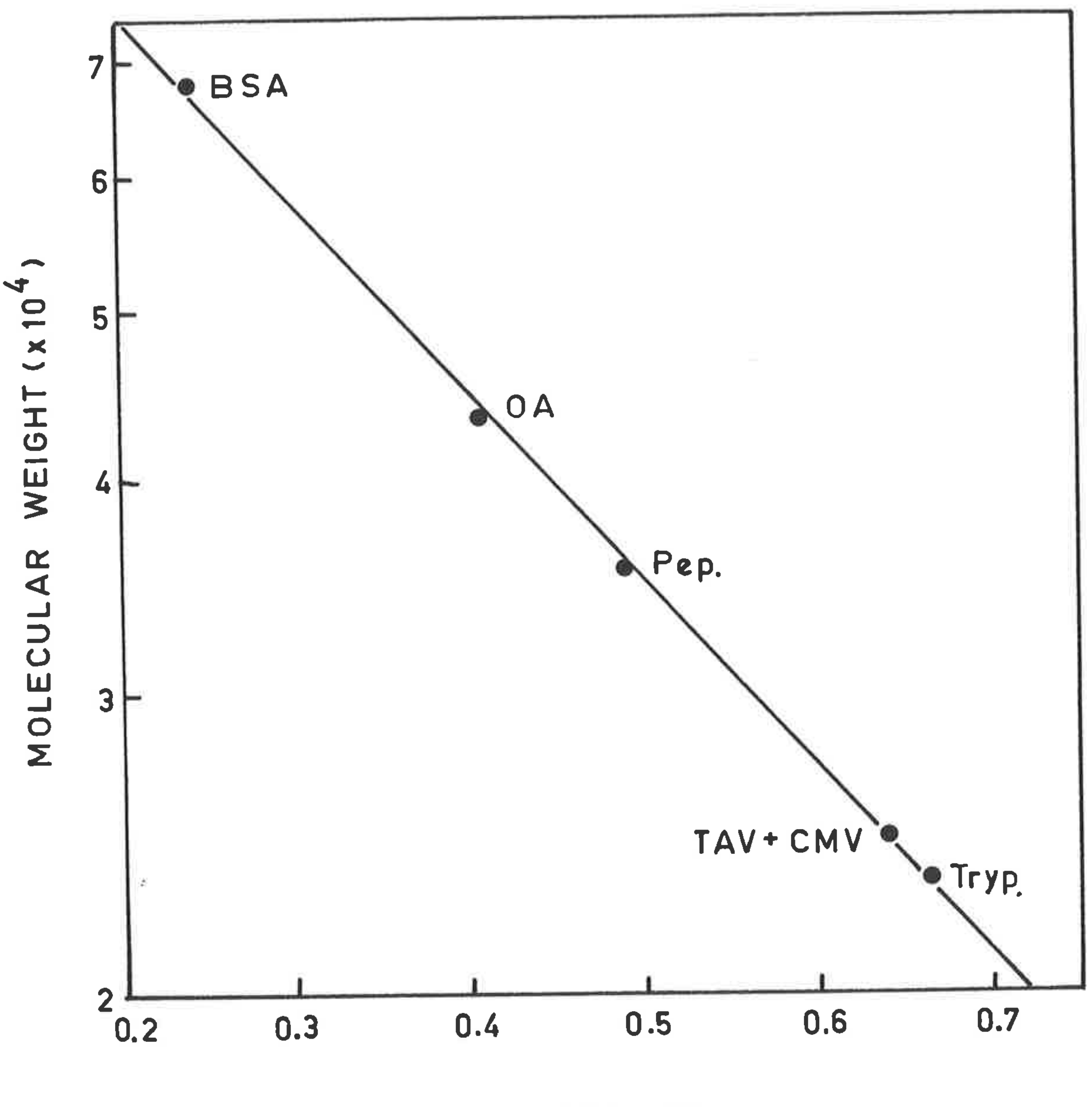
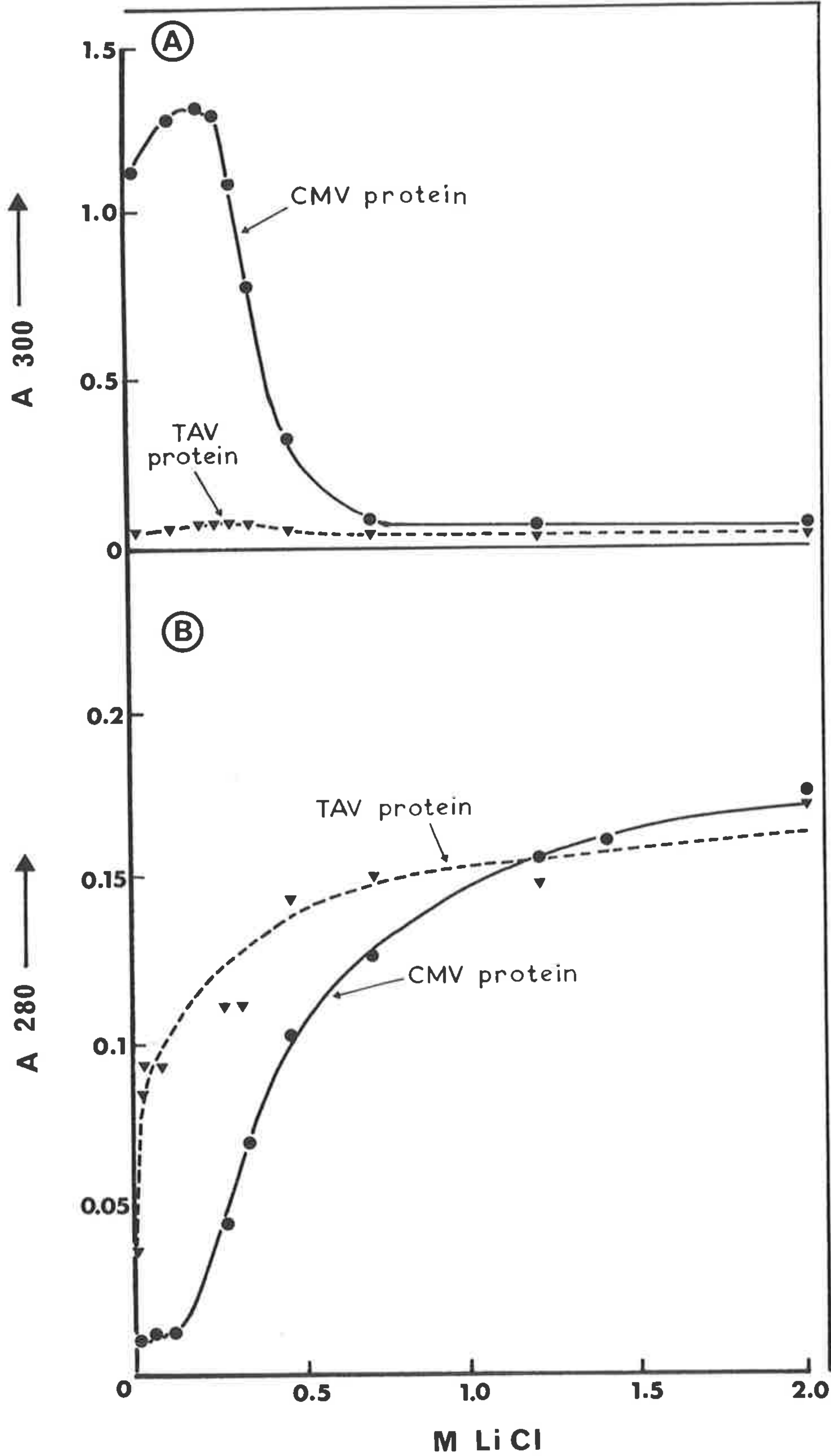

—
BSA
a
OA
1
2
3
Cyt.C
+

Fig. 3-6. Molecular weight determination of TAV and CMV proteins by PAG electrophoresis. Mobilities of the proteins in 10% PAGs were determined relative to that of cytochrome *c* which was used as an internal marker. Marker proteins used were bovine serum albumin (BSA), ovalbumin (OA), pepsin (Pep), and trypsin (Tryp).



MOLECULAR WEIGHT (x10^4)
7
6
5
4
3
2
BSA
OA
Pep.
TAV + CMV
Tryp.
0.2
0.3
0.4
0.5
0.6
0.7
RELATIVE MOBILITY

Fig. 3-7. Solubility of TAV and CMV proteins in various concentrations of LiCl. In (A), 0.1 ml of a protein preparation (0.3 mg) in 2 M LiCl was added to 20 mM Tris HCl, pH 7.0, incubated at 25° for 10 min, and the increase in light-scattering was measured at 300 nm. In (B), the protein preparations were dialysed exhaustively against buffers containing various concentrations of LiCl at 4° overnight. The samples were centrifuged at low speed, and the absorbance of the supernatant was measured at 280 nm.



A
1.5
1.0
0.5
0
A 300
CMV protein
TAV
protein
B
0.2
0.15
0.1
0.05
A 280
TAV protein
CMV protein
0
0.5
1.0
1.5
2.0
M LiCl

preparations were treated with formaldehyde, virus-like particles were recovered from light-scattering bands following isopycnic density-gradient centrifugation in CsCl (Fig. 3-8). The band produced by TAV was consistently narrower than that produced by CMV and in many experiments there was a suggestion that the CMV band consisted of two imperfectly separated components (Fig. 3-8 a and b). Formaldehyde-treated TAV and the two components of CMV were recovered from the density-gradients at an average buoyant density of 1.367 $\pm$ 0.007 g/cm^3; they had the appearance of typical virus particles when examined in an electron microscope, their ultraviolet absorption spectra were characteristic of the viruses, and positive serological reactions were observed with homologous antisera.

The heavy and light components of CMV recovered from CsCl density-gradients (Fig. 3-8) each produced a single sharp peak on sucrose density-gradients sedimenting at the same rate as normal CMV. A single peak was also observed when both components were mixed. RNA isolated from the light and heavy fractions of CMV and TAV by the Pronase/SDS method was examined by PAG electrophoresis (Fig. 3-9). It appears that the light fractions of both viruses are enriched in RNA 2 and the heavy fractions in RNA 1, whereas RNA 3 and RNA 4 of TAV and CMV were recovered from both light and heavy fractions (Fig. 3-9). A similar conclusion on several strains of CMV was recently made by Lot *et al.* (1974). They subjected normal virus preparations to isopycnic

Fig. 3-8. Isopycnic density-gradient centrifugation of formaldehyde-treated TAV and CMV. (a) Samples containing 1 mg of virus mixed with an equal volume of CsCl solution of density 1.72 g/cm^3. Three-millilitre samples of the virus in CsCl were overlaid with paraffin oil in 5-ml plastic centrifuge tubes and subjected to centrifugation in a Beckman SW 50.1 rotor at 38,000 rpm for 40 h at 4°. (b) Samples of virus (0.5 mg/cell) prepared as in Fig. 3-8a were subjected to centrifugation at 35,600 rpm for 17 h in an AnF rotor of a Beckman Model E ultracentrifuge maintained at 5-6°. The schlieren photograph was taken at a bar angle of 75° after 17 h of centrifugation (sedimentation from left to right).

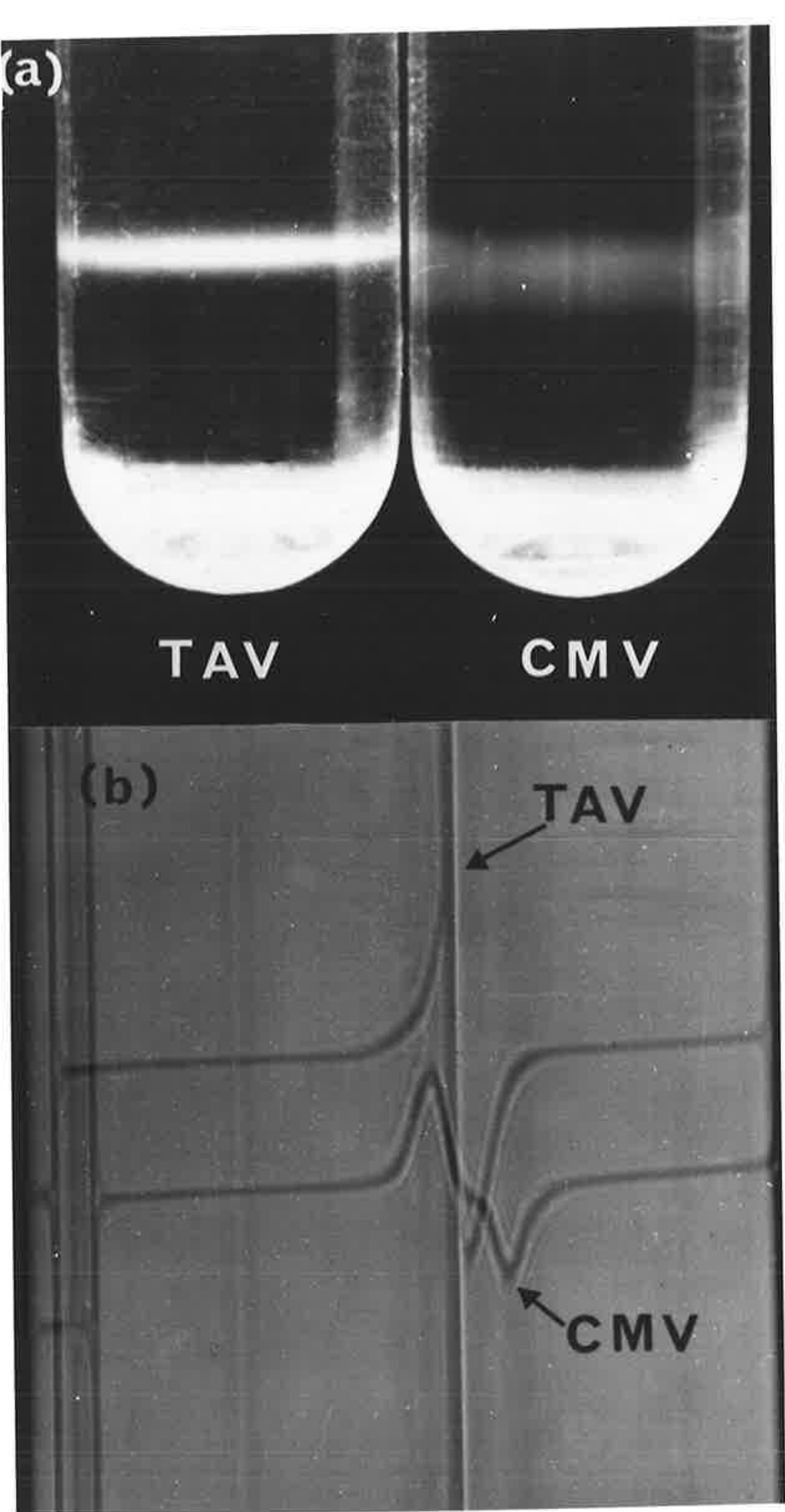
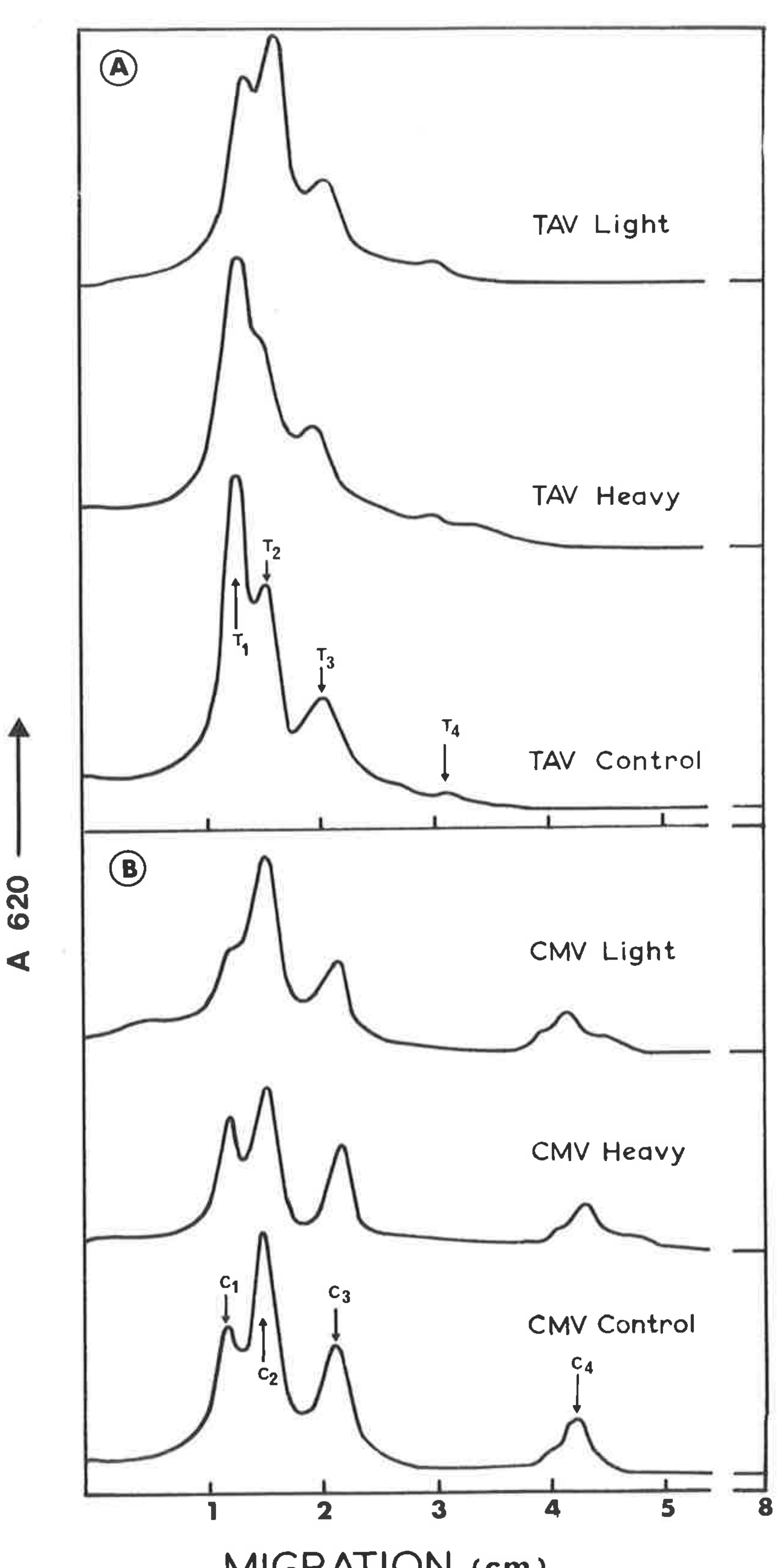

(a)
TAV
CMV
(b)
TAV
CMV

Fig. 3-9. Densitograms of PAGs of TAV-RNA (A) and CMV-RNA (B). The formaldehyde-treated virus preparations were sedimented to equilibrium in CsCl (average density = 1.36 g/cm^3) by centrifuging in a Beckman SW 50.1 rotor at 30,000 rpm for 48 h at 4°. The RNA from top (light), and bottom (heavy) fractions was isolated by Pronase/SDS method (Chapter 2); electrophoresed with unfractionated RNA and scanned as described in Fig. 3-3.



A
TAV Light
TAV Heavy
TAV Control
T1
T2
T3
T4
B
CMV Light
CMV Heavy
CMV Control
C1
C2
C3
C4
A 620
1
2
3
4
5
8
MIGRATION (cm)

centrifugation in sucrose density-gradients and analysed RNA isolated from various fractions of the gradients in PAGs.

VI. Isoelectric Points of TAV and CMV

The isoelectric points (pIs) of TAV and CMV determined in four separate experiments are summarized in Table 3-4. The results in Fig. 3-10 show the electrofocusing patterns of the two viruses. It was observed that unlike TAV, CMV aggregated in the presence of ampholine, the reason for which is unknown. On the other hand, TAV concentrated as a sharp band at pH around 5.7 (Fig. 3-10). A similar pI was obtained with the titration method (Table 3-4). The pI obtained for CMV agrees reasonably well with the value of 4.7 reported by Gibbs and Harrison (1970).

Conclusions

Some physical and chemical properties of TAV and CMV have been compared. The viruses were indistinguishable with respect to: (a) size and morphology, (b) sedimentation rate, (c) buoyant density, (d) RNA base ratio, (e) number of RNA species and molecular weights of species 1 and 2, and (f) molecular weight of the protein subunits. However, they differed in: (a) the amino acid composition of their coat proteins, (b) the isoelectric points, (c) solubility of protein subunits in low concentrations of LiCl, (d) molecular weights of RNA

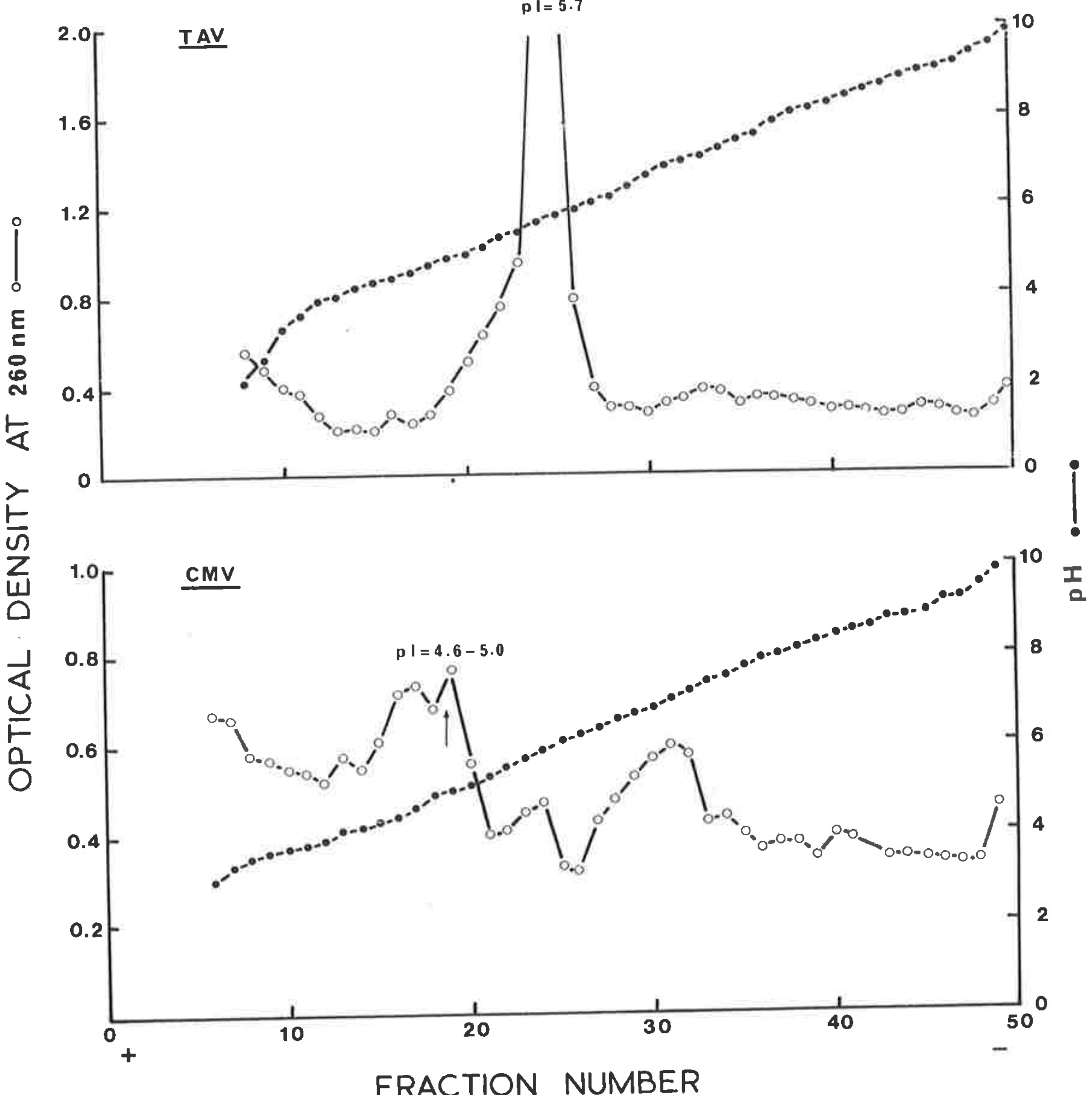
Table 3-4. Comparison of the isoelectric points of TAV and CMV.

Experiment Number[a]	Isoelectric Point (pI)	
	TAV	CMV
1	5.5	4.4-5.0
2	5.6	4.7
3	-[b]	4.5
4	5.7	4.4-5.0

a In experiments 1-3 the pIs were determined by titration in various pHs and in experiment 4 were determined by electro-focusing (see Methods for details).

b Not tested.

Fig. 3-10. Determination of the isoelectric points (pI) of TAV and CMV particles by isoelectrofocusing in sucrose gradients containing 1% ampholine (pH 3-10) as described in Methods. Two millilitre-fractions were collected for pH and optical density measurements (at 260 nm). CMV was aggregated in the presence of ampholine. The position of particles producing an opalescent band with no evidence of flocculation is indicated by arrow.


TAV
pI = 5·7
CMV
pI = 4.6 – 5.0
OPTICAL DENSITY AT 260 nm
pH
FRACTION NUMBER
2.0
1.6
1.2
0.8
0.4
0
1.0
0.8
0.6
0.4
0.2
10
8
6
4
2
0
10
20
30
40
50
+
–

3 and RNA 4 of each virus, and the proportion of RNA 4 which was consistently lower in TAV, (e) the proportion of RNA 1 to RNA 2 which was high in TAV and low in CMV, (f) isopycnic banding of formaldehyde-treated viruses, in that CMV produced a more diffuse band than TAV.

CHAPTER 4

VIRUS DISSOCIATION AND STABILITY

Introduction

Although the stability of CMV capsids has been studied extensively, this aspect of work on TAV has been neglected. CMV is readily aggregated with $MgCl_2$ and can be stabilized with EDTA (Takanami and Tomaru, 1969; Tomlinson *et al.*, 1973). CMV precipitates readily on mild heating in NaCl solutions (Francki *et al.*, 1966) and degrades in the presence of RNase (Francki, 1968; Kaper and Geelen, 1971) and low concentrations of SDS (Boatman and Kaper, 1972).

This Chapter describes experiments comparing the behaviour of TAV and CMV under a variety of treatments showing that the requirement for stability is different for the two viruses.

Methods

1. Virus incubation

In stability tests the viruses were incubated at the required temperature at concentrations between 0.5 and 1 mg/ml. To test the effect of pH, 20 mM Tris HCl buffer was used for pHs between 7.0 and 9.0 and 20 mM sodium acetate buffer for pHs below 7.0. For incubations

at 4° exceeding one day, a drop of chloroform was added to the virus suspensions to inhibit bacterial growth.

2. <u>Analysis of viruses and estimation of sedimentation rates</u>

Samples containing 30-50 µg of virus were analysed in sucrose density-gradients as described in Chapter 2. Approximate sedimentation coefficients of the viruses and virus degradation products were calculated from the sedimentation rates of tobacco ringspot virus components (Randles and Francki, 1965) and added as internal markers.

3. <u>Measurement of the percentage of virus particles stabilized with various concentrations of formaldehyde</u>

Aliquots of 0.2 ml of purified TAV and CMV (3 mg/ml) in 20 mM phosphate buffer, pH 7.6, were dialysed at 4° for 20 h against 20 ml of the same buffer containing different concentrations of formaldehyde. To each sample (0.15 ml) an equal volume of 4 M LiCl was added and the mixture was frozen at -15° for 2 h. After slow thawing, the samples were centrifuged at 5,000 *g* for 15 min. The pellet (RNA) was resuspended in 1 ml water and the optical density at 260 nm was measured. The percentage of virions stabilized was calculated for each concentration of formaldehyde from the differences in dissociation between formaldehyde-treated and non-treated virus samples, assuming 100% dissociation occurred in the absence of formaldehyde.

In some experiments the viral capsids were stabilized with formaldehyde as described in Chapter 2.

Results

I. Degradation of Viruses by Pancreatic RNase

When the effect of various concentrations of RNase on TAV and CMV were compared, it was observed that TAV was much more resistant to degradation by the enzyme than CMV (Table 4-1).

II. Precipitation of Viruses by Mild Heating in NaCl Solutions

Incubation of TAV in 0.14 M NaCl had very little effect on the virus. Even on heating at 37° for 20 min, 85% of the TAV virions survived the treatment, whereas over 50% of the CMV virions precipitated (Table 4-2).

III. Stability of Viruses in the Presence of Mg^{2+}

TAV and CMV preparations were stored in the presence and absence of 10 mM $MgCl_2$ for three weeks at 4° and were analysed at intervals by sucrose density-gradient centrifugation. Results of the experiment indicate that TAV was stable in the presence of Mg^{2+} during the first week but the homogeneity of the preparation was reduced thereafter (Fig. 4-1). The same concentration of $MgCl_2$ caused CMV to precipitate although a part of the virion population retained its integrity (Fig. 4-1). Results of another experiment, summarized in Fig. 4-2, showed that the precipitation reaction was rapid as indicated

Table 4-1. Degradation of TAV and CMV with pancreatic RNase[a]

RNase concentrations (μg/ml)	% Survival[b]			
	TAV		CMV	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
0.1	100	-[c]	-	29
1	100	-	28	23
10	95	72	3	16
100	84	32	0	-

a Virus preparations (1 mg/ml) were incubated at 25° for 1 h at 20 mM sodium phosphate buffer, pH 7.6.

b Survival of virus treated with RNase was calculated from the area under the peak of the ISCO traces after sucrose density gradient centrifugation.

c Not tested.

Table 4-2. Precipitation of TAV and CMV by mild heating in NaCl[a]

Treatments	% Survival[b]	
	TAV	CMV
-NaCl at 0°	100	100
+NaCl at 0°	100	92
-NaCl at 37°	100	70
+NaCl at 37°	85	42

a Virus preparations (1 mg/ml) were incubated at 37° for 20 min in 20 mM sodium phosphate buffer, pH 7.6, containing 0.14 M NaCl.

b Determined as described in Table 4-1. Each value is the mean of three independent experiments.

Fig. 4-1. Sedimentation profiles of TAV and CMV incubated at 1 mg/ml in 5 mM sodium borate buffer, pH 9.0, with and without 10 mM $MgCl_2$. After various times of incubation at 4°, samples of 50 µg of each virus were layered on 5 to 25% (w/v) linear sucrose density gradients in 20 mM sodium phosphate buffer, pH 7.5, and centrifuged at 4° in a Spinco SW 50.1 rotor at 50,000 rpm for 35 min. The tubes were then scanned at 254 nm with an ISCO apparatus.

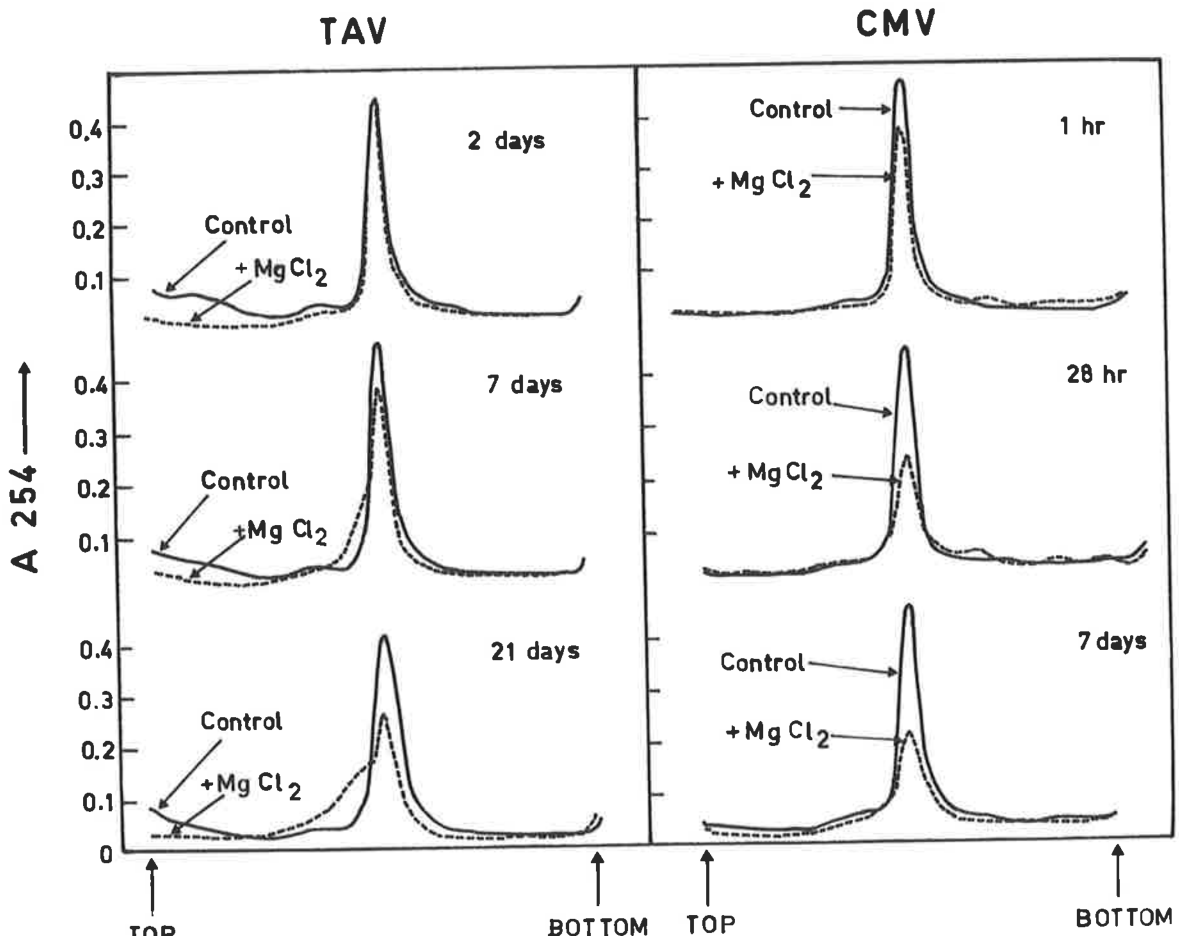
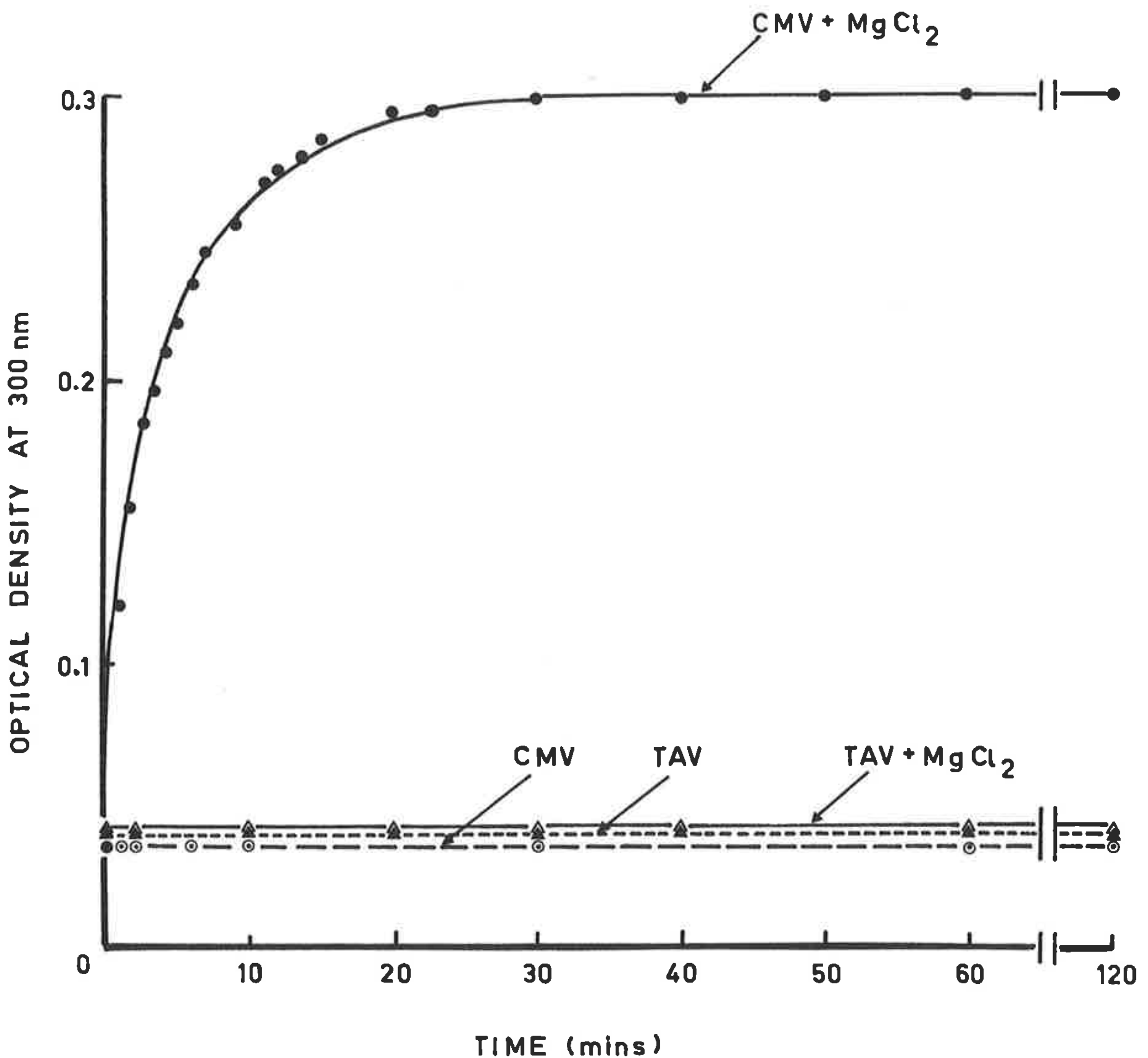

TAV
CMV
0.4
0.3
0.2
0.1
0
2 days
7 days
21 days
Control
+ Mg Cl2
1 hr
28 hr
7 days
A 254
TOP
BOTTOM
TOP
BOTTOM

Fig. 4-2. Changes in optical density at 300 nm of TAV and CMV preparations in 20 mM sodium phosphate buffer, pH 7.6, with and without 1 mM $MgCl_2$ during incubation at 25°.


CMV + MgCl2
0.3
0.2
0.1
0
OPTICAL DENSITY AT 300 nm
CMV
TAV
TAV + MgCl2
10
20
30
40
50
60
120
TIME (mins)

by rapid increase in light scattering by the virus; the reaction reached completion within 20–30 min. However, light scattering did not develop when formaldehyde-treated CMV was exposed to Mg^{2+}.

It has been suggested (see Chapter 8) that TAV and CMV preparations each contain three distinct types of particles each containing different species of RNA. The possibility was considered that Mg^{2+} selectively precipitates a particular population of CMV particles. Analysis of RNA isolated from the Mg^{2+} soluble and insoluble fractions of CMV by PAG electrophoresis showed that the soluble fraction was deficient in the largest species of CMV-RNA (C_1) whereas the insoluble fraction contained all four species of RNA (Fig. 4–3).

Although 10 mM $MgCl_2$ failed to precipitate TAV at 4°, it was demonstrated that at higher concentrations of the salt at 37°, a proportion of the virus particles was precipitated (Fig. 4–4). The $MgCl_2$ soluble fraction of TAV remained in solution at concentrations of the cation as high as 100 mM (Fig. 4–4). PAG electrophoresis of RNA isolated from the Mg^{2+} soluble and insoluble TAV failed to detect any quantitative differences. The effect of $MgCl_2$ of TAV was similar when the virus was suspended in buffers of pH between 6 and 9.

