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CACTUS VIRUS.

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Botany

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CHARACTERIZATION AND IDENTIFICATION OF
CACTUS VIRUS

By

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Presented in partial fulfillment of the requirements for

the degree of

Doctor of Philosophy

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Two previously unreported virus isolates were characterized and identified.

Isolate CaV 196, a TMV-like virus produced host reactions different from those induced by all other strains of TMV. In crude sap, the thermal inactivation point was 85°-90°C at 10 min., the dilution end point 10^{-8} - 10^{-9} , and resistance in vitro was more than two weeks at room temperature. It was purified by polyethylene glycol precipitation and gave UV absorption spectrum typical of nucleoprotein with a minimum at 244-246 and maximum at 260-262 nm. Electron microscopy revealed rigid, elongated rods with a normal length of 302 ± 5 nm. The isolate exhibited a sedimentation coefficient ($s_{20,w}$) of 183S and a partial specific volume (\bar{v}) of 0.72-0.73 ml/g. A-protein preparation exhibited a dimer with $s_{20,w}$ of 3.48S and an aggregation intermediate with $s_{20,w}$ of 15S. The isolate is serologically similar to common TMV (U1) but not identical to it. It is concluded that the isolate is a new strain of TMV.

Isolate ZyV 58 has been found in the cultivated Christmas cactus, Zygocactus sp. The virus infected only 6 species of the family Chenopodiaceae. In crude sap the thermal inactivation temperature was 75°-80°C at 10 min., the dilution end point was 10^{-5} - 10^{-6} and aging in vitro was 6-7 days at room temperature. Virus was purified by a combination of chloroform-butanol treatment and differential ultracentrifugation. The purified virus was highly infectious and gave a typical nucleoprotein ultraviolet absorption with minimum at 246 and broad maximum around 260 nm. Electron microscopy revealed elongated, slightly flexuous particles with a NML of 519 ± 10 nm. The virus has a sedimentation coefficient ($s_{20,w}$) of 120-123S. The virus was found to be serologically distantly related to cactus virus X and potato virus X but not to Casper's Zygocactus virus. It is concluded that it is a new member of PVX group but similar to CaVX.

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CHAPTER I

INTRODUCTION

In less than a century the science of virology has become of paramount importance in the life sciences. Viruses have been used extensively for studies in molecular genetics and in molecular and developmental biology. There are three major virus groups: plant, animal, and bacterial. Tobacco mosaic virus (TMV), belonging to the plant group, was the first virus to be discovered (Beijerinck, 1886).

Historical

In 1886 Mayer described a disease of tobacco plants which he called the "Mosaikkrankheit." Mayer (1892) showed that this mosaic disease could be transmitted to a healthy tobacco plant by inoculation with the sap from an infected plant, and it was during this period that the first scientific proof of the existence of a virus was given. Iwanowski (1892), working on the mosaic disease of tobacco, proved that sap from diseased tobacco plants was capable of inducing the mosaic disease in healthy tobacco plants after it had been passed through a bacteria proof filter. But Iwanowski himself failed to grasp the true significance of this, and his discovery was unnoticed until the work was repeated seven years later by Beijerinck (1898), who then propounded his theory of a "contagium vivum fluidum."

Over the next thirty years many plant diseases thought to be due to filterable viruses were described. During that time, viruses were recognized solely by the disease they produced, viz., by their biological

activity. Viruses were set apart from other infectious disease-producing agents by their small size as evidenced by their ability to pass through filters which could hold back ordinary bacteria. For a time, new plant and animal viruses were found every year and, with the discovery of bacterial viruses in 1915, it became obvious that viruses could infect almost every type of living cell. Still, the major interest was in the diseases caused by the viruses and not in the nature of viruses themselves. Other characteristics of their physical and chemical nature were unknown because adequate methods for characterizing viruses had not been developed.