In another series of experiments, it was demonstrated that 1 mM $MgCl_2$ had a significant effect on the susceptibility of TAV to RNase digestion (Fig. 4–5). At pH between 7 and 9 the addition of Mg^{2+} to

Fig. 4-3. PAGs run with CMV-RNA isolated by Pronase/SDS method (Chapter 2). a. RNA from $MgCl_2$-insoluble CMV preparations, b. RNA from $MgCl_2$-soluble fractions, c. Mixture of a + b, and d. RNA from unfractionated CMV preparations. C_1 to C_4 refer to various RNA species. The electrophoresis condition was as described in Fig. 3-3. The gels were stained in toluidine blue and destained in water.

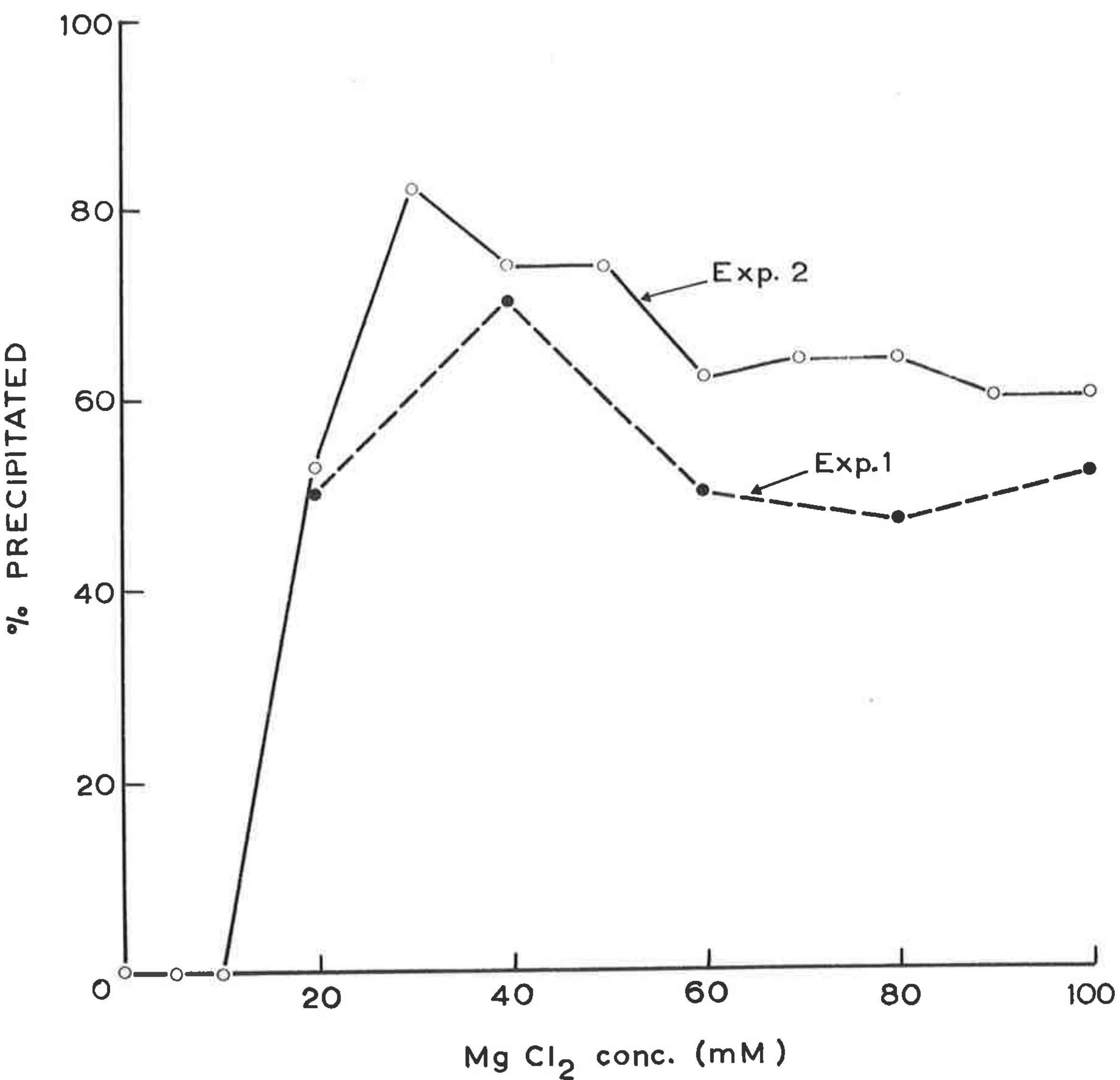
a b c d

—

C_1
C_2
C_3
C_4

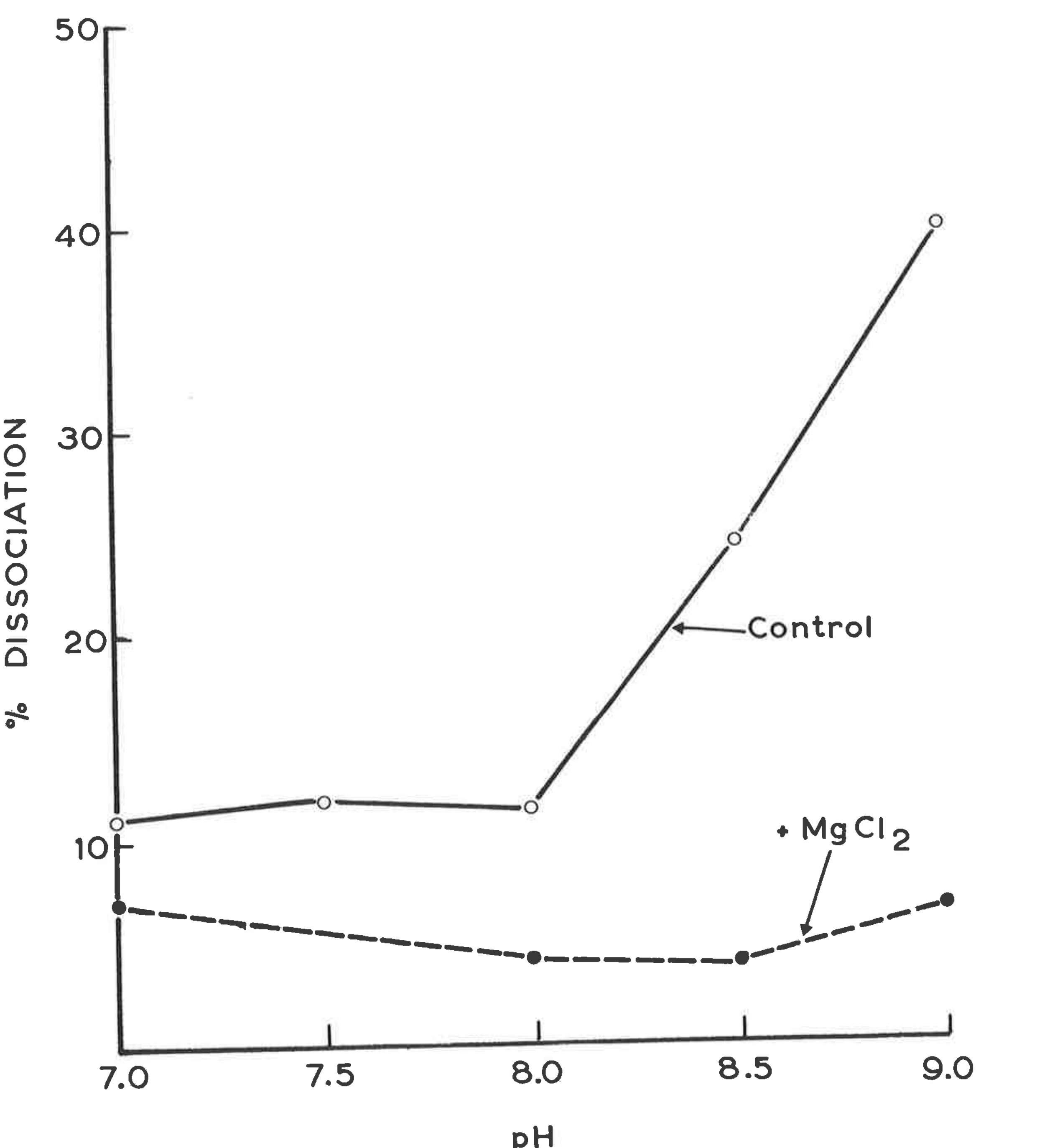
+

Fig. 4-4. Precipitation of TAV with $MgCl_2$. TAV (100 µg/ml) was incubated in 5 mM sodium borate buffer, pH 9.0, containing various concentrations of $MgCl_2$ at 37° for 30 min. The mixture was then centrifuged at 3,000 *g* for 15 min. The precipitated virus was measured by difference from that remaining in the supernatant.



100
80
60
40
20
0
% PRECIPITATED
Exp. 2
Exp. 1
20
40
60
80
100
Mg Cl2 conc. (mM)

Fig. 4-5. Degradation of TAV with pancreatic RNase in the presence and absence of $MgCl_2$. TAV (0.5 mg/ml) was incubated with 10 μg/ml RNase in 20 mM Tris HCl of pH 7.0 to 9.0 with and without 1 mM $MgCl_2$. The samples were subjected to density-gradient centrifugation as described in Fig. 4-1.


50
40
30
20
10
% DISSOCIATION
Control
+ MgCl2
7.0
7.5
8.0
8.5
9.0
pH

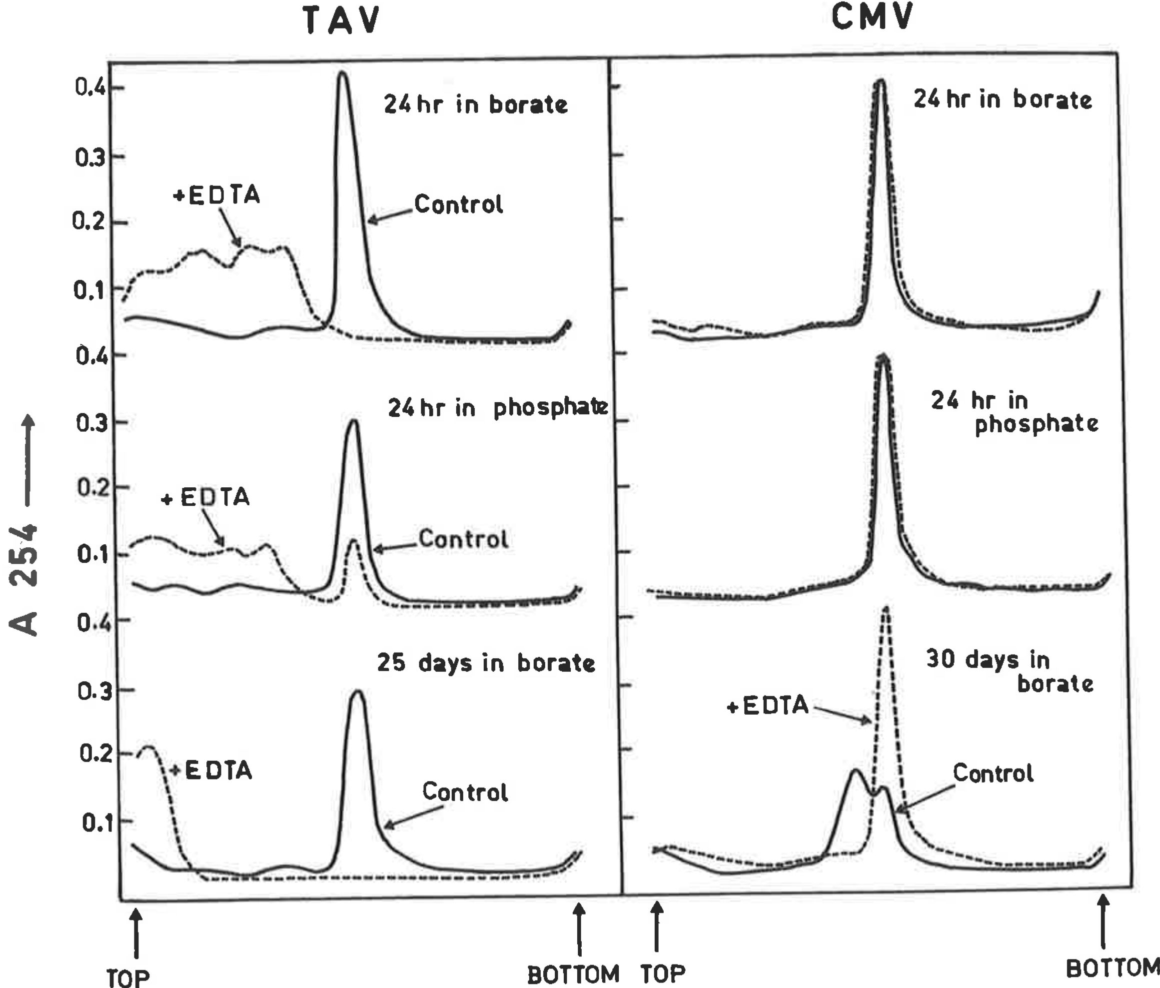
TAV had a slight but significant protective effect which increased considerably at pH above 8.0 (Fig. 4-5). The increased susceptibility of TAV to RNase at pH above 8.0 in the absence of Mg^{2+} (Fig. 4-5) is unlikely to have been due to a swelling of the virus particles as reported for cowpea chlorotic mottle virus (Bancroft *et al.*, 1967) as there was no significant change in the sedimentation coefficient of TAV when the virus was suspended in buffers between pH 6.0 and 9.0. Addition to $MgCl_2$ also failed to affect the sedimentation properties of the virus.

IV. Stability of Viruses in the Presence of EDTA

TAV and CMV were stored in the presence and absence of 10 mM EDTA for 30 days at 4° and analysed at intervals by sucrose density-gradient centrifugation. Results of the experiment indicate that EDTA causes rapid degradation of TAV, but apparently stabilizes CMV (Fig. 4-6). An infectivity test was carried out on CMV preparations being stored for 23 days. An average of 100 local lesions per half-leaf were produced on cowpeas by the virus in EDTA, whereas only 2 lesions were produced by the control preparation.

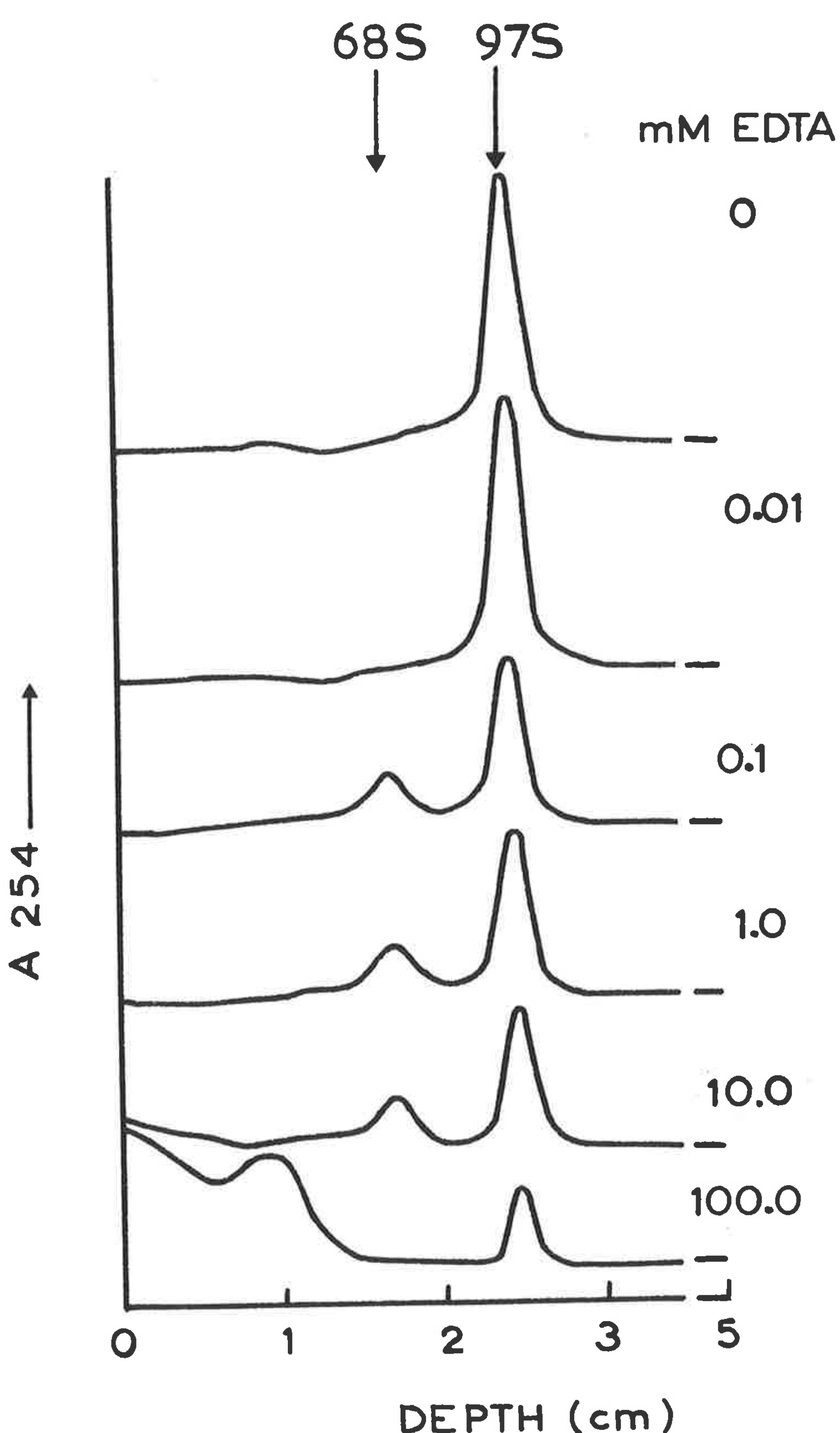
TAV was more resistant to degradation in EDTA when suspended in phosphate buffer, pH 7.6, than in borate buffer, pH 9.0 (Fig. 4-6). A subsequent experiment demonstrated that EDTA concentrations as low as 0.1 mM had a disruptive effect on TAV at pH 7.8 (Fig. 4-7). After

Fig. 4-6. Sedimentation profiles of TAV and CMV in sucrose-density gradients in the presence and absence of EDTA. Samples (1 mg/ml) were incubated in 20 mM sodium phosphate buffer, pH 7.6 and 5 mM sodium borate buffer, pH 9.0, at 4° with and without 10 mM EDTA. The samples were analysed as described in Fig. 4-1.



TAV
CMV
A 254
0.4
0.3
0.2
0.1
24 hr in borate
+EDTA
Control
24 hr in phosphate
+EDTA
Control
25 days in borate
+EDTA
Control
24 hr in borate
24 hr in phosphate
30 days in borate
+EDTA
Control
TOP
BOTTOM
TOP
BOTTOM

Fig. 4-7. Sedimentation profiles of TAV incubated in 20 mM sodium phosphate buffer, pH 7.8, with various concentrations of EDTA. The samples (1 mg virus/ml) were incubated at 4° for 24 h. Other conditions as in Fig. 4-1.



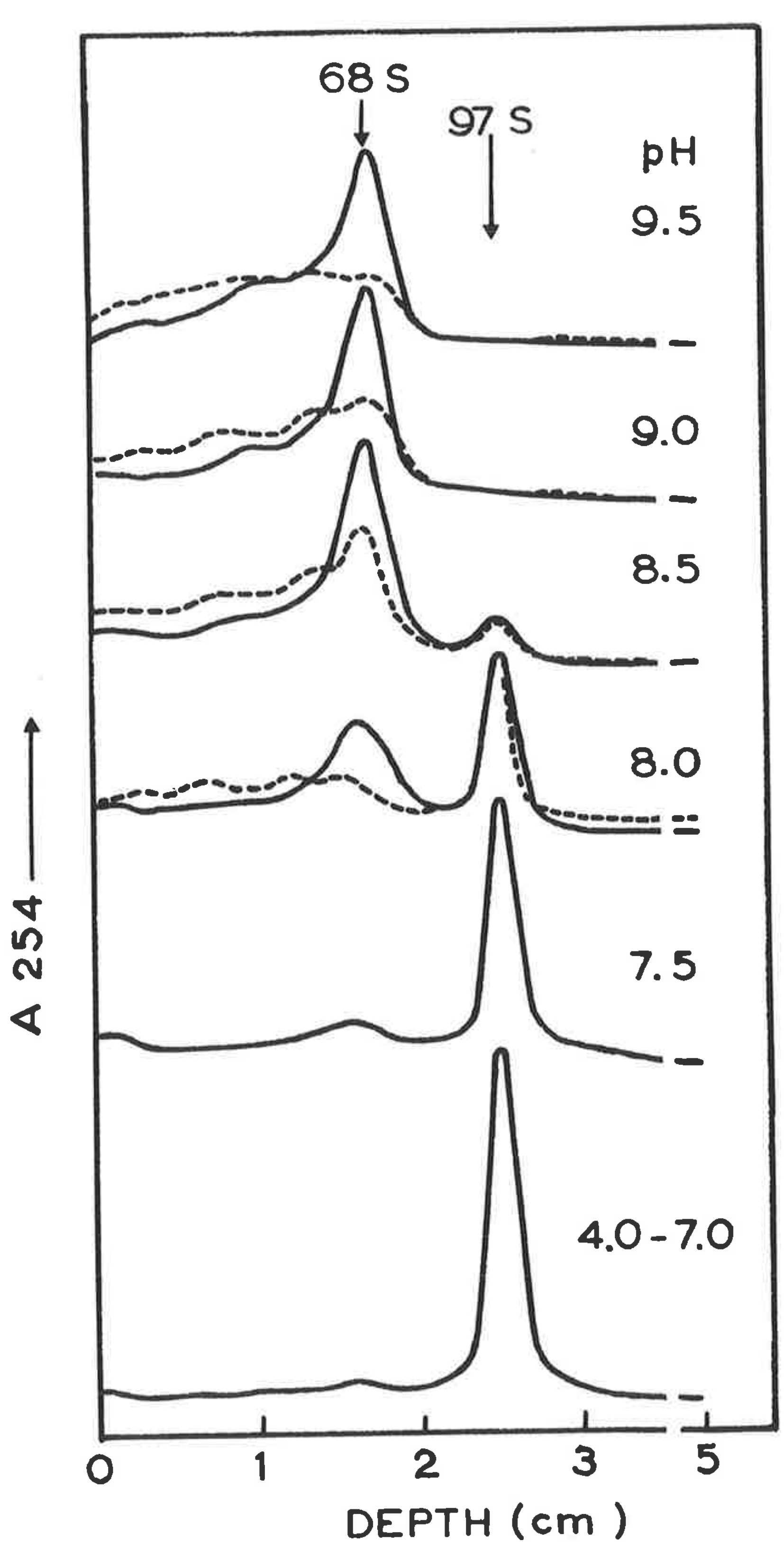
68S
97S
mM EDTA
0
0.01
0.1
1.0
10.0
100.0
A 254
0
1
2
3
5
DEPTH (cm)

treatment of TAV with EDTA at concentrations between 0.1 and 10 mM, sub-viral material with a sedimentation coefficient of about 68S was observed (Fig. 4-7). This material was recovered from sucrose gradients and shown to have an ultraviolet absorption spectrum characteristic of nucleoprotein. The material was unstable and defied efforts at further purification and examination in an electron microscope. However, when tested against TAV antiserum in gel-diffusion tests, immuno-precipitin bands were observed.

In subsequent experiments, it was demonstrated that the disruptive effect of EDTA on TAV is pH-dependent (Fig. 4-8). When 1 mM EDTA was added to TAV preparations in buffers of pH between 4 and 7, no significant effect on the virus was observed. However, at pH 7.5 and above, virus degradation increased with pH and higher amounts of 68S material were detected at the expense of 97 S virus (Fig. 4-8). The experiments also demonstrate that the degradation of TAV by EDTA is irreversible.

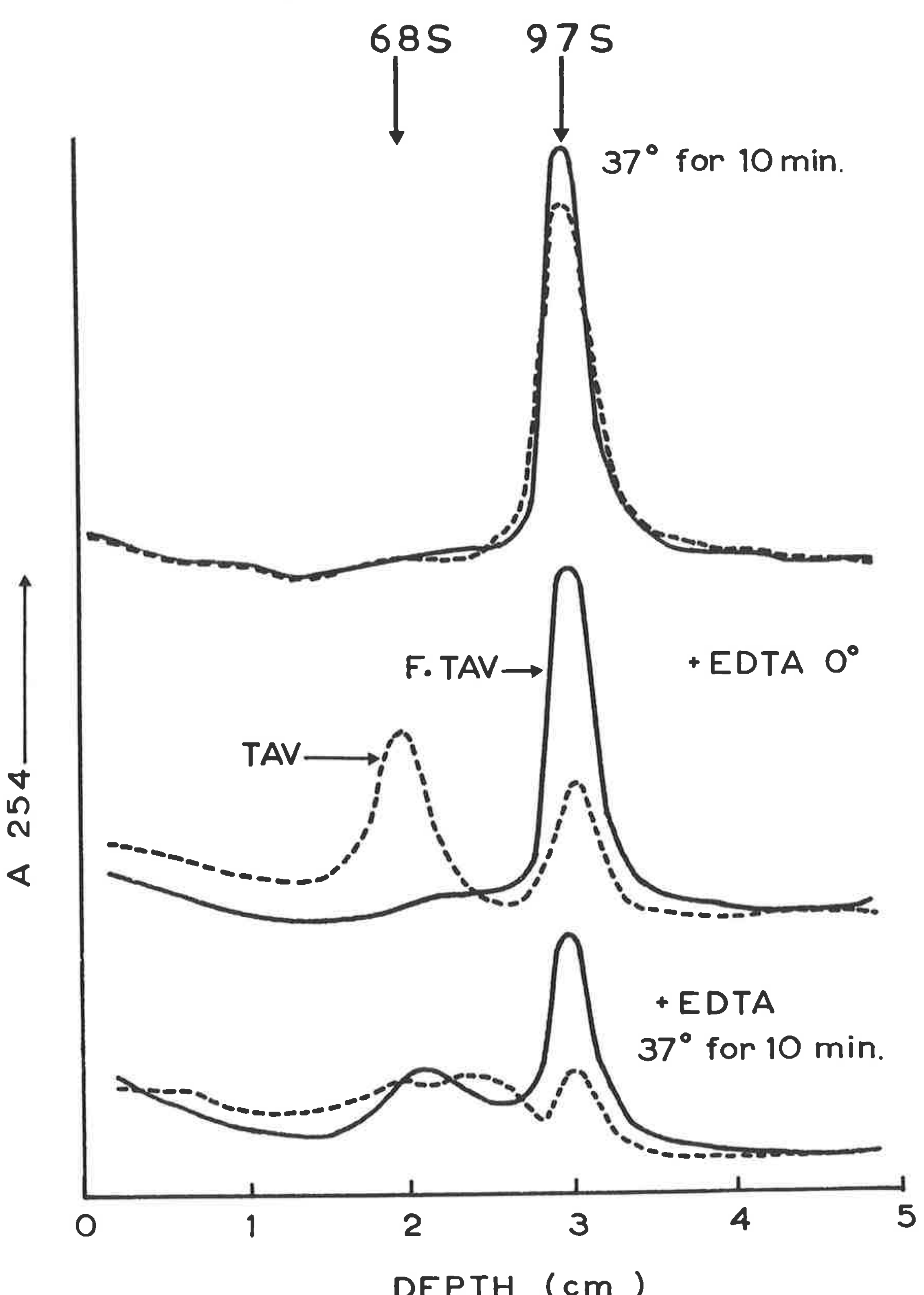
Francki and Habili (1972) showed that the capsid structure of CMV could be stabilized by treatment with formaldehyde and it was suggested that this resulted from the cross-linking of the amino groups of the subunits forming the protein shell of the capsids. Hiebert and Bancroft (1969) showed that spermidine stabilized the protein shells of cowpea chlorotic mottle virus. Results of the experiments summarized in Figs. 4-9 and 4-10 indicate that both formaldehyde and spermidine

Fig. 4-8. Sedimentation profiles of TAV incubated in EDTA at various pH. Tris HCl (20 mM) was used to make up pH above 7.0 and Na-acetate (20 mM) for pH below 7.0. The samples at 1 mg/ml in 1 mM EDTA were incubated at 4° for 16 h. The dashed lines of pH 8.0 to 9.5 were obtained after the preparations (solid lines) of individual pH were dialysed at 4° for 24 h against 20 mM Tris HCl, pH 7.0.



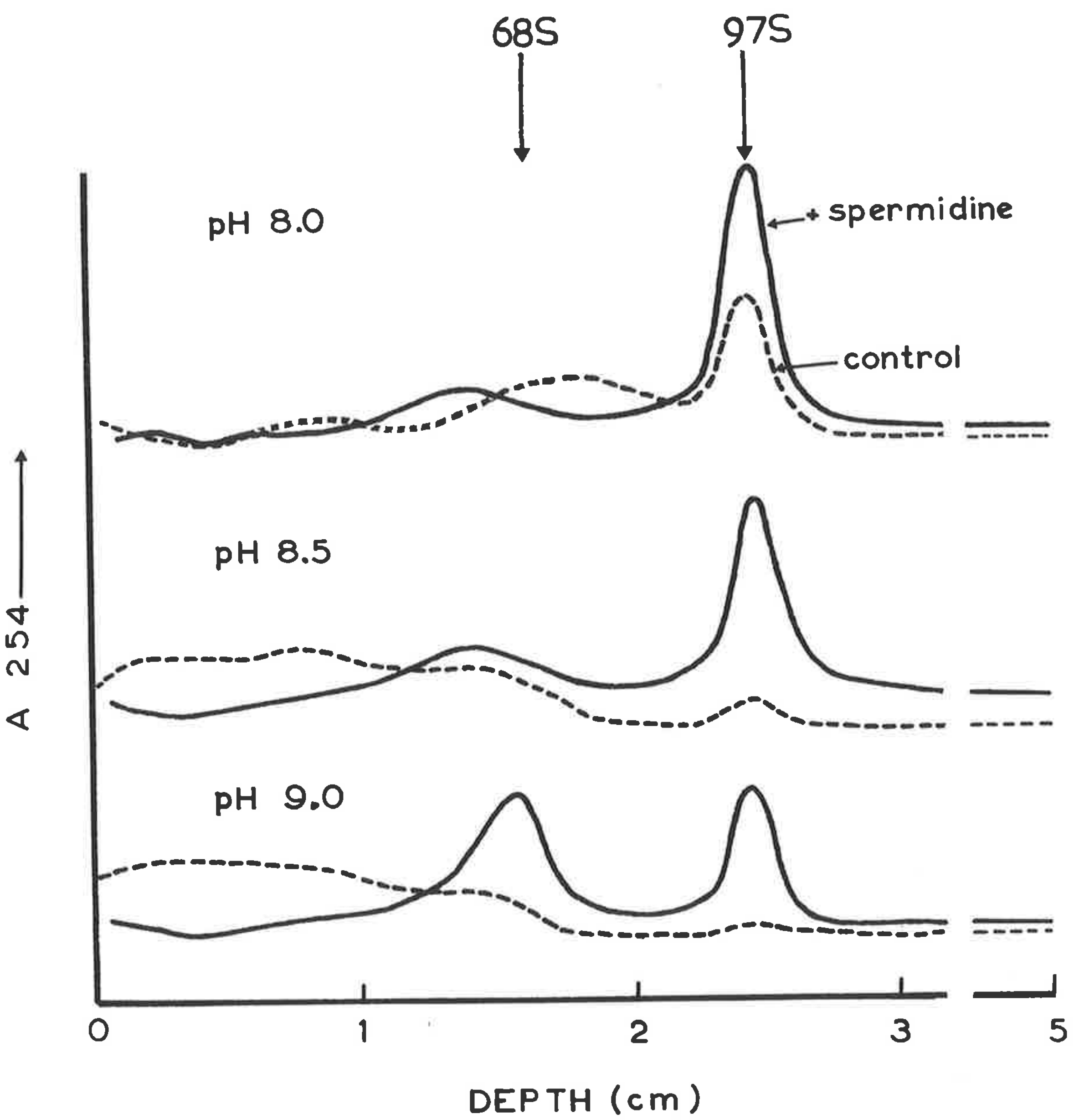
68 S
97 S
pH
9.5
9.0
8.5
8.0
7.5
4.0 - 7.0
A 254
0
1
2
3
5
DEPTH (cm)

Fig. 4-9. Sedimentation profiles of TAV (— — —) and the formaldehyde-treated virus (F·TAV) (———) incubated at 0° and 37° for 10 min in 10 mM EDTA in 20 mM sodium phosphate buffer, pH 7.6. The virus was treated with 0.2% formaldehyde as described in Chapter 2.



68S
97S
37° for 10 min.
A 254
F. TAV
+ EDTA 0°
TAV
+ EDTA
37° for 10 min.
0
1
2
3
4
5
DEPTH (cm)

Fig. 4-10. Sedimentation profiles of TAV (1 mg/ml) (— — —) and spermidine-treated virus (———) incubated in 1 mM EDTA at 20° for 30 min in 20 mM Tris HCl of various pH. The virus was treated with spermidine by dialysing at 4° overnight against 20 mM Tris HCl containing 1 mM spermidine at the pH indicated. Control preparations were treated similarly but without spermidine.


68S
97S
pH 8.0
+ spermidine
control
pH 8.5
pH 9.0
A 254
0
1
2
3
5
DEPTH (cm)

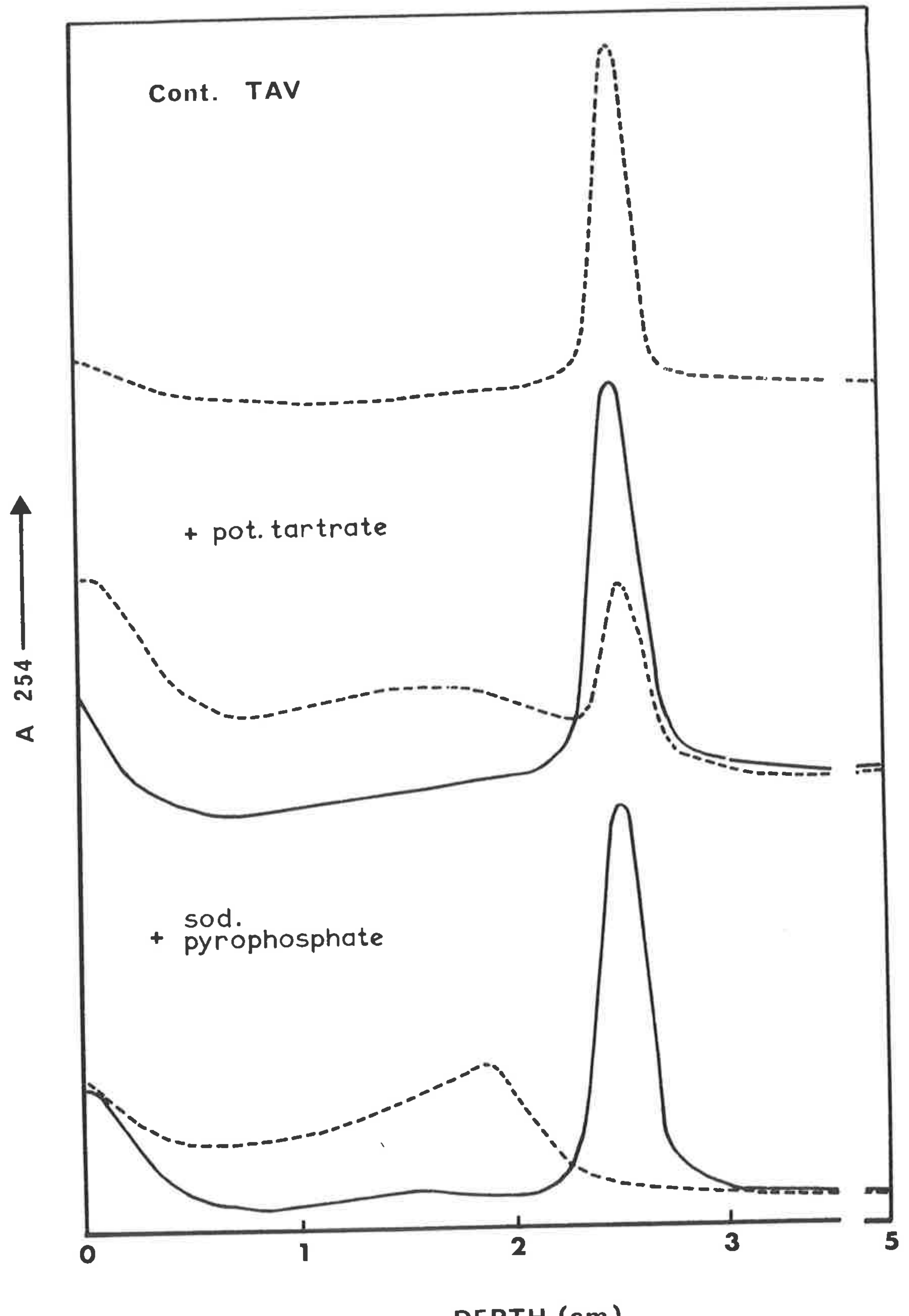
treatment of TAV render the virus more resistant to dissociation by EDTA.

The stability of TAV in the presence of other chelating agents, e.g. tetra-sodium pyrophosphate and potassium tartarate was also examined at pH 7.0 and 9.0. Both reagents disrupted the virus at pH 9.0 in the same fashion as EDTA (Fig. 4-11).

V. Stability of Viruses in the Presence of SDS

Boatman and Kaper (1972) suggested that low concentrations of SDS are capable of dissociating protein-RNA linkages of viruses. The effect of SDS on TAV and CMV was compared and showed that TAV is more readily dissociated by the reagent than CMV (Fig. 4-12). Both viruses are more stable in SDS at pH 9 than at pH 7, and it would appear that the type of buffer determines how easily the virus is dissociated (Fig. 4-12). TAV dissociates more readily in Tris HCl buffer than in borate at pH 9, whereas CMV dissociates more readily in borate buffer (Fig. 4-12). The dissociation of TAV takes a different course in the presence and absence of Mg^{2+}. When 1 mM $MgCl_2$ was added to TAV in 0.015% SDS, a distinct peak of 24S material was detected in sucrose gradients which was absent in preparations to which no Mg^{2+} was added (Fig. 4-13). The 24S material recovered from the gradients had ultraviolet absorption spectra characteristic of nucleoprotein. It was also observed that the stability of TAV with SDS in the presence of

Fig. 4-11. Sedimentation profiles of TAV analysed in sucrose gradients after incubating the virus at 37° for 10 min in 20 mM Tris HCl, pH 7 (———), and pH 9 (– – –) without chelating agents (cont·TAV), or in the presence of 20 mM potassium tartarate, and 20 mM sodium pyrophosphate.



Cont. TAV
+ pot. tartrate
+ sod. pyrophosphate
A 254
0
1
2
3
5
DEPTH (cm)

Fig. 4-12. Dissociation of TAV and CMV with SDS calculated from the area under sucrose density-gradient peaks. Increasing concentrations of SDS were mixed with the virus preparations (final virus concentration was 0.5 mg/ml) and incubated at 20° for 30 min. Tris buffer (20 mM) was adjusted with HCl to the required pH, and 5.0 mM sodium borate, pH 9.0, were used.

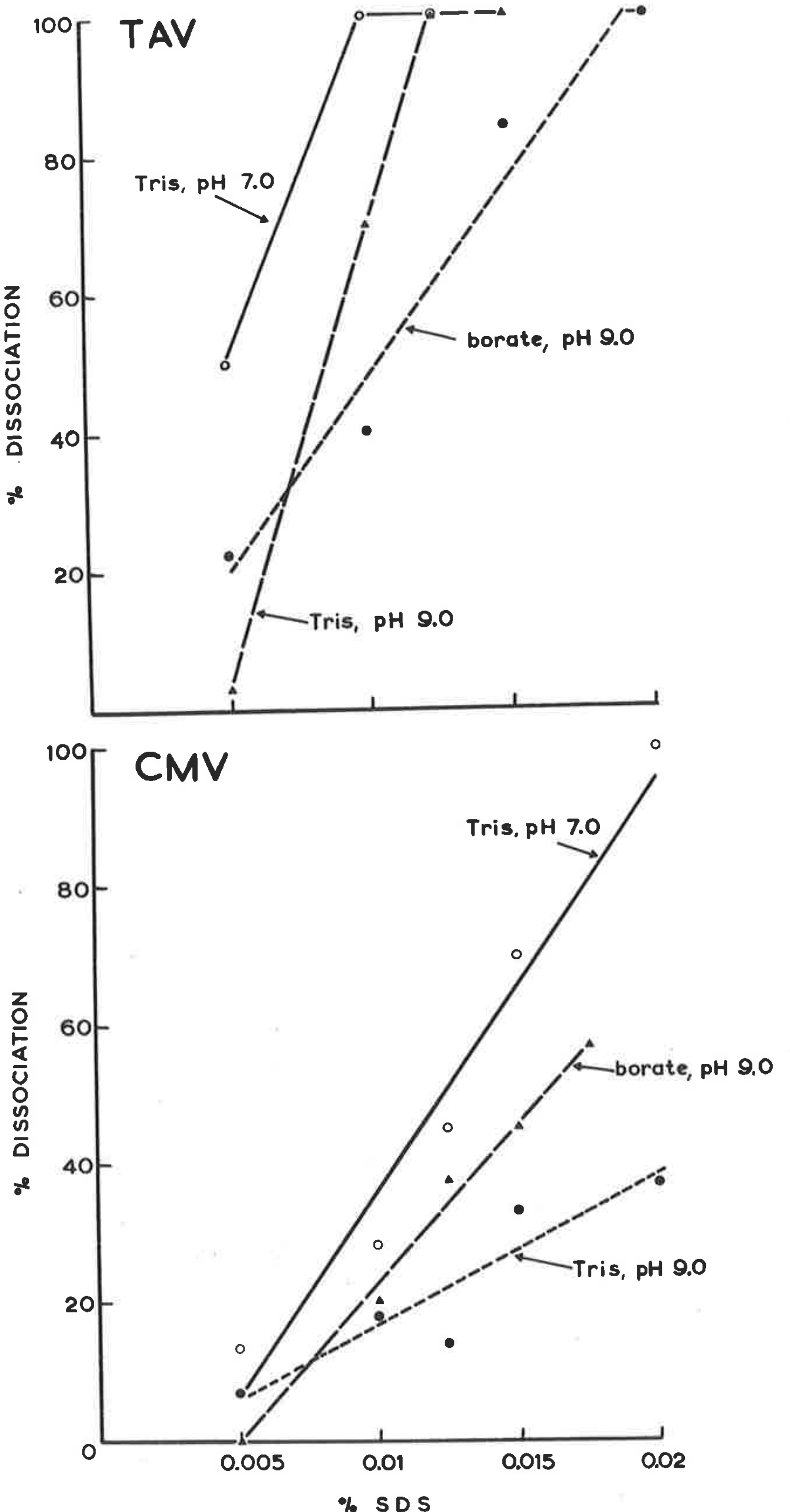
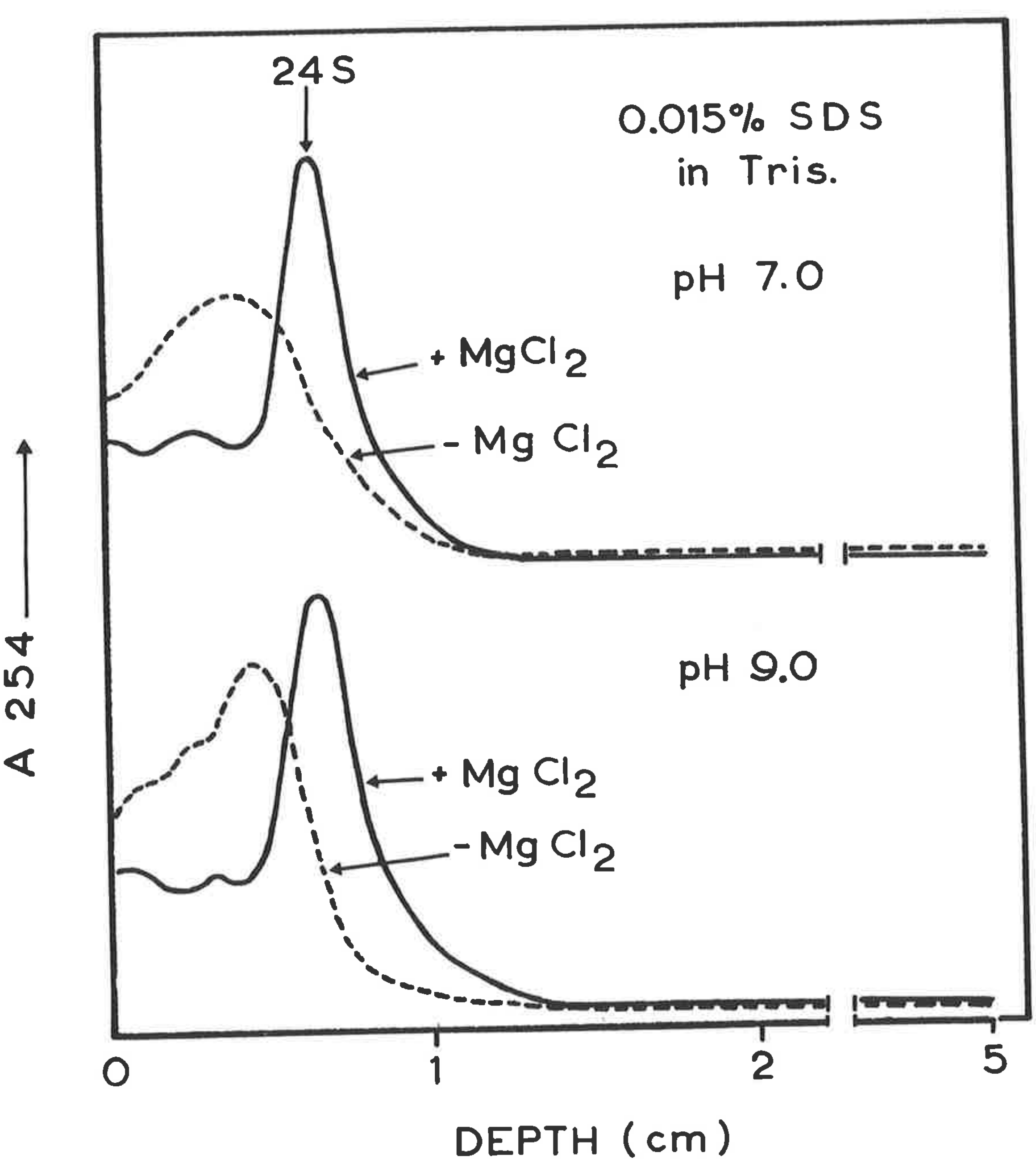

TAV
100
80
60
40
20
% DISSOCIATION
Tris, pH 7.0
borate, pH 9.0
Tris, pH 9.0
CMV
100
80
60
40
20
0
% DISSOCIATION
Tris, pH 7.0
borate, pH 9.0
Tris, pH 9.0
0.005
0.01
0.015
0.02
% SDS

Fig. 4-13. Sedimentation profiles of TAV dissociated with SDS in 20 mM Tris HCl, pH 7.0 and 9.0, in the presence and absence of 1 mM $MgCl_2$. Other conditions as described in Fig. 4-1.


24S
0.015% SDS
in Tris.
pH 7.0
+ MgCl2
- Mg Cl2
pH 9.0
+ Mg Cl2
- Mg Cl2
A 254
0
1
2
5
DEPTH (cm)

Mg^{2+} was similar at pH 7 and 9.

VI. Stabilization of the Capsids by Formaldehyde

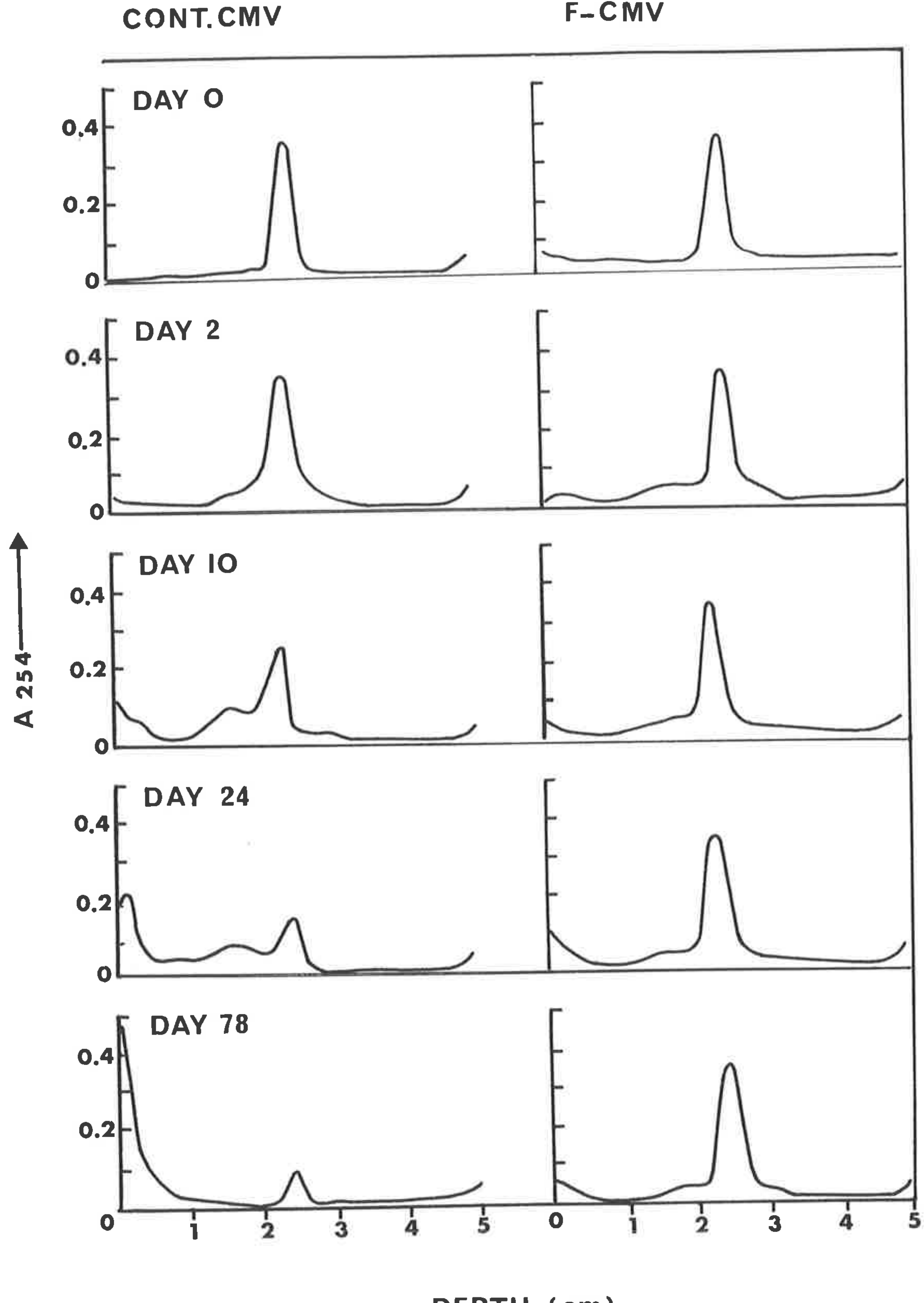
Studies on the stabilization of CMV capsid structure by formaldehyde have been reported previously (Francki and Habili, 1972). In subsequent experiments, CMV preparations were treated with formaldehyde and stored at 4° for up to 11 weeks. Samples analyzed in sucrose density-gradients at various time intervals showed no sign of degradation, although the control virus started to degrade within the first week of storage (Fig. 4-14).

To determine the optimum amount of formaldehyde required by the capsids of TAV and CMV to achieve maximum stability, the experiments summarized in Fig. 4-15 were carried out. At a concentration of about 10 mM formaldehyde each capsid was apparently saturated with H.CHO molecules and attained maximum stability. The fact that 100% stabilization of particles was not achieved, even with 0.1 M formaldehyde, could be due to errors involved in measuring the optical densities (see Methods). It is not known why CMV responded better to stabilization by formaldehyde (more than 90% of the particles being stabilized) than TAV (80% stabilization achieved) (Fig. 4-15).

Conclusions

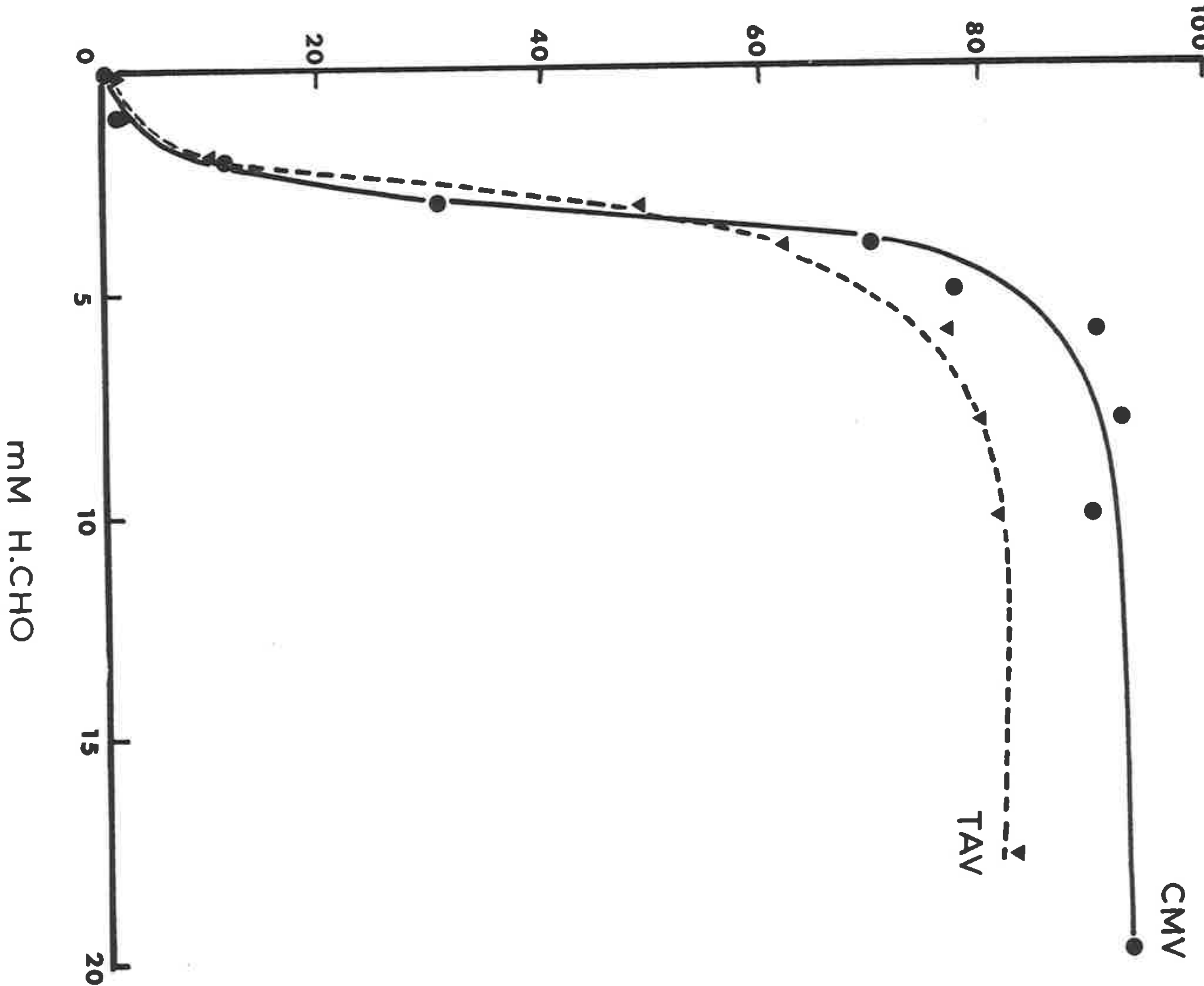
Although TAV and CMV are structurally similar in many respects, the conditions required for their stability differ considerably. TAV is

Fig. 4-14. Sedimentation profiles of normal CMV (cont·CMV) and the formaldehyde-treated virus (F-CMV) in sucrose density-gradients. The virus preparations in 5 mM borate buffer, pH 9.0, were incubated at 4°. At various times indicated, 50 µg of each sample was layered on the gradients and analysed as described in Fig. 4-1. CMV was purified by the method of Scott (1963) and treated with 0.2% formaldehyde as described in Chapter 2.



CONT.CMV
F-CMV
DAY 0
DAY 2
DAY 10
DAY 24
DAY 78
0.4
0.2
0
A 254
0
1
2
3
4
5
DEPTH (cm)

Fig. 4-15. Stabilization of TAV and CMV capsids in various concentrations of formaldehyde. The virus preparations were dialysed against different concentrations of formaldehyde (0-20 mM) in 20 mM phosphate buffer, pH 7.6, at 4° overnight. The percentage of particles stabilized was measured after incubation in 2 M LiCl and precipitation of RNA as described in Methods.



% STABILIZED PARTICLES
0
20
40
60
80
100
mM H.CHO
5
10
15
20
TAV
CMV

more resistant than CMV to degradation by pancreatic RNase and to precipitation by NaCl. Whereas TAV is stabilized by Mg^{2+}, CMV precipitates in the presence of the cation. CMV is stabilized, but TAV is degraded by EDTA. Both viruses are dissociated by low concentrations of SDS and can be stabilized by formaldehyde treatment indicating that their capsid structures depend on protein-RNA interactions.

Data presented in this Chapter may serve as an example to show that two viruses which are similar enough to be included in the same taxonomic group (Harrison *et al.*, 1971; Stace-Smith and Tremaine, 1973) require quite different conditions for maintaining their structural integrity.

CHAPTER 5

IN VITRO RECONSTITUTION

Introduction

Kaper (1969) dissociated the S strain of CMV in 2 M LiCl containing 2.5 mM Cleland reagent. When the dissociated preparation was dialysed against 20 mM Tris.HCl, 80 mM KCl, and 1 mM Cleland reagent, pH 7.2 (TKC), the virus was observed to reconstitute. Kaper and Geelen (1971), later reported that the reconstituted virus had physical and biological properties similar to those of normal virus.

As yet, the *in vitro* reconstitution of TAV has not been reported. Since the results summarized in the previous Chapter showed that TAV and CMV required different conditions for stability, it was thought that the requirements for reconstitution of the two viruses may differ. In this Chapter conditions required for TAV reconstitution have been investigated and compared to those reported by Kaper (1969) and Kaper and Geelen (1971) for CMV.

Methods

1. Dissociation of the viruses

TAV and CMV preparations at 4 mg/ml were mixed with equal volumes of a dissociating reagent consisting of 4 M LiCl, 10 mM Cleland

reagent and 20 mM Tris.HCl, pH 7.0. The mixture was frozen at -15° for at least 1 h, and thawed slowly to facilitate precipitation of RNA. Depending on the purpose of the experiment, the RNA and protein were either separated by centrifugation at 5,000 *g* for 10 min, or the dissociated mixture was directly used for the reconstitution. To ensure that the dissociation was complete, aliquots of separated RNA and protein were analysed by sucrose density gradient centrifugation. The lack of a virus peak indicated that the preparation was completely dissociated (see also Results).

2. Reassociation of protein and RNA

a. Dialysis buffers

Dissociated TAV and CMV preparations or stoichiometrically mixed protein and RNA (4 parts by weight of protein to 1 part of RNA) were dialysed against the following buffers:

1. TKC: 20 mM Tris-HCl, 80 mM KCl, 1 mM Cleland reagent, pH 7.2. Used by Kaper (1969) for the reconstitution of CMV.

2. TKM: 10 mM Tris.HCl, 10 mM KCl, 5 mM $MgCl_2$, pH 7.4. Used by Bancroft (1970) for the reconstitution of cowpea chlorotic mottle virus.

3. TKCM: 20 mM Tris.HCl, 50 mM KCl, 1 mM Cleland reagent, 1 mM $MgCl_2$, pH 7.2.

4. TC: 20 mM Tris.HCl, 1 mM Cleland reagent, pH 7.0 to 9.0. The effect of EDTA, $MgCl_2$ and KCl at various concentrations on the reconstitution was tested by adding these chemicals to this buffer where required.

b. Reconstitution method

The dissociated virus preparations or mixture of protein and RNA in 0.5 M LiCl and 2.5 mM Cleland reagent (final nucleoprotein concentration was 1 mg/ml) were dialysed against the above buffers overnight at 4°, with gentle shaking. The shaking was found to be necessary to prevent nonspecific precipitation of RNA and protein. Aliquots of reconstituted virus were then analysed by sucrose density-gradient centrifugation as described in Chapter 2.

3. Stability of the viruses to RNase

Normal and reconstituted TAV (R-TAV) were isolated by centrifugation in sucrose density-gradients and the concentrations were adjusted to 1 mg/ml in 20 mM phosphate buffer, pH 7.6. Pancreatic RNase at various concentrations was added to the preparations and incubated at required temperatures, depending on the purpose of experiment.

Results

I. Reconstitution of TAV

1. Absence of orderly assembled material after dialysing TAV protein and RNA separately

No reconstituted material sedimenting at the rate of normal virus was detected in sucrose gradients when either TAV protein or TAV-RNA were dialysed alone against TKC or TKM buffer (Fig. 5-1).

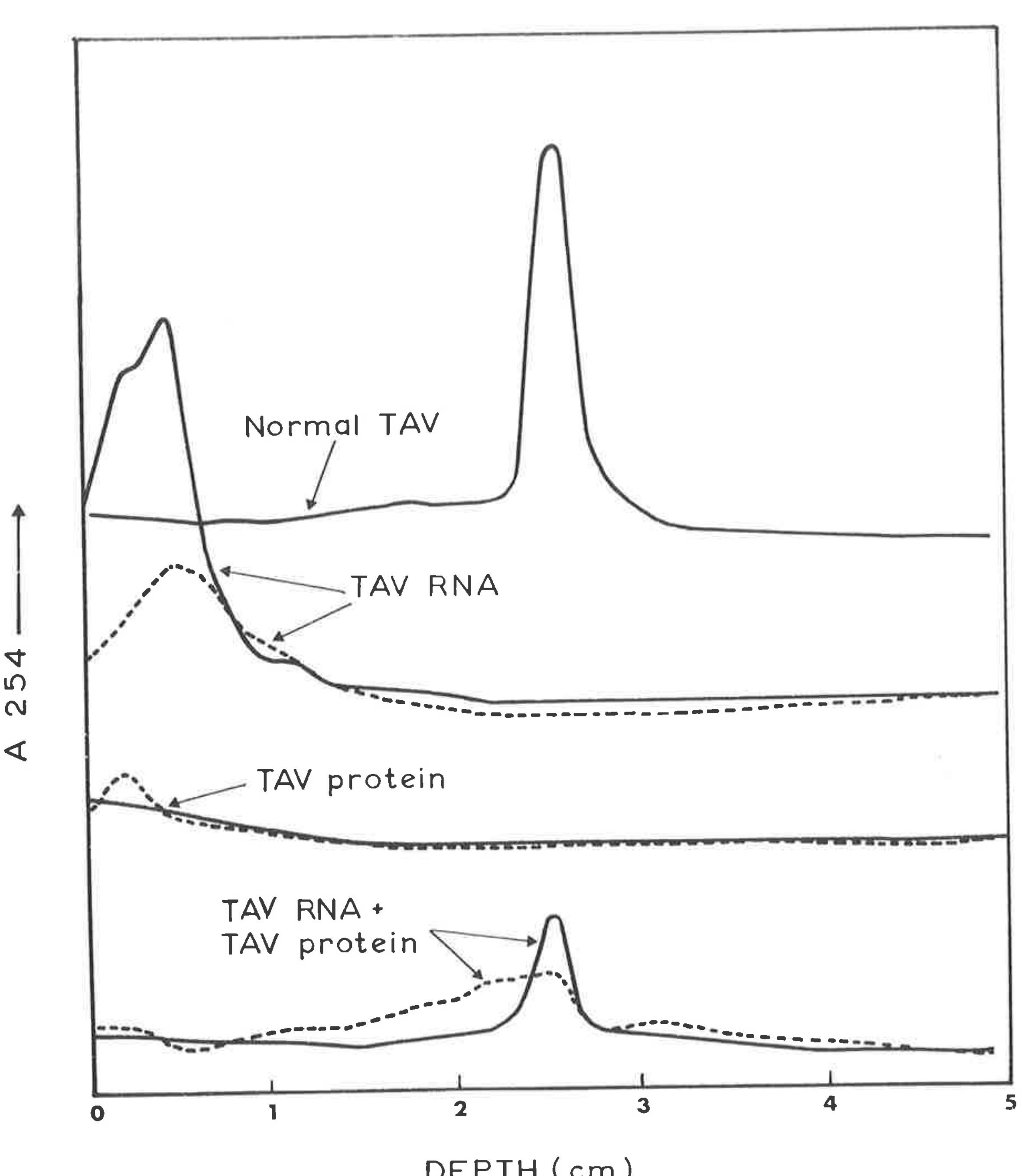
2. Effect of two kinds of buffers on the efficiency of TAV reconstitution

The stoichiometrically mixed protein and RNA of TAV were dialysed against TKC and TKM buffers. The results summarized in Fig. 5-1 show that TAV reconstituted more efficiently in TKC than in TKM buffer. The poor efficiency of reconstitution of TAV in TKM buffer may be due to the absence of Cleland reagent.

3. Effect of pH on TAV reconstitution

Dissociated TAV preparations were dialysed against TC buffer at pH 7.0 and 9.0, and the product was subjected to centrifugation in sucrose density-gradients. The reconstituted virus appeared to be more homogeneous in sedimentation behaviour at pH 7.0 than pH 9.0 (Fig. 5-2a). In the electron microscope the nucleoproteins produced

Fig. 5-1. Sedimentation profiles of TAV-RNA, TAV protein and reconstituted virus on sucrose density gradients. TAV was dissociated to protein and RNA in 2 M LiCl as described in Methods. The RNA was sedimented by a low speed centrifugation (5,000 *g* for 10 min) and resuspended in water. Protein and RNA alone, or after being mixed stoichiometrically were dialysed against TKC (———) and TKM (— — —) buffers, at 4°, overnight and analysed as described in Fig. 4-1.



Normal TAV
TAV RNA
TAV protein
TAV RNA +
TAV protein
A 254
0
1
2
3
4
5
DEPTH (cm)

from dialysis at pH 7.0 appeared more homogeneous in size than those reconstituted at pH 9.0.

4. Reconstitution in the presence of Mg^{2+}

When TAV was reconstituted in the presence of 1 mM $MgCl_2$, the product was more homogeneous than in the absence of Mg^{2+} (Fig. 5-2a and b). With the cation, a more homogeneous product was obtained at pH 9.0 than at pH 7.0 (Fig. 5-2b). The results summarized in Table 5-1 and Fig. 5-3 indicate that Mg^{2+} is required for efficient reconstitution of TAV and best results are obtained with 1 mM $MgCl_2$ resulting in over 40% reconstitution.

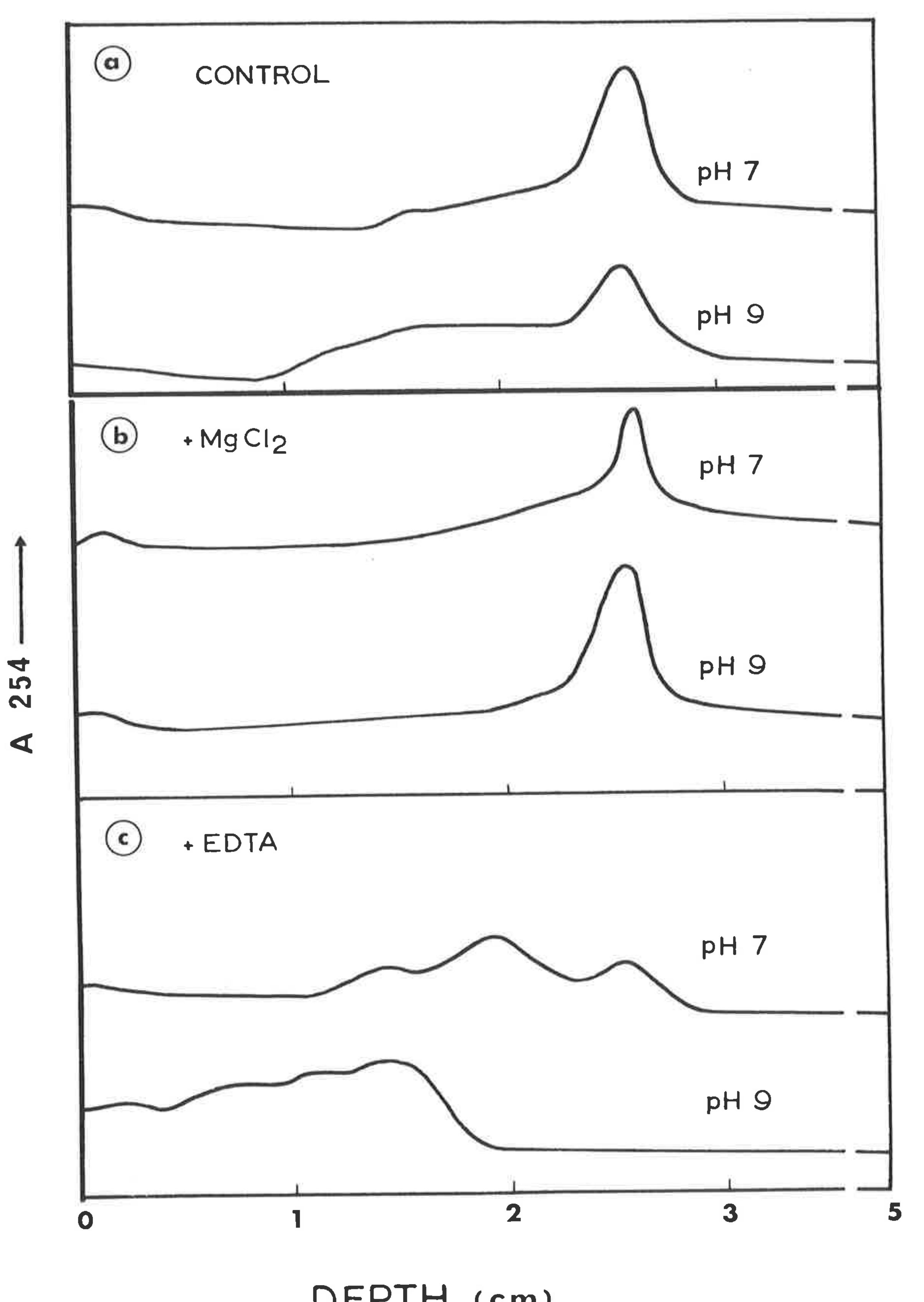
5. Reconstitution in the presence of EDTA

TAV was reconstituted in TC buffer containing 1 mM EDTA. At pH 7.0, heterogeneous material was observed. When the pH was increased to 9.0 no reconstituted material could be recovered in the region expected for TAV (Fig. 5-2c).

6. Reconstitution of TAV in the presence of different KCl concentrations

In early experiments, the dissociated TAV was routinely dialysed against TC buffer. When KCl was added to the buffer at pH 7.2, more reconstituted virus was obtained. The effect of KCl concentration between 50 mM and 0.15 M was examined. It was found that

Fig. 5-2. Sedimentation profiles of TAV reconstituted by dialysis against: 20 mM Tris HCl, 1 mM Cleland reagent, pH 7 and 9 (a); the same as (a) containing 1 mM $MgCl_2$ (b); and the same as (a) containing 1 mM EDTA (c). The samples were analysed as described in Fig. 4-1.



a
CONTROL
pH 7
pH 9
b
+ MgCl2
pH 7
pH 9
c
+ EDTA
pH 7
pH 9
A 254
0
1
2
3
5
DEPTH (cm)

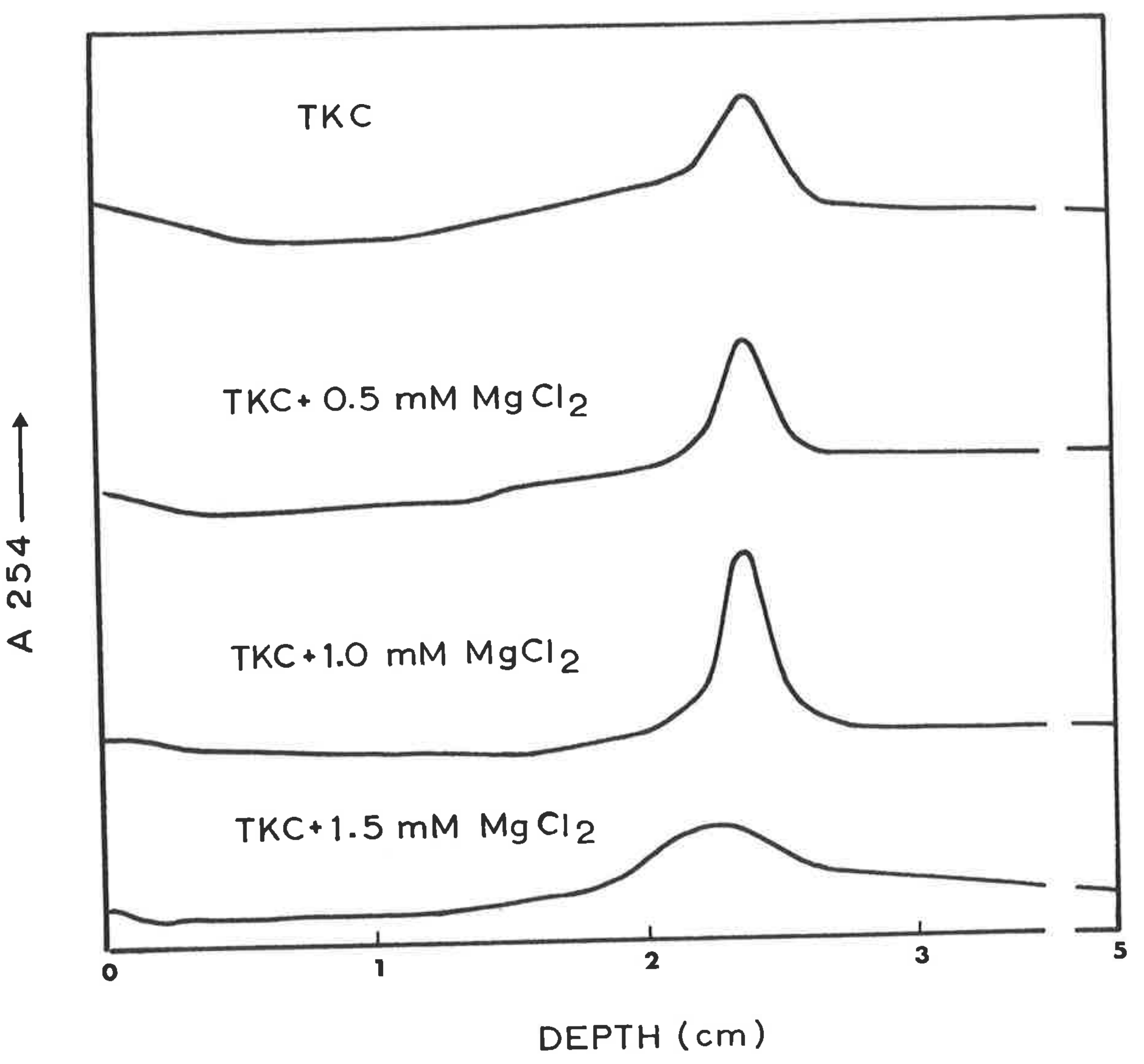
Table 5-1. Effect of $MgCl_2$ at various concentrations on the *in vitro* reconstitution of TAV.

mM $MgCl_2$[a] added	% Virus[b] reconstituted
0	33.4
0.1	37.4
1.0	41.5
10.0	18.8
100.0	0

a $MgCl_2$ was added to the dialysis buffer consisting of 20 mM Tris-HCl, 50 mM KCl, and 1 mM Cleland reagent, pH 7.2.

b Determined by the difference in areas under the ISCO traces of normal and reconstituted virus.

Fig. 5-3. Sedimentation profiles of reconstituted TAV resulted from dialysing the mixture of protein and RNA, at 4°, against TKC (20 mM Tris HCl, 80 mM KCl, 1 mM Cleland reagent, pH 7.2) and TKC containing 0.5, 1.0, and 1.5 mM $MgCl_2$.


TKC
TKC+ 0.5 mM MgCl2
TKC+1.0 mM MgCl2
TKC+1.5 mM MgCl2
A 254
0
1
2
3
5
DEPTH (cm)

the efficiency of the reconstitution was not affected by the range of KCl concentrations tested. However, normal virus started to precipitate in the presence of salt concentrations higher than 0.1 M as was previously observed (Chapter 4). Therefore, the use of high KCl concentrations was avoided.

7. Electron microscopy of R-TAV

TAV was reconstituted in the presence of TKCM buffer and observed in the electron microscope. The size and morphology of TAV and R-TAV were indistinguishable (Fig. 5-4).