Obviously, other approaches to this problem were needed. These were begun during the 1920s with the discovery that TMV could be precipitated by protein precipitants and resuspended without losing its infectivity (Mulvania, 1926). Reasonably active and colorless preparations of TMV were obtained by Vinson and Petri (1929, 1931). They concluded that it was a nitrogen-containing substance. The attempts to obtain the virus in sufficient quantity for further characterization were still unsuccessful. The methods of quantitative assay were just beginning to be developed. Holmes (1928, 1929) pointed out that the necrotic local lesions produced on Nicotiana glutinosa could be used to compare the relative infectivity of inocula. The serological techniques, first applied to virus infected plants by Dvorak (1927), were furthered by the work of Purdy Beale (1928, 1929, 1931) who gave a more reliable basis for estimating the virus identity and content in sap. Chester (1935), using these techniques, reached the more accurate conclusion that infective sap might contain from 0.1 to 1 mg

of TMV per ml.

A significant breakthrough came when Stanley (1935, 1936), by using the methods employed in isolating proteolytic enzymes, obtained substantial amounts of what he called "a crystalline protein possessing the properties of tobacco mosaic virus." Curiously enough, his original description contradicted the present-day knowledge of the properties of TMV. His statement that the protein was a globulin, containing 20 per cent nitrogen with no phosphorus and carbohydrate and partially digestible with pepsin, suggested that his preparations were either highly contaminated or consisted largely of inactive virus (Bawden, 1964).

Bawden, et al. (1936) described the isolation from TMV infected plants of a liquid crystalline nucleoprotein preparation containing nucleic acid of the ribose type. They showed that the particles were rod-shaped, thus confirming the earlier suggestion of Takahashi and Rawlins (1932) based on the observation that solutions containing TMV showed anisotropy of flow. The isolation of other rod-shaped viruses and spherical viruses which formed true crystals soon followed. All were found to consist of ribonucleic acid and protein. All viruses yet isolated from flowering plants resemble TMV in containing nucleic acid of ribose type with the exception of cauliflower mosaic virus (Shepherd, 1968) and potato leaf roll virus (Sarkar, et al., 1973) which contain deoxyribose nucleic acid. Also, most viruses that attack bacteria, or that attack animals, contain deoxyribose nucleic acid.

Electron microscopy and x-ray crystallography were next to be used

to explore the architecture of virus particles. Early electron micrographs (Kausche and Stubbe, 1939) confirmed that TMV was rod-shaped and provided its approximate dimensions, but they were not good enough to reveal a significant amount of information due to the lack of contrast between the virus particles and supporting membrane. The development of shadow casting with heavy metals greatly increased the usefulness of the electron microscopy for determining the overall size and shape of virus particles. With the development of high resolution microscopes and of negative staining in the 1950s, electron microscopy has become an important tool for studying virus substructure.

Work on physico-chemical properties of virus nucleoprotein followed. Early sedimentation studies (Eriksson-Quensel and Svedberg, 1936; Wyckoff, 1937) demonstrated that TMV had a very high molecular weight. The first reasonably accurate model of TMV was obtained by combining viscosity and sedimentation data (Lauffer, 1938). Many values regarding molecular weight and size of the particle were reported by different workers, based on diffusion, sedimentation, viscosity and x-ray diffraction studies. Frequently quoted values were 300 Å for length, 149 Å for diameter and 39 ± 1.2 million for the molecular weight (Boedker and Simmons, 1958).

Markham (1951), after studying the physico-chemical properties of virus nucleoprotein and empty viral protein shells found in turnip yellow mosaic virus (TYMV), concluded that the RNA of the virus must be held inside a shell of protein, a view that has since been amply confirmed for this and other viruses by x-ray crystallography. After the classic experi-

ments of Hershey and Chase (1952) on bacterial viruses, it was realized that the viral nucleic acid was the only infectious component. Later studies by Gierer and Schramm (1956), Fraenkel-Conrat and Williams (1955) and Fraenkel-Conrat (1956) demonstrated the infectivity of naked TMV-RNA and the protective role of protein coat. Subsequent work has shown that for viruses like TMV, RNA is present as a single strand and one cleavage in this strand leads to inactivation of the virus particle.

A significant number of studies has been done on the protein part of the viruses. The full sequence of 158 amino acids in the subunit of the coat protein of TMV is known (Anderer, et al., 1960; Wittmann and Wittmann-Liebold, 1966) and many naturally occurring strains and artificially induced mutants have been determined.