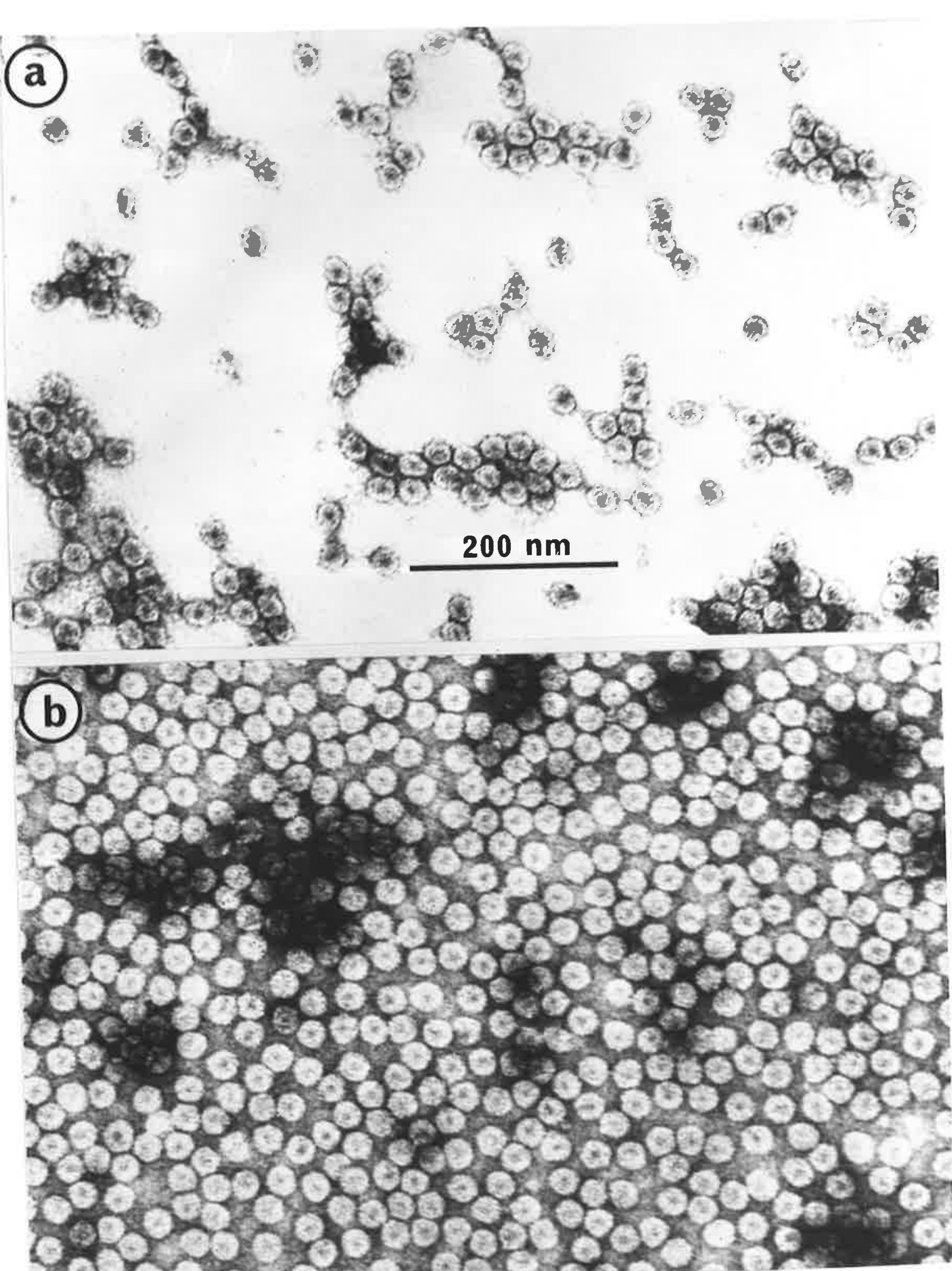
8. Infectivity of R-TAV

TAV was reconstituted by dialysis against TKCM buffer and the product was subjected to centrifugation in sucrose gradients. The material under the peak (R-TAV) was recovered and diluted to 50 µg/ml for infectivity assay. Most preparations of R-TAV had specific infectivity not significantly lower than that of normal virus. In a typical experiment R-TAV (50 µg) produced an average of 75 lesions per half-leaf of *C. amaranticolor* as compared to 84 produced by the same concentration of normal virus.

9. Comparison between RNA electrophoretic patterns of TAV and R-TAV

RNA preparations from R-TAV and TAV were analysed by PAG electrophoresis. The results summarized in Table 5-2 show that T_4 was not detected in RNA preparations of the reconstituted virus. The

Fig. 5-4. Electron micrographs of particles of R-TAV isolated from sucrose density-gradients (a), and normal TAV (b), stained with 2% uranyl acetate. The reconstituted virus was obtained by dialysing stoichiometrically mixed protein and RNA preparations against TKCM buffer as described in the Methods of this Chapter.


a
200 nm
b

results suggest that T_4 is probably not encapsidated in R-TAV.

10. RNase sensitivity of R-TAV

The results of an experiment in which normal virus and R-TAV were treated with RNase and subjected to sucrose density gradients are shown in Fig. 5-5. It appears that R-TAV is only slightly more susceptible to the enzyme than normal virus.

In a subsequent experiment, TAV, R-TAV and TAV-RNA were subjected to digestion with 2 μg/ml RNase at 37° for 10 min. The RNA was extracted from each sample and analysed by PAG electrophoresis. The electrophoretic patterns of RNA isolated from RNase-treated R-TAV were similar to those of untreated R-TAV. The same results were obtained with normal virus.

The infectivity of TAV, reconstituted TAV, and TAV-RNA after incubation with various concentrations of RNase was compared (Table 5-3). The results indicate that in the reconstituted virus, TAV-RNA is protected by the coat protein against the enzyme attack, although the protection provided is not as efficient as that of normal virus (Table 5-3).

II. Comparison Between Reconstitution of TAV and CMV

1. Reconstitution of CMV in the presence of Mg^{2+}

The reconstitution of the S strain of CMV in the presence of

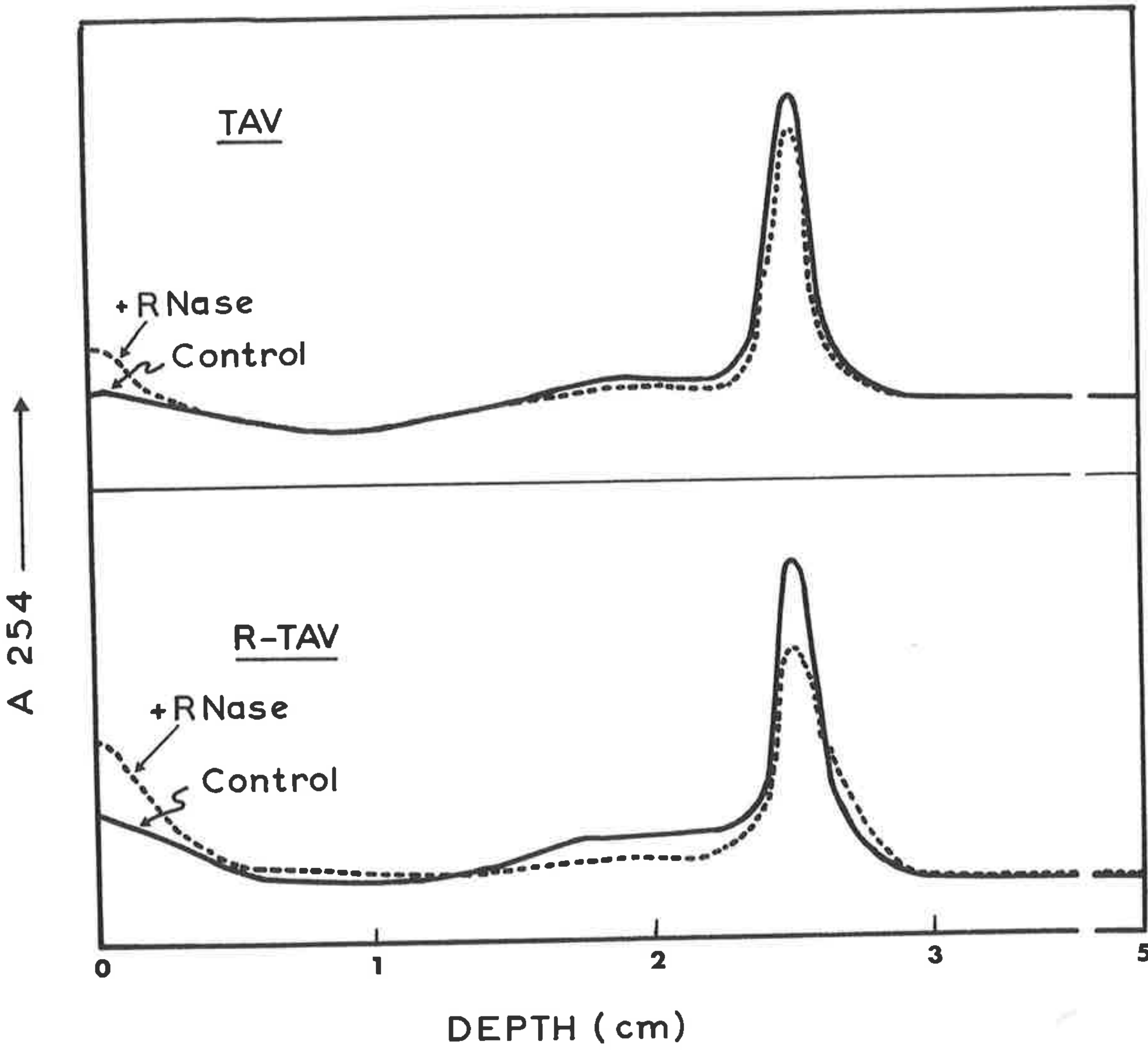
Table 5-2. Comparison between relative amounts of various RNA species in normal and reconstituted TAV[a].

	RNA species	Relative amount of RNA species	
		TAV-RNA	R-TAV-RNA
Experiment 1[b]	1+2	1.00	1.00
	3	0.19	0.22
	4	0.10	0
Experiment 2	1+2	1.00	1.00
	3	0.24	0.28
	4	0.04	0

a R-TAV was obtained from dialysis against TKCM buffer (see Methods). The preparation was subjected to sucrose density gradient centrifugation to separate unassembled RNA and protein. RNA isolated from normal virus and R-TAV was analysed by PAG electrophoresis. The gels were stained and scanned in a densitometer. Area under the peaks of RNA 3 and RNA 4 relative to that of species 1+2 was measured.

b In each experiment the RNA preparations were analysed in duplicates.

Fig. 5-5. Sedimentation profiles of RNase treated (— — —) and untreated (———) TAV and R-TAV. Virus preparations at 1 mg/ml in 20 mM phosphate buffer, pH 7.6, were incubated at 20° for 30 min in the presence and absence of 10 μg/ml pancreatic RNase and analysed as described in Fig. 4-1.



TAV
+RNase
Control
R-TAV
+RNase
Control
A 254
0
1
2
3
5
DEPTH (cm)

Table 5-3. Infectivity of reconstituted TAV, TAV, and TAV-RNA before and after treatment with RNase[a].

RNase concentration (μg/ml)	Reconstituted TAV		TAV		TAV-RNA	
	Control	+RNase	Control	+RNase	Control	+RNase
1	254[b]	0	34	0	130	0
0.1	251	1	135	2	75	0
0.01	129	60 (47%)[c]	137	119 (87%)	102	0 (0%)

a 50 μg/ml of each nucleoprotein (or 10 μg/ml of RNA) in 20 mM phosphate buffer, pH 7.6, was incubated with or without RNase at 0° for 1 h before inoculation.

b Average of local lesions in four half-leaves of *C. amaranticolor*.

c Percentage infectivity retained.

Mg^{2+} was not examined by Kaper (1969) or Kaper and Geelen (1971). In this work, when CMV was reconstituted in the presence of the cation some aggregation of the product was detected (Fig. 5-6).

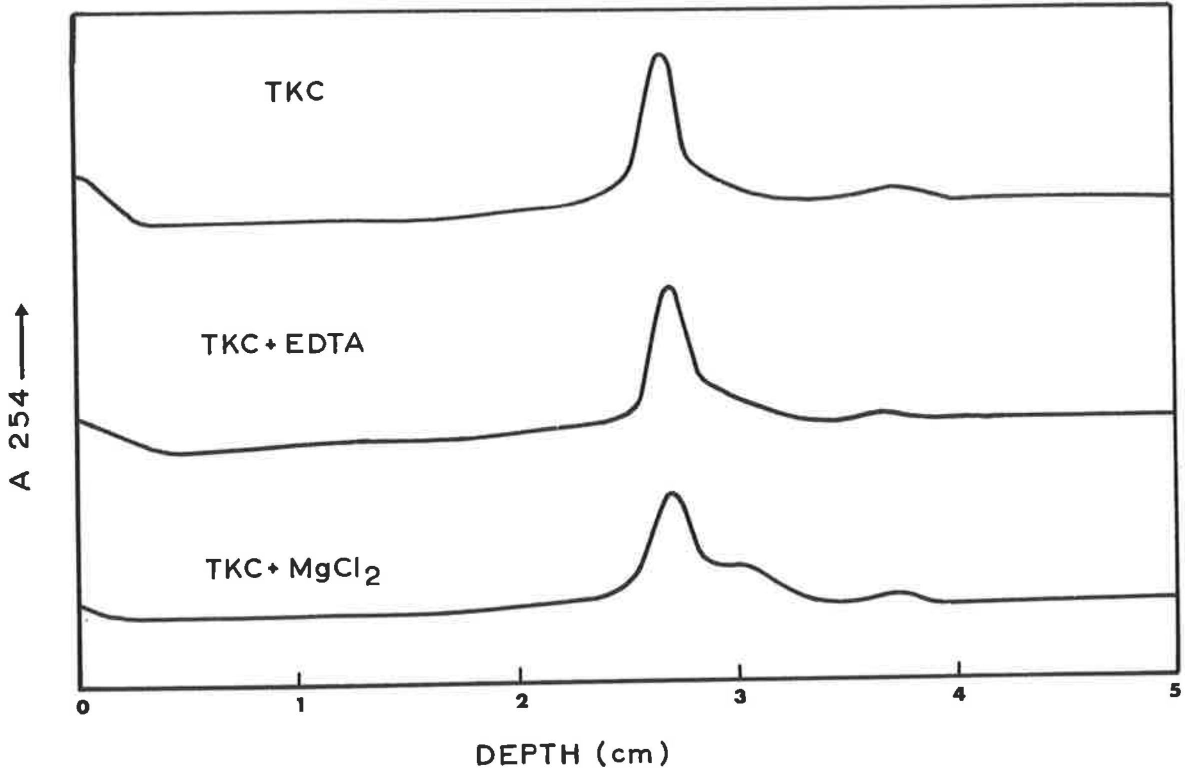
2. Reconstitution of CMV in the presence of EDTA

The effect of EDTA on the reconstitution of CMV has not been reported. CMV was reconstituted in TKC buffer in the presence of 1 mM EDTA. The efficiency of the reconstitution was similar in the presence and absence of EDTA (Fig. 5-6).

3. Homologous and heterologous reconstitution of TAV and CMV

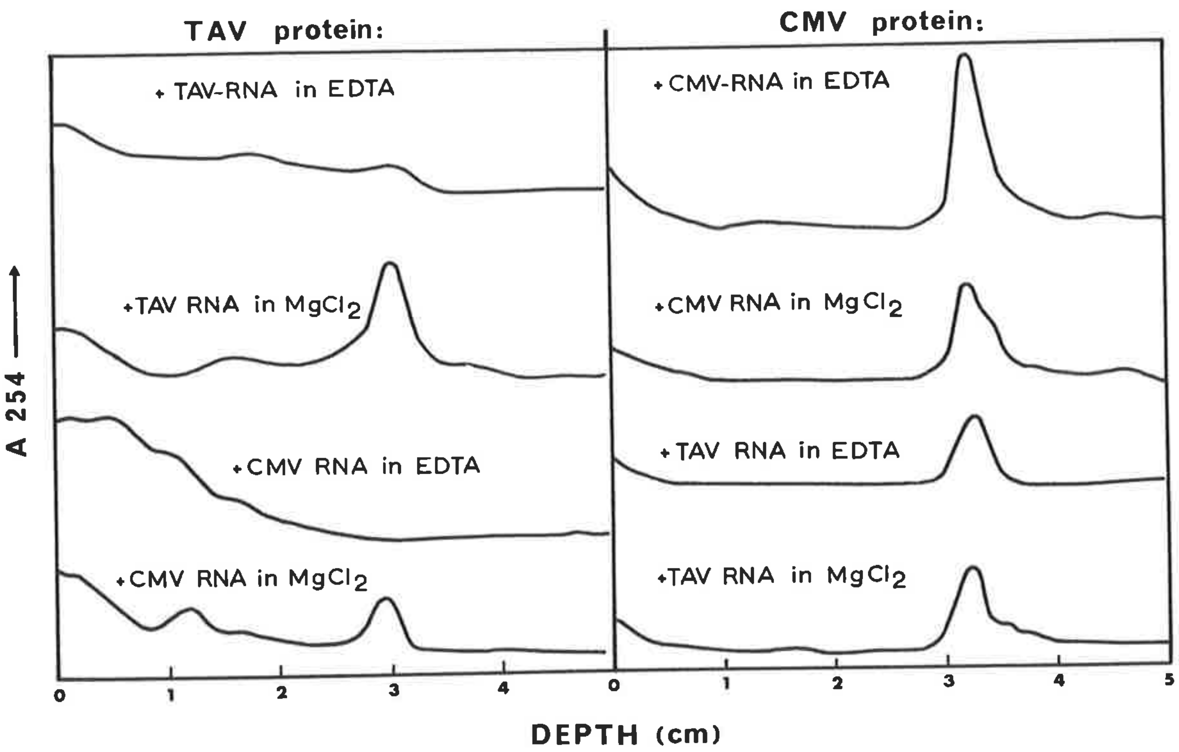
Reconstitution was carried out by mixing TAV protein with CMV-RNA and CMV protein with TAV-RNA in TKC buffer in the presence of Mg^{2+} and EDTA. Results of homologous and heterologous reconstitution are summarized in Fig. 5-7. It appears that with TAV protein, particles with sedimentation properties of virus were produced only when Mg^{2+} was present, and that the reconstitution was more efficient with homologous than with heterologous RNA. However, with CMV protein, particles were reconstituted in the presence of either Mg^{2+} or EDTA. The reconstitution process was more efficient with homologous RNA in the presence of EDTA. The results indicate that the requirement of Mg^{2+} and EDTA in virus reconstitution is determined by the virus protein rather than RNA.

Fig. 5-6. Sedimentation profiles of CMV reconstituted in TKC (20 mM Tris HCl, 50 mM KCl, 1 mM Cleland reagent, pH 7.2), TKC containing 1 mM EDTA, and TKC containing 1 mM $MgCl_2$.



TKC
TKC + EDTA
TKC + MgCl2
A 254
0
1
2
3
4
5
DEPTH (cm)

Fig. 5-7. Sedimentation profiles of TAV and CMV reconstituted by mixing protein and RNA of each virus homologously or heterologously. Protein and RNA of TAV and CMV were mixed stoichiometrically and dialysed against TKC buffer containing 1 mM $MgCl_2$ or 1 mM EDTA.



TAV protein:
CMV protein:
+ TAV-RNA in EDTA
+TAV RNA in MgCl2
+CMV RNA in EDTA
+CMV RNA in MgCl2
+ CMV-RNA in EDTA
+CMV RNA in MgCl2
+TAV RNA in EDTA
+TAV RNA in MgCl2
A 254
0
1
2
3
4
5
DEPTH (cm)

Conclusions

The results reported in this Chapter confirm those obtained in Chapter 4, that the requirement for capsid integrity of TAV and CMV differs considerably. Mg^{2+} enhances the efficiency of reconstitution of TAV and in this respect TAV is similar to the bromoviruses (Bancroft, 1970). The relatively efficient reconstitution of TAV in the absence of Mg^{2+} (Table 5-1) may be due to traces of divalent cations in the reconstitution buffer. Inhibition of the process by EDTA would support this conclusion. Unlike bromoviruses (Bancroft, 1970), TAV cannot produce empty protein shells *in vitro* (Fig. 5-1).

Although the reconstitution of CMV does not seem to be inhibited by Mg^{2+}, aggregated material was observed in sucrose gradients. Reconstitution of CMV in the presence of EDTA appears to be very efficient. Hull (1970) observed that the reconstitution of alfalfa mosaic virus proceeded normally in the presence of EDTA.

CHAPTER 6

SEROLOGY

Introduction

It is generally agreed that CMV is a poor immunogen (Francki *et al.*, 1966; Scott, 1968), whereas TAV has been claimed to be both a good (Hollings and Stone, 1971) and a poor immunogen (Stace-Smith and Tremaine, 1973). The results of Chapter 4 suggest that TAV is more stable than CMV to salt, heat, and RNase treatments and hence it could be expected to be a better immunogen.

In this Chapter the immunogenicity of CMV was compared on the one hand to TAV, and on the other to TMV, which is known to be a good immunogen (Rappaport, 1965). Antisera to TAV and CMV were used to study the antigenicity of the two viruses and to test for a serological relationship between them.

Methods

1. Animals and immunization

Immunogenicity of viral antigens was studied in an outbred strain of Swiss mice. In all experiments five mice of the same sex were used for each treatment and kept in 28 x 16 x 10 cm cages in a

ventilated room at temperatures ranging between 20° and 30°. Adult animals, weighing between 20 to 30 g were injected intraperitoneally with purified antigens. When adjuvant was used, the antigen in 0.1 ml buffer was emulsified with an equal volume of Freund's complete adjuvant. Blood samples (0.05 - 0.5 ml) were collected by puncturing the retro-orbital plexus with a Pasteur pipette and the blood was transferred to centrifuge tubes. Both tubes and pipettes were heparinized by allowing a drop of heparin solution (2 mg/ml) to dry in them at 100° to prevent the blood clotting. In this way the maximum volume of serum (up to 0.2 ml) was recovered. The blood samples were centrifuged at 2,000 *g* for 20 min, and the sera were stored at -15°.

Toads (*Bufo marinus*) collected in the field at Innisfail, Queensland, were also immunized with viral antigens. The animals were kept in either moist wood shavings or a water bath at the required temperatures and force-fed with live grasshoppers. A total of 0.2 ml antigen (200 μg) emulsified in adjuvant was administered intraperitoneally. The animals were bled by heart puncture using a 1-ml syringe with a 23 gauge needle. The sera were isolated as described above.

2. Assay of precipitating antibodies

Antisera were titrated by preparing two-fold dilution series in 20 mM phosphate buffer, pH 7.2, and testing against antigens at a concentration of 200 μg/ml. In the case of TMV the virus was sonicated

for 10 min at 0° so as to fragment the elongated particles to enhance diffusion through agar (Tomlinson and Walkley, 1967). Unless otherwise stated, experimental results are expressed as geometric mean titres, the integers 1, 2, 3, ..., corresponding to a visible precipitin line being observed at antiserum dilution of 1/2, 1/4, 1/8, ... Statistical analyses were carried out using Kolmogarov-Smirnov two-sample tests (Siegel, 1956).

To study the serological relationship between TAV and CMV, the antigens at 1 mg/ml were reacted against homologous antisera (in 50% glycerol) as well as antisera to other virus isolates obtained from other workers. Extracts from healthy tobacco and cucumber tissues were tested in the gels as control antigens.

3. Preparation and serological assay of TAV and CMV proteins

Protein was isolated from each virus by its dissociation in 2 M LiCl. The RNA was sedimented at 5,000 *g* for 10 min. The supernatant, containing mainly protein, was centrifuged at 220,000 *g* for 1 h to sediment any undissociated virus particles. The protein preparations were dialysed exhaustively against phosphate-buffered saline (20 mM sodium phosphate, 0.14 M NaCl, pH 7.6), at 4°, overnight. They were diluted in the same buffer to a concentration of 1 mg/ml and stored at -15°. To induce an immune response in mice 0.1 ml of

a protein preparation was emulsified with an equal volume of adjuvant and injected intraperitoneally. The serological assays were done as described for the viral antigens, except that dilution series were made in buffered saline.

4. Separation of IgG and IgM antibodies

Anti-TAV and anti-CMV sera obtained from several mice were pooled and the antibodies were concentrated by precipitation with ammonium sulphate as described by Spendlove (1967). One volume of saturated ammonium sulphate (0.76 g ammonium sulphate in 1 ml water) neutralized with NaOH was added drop-wise to ice-cooled serum, shaken, and left on ice for 1 h. The precipitate was then centrifuged at 5,000 *g* for 15 min. The pellet was resuspended in distilled water and the salt precipitation was repeated twice more; the final pellet was resuspended in the required volume of water and dialysed against phosphate-buffered saline. The preparation was centrifuged in 10-40% sucrose density-gradients in the buffered saline using either a SW 50.1 rotor at 48,000 rpm for 9.5 h, or a SW 41 rotor at 36,000 rpm for 12 h. The gradients were scanned at 254 nm and fractionated with an ISCO density gradient fractionator. The IgG and IgM fractions were pooled and either used directly or concentrated as above before testing by immunodiffusion.

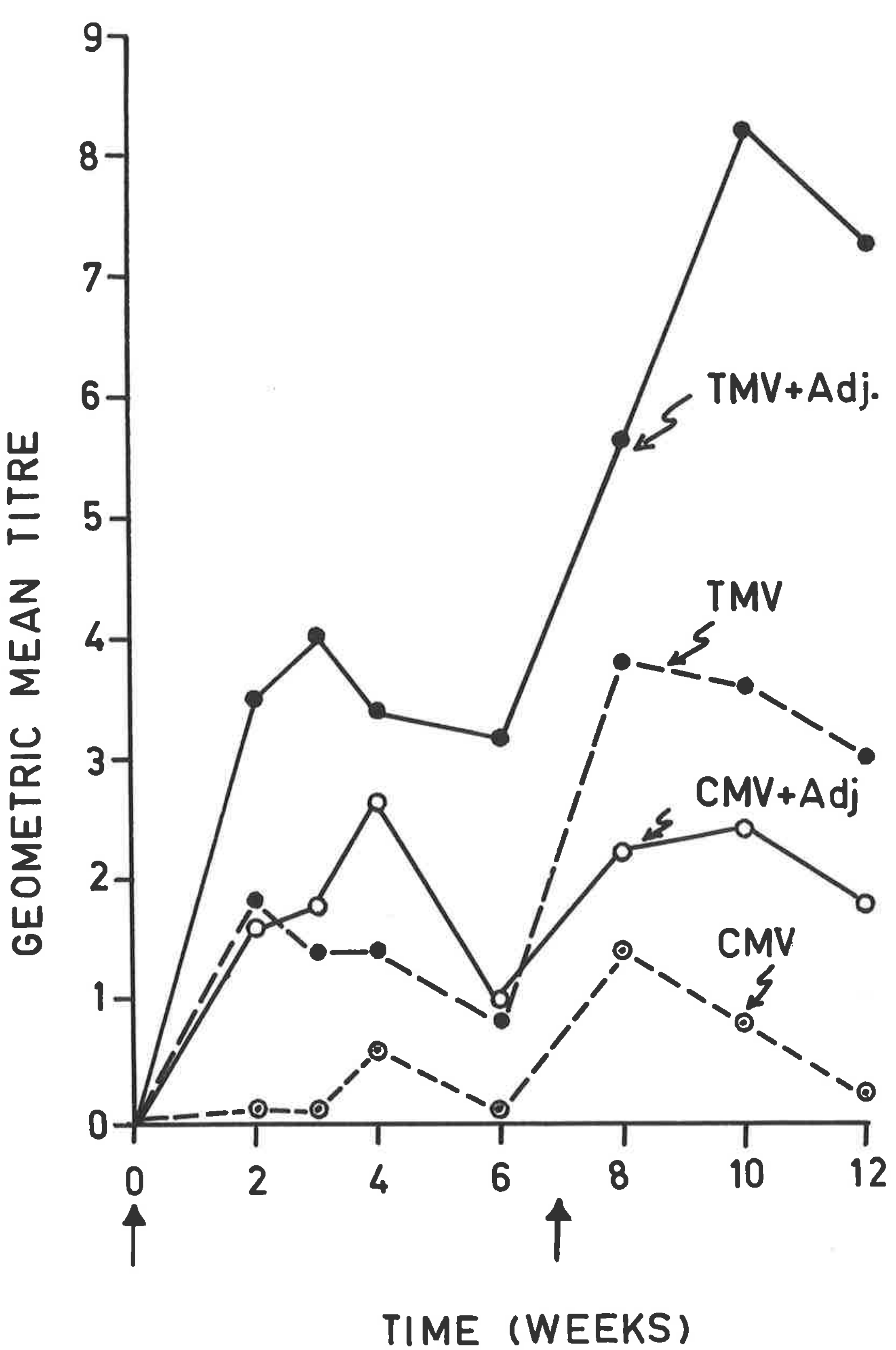
Results

I. Relative Immunogenicity of CMV and TMV

Groups of mice were immunized with CMV and TMV, and the responses of animals to the viral antigens were analysed quantitatively. The results in Fig. 6-1 show that TMV is far more immunogenic than CMV. Statistical analyses of the results showed significant differences ($P < 0.01$) between the titre of sera to CMV and TMV whether injected with or without adjuvant. The differences in the response to both antigens were significantly greater ($P < 0.01$) when injected in the presence of adjuvant. In another experiment mice were injected three times at weekly intervals with a mixture of CMV and TMV containing 50 µg of each antigen per injection. No detectable response to CMV was observed in any of the mice, whereas a geometric mean titre of 7.2 was obtained with TMV.

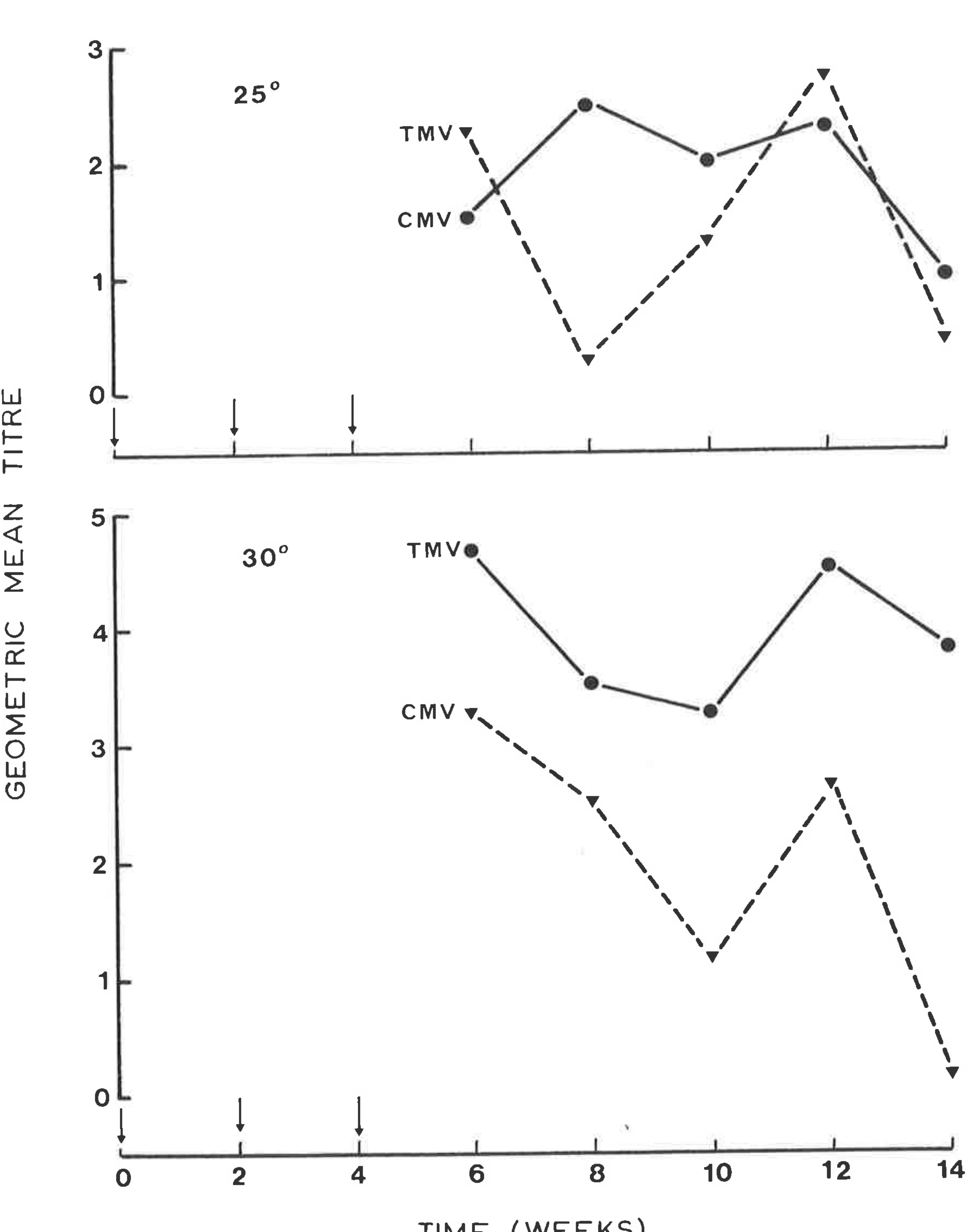
To study the effect of temperature on the immunogenicity of CMV, two groups of toads were immunized with an equal mixture of CMV and TMV. One group was kept at 25° and the other at 30°. There was no significant difference between the immune response of CMV and TMV at 25° ($P > 0.05$) (Fig. 6-2). However, at 30° the response to TMV was significantly higher ($P < 0.01$) than to CMV (Fig. 6-2). The results suggest that at 30° CMV induces less antibody response than at 25°.

Fig. 6-1. The immune response of mice to TMV and CMV injected in the presence (+ Adj) and absence of adjuvant. All mice were injected at the commencement of the experiment and seven weeks later (indicated by vertical arrows), each time with 20 μg of antigen per animal.



GEOMETRIC MEAN TITRE
9
8
7
6
5
4
3
2
1
0
TMV+Adj.
TMV
CMV+Adj
CMV
0
2
4
6
8
10
12
TIME (WEEKS)

Fig. 6-2. The immune response of toads (8 animals per treatment) to a mixture of TMV and CMV injected simultaneously. TMV (250 μg) and CMV (250 μg) in 0.1 ml buffer were emulsified in adjuvant (0.1 ml) and injected at the times indicated by arrows. The groups of animals were kept at 25° and 30°, and bled by heart puncture. The sera were titrated as described in Methods.



GEOMETRIC MEAN TITRE
25°
TMV
CMV
30°
TMV
CMV
3
2
1
0
5
4
3
2
1
0
0
2
4
6
8
10
12
14
TIME (WEEKS)

Fig. 6-3. The immune response of toads (5 animals per treatment) to TAV and CMV. Each viral antigen (200 μg) in 0.1 ml buffer was emulsified with an equal volume of adjuvant and injected as shown by arrows. The animals were kept at 28° and the sera were collected and analysed as described in Methods.

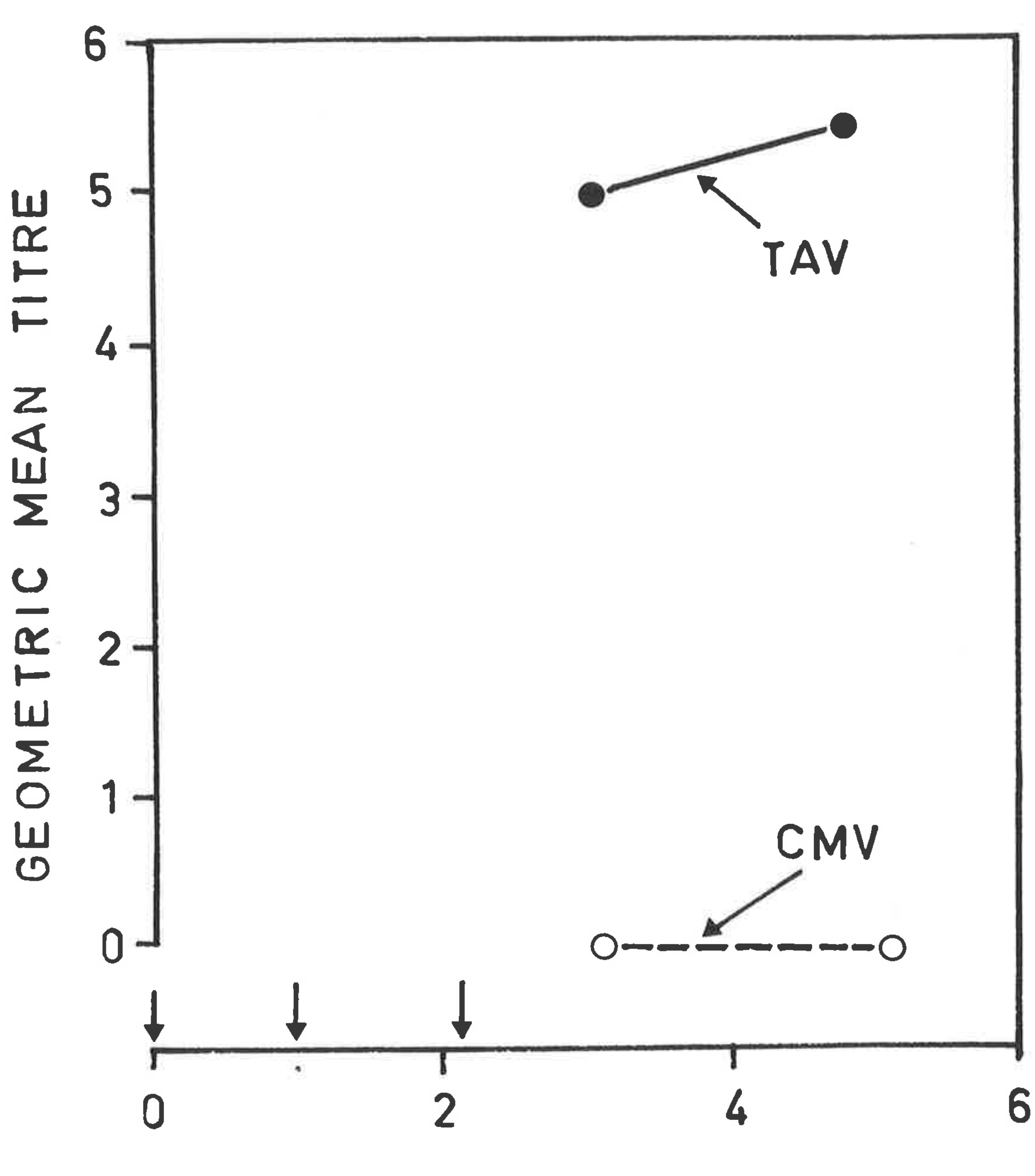

GEOMETRIC MEAN TITRE
6
5
4
3
2
1
0
TAV
CMV
0
2
4
6
TIME (WEEKS)

Fig. 6-4. The immune response of mice to a mixture of TAV and CMV injected simultaneously. TAV preparations at the quantities indicated (5 to 40 µg) were mixed with equal amounts of CMV and injected in the presence of adjuvant at the times indicated by arrows. Titration of each serum against TAV (- - -) and CMV (———) was made simultaneously as described in Methods.