Since the discovery of TMV, several elongated and spherical viruses have been found which infect higher plants. Each year new plant viruses are described. Since the present study covers elongated viruses, it may be worthwhile to mention the different groups of elongated plant viruses. Brandes and Wetter (1959) described six different groups of elongated viruses on the basis of their morphology and serological properties:

1. Tobacco rattle virus group with normal length from 130 to 210 nm;
2. Tobacco mosaic virus group with normal length from 300 to 320 nm;
3. Potato virus X group with normal length from 480 to 580 nm;
4. Potato virus S group with normal length from 620 to 690 nm;
5. Potato virus Y group with normal length from 730 to 750 nm; and
6. Beet yellow virus group with normal length of 1250 nm.

One of the viruses in the present study seems to be a strain of TMV, therefore it will be worthwhile to describe some of the reported strains.

Strains of Tobacco Mosaic Virus

When TMV was first discovered, the genetic determination of virus variants was not possible because techniques for physical and chemical identification and characterization were unknown. After the crystallization of virus particles (Stanley, 1935), these variants attracted attention because of the markedly different symptoms caused in their host plants. It is a common practice for most TMV researchers to consider the following criteria as characteristics of strains of TMV (Hennig and Wittman, in Kado and Agrawal, 1972):

1. Morphology: Rod-shaped particles with a length of about 300 nm and a diameter of about 15-20 nm.
2. Biochemistry: 5 per cent RNA with the base group composition A:G:C:U 1.2: 1.0: 0.7: 1.1 and 95 per cent protein with larger amounts of Aspartic acid, Threonine, Serine, Glutamic acid, Alanine, Valine, and Leucine each; and smaller amounts of Cysteine, Methionine, Lysine, Histidine, and Tryptophane each.
3. Physical: Particle weight of intact virus is about 40×10^6 daltons, sedimentation coefficient ($s_{20,w}$) is about 200S, molecular weight of RNA is about 2×10^6 and molecular weight of protein subunits is about 17,500.
4. Serology: Cross reaction with antiserum made against the common strain (U1), or another strain, which is definitely known to belong to the TMV group.
5. Amino acid composition: The protein from almost all the TMV strains have been found to contain 158 amino acids.

Besides the above-mentioned characteristics, the host reaction is very important and constitutes a preliminary aspect to differentiate one

strain from another. For example: among the set of eight strains of TMV studied by Siegel and Wildman (1954), two (U2 and U7) caused the same mild mosaic disease in Turkish tobacco, but differed markedly in four other properties. One of those four properties was their differential reaction on N. sylvestris. The U2 strain produced local lesions whereas U7 produced a systemic infection in N. sylvestris. Symptoms produced by different virus strains in the same species and variety of host plant may range from a symptomless carrier state through mosaic diseases of varying degrees of severity.

According to the above criteria (Hennig and Wittman, 1972) many strains have been reported by various workers such as: Common mosaic (U1) from tobacco (Iwanowsky, 1892); Aucuba mosaic (YA) from tomato (Bewley, 1924); Cucumber mosaic 3 and 4 (CV3 and C4) from cucumber (Ainsworth, 1935); Dahlemense from tomato (Melchers, et al., 1940); Holmes rib-grass (Hr) from rib-grass (Holmes, 1941); Southern sunnhemp mosaic from sunnhemp (Capoor, 1950); Mild mosaic (U2) from tobacco (Singer, et al., 1954); Odontoglossum ring spot (ORSV) from orchid (Jensen and Gold, 1951); U5 from tobacco (Siegel and Wildman, 1954); OM from tobacco (Hiruki, et al., 1954); Cowpea virus (CPV) from cowpea (Lister and Thresh, 1955); Sammons' Opuntia virus (SOV) from cactus (Sammons and Chessin, 1961); Green tomato atypical mosaic (G-TAMV) and Yellow tomato atypical mosaic (Y-TAMV) from tomato (Knight, et al., 1962); CV60, CV61, American collection No. 9 (AC-9), Hall Davis (HD), San Joaquin (SJ), Ventura (Ven), Australian II (Aus II), Dutch I (DUT I), Yellow leaf GP (YLGP), K-1, PTA,

PTV and South African (SAF) from tomato (Wang and Knight, 1967); Cucumber green mottle virus (CGMV) from cucumber (Inouye, et al., 1967); Lychnis strain from Lychnis (Chessin, et al., 1967); 01 through 07 from orchid (Kado, et al., 1968); and B-TMV 1 and 2 from pear tree (Opel, et al., 1969).

Many other strains are not included here but may be found elsewhere in literature (Plant Virus Names, a C. M. I. Publication, 1968 and 1971).

Previous Research on Cactus Viruses

In 1885, Molisch, a famous German botanist, found in several species of cacti, inclusion bodies which he called "protein spindles." These proteinaceous spindles were possibly viral aggregate. In 1920, Weingart demonstrated that the agent responsible for the formation of these bodies was graft transmissible to previously spindle-free cactus. This agent was ultimately shown to be viral in nature (Rosenzopf, 1951). Later Amelunxen (1958) demonstrated that the characteristic cigar or spindle-shaped inclusion bodies which could be seen in the cells of certain Opuntia species with the aid of an ordinary microscope were of virus origin.

The first detailed electron microscopic and chemical study of a cactus virus was made by Amelunxen (1958) on the cactus virus X. He demonstrated that cactus virus X from O. monocantha was a typical RNA-containing plant virus and was related to members of the potato virus X group.

Brandes and Wetter (1959) found two elongated viruses: cactus virus 1 with a normal length of 515 nm, and cactus virus 2 with a normal length of 650 nm. Cactus 1, they mentioned, was identical to the virus described by Amelunxen (1958).

In 1961, Sammons and Chessin reported that several prickly pear Opuntia species cultivated in the United States contained a virus with rigid rod-like particles similar in morphology to TMV. This was later named Sammons' Opuntia virus (Brandes and Chessin, 1965). A subsequent, more detailed study showed that this virus was (1) serologically distantly related to TMV (Wetter and Paul, 1967), (2) restricted in host range to cacti and several Chenopodium sp., and (3) possessed a normal length significantly greater than that of TMV (Brandes and Chessin, 1965).

In 1969, R. Casper and his associates of Braunschweig, Germany, isolated an elongated, slightly flexible virus of the potato virus X group with the normal length of 580 nm from Zygocactus sp. Casper and Lesemann (1971) isolated one isometric virus from cultivated Opuntia tuna showing witches' broom symptoms. Milbrath and Nelson (1972) have reported another isometric virus from the giant saguaro cactus, Carnega gigantia.

In this study a virus of the X group was isolated from cultivated Zygocactus which differs from Casper's Zygocactus virus (Casper and Brandes, 1969) and cactus virus X. Its closest resemblance serologically is to the white clover mosaic virus of the potato virus X group (Giri and Chessin, 1972).

Statement of the Problem

In the Fall of 1970, during a routine survey of viruses in wild cacti collected by Dr. M. Chessin in the Southwestern United States, TMV-like particles were found in several pads. One of the cactus samples with TMV-like particles, called Cactus (CaV) 196, was selected for further characterization. The purpose of the characterization of this isolate was to see whether this virus was a common TMV, or some strain of it, or Sammons' Opuntia virus (Brandes and Chessin, 1965). At the same time, another virus was isolated from Zygocactus which was arbitrarily named ZyV 58. An effort was made to characterize this second isolate also to determine whether it was the same virus reported by Casper and Brandes (1969), or a typical cactus virus X (Amelunxen, 1958), or a new member of the potato virus X group.

To characterize and identify any plant virus, the following criteria are considered important:

1. The reaction of the virus in different host plants;
2. Physical and chemical properties;
3. Morphology and size of the virus particles; and
4. Serology.

In the present study an effort was made to characterize the two isolates with respect to the above-mentioned properties. Unfortunately, due to insufficient yield of ZyV 58 and a considerably longer time required for symptom appearance, it was not possible to do as much work on ZyV 58 as was done on CaV 196.