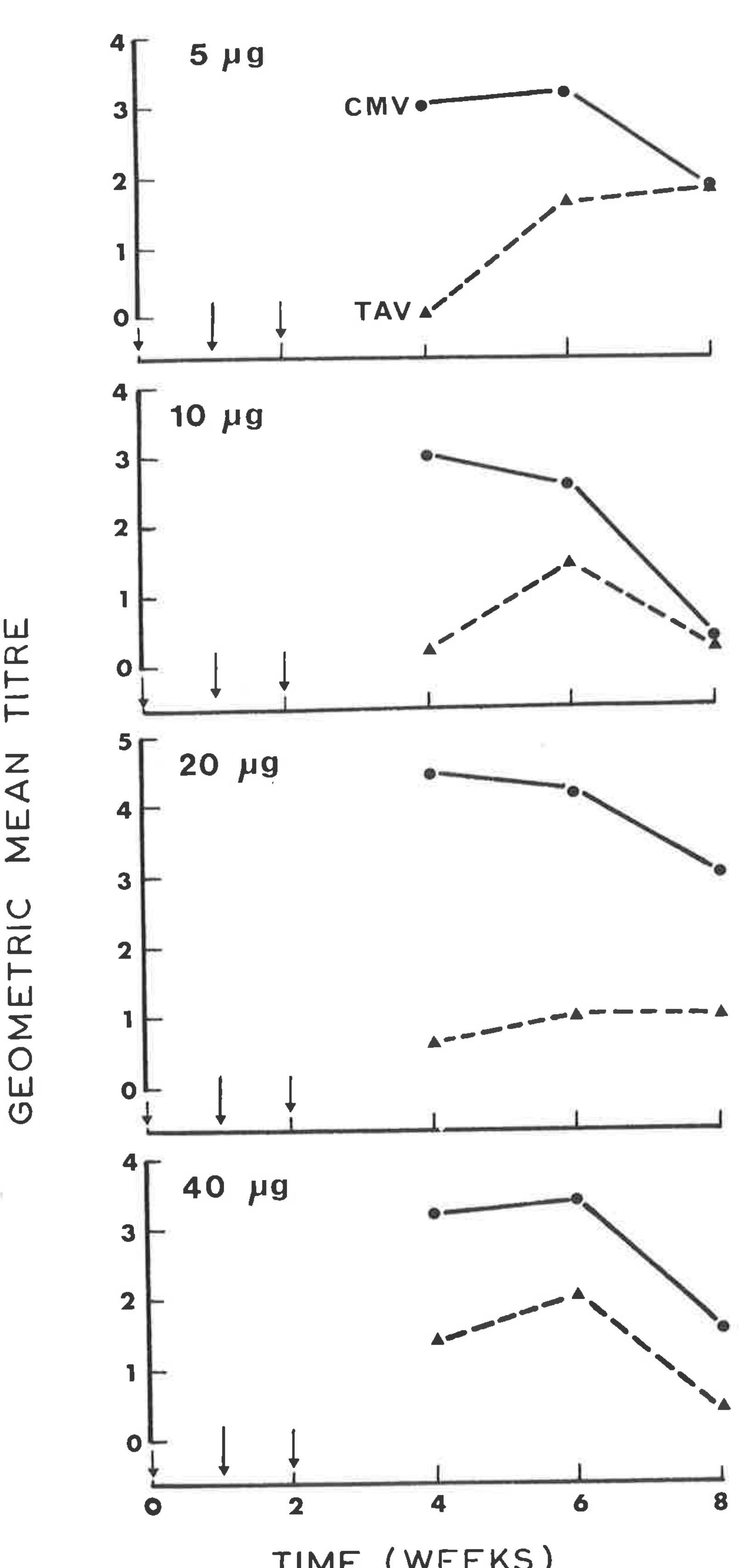
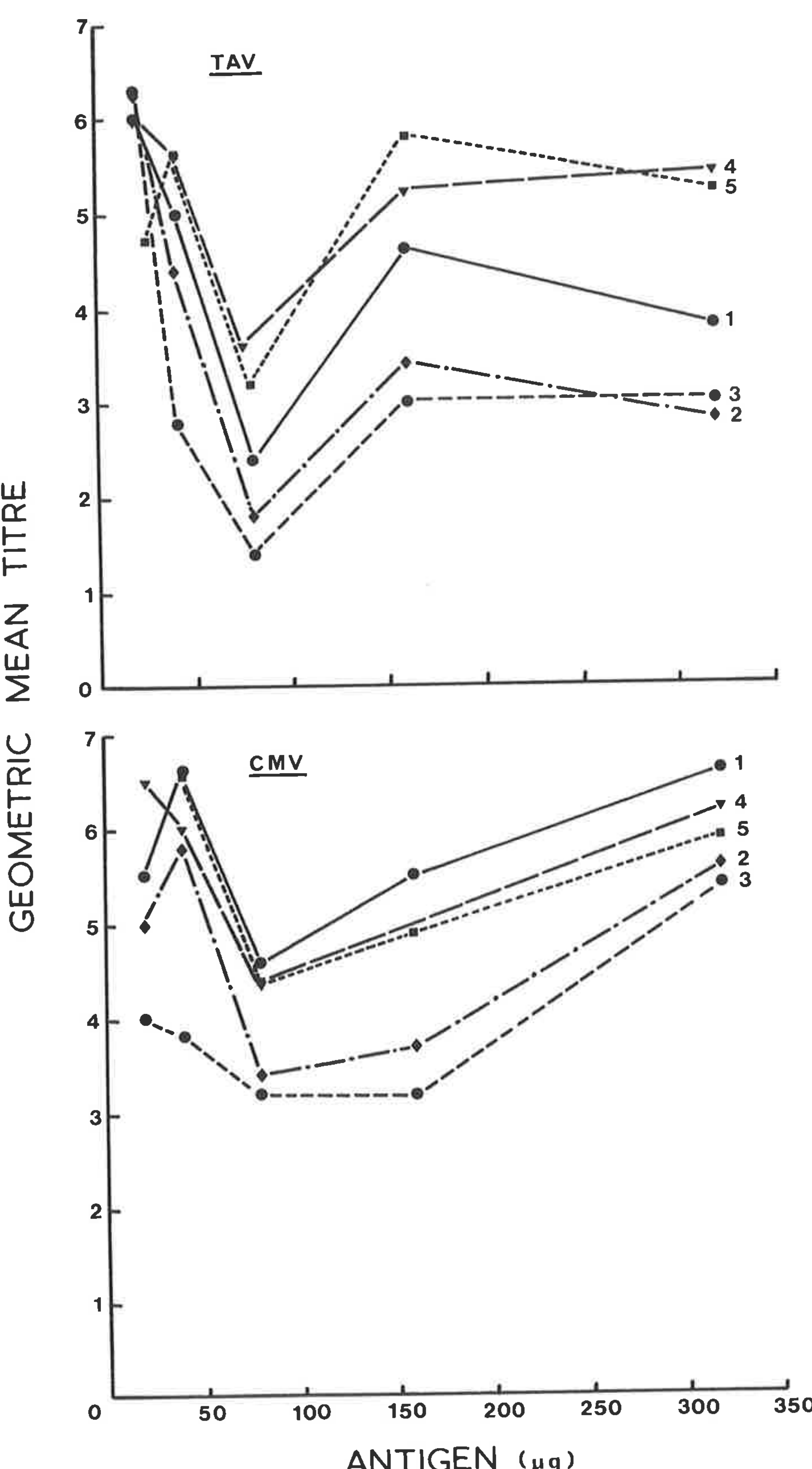

5 µg
CMV
TAV
10 µg
20 µg
40 µg
GEOMETRIC MEAN TITRE
TIME (WEEKS)
0
1
2
3
4
5
6
8

Fig. 6-5. The immune response of mice to various doses (20-320 µg) of TAV and CMV antigens. The animals were injected at the commencement of the experiment and after 1, 2 and 8 weeks, and bled five times at weekly intervals. The first bleeding (1) was 4 weeks after immunization started.



TAV
CMV
GEOMETRIC MEAN TITRE
ANTIGEN (μg)
0
1
2
3
4
5
6
7
50
100
150
200
250
300
350

II. Comparative Immunological Studies on TAV and CMV

1. Relative immunogenicity of TAV and CMV

The immunogenicity of TAV and CMV was compared in toads and mice. Results in Fig. 6-3 show that when toads were used, a relatively high response was observed for TAV. However, no response could be detected against CMV in either of the two bleedings. In another experiment mice were injected with the two antigens mixed in equal proportions and various doses (Fig. 6-4). In all groups of animals CMV was more immunogenic; being significantly more so in two groups when 10 µg ($0.05 > P > 0.01$) and 20 µg ($P < 0.01$) of each antigen were administered. In the experiment summarized in Fig. 6-6 where the immunogenicity of TAV and CMV was compared after injecting two separate groups of mice with each antigen, there was no significant difference ($P > 0.05$) in the response of the animals to the antigen. The results indicate that TAV was not a better immunogen than CMV.

To further investigate the effect of antigen dose on the immune response, experiments were done in which a wider range of antigen dose was used. The results summarized in Fig. 6-5 indicate that there is no significant difference ($P > 0.05$) in the response of animals to doses between 20 µg to 320 µg of either antigen. It was often observed that the immuno-precipitin lines were sharper when antisera from animals injected with lower doses of antigen (20-40 µg) were tested than those

receiving higher doses (160-320 μg). The reasons for this phenomenon were not investigated further.

2. Immunogenicity of TAV and CMV proteins

The immunogenicity of TAV, CMV and their respective isolated proteins were compared. No response was detected against CMV protein (Fig. 6-6). However, a slight response was induced with TAV protein. Nevertheless, the response was very much lower than that produced against intact capsids (Fig. 6-6).

3. Enhancement of immunogenicity of CMV and TAV by formaldehyde treatment

Results of one experiment in which the immunogenicity of formaldehyde-treated CMV was compared to that of normal virus are summarized in Fig. 6-7. Sera from animals immunized with formaldehyde-treated CMV had a significantly higher titre ($P < 0.01$) than those immunized with normal virus, whether titrated against homologous or heterologous antigen.

Treatment of TAV with formaldehyde also enhanced immunogenicity of the virus in mice. The results in Fig. 6-8, where the immunogenicity of normal and formaldehyde-treated TAV and CMV are compared, indicate that the titres of sera increased after treating the antigens with formaldehyde. TAV induced a slightly higher but not significant

Fig. 6-6. The immune response of mice to TAV, CMV and their respective isolated proteins. The viral antigens were suspended in 20 mM phosphate buffer, pH 7.6, and the viral proteins in the same buffer containing 0.14 M NaCl. Each animal was injected with 100 µg of antigen emulsified in adjuvant at the times indicated by arrows. The antisera were titrated against homologous antigens.

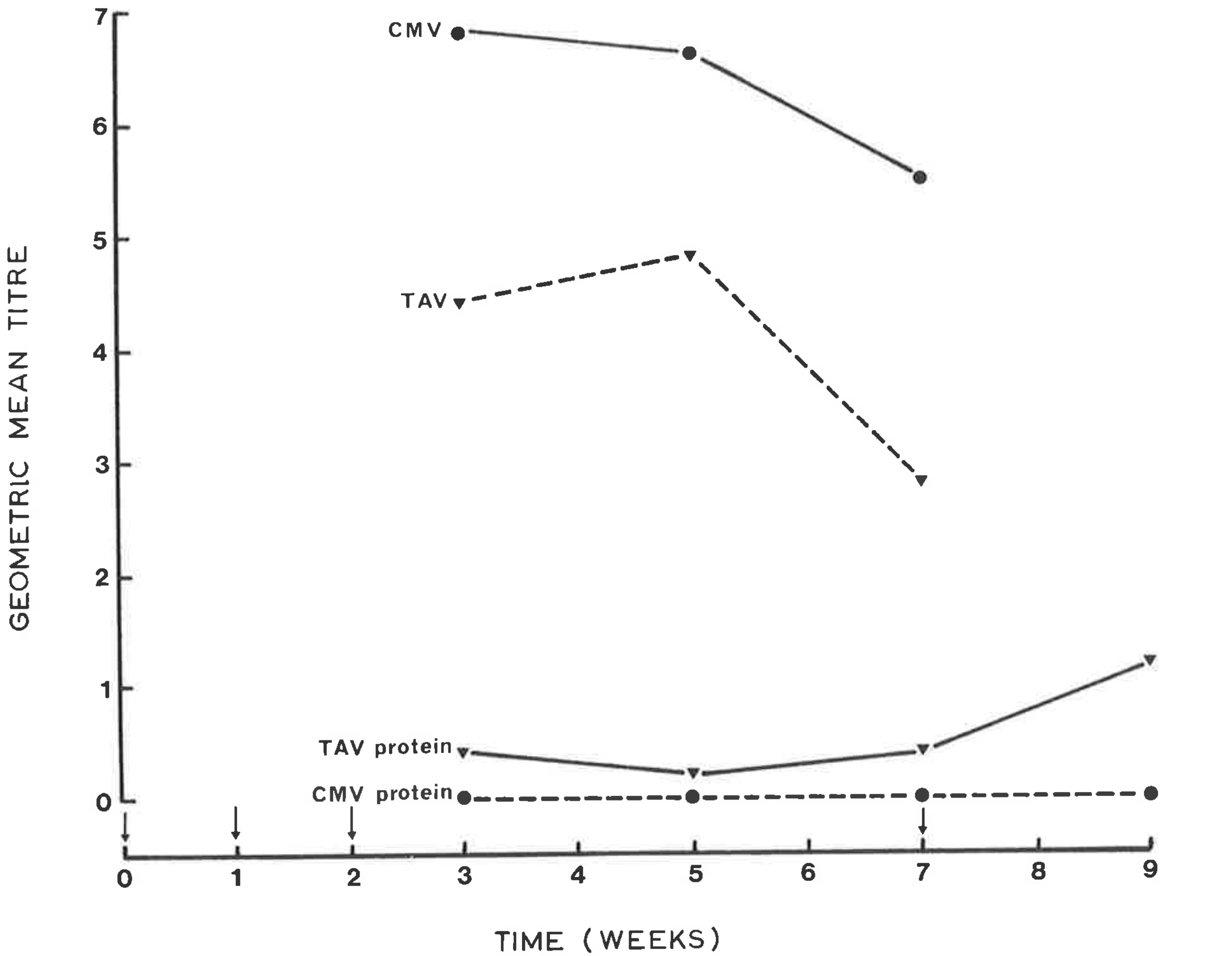
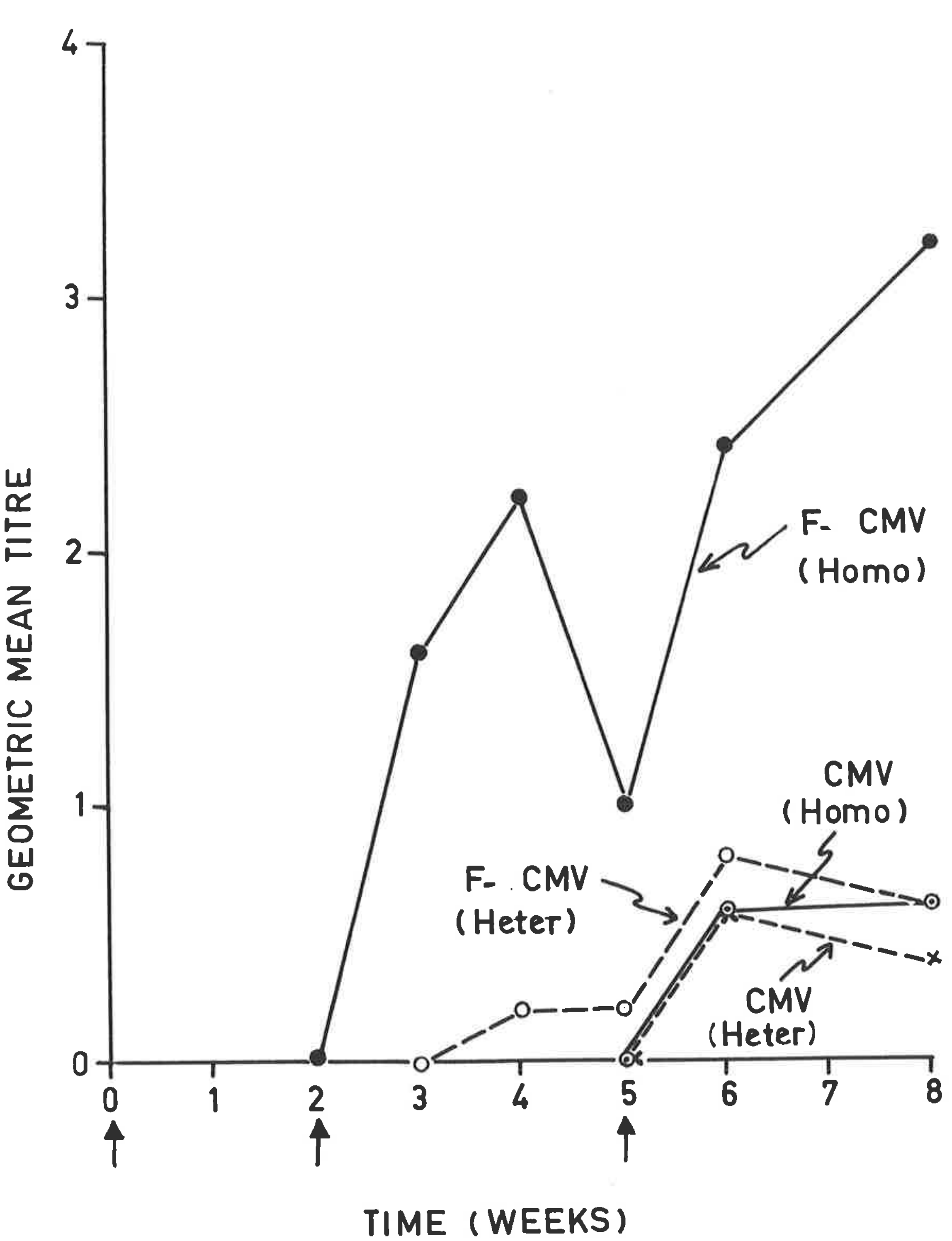
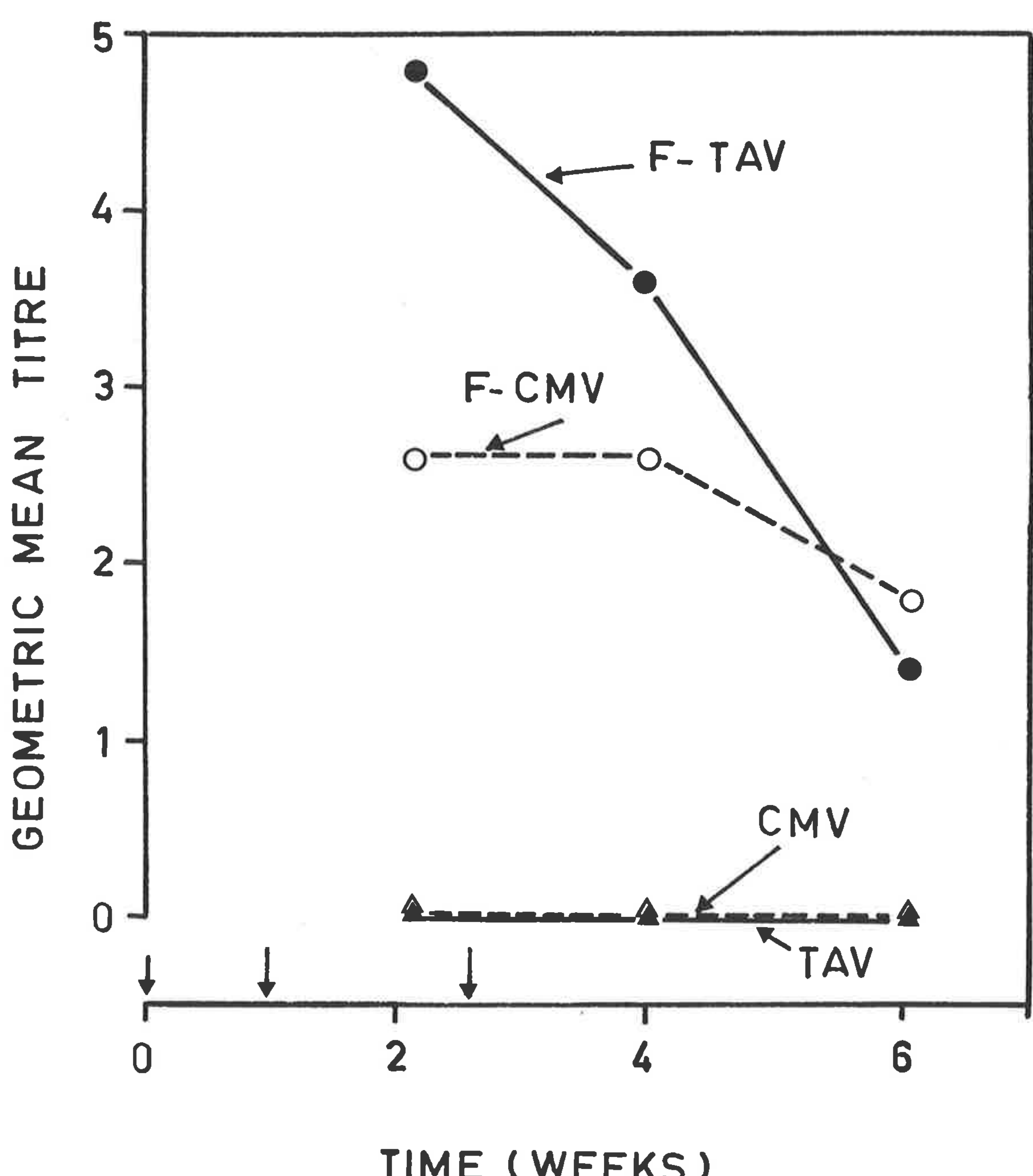

CMV
TAV
TAV protein
CMV protein
GEOMETRIC MEAN TITRE
7
6
5
4
3
2
1
0
0
1
2
3
4
5
6
7
8
9
TIME (WEEKS)

Fig. 6-7. The immune response of mice to normal (CMV) and formaldehyde treated (F-CMV) virus in the presence of adjuvant. Solid lines indicate serum titres when assayed against homologous antigen (Homo) and broken lines indicate titres when assayed against heterologous antigen (Heter). All animals were injected at the commencement of the experiment and two and five weeks later (indicated by vertical arrows), each time with 20 μg of antigen per animals.



GEOMETRIC MEAN TITRE
4
3
2
1
0
F- CMV
(Homo)
CMV
(Homo)
F- CMV
(Heter)
CMV
(Heter)
0
1
2
3
4
5
6
7
8
TIME (WEEKS)

Fig. 6-8. The immune response of mice to normal and formaldehyde-treated (F-TAV and F-CMV) TAV and CMV. All animals were injected with 20 μg of antigen in adjuvant at the times indicated by arrows. F-TAV and F-CMV were treated with formaldehyde as described in Chapter 2.



GEOMETRIC MEAN TITRE
5
4
3
2
1
0
F- TAV
F- CMV
CMV
TAV
0
2
4
6
TIME (WEEKS)

response than CMV ($P > 0.05$).

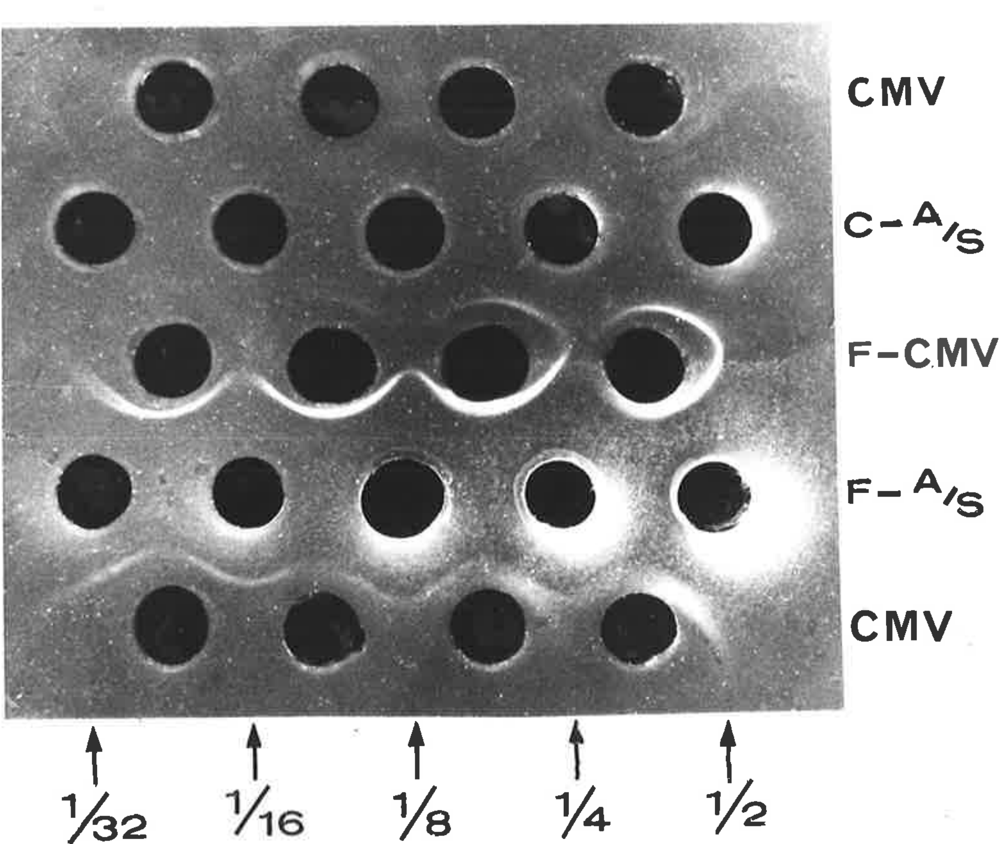
The differences in titre of sera from animals immunized with formaldehyde-treated TAV and CMV, when tested against formaldehyde-treated and normal antigens, is at least in part due to the treated viruses being better test antigens in gel diffusion. To study the behaviour of normal and formaldehyde-treated CMV in agar gels, the following experiment was done: The gels were solidified in Pasteur pipettes (0.7 cm in diameter) to a depth of 2 cm. The virus preparations at 1 mg/ml (0.1 ml) were layered on the agar gels and allowed to diffuse into the agar column for 24 h at 25°. The columns were removed from Pasteur pipettes and transferred to an agar gel plate in a groove next to an antiserum reservoir. With normal virus a heavy precipitin band was observed adjacent to the top of the column, whereas with the formaldehyde-treated antigen the intensity of the band was uniform throughout the length of the column.

It was observed that in many sera antibodies elicited in response to either formaldehyde-treated or normal CMV were more readily detected when formaldehyde-treated antigen was used (Fig. 6-9).

4. Comparison of immunodiffusion lines produced by TAV and CMV and their homologous antisera

While studying the immune response of mice to various doses of TAV, it was observed that sera of certain mice contained antibodies

Fig. 6-9. Immunodiffusion test between CMV and its antiserum (C-A/S), the formaldehyde-treated virus (F-CMV) and its antiserum (F-A/S). All wells contained the same concentration of antigen (200 μg/ml) but the sera were diluted as indicated by arrows.



CMV
C-A/S
F-CMV
F-A/S
CMV
1/32
1/16
1/8
1/4
1/2

which produced a straight precipitin line nearer the antiserum well in addition to a curved line always present nearer the antigen well (SC-antisera) (Fig. 6-10a). The titre of antibodies producing the straight line was always lower than that producing the curved line (Fig. 6-11). There was a linear relationship between the titre of antibodies producing the straight line and those producing the curved line (Fig. 6-12). However, in some animals, although a relatively high titre of antibodies producing the curved line was obtained, no antibodies producing the straight line were detected (Figs. 6-11 and 6-12). Production of double immunodiffusion lines by SC-antisera could result from either; (a) The production of a curved line by the reaction of antigen with 7 S (IgG), and that of straight line with 19 S (IgM) antibodies. The rapid disappearance of antibodies producing the straight line from the sera of immunized animals (Fig. 6-11) suggested that the antibodies involved may have been of the IgM class; or (b) The dissociation of virus particles and the reaction of protein subunits with specific antibodies produced the straight line in addition to the curved line which resulted from reaction of antibodies with intact virus particles.

To differentiate between the above possibilities the following experiments were done;

1. The SC-antiserum was incubated at 37° for 1 h in the presence of 0.1 M 2-mercaptoethanol, a treatment which destroys IgM leaving the

Fig. 6-10. Immunodiffusion test between TAV (T), TAV protein (T_p) (10 µg of each antigen per well) and TAV antiserum. (a) The antiserum (ts) was from a mouse producing a straight precipitin line (s) and a curved line (c) (SC-antiserum). The faint line (c_1) probably resulted from a reaction between residual virus particles, or an aggregated product of the protein subunits and the antiserum. (b) The antiserum (ts_1) was obtained from a mouse immunized with formaldehyde-treated TAV. The blank wells were not charged with any reactant.

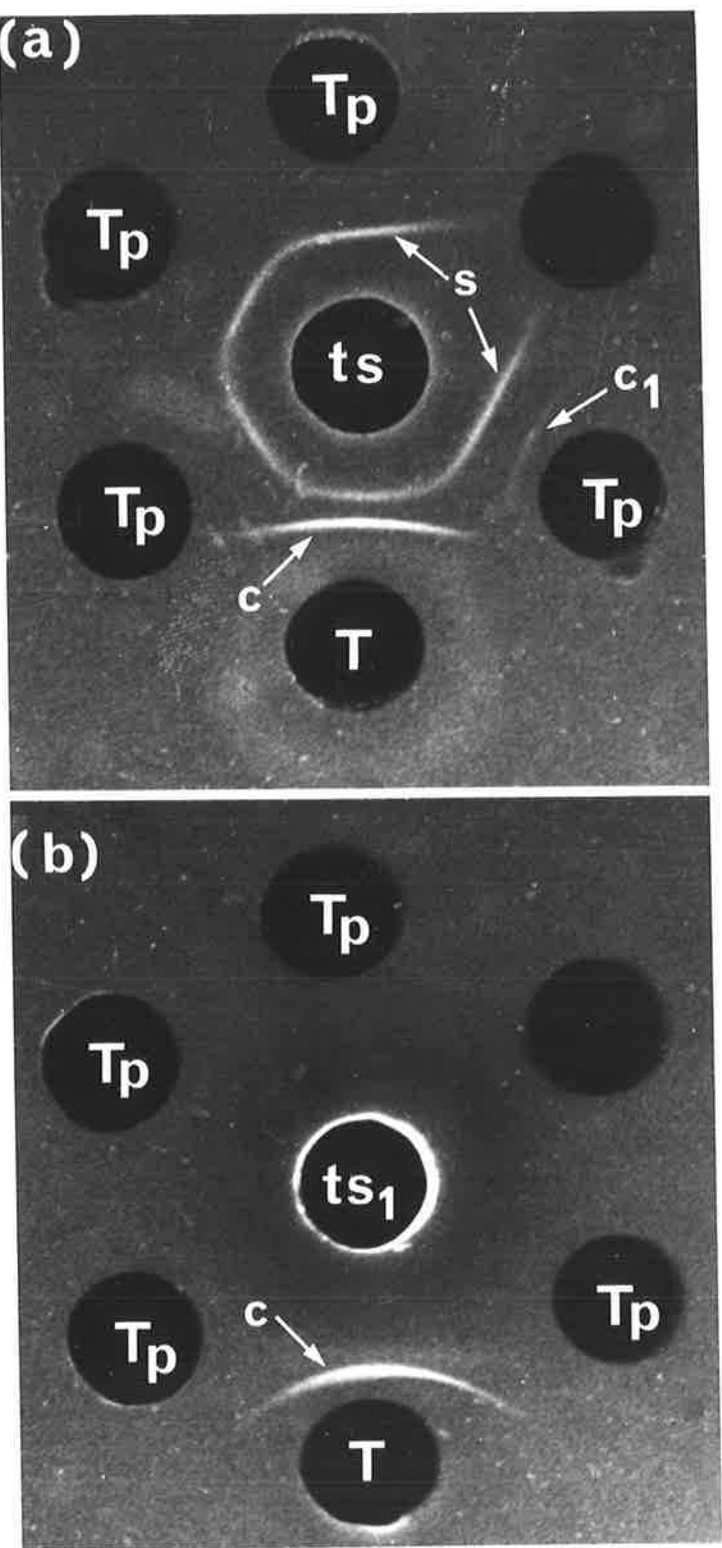

(a)
Tp
Tp
s
ts
c1
Tp
Tp
c
T
(b)
Tp
Tp
ts1
c
Tp
Tp
T

Fig. 6-11. The immune response of mice to 20 (group A), 40 (B), 80 (C), 160 (D) and 320 (E) μg of TAV injected per animal at the times shown by arrows. The results summarized in this Figure are those in Fig. 6-5, except that responses of individual mice are recorded. The titre of antibodies producing the curved line against homologous antigen are shown by empty histograms. The filled histograms represent the titres of antibodies producing the straight lines (see also Fig. 6-10a). The mice were injected at the times indicated by arrows.

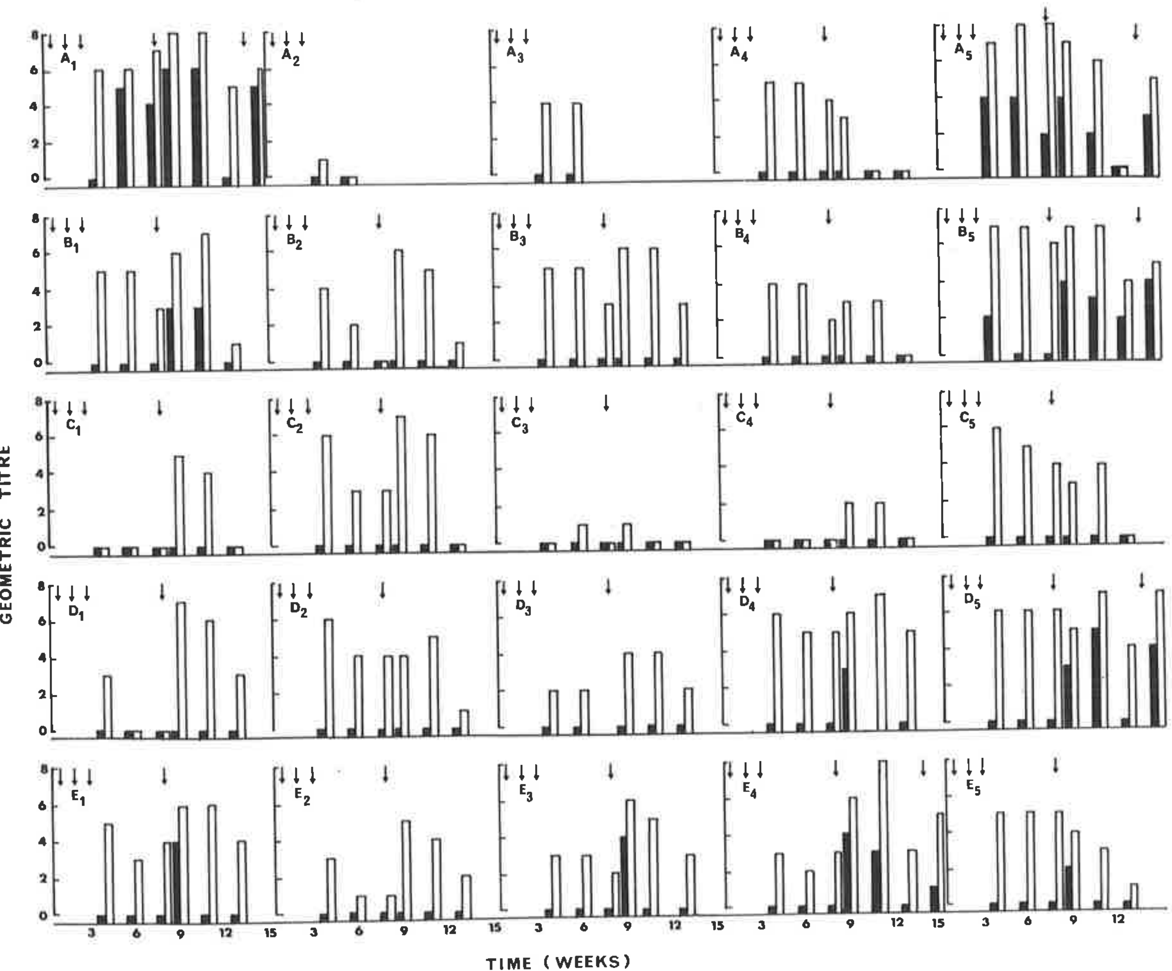
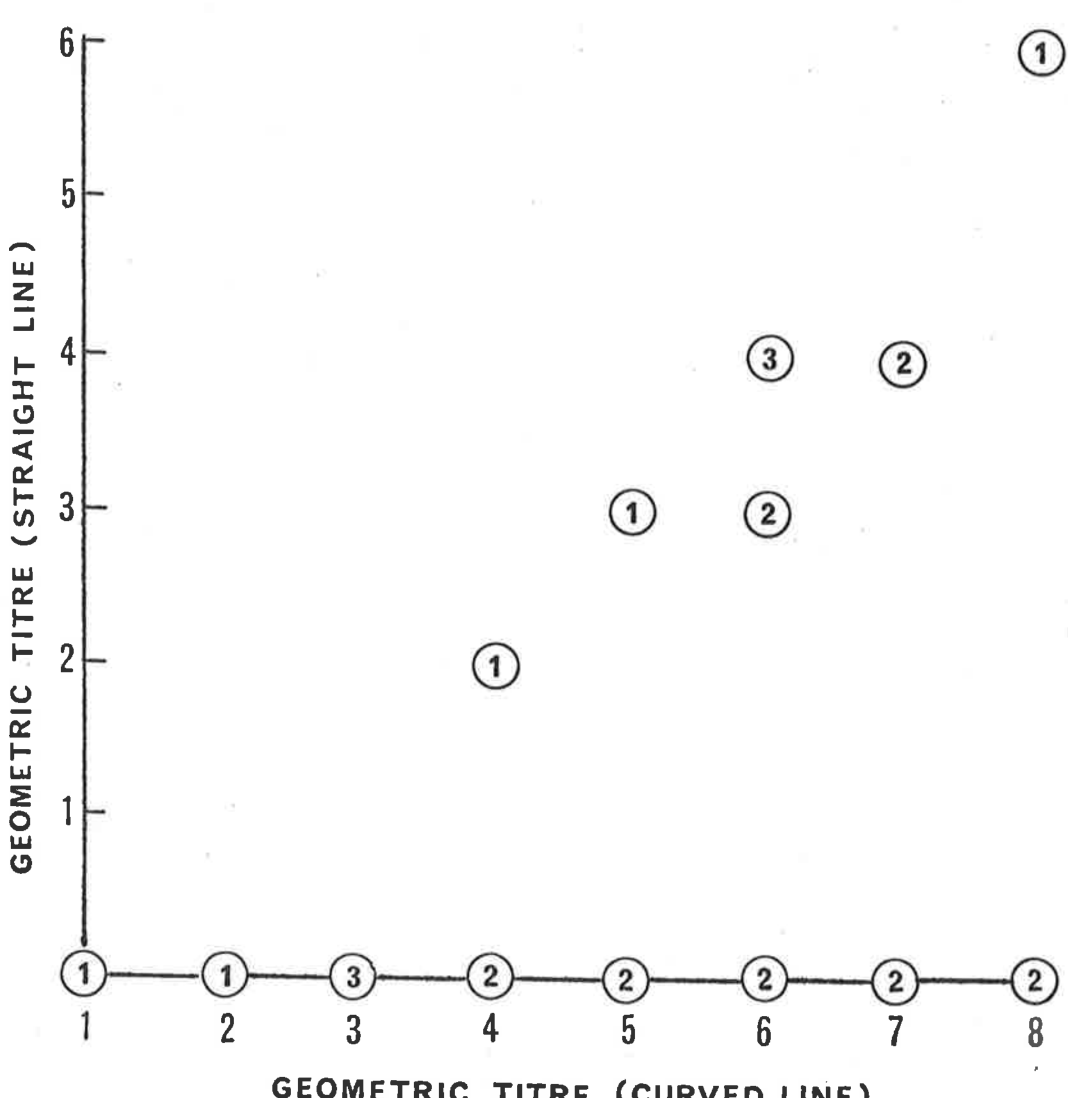

GEOMETRIC TITRE
TIME (WEEKS)
A_1
A_2
A_3
A_4
A_5
B_1
B_2
B_3
B_4
B_5
C_1
C_2
C_3
C_4
C_5
D_1
D_2
D_3
D_4
D_5
E_1
E_2
E_3
E_4
E_5
0
2
4
6
8
3
6
9
12
15

Fig. 6-12. Relationship between the titre of antibodies producing the straight line and those producing the curved line present in mouse anti-TAV sera. The results summarized in this Figure are taken from some of the data presented in Fig. 6-11. In this Figure the data from the antisera obtained after the first booster injection (week 8) are used. Numbers in the circles represent the number of mice whose antiserum shows the given characteristic.