CHAPTER II

MATERIALS AND METHODS

Virus and Plant Materials

The names CaV 196 and ZyV 58 are given to the two isolates are descriptive. The isolate CaV 196 was isolated from Opuntia sp., a wild cactus, and ZyV 58 from cultivated Zygocactus sp. Both isolates were inoculated from cactus to Chenopodium quinoa L. Pieces about 1.5 x 5 cm in size were removed from the cactus pad and ground in a mortar with 0.1 M pH 7.0 phosphate buffer. All the inoculations were made with the forefinger, employing carborundum (200-400 mesh) as an abrasive. The inoculated leaves were rinsed with distilled water. Three-to four-week old C. quinoa plants were found suitable. All plants were raised and kept in the greenhouse at a temperature of about 65°-70°F, except for a few days in the summer when the temperature went considerably higher.

Single Lesion Isolation

Local necrotic lesions contain the progeny of a single virus particle. Therefore, to maintain the identity and uniformity of the isolates, single chlorotic or necrotic lesions produced on C. quinoa, after inoculation from cacti, were used for further inoculations. Subsequently, isolates CaV 196 and ZyV 58 were multiplied on Nicotiana tabacum var. Turkish systemic and Chenopodium quinoa, respectively. For single lesion isolation from inoculated leaves, 5 x 5 mm pieces with single lesions were removed with the help of a razor blade and ground in a small mortar with 1 ml of

0.05 M pH 7.0 phosphate buffer and inoculated to a suitable systemic host. Several single lesion transfers were made to reduce the chances of the isolate being contaminated by other viruses or by strains of the same virus.

Host Range Study

Several plant species in the families Amaranthaceae, Chenopodiaceae, Solanaceae, and Leguminosae were tested. The results were generally recorded four weeks after the inoculation and 8-10 plants of each species were used for each test. Back inoculations were made from all those plants which did not show any conspicuous symptoms, to check if virus could be recovered from them and if any were symptomless carriers of the virus. Sometimes back inoculations were made even from those plants which did show some symptoms, to observe expected characteristic symptoms on Chenopodium quinoa. The experiments were repeated and final observations were made on the basis of the results obtained.

Cytology

Some viruses cause cytological changes in the host plant cell by producing inclusion bodies. The presence and absence of inclusion bodies were studied in parent host plants such as wild cactus and Zygocactus sp. Examination of paradermal sections was made to check for inclusion bodies, following the method used by Rubio-Huertos (1962). The strips were stained with a stain combination comprised of phloxin-methylene blue (1:20), without

previous fixation, mounted in water and observed under the light microscope.

Physical Properties of Virus in Plant Sap

Thermal Inactivation Temperature

The critical range of inactivation temperatures vary from virus to virus. In this study virus in crude sap from infected plants was treated at different temperatures and infectivity was assayed by inoculation on local lesion hosts. The sap was extracted from systemically infected plants without adding any diluent each time for each treatment. Two ml of distilled water was kept in a water bath for 10 minutes at the temperature to be used in this experiment. Two ml of extracted sap was quickly mixed with the water bath heated distilled water in a thin-walled glass vial and kept at the same temperature for another 10 minutes. After each treatment 10 plants of C. quinoa of the same age and size were inoculated. In the case of isolate CaV 196, Nicotiana glutinosa was also used.

Tolerance to Dilution

The infectivity of viruses is generally quantitatively related to the dilution. A series of dilutions from 10^{-1} to 10^{-10} from the sap of the viruses were prepared and inoculated to local lesion hosts. The dilutions of the inoculum were prepared in distilled water or 0.05 M pH 7.0 phosphate buffer. Ten plants of C. quinoa or N. glutinosa were inoculated with each dilution.

Aging in Vitro

Viruses have different survival potential at various temperatures. The crude sap was extracted from infected plants and diluted 1:10 in 0.01 M phosphate buffer, pH 7.0, and stored at room temperature as well as in the refrigerator. Each fraction of sap was inoculated to 10 plants of C. quinoa or N. glutinosa at 24-hour intervals, to assay the longevity in vitro.

For all three of the above-mentioned experiments the same method of inoculation was applied, adding 0.25 g celite/5 ml as abrasive to the inoculum. Observations were taken 4-10 days after inoculations. Experiments were repeated and final results were prepared by using the mean value of two experiments.

Buffer Solutions and ReagentsStock Solutions

1. Phosphate Buffer:
 - A. 0.2 M sodium phosphate monobasic
 - B. 0.2 M sodium phosphate dibasic
2. Citrate Phosphate Buffer:
 - A. 0.1 M citric acid
 - B. 0.2 M sodium phosphate dibasic
3. Borate Buffer:
 - A. 0.2 M solution of boric acid
 - B. 0.5 M solution of borax
4. Citrate Buffer:
 - A. 0.1 M solution of citric acid
 - B. 0.1 M solution of sodium citrate
5. Acetate Buffer:
 - A. 0.2 M solution of acetic acid
 - B. 0.2 M solution sodium acetate

Preparation of Buffers

The buffer solutions of different molarity and pH required for the

specific experiments were prepared from the stock solutions by using the tables of buffer preparation described in Gomori (1955).

Purification of Virus

The virus isolates were purified by using several methods with occasional modifications. Nicotiana tabacum var. Turkish systemic infected with CaV 196 and Chenopodium quinoa leaves infected with ZyV 58 were harvested about 25 days after inoculation and stored in a deepfreeze for 24 to 48 hours. Although all the steps were carried out at room temperature, sap and virus solutions were always kept in an ice bath.

Extraction

The frozen leaves were thawed and homogenized in a Waring blender with suitable buffer solution 2 ml/g of leaf material. To prevent inactivation of virus in sap by phenolic compounds, reducing agents ascorbic acid and sodium sulfite were added to buffer to a concentration of 1 per cent (Francki, 1970). After 5 min. homogenization, the homogenate was filtered through four layers of cheesecloth. The extracted sap was subjected to a low speed centrifugation at 4000 \times g for 5 min.

Clarification

The extracted sap was clarified by using previously described methods with minor modifications (Steere, 1956; Wetter, 1961; Tomlinson, 1963).

Chloroform-Butanol Method. One volume of butanol and one volume of chloroform were mixed with two volumes of extracted crude juice with continuous stirring. The mixture was stirred for one hour and then centrifuged in a Sorvall-type RC2-B centrifuge for 5 min. at 4000 \times g. The top layer consisting of the aqueous phase and containing the virus was removed and saved. This was further clarified by centrifuging for 10 min. at 1200 \times g. The pellet was discarded and the supernatant saved for purification.

Ether and Butanol Method. An equal volume of cold ether was mixed with extracted crude juice, shaken for 10-15 min. and centrifuged for 10 min. at 4000 \times g in the Sorvall-type RC2-B centrifuge. The pellet was discarded and the supernatant was treated with an equal volume of cold carbon tetrachloride to remove the ether followed by addition of n-butanol to a final concentration of 8 per cent, stirred for 30 min. and clarified by low speed centrifugation. The clear yellow supernatant was used for further purification.

Chloroform Method. In this method the extracted juice was treated with an equal volume of cold chloroform only, stored overnight in the refrigerator, centrifuged at low speed and the aqueous phase containing virus was saved for further purification.

Further Purification or Concentration

Several methods were applied to purify the virus from clarified sap.

Each will be described individually.

Differential Centrifugation. The clarified sap was centrifuged in a Spinco modl L ultracentrifuge at 35,000 rpm ($100,000 \times g$) for 2 hrs. using type 40 rotor. The supernatant was discarded and the pellet was dissolved in 0.05 M pH 7 phosphate or borate buffer and the resulting suspension was clarified by centrifuging for 10 min. at $12,000 \times g$. The clarified virus solution was again subjected to ultracentrifugation at 35,000 rpm for 2 hrs. Three or four similar cycles of ultra and low speed centrifugations were given. The final pellet was dissolved in water or 0.05 M buffer at pH 7.0. The virus preparation was stored frozen. The complete procedure is outlined in the flow diagram (Scheme 1).

Purification by Adsorption Chromatography. The virus isolates were purified by a modified method of Venekamp and Mosch (1963). In this method, a measured quantity of clarified sap was used. Polyethylene glycol (PEG) and NaCl were added to the clarified sap to a final concentration of 3 per cent and 2.25 per cent, respectively, as a preparation for the adsorption of the virus to cellulose.