GEOMETRIC TITRE (STRAIGHT LINE)
GEOMETRIC TITRE (CURVED LINE)
6
5
4
3
2
1
1
2
3
4
5
6
7
8

IgG antibodies intact (Katsura, 1972). When the heated serum was retested against TAV antigen the straight line was still present indicating that IgM was probably not involved.

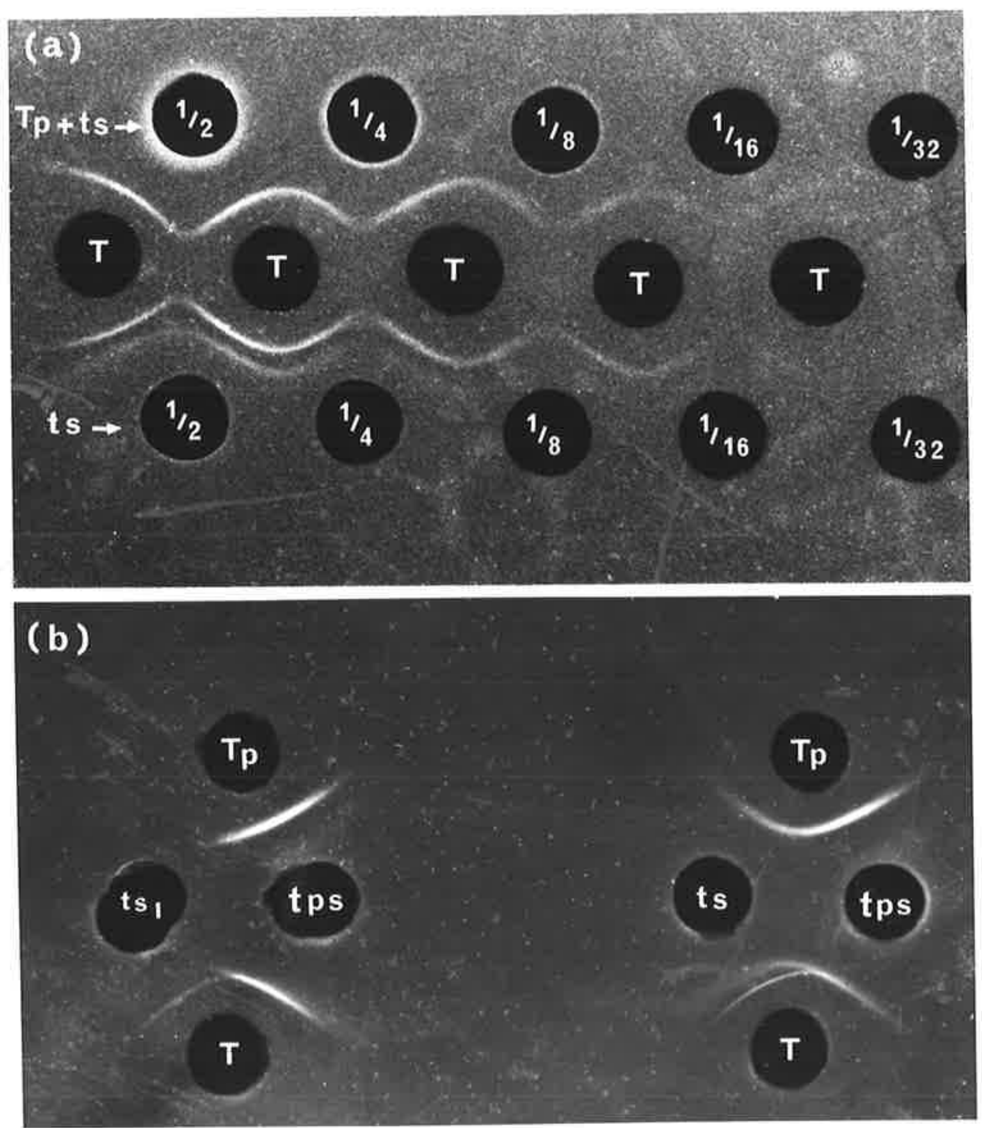
2. The 7 S (IgG) and 19 S (IgM) immunoglobulins were isolated by centrifugation in sucrose gradients. The IgG fraction produced both straight and curved lines. However, no detectable precipitin lines could be observed with the IgM fraction, again indicating that IgM antibodies were not involved.

3. When intact TAV and its isolated protein were reacted against the SC-antiserum in immunodiffusion tests, the straight line produced between the virus antigen and the homologous antiserum was confluent with a single line produced by the reaction of TAV protein and the SC-antiserum (Fig. 6-10a).

4. In intragel absorption tests, the straight line was not detected when the antiserum was absorbed with TAV protein (Fig. 6-13a). Results of these experiments indicate that the antibodies producing the straight line were specifically elicited against TAV protein. Isolated TAV protein reacted with homologous- and with SC-antisera (Fig. 6-13b). However, no reaction was observed between TAV protein and TAV antisera obtained from toads (Fig. 6-13b). Similarly, antisera produced in over 50% of the mice in the experiments summarized in Fig. 6-11 and those

Fig. 6-13. a. Results of intragel-absorption tests between TAV (T), TAV protein (T_p), and TAV antiserum (ts). TAV protein (8 μg) was allowed to diffuse from each well for 24 h before introducing the other reactants to their respective wells. Each well was charged with 10 μg of TAV, but the antiserum was diluted as indicated. Note the absence of bands nearer the antiserum wells (straight bands) which were precharged with TAV protein.

b. Immunodiffusion test between TAV, TAV protein and their antisera. Antiserum against TAV protein (tps) was produced in mice. Antiserum against TAV (ts) was the one used in Fig. 6-10a, and ts_1 was obtained from toads.


(a)
Tp+ts
1/2
1/4
1/8
1/16
1/32
T
T
T
T
T
ts
1/2
1/4
1/8
1/16
1/32
(b)
Tp
Tp
ts1
tps
ts
tps
T
T

elicited against formaldehyde-treated TAV failed to react with TAV protein (Fig. 6-10b). These antisera produced only the curved line when reacted against TAV antigen (Figs. 6-10b and 6-13b).

All antisera from mice immunized with CMV revealed only the curved line when reacted against homologous antigen (Fig. 6-9). No reaction was detected between CMV protein in the presence of various concentrations of NaCl and anti-CMV serum. The salt was used to prevent aggregation of the protein (Scott, 1968).

5. Tests for a serological relationship between TAV and CMV

Antisera prepared in mice against TAV (maximum titre = 1/128) and those against CMV (maximum titre = 1/256) reacted positively with their respective antigens. However, no cross-reactions were observed in any of the tests. Similarly, only homologous reactions against the two antigens were observed with TAV and CMV antisera obtained from other workers (Table 6-1).

Conclusions

The serological studies carried out indicate that:

(1) Compared to TMV, both TAV and CMV are poor immunogens. No significant difference in the immune response was detected when mice were injected with various doses (20-320 μg) of TAV or CMV.

Table 6-1. Serological relationships between TAV and CMV isolates.

Virus	Isolate	Reference	Serological reaction with antigen	
			TAV (V strain)	CMV (Q strain)
TAV	Strain 1	Hollings (Private communication)	+[a]	-[b]
	Strain 2	"	+	-
	Hitchborn's	Grogan *et al.* (1963)	+	-
	Till's	"	+	-
	Canadian	Stace-Smith and Tremaine (1973)	+	-
	Strain V	This study	+[c]	-
CMV	South African (Strain S)	van Regenmortel (1966b)	-	+
	Price's Yellow	Hollings (Private communication)	-	+
	Beta vulgaris	"	-	+
	Wisconsin yellow	"	-	+
	Strain R	Devergne and Cardin (1970)	-	+
	Strain T	"	-	+
	Imperial 78	Grogan *et al.* (1963)	-	+
	Canadian	Stace-Smith and Tremaine (1973)	-	+
	Strain Q	This study	-	+[c]

a Visible band formed in immunodiffusion tests when antigen at a concentration of 1 mg/ml was used.

b No reaction observed in immunodiffusion tests.

c Homologous reaction.

(2) The immunogencity of both viruses can be improved either by formaldehyde treatment or by using cold-blooded animals kept at temperatures below 30°.

(3) Mouse anti-TAV sera sometimes contained antibodies specific to TAV protein as well as to intact capsids, whereas with mouse anti-CMV sera only antibodies to intact capsids were detected.

(4) Although the difference in the immunogenicity of intact TAV and CMV is not significant, isolated TAV but not CMV protein induced a detectable immune response in mice.

(5) There appears to be no serological relationship between TAV and CMV.

CHAPTER 7

GENOME PROPERTIES AND INTERACTION

Introduction

Recently, it has been shown that RNA 1, 2 and 3 of CMV are all necessary for infectivity (Peden and Symons, 1973; Lot *et al.*, 1974). However, the properties of the TAV genome have not been reported. TAV and CMV are readily distinguishable serologically (Chapter 6), by their differential stability in Mg^{2+} and EDTA (Chapter 4), and by the difference in electrophoretic mobility of RNA3 and RNA4 in PAG (Chapter 3). Using these criteria as markers, it seemed worthwhile to try to construct new virus variants by *in vitro* mixing of their RNA species. Viruses whose genomes are derived from two parental types have been referred to as 'hybrids' (Bancroft, 1972; Dingjan-Versteegh *et al.*, 1972; van Kammen, 1972). However, since this term carries connotations of a diploid organism it is perhaps better to refer to such viruses as pseudorecombinants as suggested by Gibbs and Harrison (1974).

This Chapter describes a comparison made of the nucleotide base sequences of TAV- and CMV-RNAs by RNA-RNA hybridization studies. The genetic functions of the RNA species have been investigated by comparing the properties of a pseudorecombinant virus with those of the parental

viruses.

Methods

1. Determination of TAV and CMV concentrations in *N. clevelandii* leaves

Four *N. clevelandii* plants were inoculated with purified preparations of either TAV or CMV (100 µg/ml) and maintained in a growth chamber (see Chapter 2). The rate of virus synthesis was determined serologically. Discs (4 mm in diameter) were excised from each leaf (2 discs/leaf) at three-day intervals starting from day 3 after inoculation. For each assay, eight discs were pulverized in 50 µl, 20 mM phosphate buffer, pH 7.6, containing 0.01% sodium sulphite. Two-fold dilutions of the extracts were prepared in the same buffer and introduced into immunodiffusion plates against homologous antisera. The point at which diluted extract was still giving reaction was recorded as an estimate of virus concentration in plant tissue.

2. Isolation of viral RNAs and separation into components

RNA was extracted from purified virus preparations with phenol-SDS and separated into components by electrophoresis in PAGs as described in Chapter 2. Tubes, 7.4 cm long and 1 cm in diameter, were used when preparative separation of RNA species from the gels was to be carried out. Up to 150 µg RNA was loaded on each gel-tube. RNA bands were located

by staining with 0.05% toluidine blue, 55 mM sodium acetate, 0.1 mM EDTA, pH 5.5 for 20 sec (Peden and Symons, 1973) and destaining with the same buffer without dye for at least 1 h at 4° in the dark. The RNA was isolated from the excised band using the procedure described by Cory *et al.* (1972) as modified by Peden and Symons (1973). The corresponding bands from up to 12 gels were placed in a 30 ml plastic syringe and forced into a 50 ml beaker. The gels were emulsified with 6 ml TNE buffer (20 mM Tris HCl, 0.1 M NaCl, 5 mM EDTA, pH 8.5) containing 1% SDS by drawing them several times through an 18 gauge needle affixed to a syringe. The needle was then replaced with a 23 gauge one, and the extrusion continued. Water-saturated phenol (6 ml) was added to the slurry and after drawing five more times, the mixture was shaken vigorously at 25° for 30 min. The aqueous phase was separated by centrifugation at 5,000 *g* for 10 min. The phenol phase was re-extracted with 6 ml of the same buffer. Both aqueous phases were combined, left on ice for 5 min, and centrifuged at 20,000 *g* for 15 min to sediment traces of polyacrylamide. The RNA was then precipitated with 2½ volumes of ethanol at -15° overnight.

3. Labelling of viral RNAs with ^{14}C

Virus-infected *N. clevelandii* plants were transferred to a desiccator and grown in the presence of $^{14}CO_2$ for between 6 to 12 days after infection at which time both TAV and CMV were actively multiplying

(Fig. 7-1). $^{14}CO_2$ was released in the desiccator by injecting 2.5 ml ^{14}C-labelled sodium bicarbonate into a beaker containing 0.1 N H_2SO_4 inside the chamber (Rezaian and Francki, 1973). The viruses were purified and their RNAs isolated with phenol as described in Chapter 2. The specific activity of the RNAs was approximately 1,200 cpm/μg.

4. Preparation of a salt-soluble nucleic acid fraction from *N. clevelandii*

Nucleic acids were extracted from virus-infected or healthy plants using a modification of the procedure described by Rezaian and Francki (1973). Up to 200 g leaf material was homogenized with a buffer containing 0.1 M Tris HCl, 0.1 M NaCl, 10 mM EDTA, 1% SDS, pH 7.0, and 78% phenol containing 0.1% 8-hydroxyquinoline (2 ml of each reagent/g leaf material). The aqueous phase was recovered by centrifugation at 5,000 *g* for 10 min, and re-extracted twice more with half a volume of phenol. The nucleic acids were precipitated from the aqueous phase by two volumes of ethanol and were left at -15° for at least 2 h. They were sedimented at 5,000 *g* for 10 min and resuspended in STE buffer (0.1 M NaCl, 50 mM Tris HCl, 1 mM EDTA, pH 6.85) (Jackson *et al.*, 1971). The traces of phenol were removed by washing the suspension with two volumes of ether. The excess ether was then evaporated under vacuum.

To prepare the salt-soluble fraction of nucleic acids, equal volumes of 3 M NaCl in STE buffer were added to the nucleic acid preparations and frozen at -15° for at least 3 h (Bishop and Koch, 1967). The mixture was thawed slowly and centrifuged at 5,000 *g* for 20 min. Two volumes of ethanol were added to the supernatant to precipitate the salt soluble nucleic acids which were then resuspended in 0.1 M Tris HCl, 0.1 M NaCl, 10 mM EDTA, pH 7.0.

Polysaccharides were removed from nucleic acid preparations by the method of Ralph and Bergquist (1967). Equal volumes of 2-methoxyethanol and of 2.5 M potassium phosphate buffer, pH 8.1, were added to the preparations at 0°, and centrifuged at 5,000 *g* for 10 min to recover the upper phase. An equal volume of 0.2 M sodium acetate and half a volume of 1% cetyltrimethylammonium bromide (CTA) were added and the preparation kept at 0° for 10 min. The CTA-nucleate was sedimented by centrifugation at 5,000 *g* for 10 min and converted to the sodium salt by washing three times with chilled 70% ethanol containing 0.1 M sodium acetate. The residual ethanol was removed from the final precipitate by evaporation under vacuum. It was resuspended in 1 x SSC (0.15 M NaCl and 0.015 M sodium citrate) and diluted to an optical density of 100 at 260 nm.

5. RNA-RNA hybridization technique

Preparations of salt-soluble nucleic acids were incubated with 2 µg of preincubated Pronase at 37° for 20 min, ^{14}C-labelled viral RNA

(and competing viral RNA if required) in 1 x SSC was added and the volume was adjusted to 200 µl with 1 x SSC. The tubes were stoppered, heated at 100° for 10 min, transferred to a hot-water bath at 85° and allowed to cool slowly to 37° overnight. RNase A (10 µg) and RNase T_1 (10 units) were added to each sample and the tubes were again incubated at 37° for 30 min. The preparations were spotted on discs of Whatman No. 3 filter papers and dried in a jet of warm air. The discs were washed three times with 5% trichloroacetic acid (TCA), twice with 80% ethanol and once with ether (Byfield and Scherbaum, 1966). Each disc was transferred to a scintillation vial, 5 ml toluene based scintillation liquid containing 0.5% PPO and 0.01% POPOP was added to each vial. The RNase resistant radioactivity was determined in a Packard Scintillation Spectrometer.

6. Infectivity assay

RNA preparations were diluted to the required concentrations with 0.1 M potassium phosphate buffer, pH 8.0, containing 2 mg/ml of EDTA-treated bentonite (Fraenkel-Conrat *et al.*, 1961) and inoculated to young leaves of either *Chenopodium amaranticolor* Coste & Reyn. or *N. tabacum* L. cv. White Burley. To increase sensitivity the tobacco plants were kept in the dark for 2-3 days before inoculation. Ten µl of the inoculum was spread over the surface of each half-leaf. After inoculation the tobacco plants were kept in a growth room at 25°

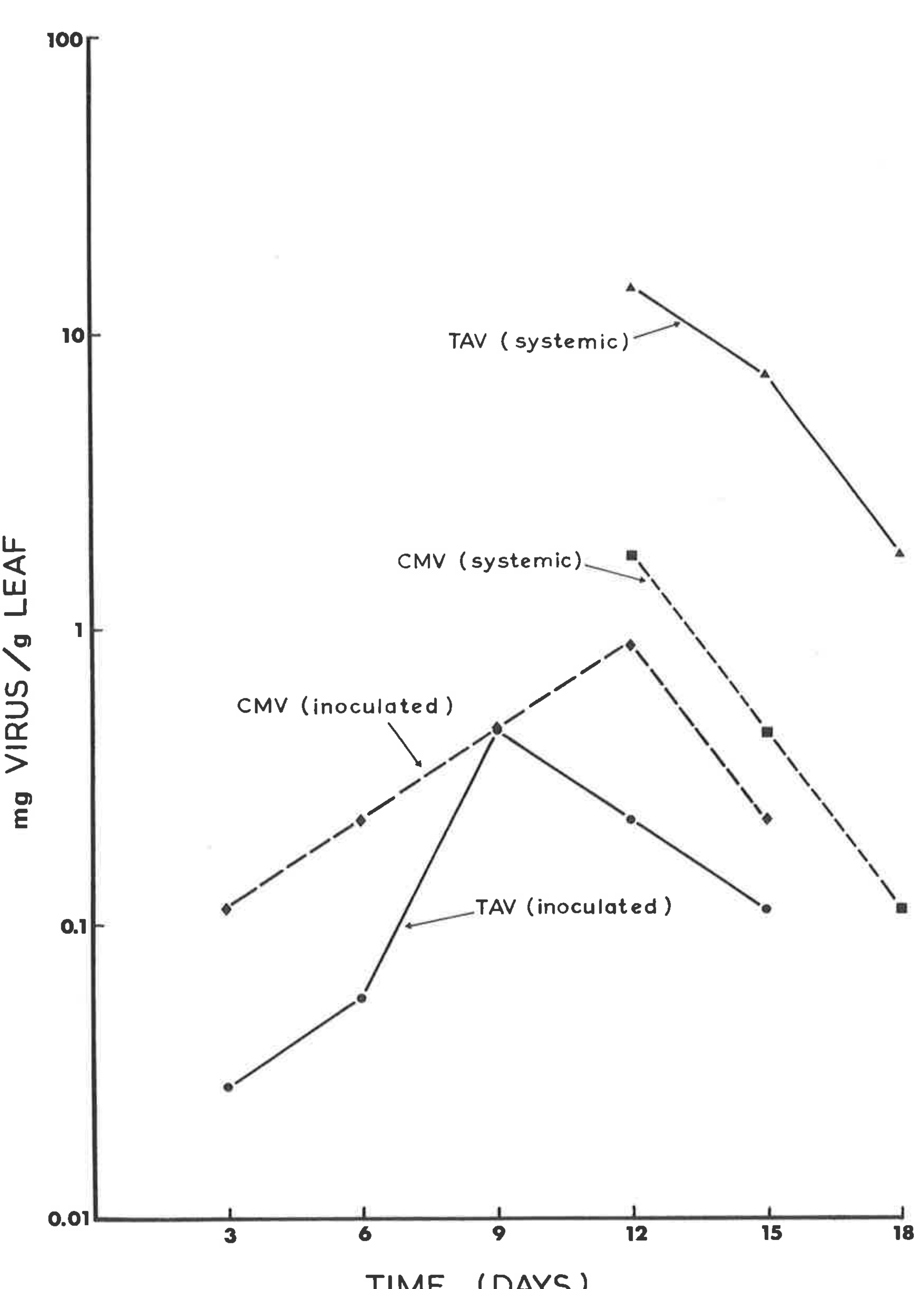
under continuous illumination of 4,500 lux from fluorescent lights. Under these conditions faint chlorotic lesions developed after 2-3 days. When excised singly and pulverized in 15 μl of 20 mM phosphate buffer, containing 0.01% sodium sulphite, pH 7.6, these lesions yielded sufficient virus for inoculating single plants or for direct serological testing by immunodiffusion.

Results

1. Multiplication of TAV and CMV in *N. clevelandii*

A preliminary experiment on the rate of multiplication of TAV and CMV *in vivo* seemed to be necessary to assess the time at which the viruses were actively multiplying. The growth curves of TAV and CMV in *N. clevelandii* are presented in Fig. 7-1. Although the quantity of CMV per gram of leaf tissue was similar in inoculated and systemic leaves, more TAV was recovered from systemically infected than from the inoculated leaves. These results are comparable to those observed by several other workers. Tomlinson *et al.* (1973), using the infectivity assay technique, observed that the amount of the W strain of CMV in *N. tabacum* cv. White Burley was highest at 10-12 days after infection. The same results were obtained by Hollings *et al.* (1968) when some strains of CMV were propagated in *N. clevelandii*. TAV was also found to reach its highest concentration 10 days after inoculation on *N. glutinosa* (Grogan *et al.*, 1963).

Fig. 7-1. Multiplication of TAV and CMV in inoculated and systemically infected leaves of *N. clevelandii* plants grown in a growth chamber with 12 h photoperiod and 22°. At various times after inoculation leaf discs were cut, pulverized and assayed serologically against homologous antisera in gel-diffusion. The lowest concentration of a purified virus preparation giving a visible immunodiffusion line (12.5 μg/ml) was used to calculate the approximate concentration of virus.



100
10
1
0.1
0.01
mg VIRUS / g LEAF
TAV (systemic)
CMV (systemic)
CMV (inoculated)
TAV (inoculated)
3
6
9
12
15
18
TIME (DAYS)

II. Tests for Nucleotide Base Sequence Homology Between TAV and CMV-RNAs

Salt soluble fractions of nucleic acid preparations from both TAV and CMV-infected *N. clevelandii* produced faint precipitin lines in immunodiffusion tests against anti-poly (I) : poly (C) serum. No such reactions were detected when similar preparations from healthy plants were tested. This indicates that the salt soluble fraction of nucleic acid preparations from infected plants contained detectable amounts of virus-specific ds-RNAs which could be used for RNA-RNA hybridization to test the degree of base sequence homology between the RNAs of the two viruses. The results summarized in Table 7-1 show that preparations from TAV-infected plants contain nucleic acids with nucleotide base sequences complementary to TAV-RNA that are absent in both healthy and CMV-infected plants. Similarly, preparations from CMV-infected plants contain nucleic acids with sequences complementary to CMV-RNA that are absent in both healthy and TAV-infected plants. These data indicate that there is negligible nucleotide base sequence homology between TAV and CMV-RNAs.

III. Interaction of RNA species from TAV and CMV

Addition of either electrophoretically purified T_3 or C_3 can greatly stimulate the infectivity of $T_1 + T_2$ (Table 7-2 and Fig. 7-2). This stimulation is specific as the highly-purified non-infectious

Table 7-1. Hybridization of salt-soluble nucleic acid fraction from *N. clevelandii* leaves with ^{14}C-labelled TAV- and CMV-RNAs.

Source of salt-soluble nucleic acid fraction used[a]	Viral RNA annealed (cpm)[b]	
	TAV-RNA[c]	CMV-RNA[d]
Nil	394	567
Healthy leaves	387	586
TAV-infected leaves	1,529	511
CMV-infected leaves	442	1,526

a 150 µl of nucleic acid adjusted to an optical density of 100 at 260 nm was used for each assay.

b Results of two independent experiments with assays carried out and in duplicate in each (see Methods for experimental details).

c 3 µg of TAV-RNA (3,000 cpm) was added in each assay.

d 3 µg of CMV-RNA (3,500 cpm) was added in each assay.

Table 7-2. Infectivity of mixtures of TAV- and CMV-RNA species in *C. amaranticolor* (Experiment 1) and *N. tabacum* cv. White Burley (Experiment 2).

Experiment number	Trial[a]	RNA species mixed[b]	Lesions per half-leaf
1[c]	A	$T_1 + T_2$	28
		$T_1 + T_2 + T_3$	136
	B	T_3	4.3
		$T_1 + T_2 + T_3$	75
	C	$T_1 + T_2$	28
		$T_1 + T_2 + C_3$	196
	D	C_3	24
		$T_1 + T_2 + C_3$	210
	E	$T_1 + T_2$	54
		$T_1 + T_2$ + TRSV-RNA_1[d]	35
2[e]	F	$T_1 + T_2$	4.5
		$T_1 + T_2 + C_3$	54
	G	C_3	0.8
		$T_1 + T_2 + C_3$	28

a In each trial, infectivity was compared on 6-8 half-leaves.

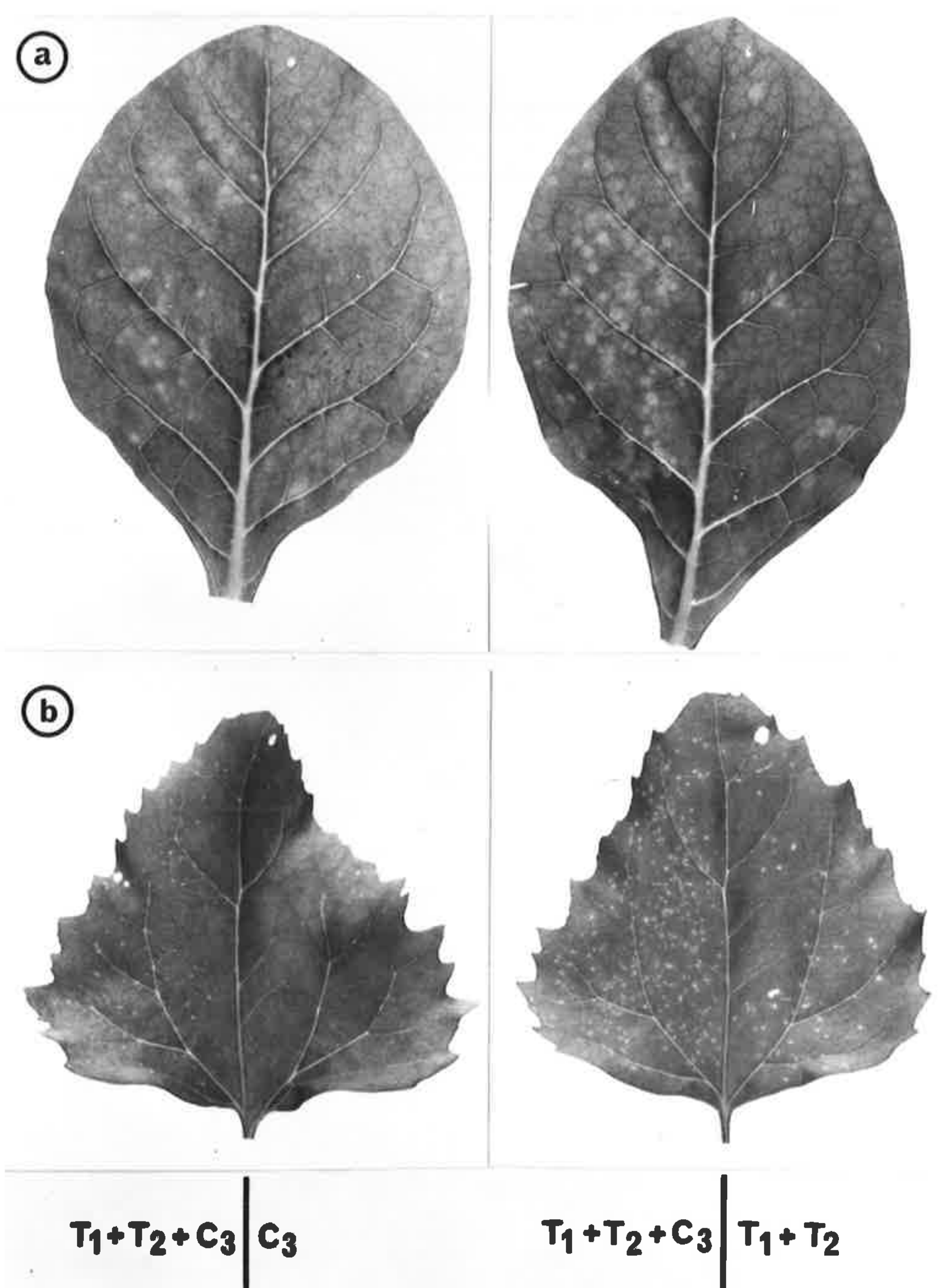
b The RNA species were adjusted to approximately the same concentrations by estimating their proportions from scans of unfractionated preparations subjected to PAG electrophoresis.

c Viral RNA preparation was heated at 75° for 5 min to minimize aggregation (Schwinghamer and Symons, 1974), and then the species were separated by PAG electrophoresis.

d Final concentration of TRSV-RNA_1 in the inoculum was 2 μg/ml.

e Viral RNA preparation was subjected to two cycles of PAG electrophoresis to separate the species.

Fig. 7-2. Local lesions developed on half-leaves of *N. tabacum* cv. White Burley (a), and *C. amaranticolor* (b) after inoculation with various combinations of RNA species of TAV and CMV, as indicated in the bottom of the Figure. Typical leaves from trials C, D, F, and G from experiments summarized in Table 7-2 are photographed.


a
b
T1+T2+C3 | C3
T1+T2+C3 | T1+T2

RNA species of tobacco ringspot virus (RNA_1) (Rezaian and Francki, 1974) had no such stimulatory effect (trial E, Table 7-2). I have been unable to demonstrate a stimulation of the infectivity of $C_1 + C_2$ by T_3. All 7 lesions developed on *N. tabacum* inoculated with $T_1 + T_2$ contained TAV antigen. However, most of the lesions induced by $T_1 + T_2 + C_3$ (trials F and G, Table 7-2) contained CMV antigen. Well separated lesions from tobacco leaves inoculated with $T_1 + T_2 + C_3$ (Fig. 7-2) were excised with a 4 mm diameter cork borer and 83 were individually tested with anti-TAV and anti-CMV sera. Extracts from 69 of these lesions produced precipitin lines with anti-CMV serum, 6 with anti-TAV serum, 2 with both, and 6 with neither of the antisera. Extracts from similar pieces of leaf tissue taken from areas between lesions failed to react with either antiserum. These data suggest that the majority of the lesions derived from infection by $T_1 + T_2 + C_3$ contained a pseudorecombinant virus whose genome was derived partly from TAV ($T_1 + T_2$) and partly from CMV (C_3). To substantiate this, the virus was subcultured and examined in detail.

IV. Isolation and Characterization of Pseudorecombinant Virus

Virus from a single lesion on tobacco inoculated with $T_1 + T_2 + C_3$ was transferred to a healthy tobacco plant. After checking that the infected leaf contained CMV antigen, the isolate was further

cloned by three more single lesion transfers and was then inoculated to *N. clevelandii* in which it induced symptoms characteristic of TAV. To minimize the risk of isolating new mutants, the virus was purified by the standard technique for TAV (Chapter 2) only from the inoculated leaves of *N. clevelandii*. The virus exhibited the following properties:

(1) Its antigenic properties were indistinguishable from those of CMV (Fig. 7-3).

(2) The virus precipitated in 1 mM $MgCl_2$ and was stabilized by 10 mM EDTA, behaviour characteristic of CMV (Chapter 4).

(3) When inoculated to a series of plant species, it induced reactions indistinguishable from those induced by TAV (Table 7-3 and Fig. 7-4).

(4) When isolated viral RNA was subjected to PAG electrophoresis, the migration and proportions of species 3 and 4 were indistinguishable from those of C_3 and C_4 (see also Fig. 3-3).

(5) The viral RNA competed with the hybridization of ^{14}C-labelled TAV-RNA and the salt soluble nucleic acid fraction from TAV-infected plants to a greater extent than with that of ^{14}C-labelled CMV-RNA and the salt soluble nucleic acid fraction from CMV-infected plants (Table 7-4). These data indicate that the RNA from the pseudorecombinant

Fig. 7-3. Immunodiffusion test between purified TAV, CMV and pseudorecombinant virus (PR) preparations and anti-TAV (ts) and anti-CMV (cs) sera. Each antigen well was charged with 10 μg of virus. Anti-TAV serum producing two immunodiffusion bands with homologous antigen has been shown to contain antibodies specific to TAV virions (band nearer antigen well) and to viral protein subunits (band nearer serum well) (see Chapter 6).

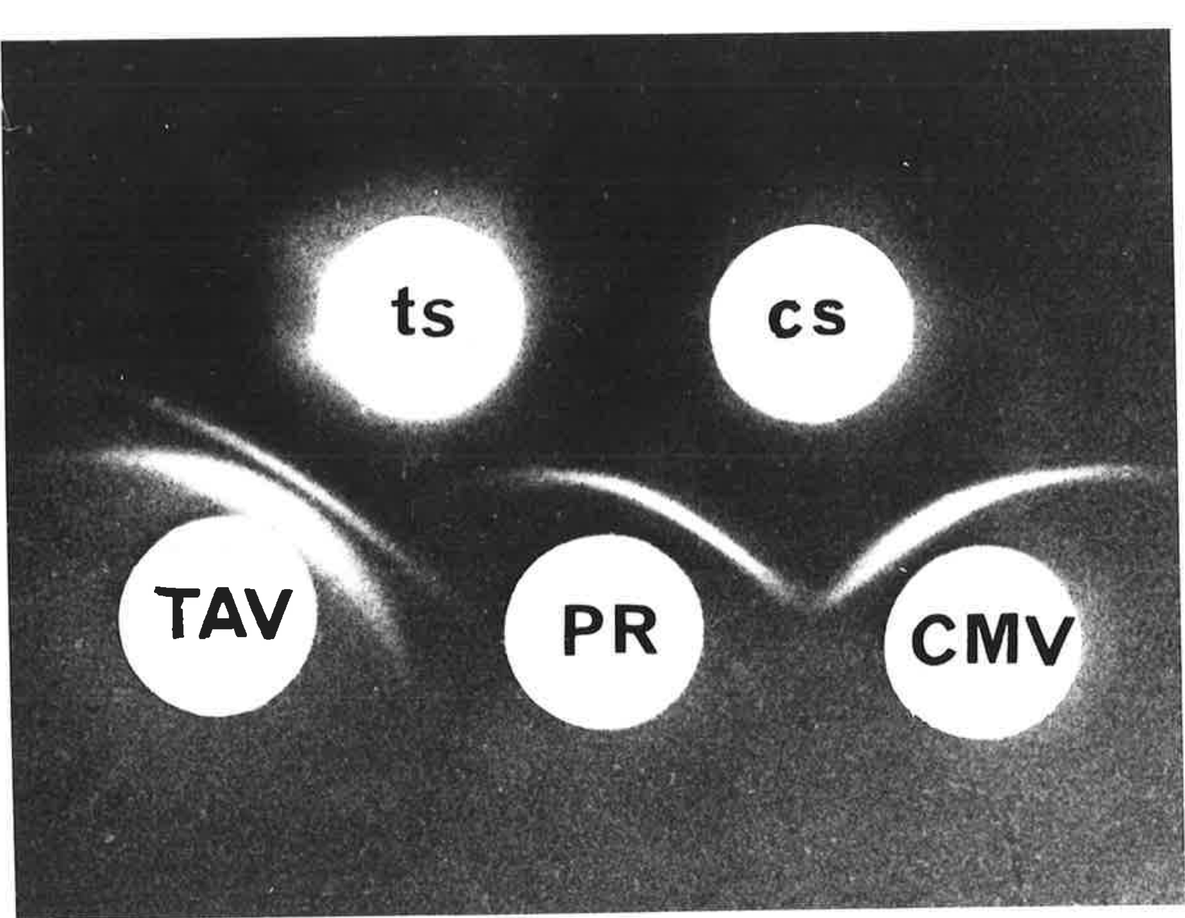

ts
cs
TAV
PR
CMV

Table 7-3. Host-range and symptoms induced by TAV, CMV and the pseudorecombinant on selected plant species.

Host plant	Symptoms produced by[a]	
	TAV and pseudorecombinant	CMV
Nicotiana clevelandii A. Gray	Necrotic lesions, severe mosaic and leaf distortion	Mild mosaic
N. excelsior J.M. Black	Mosaic	Mild mosaic
N. glutinosa L.	Severe mosaic and leaf blistering	Mild mosaic
N. tabacum L. cv. White Burley	Severe mosaic	Mosaic
Cucumis sativus L.	Immune[b]	Mosaic
Vigna sinensis (L.) Endl. cv. Blackeye	Small necrotic local lesions	Small necrotic local lesions
Chenopodium amaranticolor Coste & Reyn.	Small necrotic local lesions	Small necrotic local lesions

a Purified preparation of viruses containing 100 µg/ml nucleoprotein in 20 mM phosphate buffer used as inoculum. The plants were inoculated and maintained in a glasshouse with natural illumination. Symptoms were recorded 12 days after inoculation.

b No virus could be recovered from inoculated plants.

Fig. 7-4. Symptoms induced by CMV (column 1), TAV (column 2) and the pseudorecombinant virus (column 3) on *N. tabacum* cv. White Burley (a), *N. clevelandii* (b), and *N. glutinosa* (c). The plants were held in a glasshouse under natural illumination and photographed 12 days after inoculation.

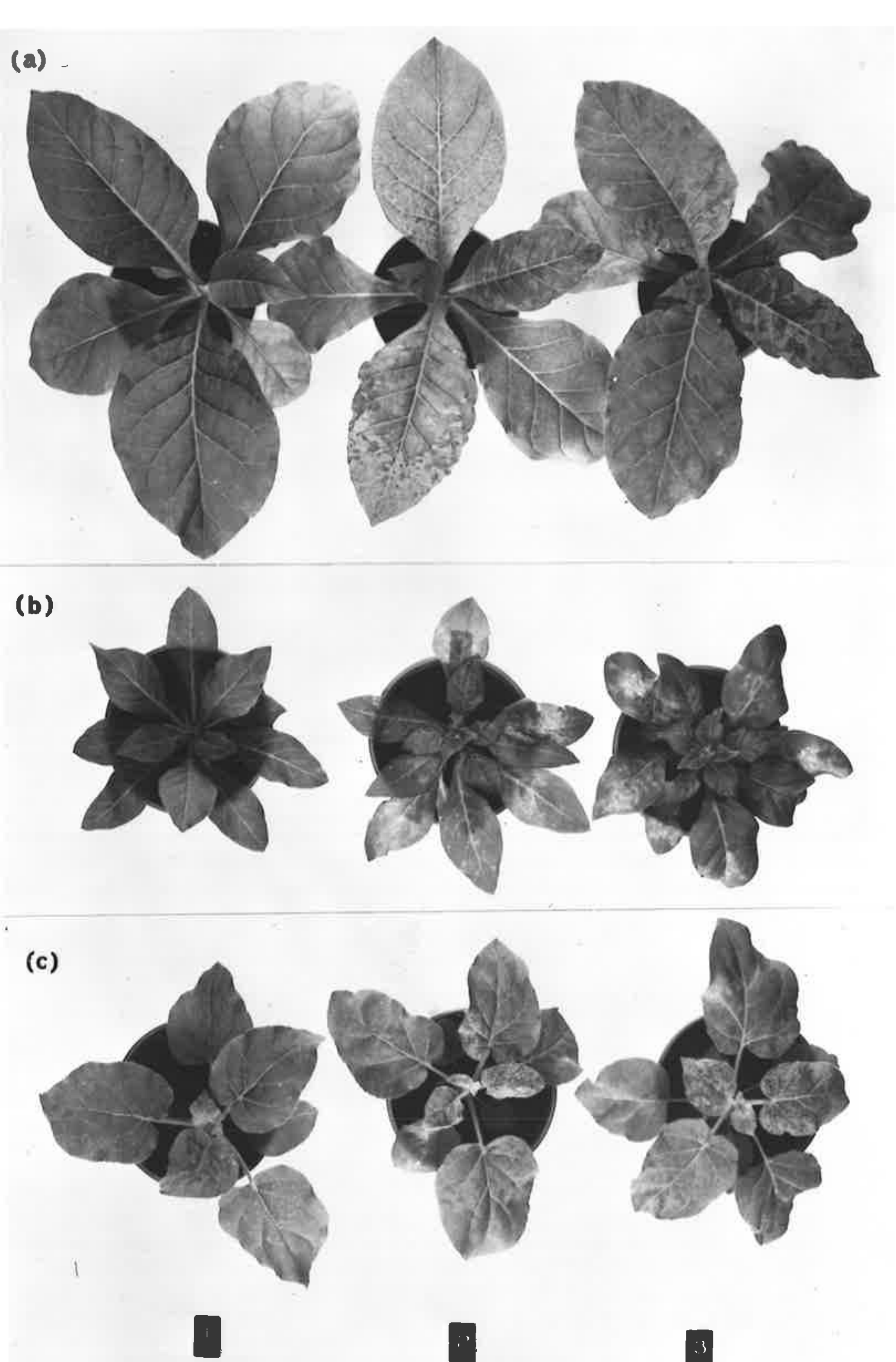

(a)
(b)
(c)
1
2
3

Table 7-4. Tests for base sequence homologies between pseudorecombinant and parental viral RNAs by competition hybridization.

Unlabelled RNA added as competitor[a]	TAV-RNA annealed (cpm)[b]	CMV-RNA annealed (cpm)[b]
Nil	1,186	1,088
CMV-RNA	1,014	248
TAV-RNA	332	1,011
PR-RNA[c]	392	708

a Competitor RNA (30 μg) was added to ^{14}C-labelled RNA (3 μg) and the salt-soluble nucleic acid fraction from virus-infected *N. clevelandii*.

b Experimental details as described in Table 7-1 and Methods.

c RNA isolated from a preparation of purified pseudorecombinant virus.

virus has more extensive nucleotide base sequence homology with TAV-RNA than with CMV-RNA.

(6) The quantity of the virus synthesised in *N. clevelandii* tissue was as high as that of TAV. In this host TAV yields 8-10 times more virus per gram of leaf tissue than CMV (Fig. 7-1; see also Chapter 2).

Conclusions

Studies reported in this Chapter indicate that:

(1) The RNAs of TAV and CMV do not appear to have any significant base sequence homology (Tables 7-1 and 7-4), and yet they can produce a pseudorecombinant virus.

(2) The RNA of the pseudorecombinant virus has greater nucleotide base sequence homology with TAV-RNA than with CMV-RNA (Table 7-4), and its RNA 3 and RNA 4 have electrophoretic mobilities in PAGs characteristic of CMV-RNA. This leads to the conclusion that it is a pseudorecombinant whose genome consists of $T_1 + T_2 + C_3 + C_4$.

(3) The coat protein cistron must be located on RNA 3 of TAV and CMV, since the pseudorecombinant virus is serologically indistinguishable from CMV (Fig. 7-3).

(4) Since the pseudorecombinant virus induces host reactions characteristic of TAV, it follows that the cistron or cistrons

regulating the host reactions studied must be located on RNA 1 or RNA 2 or are distributed between them. It appears that the cistron or cistrons determining yield of the viruses are also located on RNA 1 or RNA 2.

(5) These conclusions indicate that RNA species 1, 2 and 3 contain some unique segments of the TAV and CMV genomes.

CHAPTER 8

GENERAL DISCUSSION

I. Physical and Chemical Properties of TAV and CMV

1. Properties of virus particles

Studies carried out on the properties of TAV and CMV have shown that they are indistinguishable on the basis of their particle morphology and sedimentation rate (Chapter 3). Although preparations of each virus produce a single homogeneous peak in sucrose density gradients, at least four RNA species with a total molecular weight of over three million daltons are associated with each virus (Figs. 3-3 and 3-4). Since each virus particle is capable of enclosing RNA with a molecular weight of only about one million daltons (Kaper, 1968), three types of nucleoprotein particles are expected to be present in purified preparations of each virus (Fig. 8-1), similar to those found in preparations of the bromoviruses (Lane and Kaesberg, 1971; Bancroft and Flack, 1972). The presence of three types of nucleoprotein particles in preparations of several strains of CMV and one strain of TAV has already been suggested (Kaper and West, 1972; Peden and Symons, 1973; Lot and Kaper, 1973; Lot *et al.*, 1974). It is believed that RNA 1 and RNA 2 are each encapsidated separately, whereas RNA 3 and RNA 4 are enclosed in the same capsid (Fig. 8-1).

Some support for such a model comes from the isopycnic centrifugation of formaldehyde-treated TAV and CMV preparations in CsCl carried out in this study (Figs. 3-8 and 3-9) and from similar studies of untreated virus in D_2O-sucrose (Lot *et al.*, 1974). From the poorly resolved bands in CsCl gradients (Fig. 3-8a) it was possible to recover more of RNA 1 from the bottom fractions (particles A: Fig. 8-1) and more of RNA 2 from the top fractions (particles B). However, species 3 and 4 were found to be equally distributed between both fractions (particles C), which is difficult to reconcile with the proposed model.

Attempts to demonstrate the presence of three types of nucleoprotein particles in preparations of TAV and CMV by separating them according to suspected differences in their densities were inconclusive. This was not altogether surprising, since both TAV and CMV were unstable in CsCl and had to be fixed with formaldehyde, a treatment which could have affected capsid structure sufficiently to alter their densities. An alternative approach to the problem may be to reconstitute separated RNA species in various combinations with viral protein and compare the physical properties (sedimentation rate and morphology) of the reconstituted products to those of normal virus.

If RNA 3 and RNA 4 are indeed encapsidated in one particle, then viral RNA preparations should contain equimolar proportions of the two RNA species. This appears to be so in preparations of CMV-RNA, but not in those of TAV-RNA (Chapter 3; see also Lot *et al.*, 1974). The

Fig. 8-1. Proposed structures of TAV and CMV nucleoproteins in relation to various RNA species isolated from virus preparations. The particle weights are calculated from the molecular weights of the various RNA species (Fig. 3-4) and those of the viral protein subunits as determined by PAG electrophoresis (Fig. 3-6) and the amino acid compositions (Table 3-3).

	TAV			CMV			
	A	B	C	A	B	C	
PARTICLES	1	2	3 + 4	1	2	3 + 4	
MOLECULAR WEIGHT OF RNA IN CAPSID x 10^6	1.26	1.10	0.90 + 0.43	1.26	1.10	0.77 + 0.34	MOLECULAR WEIGHT OF PROTEIN SUB-UNIT
PARTICLE WEIGHT x 10^6	5.65 5.98	5.49 5.82	5.72 6.05	5.65 5.98	5.49 5.82	5.50 5.83	24500[a] 26200[b]
% RNA IN CAPSID	21.2 22.3	18.9 20.0	22.0 23.3	21.2 22.3	18.9 20.0	19.0 20.2	24500 26200

[a] Determined by polyacrylamide-gel electrophoresis.

[b] Calculated from the amino acid analyses of TAV and CMV proteins.

lack of equimolarity of RNA 3 and RNA 4 was even more pronounced in RNA preparations from reconstituted TAV (Table 5-2). It may be that the distribution of RNA species in TAV capsids is more complex than CMV, and that the minor RNA species, i.e. RNA 5 and RNA 6 (Table 3-2) may be involved in the construction of some types of capsids.

If it is assumed that the distribution of the major RNA species in TAV and CMV capsids is similar to that summarized in Fig. 8-1, and that each protein shell of TAV and CMV consists of 180 chemical subunits (Finch *et al.*, 1967), then it is possible to calculate the particle weight of the various nucleoproteins using the molecular weight estimates of protein subunits and RNA (Chapter 3). These are summarized in Fig. 8-1 and agree reasonably well with some of the published values (Francki *et al.*, 1966; van Regenmortel *et al.*, 1972).

Chemical analyses have shown that TAV contains about 17.7% RNA (Stace-Smith and Tremaine, 1973), whereas values between 18.0 and 18.5% have been reported for CMV (Kaper *et al.*, 1965; Francki *et al.*, 1966; van Regenmortel, 1967). From the data on the size of the protein subunits and RNAs of the two viruses (Chapter 3), values calculated for the percentage RNA are somewhat higher (Fig. 8-1). The RNA contents of the two viruses have also been calculated from the buoyant densities of their particles in CsCl by the method of Sehgal *et al.* (1970) which gave a value of 23.8% for both TAV and CMV. This value is slightly higher than those obtained from data summarized in

Fig. 8-1.

TAV capsids dissociate in the presence of EDTA and are stabilized by low concentrations of $MgCl_2$ in the suspending buffer. The requirement for Mg^{2+} by TAV for maximum stability is similar to that of bromoviruses (Brakke, 1963; Bancroft, 1970). However, unlike bromoviruses, empty protein shells were not formed *in vitro* from viral protein devoid of RNA (Fig. 5-1).

In contrast to TAV, CMV capsids aggregate in the presence of Mg^{2+} and are stabilized with EDTA, a property similar to that of alfalfa mosaic virus (Hull and Johnson, 1969).

2. Properties of TAV- and CMV-RNAs

TAV-RNA and CMV-RNA have indistinguishable nucleotide base composition (Table 3-1) although no base sequence homology could be detected (Tables 7-1 and 7-4). Although RNA 1 and RNA 2 of each virus have indistinguishable electrophoretic mobilities in PAGs, species 3 and 4 differ significantly. The molecular weights of the viral RNA species determined by PAG electrophoresis are very similar when the experiments were carried out in the presence or absence of formamide (Chapter 3).

TAV isolates contain higher proportions of RNA 1 than RNA 2, whereas the CMV isolates contain more RNA 2 than RNA 1 (Chapter 3; see also Lot *et al.*, 1974). The reason for this difference is not

known although Lot *et al.* (1974) suggest that perhaps some of the RNA molecules in preparations of CMV-RNA 2 are degradation products of RNA 1.

3. Properties of TAV and CMV coat proteins

The proteins of TAV and CMV have the same molecular weights, but different amino acid compositions (Chapter 3). Based on the amino acid compositions, strains of TAV form a group readily distinguishable from those of CMV (Table 3-3; see also Stace-Smith and Tremaine, 1973). The groups appear to differ mainly in their arginine, threonine, serine, cysteine and methionine content (Table 3-3). These differences appear to affect other properties of the proteins and hence the viruses. For example, TAV protein is more soluble in low ionic strength buffers than CMV protein. The pIs of the two viruses vary considerably, indicating that different net charges are located on the surface of the capsids. There are also differences in the behaviour of TAV and CMV in the presence of Mg^{2+} and EDTA (Chapters 4 and 5).

II. Serological Properties of TAV and CMV

It was observed that CMV was significantly less immunogenic than TMV, confirming the results obtained by Francki *et al.* (1966) and Scott (1968). The immunogenicity of TAV was shown not to be

significantly different from that of CMV, supporting the view of Stace-Smith and Tremaine (1973), but not that of Hollings and Stone (1971) who claimed that TAV was a good immunogen.

1. Serological properties of TAV

Rabbit anti-TAV sera have been observed by several workers to produce two immunodiffusion lines when reacted with homologous antigen (Grogan *et al.*, 1963; Lawson, 1967; Stace-Smith and Tremaine, 1973). It was suggested that the faster diffusing antigen was a dissociated product of the capsids (Grogan *et al.*, 1963). However, the nature of these dissociated products were not characterized and were referred to as soluble antigens (van Regenmortel, 1966a). In the present studies antisera producing double lines (SC-antisera) were obtained from up to 40% of mice in some stage of their immunization with TAV (Fig. 6-11). The straight line was confluent with a single line produced by the reaction of these antisera with isolated TAV protein. Furthermore, the straight line was not observed when the SC-antisera were pretreated with TAV protein in intragel-absorption tests. These findings demonstrate that the straight line was produced by the reaction of antibodies with TAV protein, and that the capsids disintegrate in gells. Failure to detect antibodies responsible for the straight line in some animals containing high titres of antibodies against the intact immunogen (curved lines; Fig. 6-11) is difficult

to explain, but may be a genetically controlled characteristic of the mice.

Antisera producing only the curved precipitin line against TAV did not react with TAV protein in immunodiffusion tests (Figs. 6-10b and 6-13b). It seems that these antibodies were elicited specifically against antigenic determinants present on the surface of the capsids which must have been destroyed on dissociation of capsids into protein subunits (von Wechmar and van Regenmortel, 1968).

Although isolated TAV protein was immunogenic in mice, its immunogenicity was very much lower than that of the intact nucleoprotein. Furthermore, antibodies specific to intact viral capsids present in the SC-antisera had higher titres than those specific to TAV protein subunits. It may be that the presence of RNA is responsible for enhancement of the immune response. Hirata *et al.* (1972) observed that the coat protein of bacteriophage Qβ was only slightly immunogenic in rabbits, whereas the reconstituted virus as well as the coat protein monomer-succinylated lipopolysaccharide complex were very much more immunogenic. Marbrook and Matthews (1966) observed that TMV and the reconstituted virus were more immunogenic than protein subunits or rod-shaped particles reconstituted from viral protein alone. They also found that nucleoprotein particles of turnip yellow mosaic virus induced a higher response in mice than empty protein shells.

Anti-TAV protein sera produced a single precipitin line when

reacted with preparations of intact TAV which was confluent with the line produced between the antiserum and homologous antigen (Fig. 6-13). This suggests that breakdown of TAV capsids occurred in the agar gel and cryptotopes (antigenic determinants present on the surface of protein subunits; von Wechmar and van Regenmortel, 1968) were exposed and reacted with the specific antibodies present in the anti-TAV protein sera. Similar results were obtained with brome mosaic virus by von Wechmar and van Regenmortel (1968). They also observed that when formaldehyde-treated capsids reacted in immunodiffusion tests with antisera produced against brome mosaic virus protein, no visible precipitin line was detected. Under these conditions the capsids would be expected not to dissociate to release protein subunits.

2. Serological properties of CMV

Although double immunodiffusion lines were observed with some strains of CMV (Scott, 1968; Devergne and Cardin, 1970), only a single curved line was invariably detected between anti-CMV sera and the homologous antigen in the present studies. Scott (1968) observed only a curved line when antisera obtained from rabbits after intravenous injections of the Y strain of CMV were tested against homologous antigen, whereas both a curved and a straight line were detected with antisera from animals receiving intramuscular injections of the same antigen.

The protein of CMV-Y produced a precipitin line with the SC-antisera which was confluent with the straight line (Scott, 1968). However, no line was observed between CMV-Y protein and the anti-CMV-Y serum which contained antibodies only to intact virus antigen. It appears that the antigenic properties of CMV-Y are similar to those of TAV rather than to CMV used in this study.

Injection of CMV protein failed to induce detectable antibody production in mice (Fig. 6-6). This could be due to the precipitation of the protein under physiological conditions. Hirata *et al.* (1972) observed that coat protein of Qβ phage was not immunogenic in the aggregated form.

III. The Genetics of TAV and CMV

The results summarized in Chapter 3 and Chapter 7 indicate that TAV and CMV contain functionally divided genomes. The results confirm those of Peden and Symons (1973) on CMV and Lot *et al.* (1974) on both CMV and TAV (CMV-TAV-898).

TAV and CMV appear to be suitable for the genetic analysis of the viruses because of their many distinguishing features and their ability to complement each other. Some other viruses with divided genomes are more difficult to work with. For example, some members of the comovirus group do not complement each other, although a serological relationship exists between them (Swaans and van Kammen,

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1973). Tobacco rattle and pea early browning viruses are also distantly serologically related (Maat, 1963) yet no genetic complementation could be demonstrated (Lister, 1968).

A maximum of six genetically different pseudorecombinant viruses can theoretically be constructed from suitable combinations of the three largest RNA species of TAV and CMV. However, at present there appears to be no satisfactory technique of separating RNA 1 and RNA 2 quantitatively. In this study only a single pseudorecombinant virus has been constructed by mixing $T_1 + T_2$ with C_3. Attempts to form a virus from the mixture of $C_1 + C_2 + T_3$ were not successful (Chapter 7). Similarly, Bancroft (1972) failed to construct a pseudorecombinant by mixing RNA 3 of brome mosaic virus with RNA 1 and RNA 2 of cowpea chlorotic mottle virus, although the reverse combination was successful.

The pseudorecombinant virus resulting from the infection of $T_1 + T_2 + C_3$ had serological properties indistinguishable from those of CMV (Fig. 7-3). This indicates that the cistron for coat protein is located on RNA 3 of TAV and CMV. This conclusion has been confirmed by Schwinghamer and Symons (1974), who demonstrated that the translation of C_3 *in vitro* resulted in the synthesis of two proteins, one of which was indistinguishable from that of CMV coat protein. The location of the coat protein cistron on RNA 3 of cucumoviruses is similar to that of bromoviruses where the cistron is also located on RNA 3 (Lane and Kaesberg, 1971; Bancroft, 1972; Shih and Kaesberg, 1973), and that of

alfalfa mosaic virus where it is located on Tb-RNA (comparable to RNA 3) (Bol and van Vloten-Doting, 1973). It is interesting that in all plant viruses with single-stranded RNA divided genomes which have so far been adequately investigated, the coat protein cistron is located on the smallest essential RNA species (van Kammen, 1972; Harrison *et al.*, 1974).

RNA 4 recovered from the pseudorecombinant virus showed some properties similar to C_4 (Chapter 7). Since the virus was derived from inoculum containing $T_1 + T_2 + C_3$ it would appear that C_4 was either introduced as a contaminant of C_3 (although re-electrophoresis in PAG of C_3 preparations did not show any detectable contaminating RNAs), or else C_3 and C_4 are synthesized from a common replicative complex. The latter suggestion is likely since Schwinghamer and Symons (1974) have shown that in an *in vitro* system, both C_3 and C_4 are capable of programming the synthesis of CMV coat protein, hence these RNA species must both contain the same cistron. The presence of a coat protein cistron on RNA 4 of brome mosaic virus (Shih and Kaesberg, 1973) and on the comparable RNA species of alfalfa mosaic virus (Ta-RNA) (van Ravenswaay Claasen *et al.*, 1967) has also been demonstrated.

The symptoms produced by the pseudorecombinant virus on the host plants studied (Table 7-3) were all indistinguishable from those

produced by the parent providing $T_1 + T_2$ (TAV). A similar observation has been reported by Dingjan-Versteegh *et al.* (1972) with alfalfa mosaic virus. However, the symptoms produced by a pseudorecombinant virus constructed from parts of the genomes of brome mosaic and cowpea chlorotic mottle viruses were intermediate between those of the parents (Bancroft, 1972). In TAV and CMV no cistrons involved in the production of symptoms (Table 7-3) were localized on RNA 3, whereas in bromoviruses most symptoms were determined by cistrons on this species of RNA (Bancroft and Lane, 1973). At present the biochemical reactions involved in symptom development are not known. RNA 1 and RNA 2 of TAV and CMV which appear to contain cistrons determining host reactions are capable of translating between 8 to 12 molecules of proteins in the *in vitro* system (Schwinghamer and Symons, 1974). However, the identification of these proteins and their functions is yet to be studied. For those viruses in which the genes responsible for symptom expression have been found to be linked with that for coat protein (Bancroft and Lane, 1973; Harrison *et al.*, 1974), it has been suggested that a different expression of the same gene may be involved.

IV. Taxonomic Position of TAV and CMV

Although the RNAs of TAV and CMV have indistinguishable nucleotide base ratios (Chapter 3) they do not appear to have any base

sequence homology (Chapter 7). This, together with a lack of any detectable serological relationship between the two viruses (Table 6-1), argues that they are not closely related. However, the demonstration that at least parts of the two viral genomes are interchangeable and can produce a pseudorecombinant virus suggests that some sort of taxonomic relationship must exist between the two viruses. At present the exact or relative taxonomic significance of base sequence homology, serological relationship and interaction of the viral genomes is uncertain. For example, the severe and yellow strains of cowpea mosaic virus were shown to be distantly related serologically (Agrawal and Maat, 1964), but surprisingly no base sequence homology (van Kammen and Rezelman, 1972) or genetic complementation (van Kammen, 1968) could be demonstrated. This contrasts with the relationship between TAV and CMV where neither a serological relationship nor any base sequence homology was detected, and yet genome complementation was demonstrated.

TAV and CMV are cosmopolitan viruses which occur as numerous strains in a wide variety of plants (Smith, 1972). This ecological success may be at least in part, due to their divided genomes which by reassortment of components could be an important source of variation ensuring adaptability. Influenza virus epidemics probably arise by the introduction of new 'recombinants' (probably equivalent to pseudo-

recombinant as defined in this thesis) from similar reassortments of RNA segments derived from distinct virus strains (Fenner, 1973).

Conflicting reports on the serological relationship between TAV and CMV by various workers (see Chapter 1) may be at least partly due to the fact that various isolates of the two viruses were used. It would appear that virus strains with properties intermediate to those of the TAV and CMV studied here exist. The line of demarcation between TAV and CMV may well be difficult to make, especially as strains of these viruses may pseudorecombine in nature.

There seems no doubt that TAV and CMV are sufficiently similar to be assigned to the same group, the cucumovirus group, as proposed by Harrison *et al.* (1971). However, it is difficult to come to a conclusion as to whether the isolates of TAV and CMV studied here should be considered as distinct viruses or distantly related strains of the same virus. In virus classification there appears to be no concept of species comparable to that applied to higher organisms. Ill-defined terms such as 'strain' and 'serotype' (Kassanis, 1961; van Regenmortel, 1966a) have been used rather indiscriminately in the past. As no serological relationship between TAV and CMV could be demonstrated, these two virus isolates cannot be referred to as serotypes. However, the possibility remains that there may exist other virus isolates with serological properties linking the TAV and CMV used in the present study.

The ability of higher organisms to hybridize genetically is usually considered as an important criterion of close phylogenetic relationship. If this concept is extended to viruses, then TAV and CMV are undoubtedly close relatives. Thus, for the present, at least, it may be wise to retain the names TAV and CMV until taxons used in virology are better defined.

Studies reported in this thesis confirm and extend many similarities between the cucumovirus and bromovirus groups on the one hand, and the cucumovirus group and alfalfa mosaic virus on the other. These three virus groups may well be considered as candidates for a higher taxon.

APPENDIX

PUBLICATIONS

Francki, R.I.B., and Habili, N. (1972). Stabilization of capsid structure and enhancement of immunogenicity of cucumber mosaic virus (Q strain) by formaldehyde.
Virology 48, 309-315.

Habili, N., and Francki, R.I.B. (1974). Comparative studies on tomato aspermy and cucumber mosaic viruses. I. Physical and chemical properties.
Virology 57, 392-401.

Habili, N., and Francki, R.I.B. (1974). Comparative studies on tomato aspermy and cucumber mosaic viruses. II. Virus stability.
Virology (in press).

Habili, N., and Francki, R.I.B. (1974). Comparative studies on tomato aspermy and cucumber mosaic viruses. III. Further studies on relationship and construction of a virus from parts of the two viral genomes.
Virology (in press).

Habili, N., and Francki, R.I.B. (1974). Comparative studies on tomato aspermy and cucumber mosaic viruses. IV. Immunogenic and serological properties.
Virology (in preparation).

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VIROLOGY 48, 309–315 (1972)

Stabilization of Capsid Structure and Enhancement of Immunogenicity of Cucumber Mosaic Virus (Q Strain) by Formaldehyde

R. I. B. FRANCKI AND N. HABILI

Department of Plant Pathology, Waite Agricultural Research Institute, University of Adelaide, South Australia

Accepted January 4, 1972

Treatment of the Q strain of cucumber mosaic virus (QCMV) with 0.2% formaldehyde for 24 hr at 4° in 0.005 *M* borate buffer, pH 9, stabilizes capsid structure and enhances immunogenicity in mice. Treated virus remained in suspension on mild heating in the presence of electrolyte whereas normal virus precipitated under similar conditions. Protein shells of formaldehyde-treated virus retained their structure during digestion of the viral RNA with pancreatic ribonuclease while those of normal virus degraded rapidly. Formaldehyde-treated capsids retained their structure on exposure to phosphotungstic acid, pH 6.8, during preparation for electron microscopy under conditions which degraded normal virus. It is concluded that the observed changes in the physical properties of QCMV by formaldehyde treatment are the results of cross linking of reactive sites on the adjacent subunits of the protein shells rendering the structural integrity of the capsids less dependent on bonding between the viral RNA and protein. The enhanced immunogenicity of formaldehyde-treated QCMV may be due to ability of the antigen to retain its structural integrity in the tissues of the animal.

INTRODUCTION

The instability of cucumber mosaic virus (CMV) is well known; the virus is readily degraded by strong salts (Kaper *et al.*, 1965; Francki *et al.*, 1966), pancreatic ribonuclease (RNase) (Francki, 1968; Kaper and Geelen, 1971), and neutral phosphotungstic acid (PTA) (Francki *et al.*, 1966). It has also been observed that CMV is a poor immunogen (Francki *et al.*, 1966; Scott, 1968). The immunogenicity of plant viruses can sometimes be enhanced by treating the antigen with formaldehyde (Hollings and Stone, 1962), but Scott (1968) failed to achieve such an effect on the Y strain of CMV (CMV-Y). In this paper are reported experiments in which we have investigated the stabilizing effect of formaldehyde on capsids of the Q strain of CMV (QCMV) and have shown that the immunogenicity of stabilized virus in mice is significantly higher than that of untreated antigen.

MATERIALS AND METHODS

Virus isolates and infectivity assays. The Q strain of CMV was used throughout this work; it was maintained on cucumber (*Cucumis sativus* L. var. 'Polaris') and assayed on cowpea (*Vigna sinensis* Endl. type 'Black Eye') (Francki, 1964). The U1 strain of TMV (Siegel and Wildman, 1954) was also used in some experiments.

Virus purification. QCMV was purified by chloroform clarification, differential centrifugation, and density-gradient centrifugation (Francki, 1968). TMV was purified by charcoal and DEAE-cellulose clarification, filtration through Celite, and differential centrifugation (Francki and McLean, 1968).

Treatment of QCMV with formaldehyde. Formaldehyde (May & Baker Ltd., Degenham, England; 40 % w/v solution) was added to freshly purified QCMV to a concentration of 0.2 % and dialysed against 0.2 % formaldehyde in 0.005 *M* borate buffer, pH 9, for 24 hr after which excess formaldehyde

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was removed by dialysis against 0.005 *M* borate buffer, pH 9. Untreated virus preparations used as controls were subjected to similar dialysis steps against buffers without formaldehyde.

Analytical and enzymatic techniques. Spectrophotometric measurements were made in a Shimadzu QR50 spectrophotometer, and sucrose density-gradient analyses with an ISCO apparatus (Francki, 1968). Treatment of QCMV with pancreatic RNase was carried out as described by Francki (1968) except that higher enzyme concentrations (1 μg/ml) were used throughout this work.

Electron microscopy. Specimens for examination in a Siemen's Elmiskop I instrument were prepared as described by Davison and Francki (1969).

Experimental animals and collection of serum. Immunogenicity of viral antigens was studied in an outbred strain of Swiss mice. Adult animals were injected intraperitoneally (Marbrook and Matthews, 1966) with 20 μg of antigen per injection. When adjuvant was used the antigen was emulsified with an equal volume of Freund's complete adjuvant before injection. Blood samples (50–150 μl) were collected by puncturing the retro-orbital plexus with a Pasteur pipette and transferring the blood to centrifuge tubes. Both tubes and pipettes were heparinized by allowing a drop of heparin solution (2 mg/ml) to dry in them at 100° to prevent the blood clotting. In this way the maximum volume of serum was recovered. The blood samples were centrifuged at 1500 *g* for 20 min, and the sera were stored at −15°.

Assay of precipitating antibodies. Double diffusion in agar was used so that (1) small volumes of serum could be used; (2) the nonspecific precipitation of QCMV in precipitin tube tests could be avoided (Francki *et al.*, 1966); and (3) the same serum sample could be concurrently titrated against more than one antigen. The tests were carried out in glass petri dishes 10 cm in diameter containing 12 ml of 0.75% agar in 0.01 *M* phosphate buffer, pH 7.6, with 0.02% sodium azide as preservative. Holes, 3 mm in diameter and 3.5 mm apart, were cut, and each was charged with 10 μl of serum or antigen. The plates were observed for precipitin lines after 7 days' incubation at 25° in a moist atmosphere. Sera were titrated by preparing 2-fold dilution series in 0.02 *M* phosphate buffer, pH 7.2, and testing against antigens at a concentration of 200 μg/ml. In the case of TMV the virus was subjected to sonication for 10 min at 0° so as to fragment the elongate particles to enhance diffusion through agar (Tomlinson and Walkey, 1967). Experimental results are expressed as geometric mean titres (five animals per treatment), the integers 1, 2, 3, ⋯ , corresponding to a visible precipitin line being observed at antiserum dilution of ½, ¼, ⅛, ...

RESULTS

Stabilization of QCMV Capsid Structure by Formaldehyde

Precipitation by mild heating in NaCl solutions. Francki *et al.* (1966) showed that QCMV was precipitated by mild heating in NaCl solutions. Results of the experiment summarized in Fig. 1 show that QCMV is

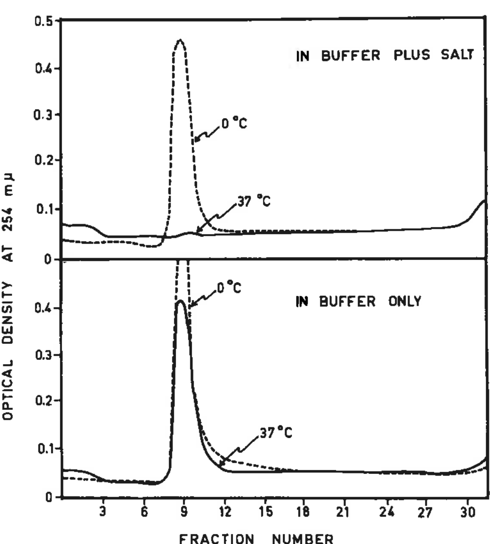

FIG. 1. Density-gradient centrifugation of QCMV after heating at 37° for 20 min in the presence (above) and absence (below) of 0.14 *M* NaCl in the suspending buffer (0.005 *M* borate, pH 9). After treatment 2 ml of each virus preparation was layered over a 5 to 25% linear sucrose density-gradient in 0.02 *M* phosphate buffer, pH 7.2, and centrifuged at 23,000 rpm for 2 hr in a Spinco SW 25 rotor.

precipitated almost completely by heating at 37° for 20 min when suspended in 0.14 *M* NaCl, whereas such heat treatment has very little effect when the virus is in 0.005 *M* borate buffer alone. The slight increase in the amount of ultraviolet absorbing material at the top of the density-gradient tubes containing heat-treated virus was probably the result of temperature-enhanced activity of traces of RNase (Francki, 1968) contaminating the virus preparations. However, the experiment shows that the bulk of the viral RNA was precipitated with the protein when heated in 0.14 *M* NaCl at 37°. The kinetics of loss of infectivity and precipitation of QCMV at 37° in the presence and the absence of 0.14 *M* NaCl are shown in Fig. 2. In the absence of salt, loss of infectivity was more rapid than virus precipitation which was again probably due to the presence of RNase in the preparations.

Infectivity assays demonstrated that the formaldehyde treatment rendered QCMV completely noninfectious. However, this treatment prevented the precipitation of viral nucleoprotein when heated in 0.14 *M* NaCl. In a typical experiment it was shown that heating untreated virus for 1 hr at 37° precipitated it completely, whereas under the same conditions formaldehyde-treated virus showed only slight signs of aggregation into dimers and trimers (Fig. 3). In other experiments the kinetics of QCMV precipitation were followed by monitoring the increase in light scattering of virus preparations spectrophotometrically during incubation at 25°. The results of a typical experiment summarized in Fig. 4 show that no increase in light scattering could be detected during a 140-min incubation of formaldehyde-treated virus whereas increase in light scattering by untreated virus was very rapid. During this time the ultraviolet absorption between 230 and 300 nm of the formaldehyde-treated virus did not change significantly whereas that of untreated virus increased substantially over this entire range as previously observed by Francki *et al.* (1966).

Degradation by pancreatic RNase. It has already been demonstrated that incubation of untreated QCMV with RNase reduces its infectivity, releases RNA from the capsids, and causes viral protein to precipitate (Francki, 1968). On comparing the effect of RNase on the ultraviolet absorption at 255 nm by QCMV and formaldehyde-treated QCMV incubated at 25°, it was shown that absorption by treated virus increased only slightly during the first hour of incubation and then remained rela-

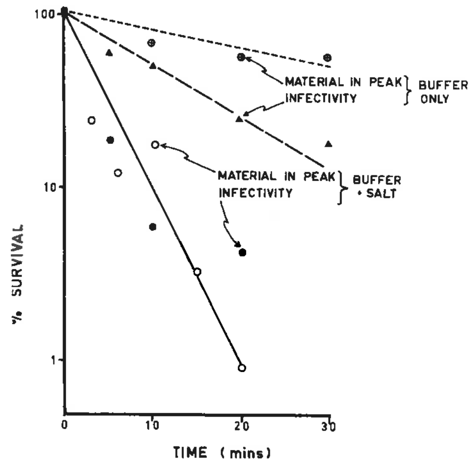

FIG. 2. Loss of infectivity and precipitation of QCMV on heating at 37° in the presence and in the absence of 0.14 *M* NaCl in the suspending buffer (0.005 borate, pH 9). Infectivity was determined by local lesion assays and precipitation of virus by sucrose density-gradient centrifugation as described in Fig. 1.

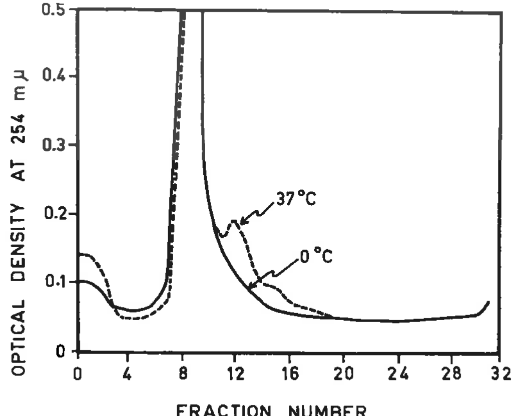

FIG. 3. Density-gradient centrifugation of formaldehyde-treated QCMV heated at 37° for 1 hr in the presence of 0.14 *M* NaCl in the suspending buffer (0.005 *M* borate, pH 9). Centrifugation conditions were as described under Fig. 1.

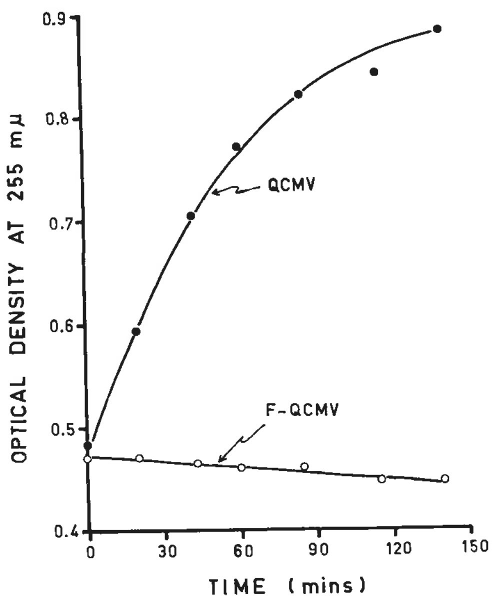

FIG. 4. Effect of 0.14 M NaCl in the suspending buffer (0.005 M borate, pH 9) on the optical density of QCMV (100 μg/ml) or formaldehyde-treated QCMV (F-QCMV) at 255 nm during incubation at 25°.

FIG. 5. Effect of RNase (1 μg/ml) in the suspending buffer (0.005 M borate, pH 9) on the optical density of QCMV (100 μg/ml) or formaldehyde-treated QCMV (F-QCMV) at 255 nm during incubation at 25°.

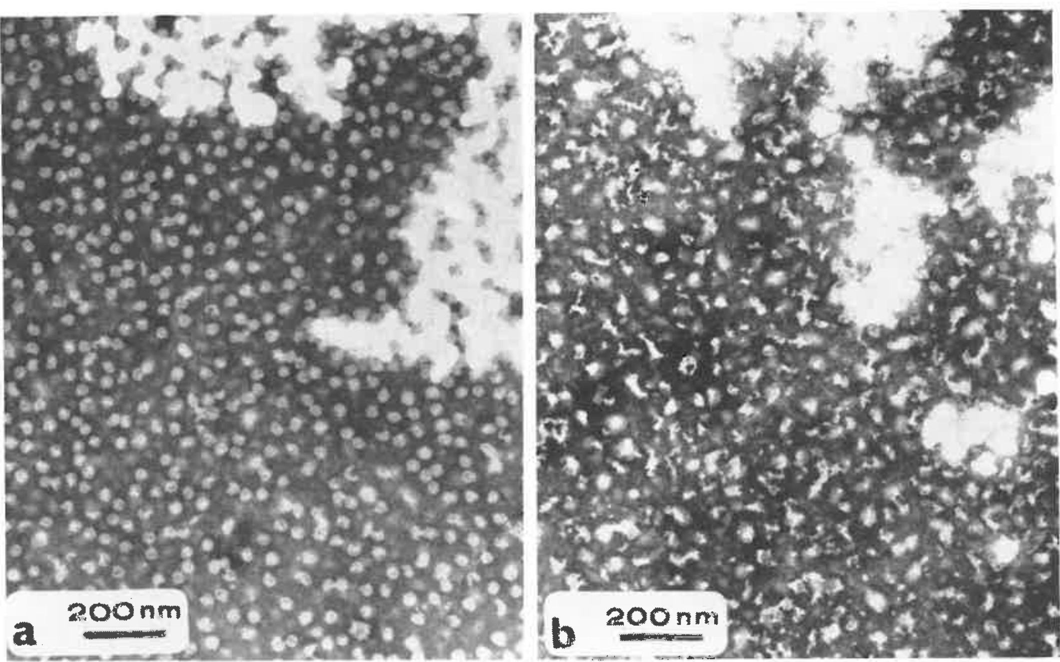

FIG. 6. Electron micrographs of formaldehyde-treated QCMV (a) and normal virus (b) negatively stained in phosphotungstic acid at pH 6.8.

tively constant (Fig. 5). However, a very marked increase in absorption was observed when untreated virus was incubated under similar conditions (Fig. 5), the shape of the curve being similar to those reported previously (Francki, 1968). Examination of absorption spectra between 230 and 300 nm of the two QCMV preparations after 60 min incubation showed that the increase in absorption by the untreated virus was over the entire range 230–300 nm, indicating precipitation of virus as previously observed (Francki, 1968). However, increase in absorption by the formaldehyde treatment was confined to the range 235–275 nm suggesting that viral RNA was digested by the RNase without precipitation of protein. This was confirmed by experiments in which formaldehyde-treated virus incubated with RNase was subjected to sucrose density gradient centrifugation. Material recovered from the tops of these gradients had an ultraviolet spectrum characteristic of nucleic acid, whereas the spectrum of material in the approximate position of the virus peak indicated the presence of nucleoprotein with relatively low amounts of RNA.

Disruption by phosphatungstic acid. Francki *et al.* (1966) failed to observe significant numbers of intact particles of QCMV in electron micrographs of virus preparations stained with phosphotungstic acid (PTA) at neutral pH. On comparing normal and formaldehyde-treated QCMV (Fig. 6), it appears that formaldehyde treatment stabilizes viral capsids which remain intact

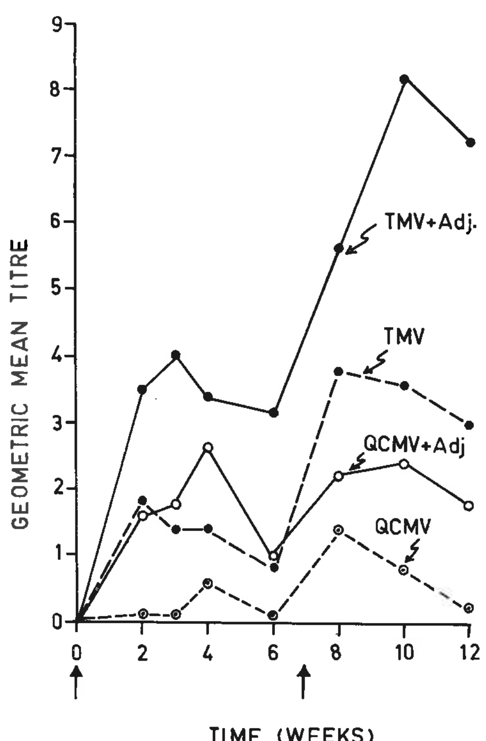

Fig. 7. The immune response of outbred Swiss mice (5 animals per treatment) to TMV and QCMV injected intraperitoneally in the presence (+ Adj) and in the absence of Freund's complete adjuvant. All mice were injected at the commencement of the experiment and 7 wk later (indicated by vertical arrows) using 20 μg of antigen per animal for each injection.

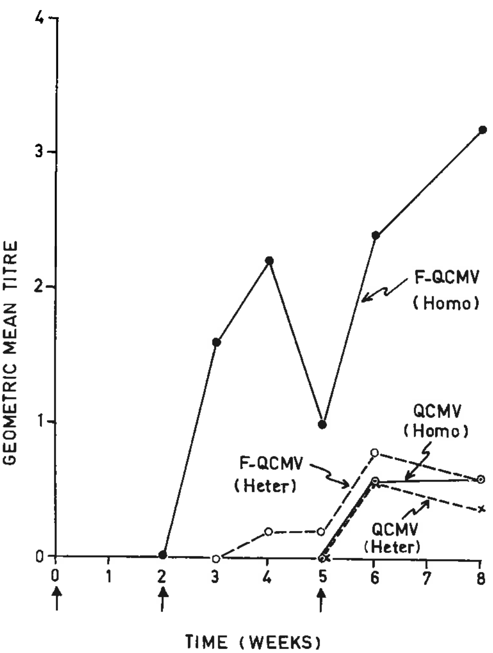

Fig. 8. The immune response of outbred Swiss mice (5 animals per treatment) to normal (QCMV) and formaldehyde-treated (F-QCMV) virus in the presence of Freund's complete adjuvant. Solid lines indicate serum titres when assayed against homologous antigen (Homo) and broken lines indicate titres when assayed against heterologous antigen (Heter). All animals were injected intraperitoneally at the commencement of the experiment and 2 and 5 wk later (indicated by vertical arrows), each time with 20 μg of antigen per animal.

during preparation for electron microscopy by PTA staining.

Immunogenicity of QCMV

Relative immunogenicity of QCMV and TMV. Although Francki *et al.* (1966) and Scott (1968) reported CMV to be a poor immunogen, its immunogenicity was not compared quantitatively to that of any other antigen. In the present study we have compared the immune response of mice to QCMV and TMV as the latter is a well characterized, highly immunogenic antigen (Rappaport, 1965). The results of one such experiment (Fig. 7) show that TMV is far more immunogenic than QCMV. Statistical analyses of these results by the Kolmogarov-Smirnov two-sample test (Siegel, 1956) showed significant differences ($P > 0.01$) in the immune response of mice to QCMV and TMV whether injected with or without adjuvant. The differences in the response to both antigens were significantly greater ($P > 0.01$) when injected in the presence of adjuvant.

Enhancement of immunogenicity of QCMV by formaldehyde treatment. Results of an experiment in which the immunogenicity of formaldehyde-treated QCMV was compared to that of normal virus are summarized in Fig. 8. Sera from animals immunized with formaldehyde-treated QCMV were significantly higher than those immunized with normal virus whether titrated against homologous or heterologous antigen. The differences in titre of sera from animals immunized with formaldehyde-treated QCMV, when tested against homologous and heterologous antigens, is at least in part due to the treated virus being a better test antigen for gel diffusion. We have observed that in many sera antibodies elicited in response to either formaldehyde-treated or normal QCMV were more readily detected when formaldehyde-treated antigen was used (Fig. 9).

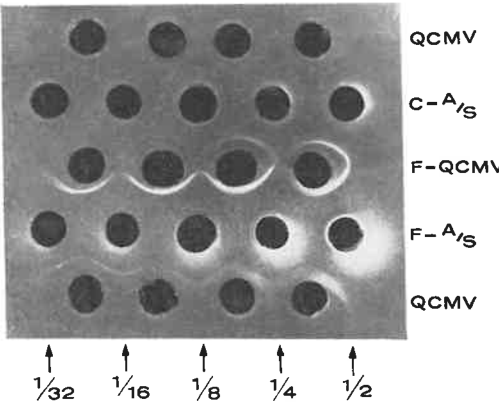

FIG. 9. Serological reactions in gel-diffusion test, between QCMV, its antiserum (C-A/S), formaldehyde-treated virus (F-QCMV) and its antiserum (F-A/S). All wells contained the same concentration of antigen (200 μg/ml) but the sera were diluted as indicated beneath the figure.

DISCUSSION

In comparison with a highly immunogenic virus such as TMV (Rappaport, 1965), QCMV is a poor immunogen (Fig. 7). This could be due to the instability of the QCMV capsid structure or its tendency to precipitate on mild heating in the presence of electrolytes (Francki *et al.*, 1966; Francki, 1968). We have now shown that the immunogenicity of QCMV can be enhanced by formaldehyde treatment (Fig. 8), which also prevents its precipitation in the presence of electrolytes (Figs. 3 and 4) and stabilizes the capsid structure against degradation by RNase (Fig. 5) and exposure to PTA (Fig. 6).

QCMV capsid structure appears to be maintained mainly by electrovalent bonding as the capsids are readily degraded in solutions of salts such as LiCl and $CaCl_2$ (Francki *et al.*, 1966). It also appears that virion structure is dependent largely on bonding between the RNA and protein as when virus preparations are incubated with RNase, the RNA is degraded and the capsid structure destroyed (Francki, 1968). Our observations on formaldehyde-treated QCMV suggest that the stabilizing effect is primarily on the protein shells as the RNA of treated capsids remains susceptible to RNase digestion whereas the protein shells show resistance to degradation (Fig. 5). It is known that the primary effect of formaldehyde on proteins involves the free amino groups with the formation of methylol groups which is probably followed by the condensation of other functional groups (Kabat and Mayer, 1961). The overall effect

on the virus capsid is probably to cross-link reactive sites tying side chains on adjacent subunits together. Such structural changes would render the capsids less dependent on protein-RNA interactions for structural integrity and would reduce the number of free functional groups on the surface of the protein shells which are probably responsible for virus precipitation in the presence of electrolytes.

The use of formaldehyde in the preparation of viral vaccines ensures loss of infectivity without significant changes in antigencity of the virus (Fraenkel-Conrat, 1969). In the case of the production of antisera to plant viruses it is not important to render the virus noninfectious. However, it appears from our work with QCMV that formaldehyde may be a very useful agent in the preparation of antisera to poorly immunogenic viruses by prolonging survival of intact immunogen in the tissues of the animal. Preliminary experiments using QCMV as antigen in gel-diffusion tests showed that formaldehyde-treated virus produced clearer precipitin lines and was more sensitive in detecting antibodies in both normal and formaldehyde-treated QCMV (Fig. 9). Thus, it appears that formaldehyde treatment may be useful in serological studies of plant viruses in stabilizing viruses for use as test antigens as well as immunogens.

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VIROLOGY 57, 392-401 (1974)

Comparative Studies on Tomato Aspermy and Cucumber Mosaic Viruses

I. Physical and Chemical Properties

N. HABILI AND R. I. B. FRANCKI

Department of Plant Pathology, Waite Agricultural Research Institute, University of Adelaide, South Australia

Accepted October 23, 1973

Some physical and chemical properties of the V strain of tomato aspermy virus (TAV) and the Q strain of cucumber mosaic virus (CMV) have been compared. The size, morphology, sedimentation rate, RNA base ratio, and buoyant density of the two viruses are indistinguishable. Preparations of RNA from both viruses were each resolved into four distinct species by polyacrylamide-gel electrophoresis. TAV-RNA preparations contained species with molecular weights of 1.26×10^6, 1.10×10^6, 0.90×10^6, and 0.43×10^6 daltons, and CMV-RNA, species of 1.26×10^6, 1.10×10^6, 0.77×10^6, and 0.34×10^6 daltons. Analysis of sodium dodecyl sulphate (SDS)-treated viral proteins by polyacrylamide-gel electrophoresis showed that both viruses have protein subunits of molecular weight 24,500 daltons. The amino acid compositions of proteins from the two viruses, although similar, were distinguishable, and the calculated molecular weights of protein subunits were 26,100 and 26,300 daltons for TAV and CMV, respectively. The two viruses were serologically distinct. On the data presented it is suggested that in preparations of both TAV and CMV three distinct particles are present in each with identical protein shells, but different RNA cores.

INTRODUCTION

It is generally agreed that tomato aspermy virus (Hollings and Stone, 1971) and cucumber mosaic virus (Gibbs and Harrison, 1970) have many properties in common and both have been assigned to the Cucumovirus group (Harrison *et al.*, 1971). However, there is lack of agreement as to whether the two are serologically related (van Slogteren, 1958; Grogan *et al.*, 1963; van Regenmortel, 1966; Lawson, 1967; Mink, 1969) and whether they cross-protect against each other (Blenocowe and Caldwell, 1949; Hollings, 1955; Holmes, 1956; Graham, 1957; Govier, 1957).

Whereas the physical and chemical properties of CMV have been investigated in some detail (Kaper *et al.*, 1965; Francki *et al.*, 1966; van Regenmortel, 1967; van Regenmortel *et al.*, 1972; Kaper and West, 1972), those of TAV are not as well known (Hollings and Stone, 1971; Stace-Smith and Tremaine, 1973). In this paper we report comparative studies on the physical and chemical properties of a strain of TAV (VTAV) and a strain of CMV (QCMV).

MATERIALS AND METHODS

Virus isolates. The Q strain of CMV (Francki *et al.*, 1966) was maintained and propagated in cucumber seedlings (*Cucumis sativus* L. cv Polaris). A strain of TAV originally isolated in Victoria, Australia from *Chrysanthemum* sp. was maintained and propagated in *Nicotiana glutinosa* L.

Virus purification. Plants were inoculated mechanically with purified virus preparations (approximately 100 μg/ml) and maintained for 2 weeks before harvesting in a growth chamber at 22° with a photoperiod

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of 12 hr at 14,000 lux. CMV was purified by the method of Lot *et al.* (1972) as modified by Peden and Symons (1973), which usually yielded about 300 mg of virus from 1 kg of cucumber leaf material. TAV was purified by the same method except that the concentration of thioglycollic acid in the extraction buffer was increased to 0.5%, and the virus was suspended in 0.02 *M* phosphate buffer, pH 7.6; usually about 2.0 g of virus was obtained from 1 kg of *N. glutinosa* leaf material. Virus concentrations were determined from their absorbance at 260 nm using $E_{260}^{0.1\%} = 5.0$ for both viruses (Francki *et al.*, 1966; Stace-Smith and Tremaine, 1973).

Isolation of viral RNA. For base ratio analyses the RNA was isolated by the LiCl precipitation method (Francki *et al.*, 1966). For electrophoretic and sedimentation studies the RNA was isolated by the phenol–sodium dodecyl sulphate (SDS) method (Peden and Symons, 1973). RNA concentrations were determined from their absorbance at 260 nm using $E_{260}^{0.1\%} = 25.0$.

Isolation of viral protein. Protein was recovered from the supernatant after precipitation of RNA with 2 *M* LiCl (Francki *et al.*, 1966). The solution was dialysed exhaustively at 4° against 10 m*M* Tris·HCl, 1 m*M* dithiothreitol, pH 7.2. The precipitated protein was recovered by slow-speed centrifugation, lyophilised and stored desiccated at 4° until required.

Electron microscopy. The viruses were negatively stained in 2% uranyl acetate and examined in a Siemens Elmiskop I instrument (Francki *et al.*, 1966).

Rate-zonal density-gradient centrifugation. Virus preparations were centrifuged in linear density-gradients containing 5–25% (w/v) sucrose in 0.02 *M* phosphate buffer, pH 7.5, at 4° for 35 min at 50,000 rpm in a Spinco Model L2-65 ultracentrifuge using a SW 50 rotor. RNA preparations suspended in TNE buffer (0.02 *M* Tris·HCl, 0.1 *M* NaCl, 1 m*M* EDTA, pH 8.5) were subjected to centrifugation in linear density-gradients containing 10–40% (w/v) sucrose in 0.02 *M* Tris·HCl, pH 7.2, for 4.5 hr as described above. Before layering the RNA samples on the gradients, 0.1 ml of 2% (w/v) EDTA-treated bentonite (Fraenkel-Conrat *et al.*, 1961) was layered on each gradient. Tobacco leaf RNA prepared as described by Francki and Jackson (1972), was used as a marker (18 and 25 S).

Isopycnic density-gradient centrifugation. The viruses were fixed with 0.2% formaldehyde as described by Francki and Habili (1972) and mixed with an equal volume of CsCl of density 1.72, in 5 m*M* borate buffer, pH 9.0. The mixtures were centrifuged at 38,000 rpm at 4°, for 20–40 hr in a Beckman SW 50 rotor, or at 35,600 rpm in a Beckman Model E ultracentrifuge for 17 hr, using an AnF rotor. After centrifugation in the preparative rotor, drop-fractions were collected by piercing the bottom of the tube and the fractions were examined spectrophotometrically for their virus content and gravimetrically for their densities.

RNA base ratio analyses. RNA samples were hydrolysed in 1 *N* HCl at 100° for 1 hr, the products were separated by paper chromatography in isopropanol-HCl and the base ratios determined as described by Markham (1955).

Amino acid analyses. Each sample of lyophilized viral protein (1–2 mg) was dissolved in 1 ml of 6 *N* HCl and a drop of 5% aqueous phenol, and was hydrolysed at 110° (DeLange *et al.*, 1969) for 24 and 72 hr. The resulting amino acids were evaporated to dryness, resuspended in 12.5% sucrose in 0.1 *N* HCl and applied to a Beckman 120C Amino Acid Analyser. Tryptophan was determined spectrophotometrically as described by Spies and Chambers (1949).

Polyacrylamide-gel electrophoresis. RNA analyses were carried out in 2.6% polyacrylamide-gels as described by Loening (1967). Samples containing 10–20 μg of viral RNA in TNE buffer and 10% sucrose were layered on the gels, and electrophoresis was carried out at room temperature using *E. coli* rRNA as markers (Peden and Symons, 1973). The gels were either scanned directly in a Joyce-Loebl Chromoscan at 265 nm or stained in toluidine blue O and scanned at 620 nm.

Protein analyses were carried out by the SDS–phosphate method and stained with Coomassie blue (Weber and Osborn, 1969; Maizel, 1971). Both viral and marker pro-

TABLE 1
SEROLOGICAL RELATIONSHIPS BETWEEN TAV AND CMV ISOLATES

Virus	Isolate	Reference	Serological reaction with antigen	
			TAV (V strain)	CMV (Q strain)
TAV	Strain 1	Hollings (private communication)	+[a]	−[b]
	Strain 2	Hollings (private communication)	+	−
	Hitchborn's	Grogan *et al.* (1963)	+	−
	Till's	Grogan *et al.* (1963)	+	−
	Canadian	Stace-Smith and Tremaine (1973)	+	−
	Strain V	This study	+[c]	−
CMV	South African (strain S)	van Regenmortel (1966)	−	+
	Price's Yellow	Hollings (private communication)	−	+
	Beta vulgaris	Hollings (private communication)	−	+
	Wisconsin yellow	Hollings (private communication)	−	+
	Strain R	Devergne and Cardin (1970)	−	+
	Strain T	Devergne and Cardin (1970)	−	+
	Imperial 78	Grogan *et al.* (1963)	−	+
	Canadian	Stace-Smith and Tremaine (1973)	−	+
	Strain Q	This study	−	+[c]

[a] Visible band formed in immunodiffusion tests when antigen at a concentration of 1 mg/ml was used.
[b] No reaction observed in immunodiffusion tests.
[c] Homologous reaction.

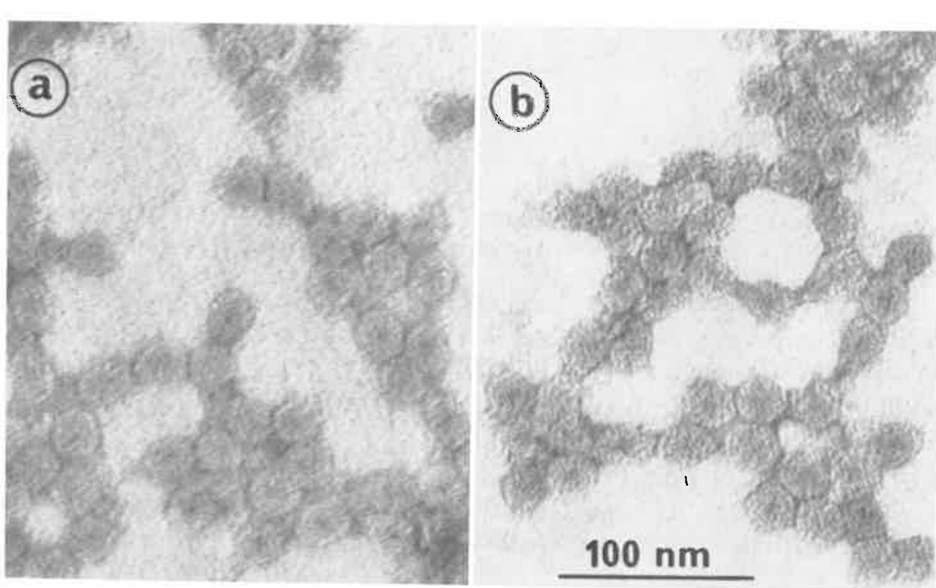

FIG. 1. Electron micrographs of TAV (a) and CMV (b) stained in 2% uranyl acetate.

teins were prepared as described by Agrawal and Tremaine (1972). Bovine serum albumin (mol. wt. 68,000), ovalbumin (mol. wt. 43,000), pepsin (mol. wt. 35,500), trypsin (mol. wt. 23,300) and cytochrome *c* (mol. wt. 12,400) were used as markers.

Serology. All serological tests were carried out by the double immunodiffusion tech-

nique as described by Francki and Habili (1972). Antisera to TAV and CMV were prepared in mice as described by Francki and Habili (1972); other antisera were obtained from sources listed in Table 1.

RESULTS

Serological Identity of the Viruses

The antisera prepared against V strain of TAV and Q strain of CMV reacted positively with their respective antigens, and no cross-reaction was observed in any case. Similarly, only homologous reactions against the two antigens were observed with TAV and CMV antisera obtained from other workers (Table 1). Thus it appears that our TAV (strain V) and CMV (strain Q) are typical isolates of these viruses, and are serologically distinct from each other.

Morphology of Virus Particles

Electron micrographs of TAV and CMV stained with uranyl acetate revealed that the particles were similar (Fig. 1). The mean diameters of the particles based on samples of 100 particles, were 29.0 ± 0.28 nm for TAV, and 28.8 ± 0.22 nm for CMV. When the two viruses were mixed and examined in the electron microscope, particles could not be distinguished into two different populations and their mean diameter was 28.4 ± 0.20 nm.

Sedimentation Properties of the Viruses

The sedimentation rates in sucrose density-gradients of TAV and CMV were indistinguishable and, when the two viruses were mixed, a single homogeneous peak was observed.

Properties of Viral RNA

The molar base ratios of TAV-RNA and CMV-RNA were similar (Table 2) and could not be distinguished by the techniques used. The values were also indistinguishable from those obtained by other workers (Table 2).

When TAV-RNA and CMV-RNA preparations were centrifuged in sucrose density gradients, most of the material sedimented as a peak with an approximate sedimentation coefficient of 22 S (peak b, Fig. 2). However, in the sedimentation profile of CMV a minor distinct peak (20 S) was observed which corresponded to a shoulder of peak b of TAV (Fig. 2).

Because of the relatively poor resolution of the RNA species of both TAV and CMV in sucrose density-gradients, RNA preparations of both viruses were subjected to polyacrylamide-gel electrophoresis. Results of these experiments are summarised in Figs. 3 and 4, and it appears that RNA of both viruses can be resolved into 4 species (Fig. 3). The two slowest migrating RNA species of TAV (T_1 and T_2) appear to be indistinguish-

TABLE 2

MOLAR BASE RATIOS OF TAV AND CMV

Virus	Reference	Mole %			
		G	A	C	U
TAV (Canadian)	Stace-Smith and Tremaine (1972)	23.7	26.4	21.2	28.7
TAV (Canadian)	Stace-Smith and Tremaine (1973)	23.1	26.2	21.6	29.0
TAV (V strain)	This work	23.9[a] ± 0.48	24.6 ± 0.47	21.4 ± 0.29	30.1 ± 0.39
CMV (Y strain)	Kaper *et al.* (1965)	23.4 ± 0.4	24.3 ± 0.5	23.2 ± 0.7	29.0 ± 0.7
CMV (Q strain)	Francki *et al.* (1966)	24.7 ± 0.33	22.4 ± 0.22	22.8 ± 0.45	30.1 ± 0.50
CMV (Q strain)	This work	24.0[b] ± 0.50	23.5 ± 0.39	21.8 ± 0.29	30.7 ± 0.39

[a] Means of ten determinations from three separate RNA preparations.
[b] Means of seven determinations from three separate RNA preparations.

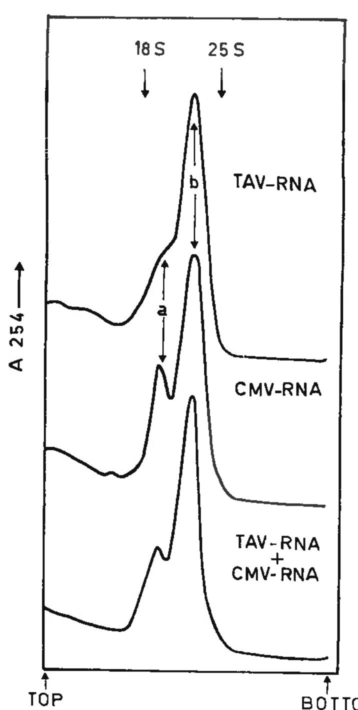

FIG. 2. Sedimentation profiles of TAV and CMV-RNA preparations in sucrose density-gradients. Of each viral RNA, 40 μg was layered on 10 to 40% linear sucrose density gradients in 0.02 *M* Tris·HCl buffer, pH 7.5, and centrifuged in the Beckman SW 50.1 rotor at 50,000 rpm for 4.5 hr at 4°.

able from those of CMV (C_1 and C_2). However, the other two components of TAV (T_3 and T_4) migrate at different rates from those of CMV (C_3 and C_4). By using *E. coli* ribosomal RNA as a marker, the molecular weights of the various RNA species of TAV and CMV have been determined (Fig. 4). T_1 and C_1 have molecular weights of 1.26 ± 0.08; T_2 and C_2, 1.10 ± 0.07; T_3 0.90 ± 0.06; C_3 0.77 ± 0.05; T_4 0.43 ± 0.03; and C_4 $0.34 \pm 0.02 \times 10^6$ daltons. Our estimates of molecular weights of the four RNA species of CMV-Q (C_1, C_2, C_3, and C_4) agree well with the values of 1.3, 1.13, 0.78, and 0.34×10^6 daltons reported by Peden and Symons (1973), but the first three species are significantly higher in molecular weight than those reported by Kaper and Waterworth (1973) for the S strain of CMV (1.07, 0.95, and 0.69×10^6 daltons).

Properties of Viral Proteins

Results of the amino acid analyses of TAV and CMV are summarised in Table 3 and compared with those of two strains of TAV (Canadian and CVL, Stace-Smith and Tremaine, 1973) and the S strain of CMV from South Africa as determined by van Regenmortel (1967) and recalculated by Stace-Smith and Tremaine (1973). It appears from the data that the amino acid compositions of all the viruses compared show similarities, and yet all can be distinguishable from each other. It can also be seen that the TAV isolates form a group distinguishable from the two CMV strains.

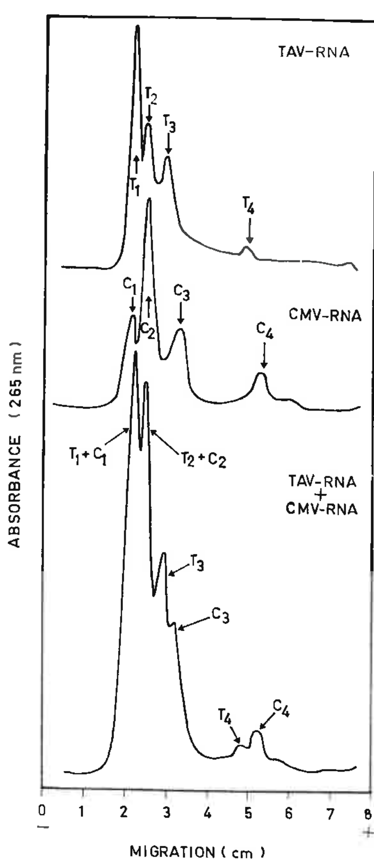

FIG. 3. Analysis of TAV-RNA and CMV-RNA preparations by polyacrylamide-gel electrophoresis. RNA samples (20 μg) were subjected to electrophoresis in 2.6% polyacrylamide-gels for 3 hr at 5 mA/gel and the gels were scanned at 265 nm with a Joyce-Loebl Chromoscan densitometer.

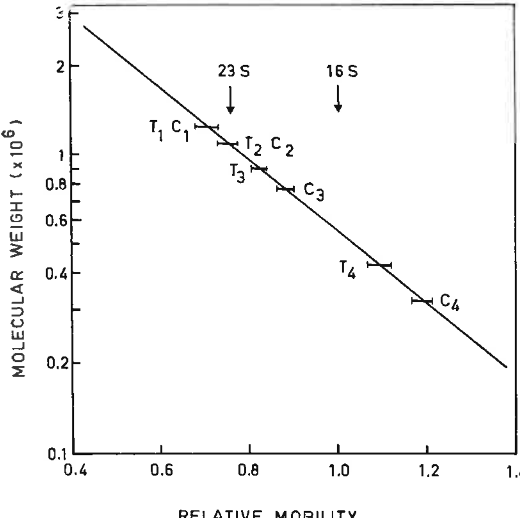

FIG. 4. Molecular weight determination of TAV and CMV-RNA species by polyacrylamide-gel electrophoresis (methods as described in Fig. 3). *Escherichia coli* rRNA was used as a marker. In each experiment the mobility of each viral-RNA relative to that of 16 S *E. coli* rRNA was determined. Molecular weights of 23 S and 16 S *E. coli* rRNAs were taken as 1.07×10^6 and 0.55×10^6 daltons, respectively (Stanley and Bock, 1965). The values in this figure are means of 16 independent determinations from 10 different RNA preparations.

The calculated molecular weights of TAV and CMV protein subunits from the amino acid analyses (Table 3) were 26,100 and 26,300 daltons, respectively. When the isolated viral proteins were subjected to 10% polyacrylamide-gel electrophoresis (Weber and Osborn, 1969) single bands were detected with very similar electrophoretic mobilities. On coelectrophoresing TAV and CMV protein preparations the two proteins migrated as a single band that could not be resolved into two components. The molecular weight of the proteins was determined by coelectrophoresis with marker proteins and shown to be 24,500 ± 270 daltons (Fig. 5), which agrees reasonably well with values of 26,300 reported by Stace-Smith and Treamine (1973), 25,200 by van Regenmortel *et al.* (1972) and 24,200 by Hill and Shepherd (1972).

Isopycnic Density-Gradient Centrifugation of the Viruses

Both TAV and CMV degraded in CsCl; however, when the virus preparations were first treated with formaldehyde, viruslike particles were recovered from light-scattering bands following isopycnic density-gradient centrifugation in CsCl (Fig. 6). The band produced by TAV was consistently narrower than that produced by CMV and in many experiments there was a suggestion that the CMV band consisted of two imperfectly separated components (Fig. 6a and b). Formaldehyde-treated TAV and the two presumed components of CMV were recovered from the density gradients at an average buoyant density of 1.367 ± 0.007 g/cm^3. They had the appearance of typical virus particles when examined in an electron microscope, their ultraviolet absorption spectra were characteristic of the viruses, and positive serological reactions were observed with homologous antisera.

The heavy and light components of CMV recovered from CsCl density gradients (Fig. 6) each produced a single sharp peak on sucrose density gradients, sedimenting at the same rate as normal CMV. A single peak was also observed when both components were mixed. Heterogeneity was also observed when high concentrations of TAV (4–8 mg/tube) were examined. Under such conditions, three sharp bands were detected and most of the material was associated with the middle band.

Attempts to extract RNA from formaldehyde-treated TAV and CMV preparations after isopycnic density-gradient centrifugation in CsCl failed. Streaking and aggregation of RNA was observed upon polyacrylamide-gel electrophoresis, possibly due to cross-linking of RNA and protein by formaldehyde.

DISCUSSION

TAV and CMV have indistinguishable particle morphology (Fig. 1), sedimentation coefficients, RNA base ratios (Table 2) and buoyant densities (Fig. 6). The RNAs of both viruses separate into four distinct species based on polynucleotide chain length,

TABLE 3

Amino Acid Composition of TAV and CMV

Amino acid	TAV strains				CMV Strains		
	VTAV		Canadian TAV[b]	CVL[b]	QCMV		S-CMV[c]
	Relative molar ratio[a]	Integer value			Relative molar ratio[a]	Integer value	
Lys	14.5	15	15	14	15.0	15	15
His	5.0	5	4	4	3.6	4	3
Arg	15.9	16	17	17	20.3	20	20
Asp	26.6	27	29	29	24.0	24	24
Thr[d]	19.4	19	17	17	13.9	14	14
Ser[d]	23.1	23	21	19	26.2	26	26
Glu	18.6	19	19	19	15.6	16	16
Pro	14.3	14	15	15	17.6	18	15
Gly	13.5	14	13	13	13.7	14	13
Ala	19.2	19	15	16	14.4	14	14
Cys	0.8	1	2	2	0	0	0
Val	16.6	17	16	17	17.6	18	18
Met	2.1	2	2	4	5.3	5	6
Ile	13.1	13	15	14	11.6	12	13
Leu	19.2	19	20	20	21.7	22	22
Tyr[d]	7.9	8	7	7	9.4	9	9
Phe	6.6	7	7	7	5.8	6	6
Trp[e]	1.4	1	1	1	1.2	1	1
Total	—	239	235	235	—	238	235

[a] Means of 24 and 72 hr hydrolyses of three separate protein preparations.
[b] Data from Stace-Smith and Tremaine (1973).
[c] Data from van Regenmortel (1967).
[d] Extrapolated to zero hydrolysis time.
[e] Determined by colorimetric analysis as described by Spies and Chambers (1949).

and although two of them (species 1 and 2) are indistinguishable by polyacrylamide-gel electrophoresis, the other two (species 3 and 4) differ significantly (Figs. 3 and 4). Although the protein subunits of the two viruses are indistinguishable in molecular weight (Fig. 5), they differ significantly in their serological properties (Table 1) and amino acid composition (Table 3). From these data we conclude that TAV and CMV can be considered as distinct viruses; however, they are sufficiently similar to be included in the same virus group.

It has already been reported by Kaper and West (1972) that CMV contains at least four species of RNA and Peden and Symons (1973) have determined that the three largest species (C_1, C_2, and C_3) are required for infectivity. Peden and Symons (1973) also concluded that three different particles, based on the RNA species which they enclosed, must be present in preparations of CMV. Data reported in this paper indicate that TAV is similar to CMV in its capsid structure in relation to their four RNA species (Fig. 7). A similar relationship of RNA species has been proposed for brome mosaic virus (Lane and Kaesberg, 1971).

Stace-Smith and Tremaine (1973) reported a particle weight of 5.3×10^6 daltons for TAV and values ranging from 5.5 to 6.8×10^6 daltons have been reported for that of CMV (Francki *et al.*, 1966; van Regenmortel, 1967; Finch *et al.*, 1967; van Regenmortel *et al.*, 1972). If it is assumed that each protein shell of TAV and CMV consists of 180 chemical subunits (Finch *et al.*, 1967), then it is possible to calculate the particle weights using our estimations of protein subunit and RNA molecular

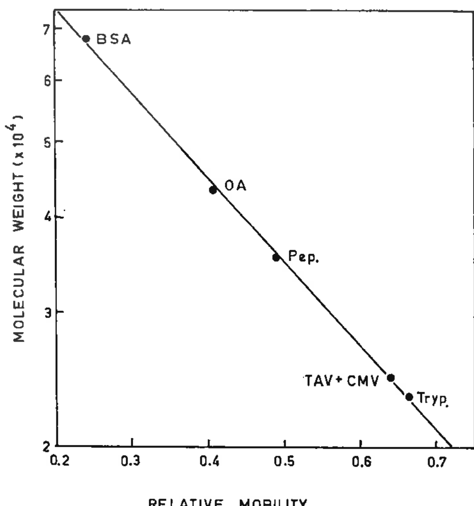

FIG. 5. Molecular weight determination of TAV and CMV proteins by polyacrylamide-gel electrophoresis. Mobilities of the proteins in 10% polyacrylamide-gels were determined relative to that of cytochrome *c* which was used as an internal marker. Marker proteins used were bovine serum albumin (BSA), ovalbumin (OA), pepsin (Pep), and trypsin (Tryp).

weights. These are summarised in Fig. 7 and agree reasonably well with some of the published determinations (Francki *et al.*, 1966; van Regenmortel *et al.*, 1972).

Chemical analyses have shown that TAV contains about 17.7% RNA (Stace-Smith and Tremaine, 1973), whereas values between 18.0 and 18.5% have been obtained for CMV (Kaper *et al.*, 1965; Francki *et al.*, 1966; van Regenmortel, 1967). From our data on the size of the protein subunits and RNAs of the two viruses, calculated values for the percentage RNA are somewhat higher (Fig. 7). The RNA content of the two viruses has also been calculated from the buoyant density of the particles in CsCl by the method of Sehgal *et al.* (1970) which gave a value of 23.8% for both TAV and CMV. This value is slightly higher than that obtained from data summarised in Fig. 7.

The appearance of CMV as two bands in CsCl isopycnic centrifugation can be interpreted as meaning that the top component consists of B and C particles (Fig. 7), and that A particles sediment as a bottom component because of their higher RNA content. It was observed that the proportion of RNA species C_1 varied in different preparations, as was also observed by Kaper and Waterworth (1973). Similarly, the amounts of the corresponding particles (particles A) were observed to vary proportionally.

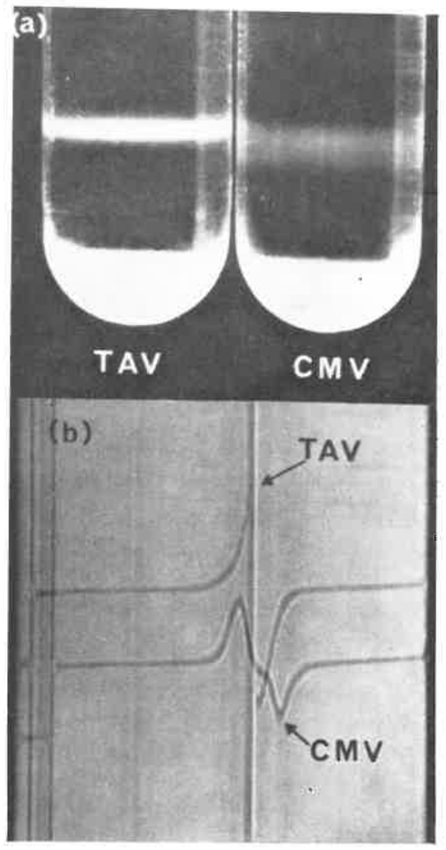

FIG. 6. Isopycnic density-gradient centrifugation of formaldehyde-treated TAV and CMV. (a) Samples containing 1 mg of virus mixed with an equal volume of CsCl solution of density 1.72 g/cm³. Three-milliliter samples of the virus in CsCl were overlaid with paraffin oil in 5-ml plastic centrifuge tubes and subjected to centrfugation in a Beckman SW 50.1 rotor at 38,000 rpm for 40 hr at 4°. (b) Samples of virus (0.5 mg/cell) prepared as in Fig. 6a were subjected to centrifugation at 35,600 rpm for 17 hr in an AnF rotor of a Beckman Model E ultracentrifuge maintained at 5–6°. The schlieren photograph was taken at a bar angle of 75° after 17 hr of centrifugation (sedimentation from left to right).

	TAV A	TAV B	TAV C	CMV A	CMV B	CMV C	
PARTICLES	1	2	3 + 4	1	2	3 + 4	
MOLECULAR WEIGHT OF RNA IN CAPSID $\times 10^6$	1.26	1.10	0.90 + 0.43	1.26	1.10	0.77 + 0.34	MOLECULAR WEIGHT OF PROTEIN SUB-UNIT
PARTICLE WEIGHT $\times 10^6$	5.65 5.98	5.49 5.82	5.72 6.05	5.65 5.98	5.49 5.82	5.50 5.83	24500[a] 26200[b]
% RNA IN CAPSID	21.2 22.3	18.9 20.0	22.0 23.3	21.2 22.3	18.9 20.0	19.0 20.2	24500 26200

[a] Determined by polyacrylamide-gel electrophoresis.

[b] Calculated from the amino acid analyses of TAV and CMV proteins.

FIG. 7. Proposed structures of TAV and CMV nucleoproteins in relation to various RNA species isolated from virus preparations. The particle weights are calculated from the molecular weights of the various RNA species (Fig. 4) and those of the viral protein subunits as determined by polyacrylamide-gel electrophoresis (Fig. 5) and the amino acid compositions (Table 3).

In all preparations of TAV-RNA, the proportion of RNA T_1 was highest (Fig. 3), resulting in a predominant amount of particles A (Fig. 7) which gave only one sharp peak on isopycnic density-gradient centrifugation (Fig. 6). However, when a higher concentration of the virus was used, three bands were observed. The top band probably corresponds with particles B and the bottom band with particles C of Fig. 7. The majority of the virus population (particles A) banded as a middle component.

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