To prepare the chromatographic column, 5 gm of cellulose powder (Whatman Column Chromadia) was suspended in 25 ml 6 per cent PEG made in 4.5 per cent NaCl solution. A column with 2 cm inner diameter and mantle (jacket) for circulation of cold water around the column was used. To improve percolation, a 2-4 cm layer of glass wool and washed white sand at the bottom and another 2-3 cm layer of white sand at the top were added.

Clarified Sap

1. Centrifuged 2 hrs. at
35,000 rpm (100,000 x g)

Pellet Supernatant
Resuspended in borate or discarded
phosphate buffer 0.05 M pH 7

Clarified 10 min. 10,000 rpm

Supernatant Pellet
discarded

2. Centrifuged 2 hrs. at
35,000 rpm (100,000 x g)

Pellet Supernatant
Resuspended in buffer discarded

Clarified 10 min. 10,000 rpm

Supernatant Pellet
discarded

3. Centrifuged 2 hrs. at
35,000 rpm (100,000 x g)

Pellet Supernatant
Resuspended in buffer discarded

Clarified 10 min. 10,000 rpm

Supernatant Pellet
discarded

4. Centrifuged 2 hrs. at
35,000 rpm (100,000 x g)

Pellet Supernatant
Resuspended in water or buffer discarded

Clarified 10 min. 10,000 rpm

Virus Solution

Scheme 1. Diagram for Virus Purification by Differential Centrifugation.

The layer of washed white sand at the top was important to prevent the flat surface of the cellulose from being disturbed. The column was equilibrated with 6 per cent PEG in 4.5 per cent NaCl.

The virus suspension was added to the column and developed first by passing 25 ml of 6 per cent PEG in 4.5 per cent NaCl solution. Brown colored and strongly UV absorbing substances were eluted. The percolation was continued by passing successive 25 ml each of the following solutions:

4	per cent PEG in 3	per cent NaCl solution
3.5	per cent PEG in 2.6	per cent NaCl solution
3	per cent PEG in 2.5	per cent NaCl solution
2.5	per cent PEG in 1.9	per cent NaCl solution
2	per cent PEG in 1.5	per cent NaCl solution
1.5	per cent PEG in 1.12	per cent NaCl solution
1	per cent PEG in 0.75	per cent NaCl solution
0.5	per cent PEG in 0.37	per cent NaCl solution
	water	

Approximately 2 ml fractions were collected in each tube and absorption at 254 nm was recorded automatically with an ISCO UV adsorption monitor. Individual UV spectra of some representative fractions were also recorded in a Shimadzu MPS-50L multipurpose recording spectrophotometer.

In a separate experiment, the method of Venekamp and Mosch (1963) was strictly followed to compare the preparations of virus. To prepare the sap of healthy and infected plants, 25 g of leaves were homogenized in 25 ml of pH 7, 0.1 M phosphate citrate buffer by grinding it in mortar with white sand. The extracted sap was shaken with 5 ml of chloroform and centrifuged at 6000 rpm for 10 min. in a Sorvall-type RC2-B centrifuge. A clear brown colored supernatant separated after centrifugation. Careful

addition of concentrated acetic acid to reduce the pH to 3.4 produced precipitate. The precipitate was collected by centrifugation at 8,000 rpm for 5 min. and dissolved in 0.1 M phosphate citrate buffer pH 7, by placing it overnight in the refrigerator. The undissolved precipitate was removed by centrifuging the suspension at 6,000 rpm for 5 min. To prepare for Chromatography, PEG, concentration 6 per cent, and NaCl, concentration 4.5 per cent, were added to the supernatant. This method of sample preparation gave similar results to those described above.

Purification by Precipitation with Polyethylene Glycol. The virus was purified by PEG precipitation (Hebert, 1963) and density gradient centrifugation. Clarified juice was treated with an equal volume of cold chloroform for 30 min. and then centrifuged for 10 min. at 10,000 \times g. PEG (mol. wt. 6,000) in 0.1 NaCl was then added to the aqueous phase with continuous stirring to give a concentration of 5 per cent. The mixture was allowed to stand for an hour in the refrigerator before low speed centrifugation at 10,000 \times g for 10 min. The supernatant was discarded and the pellets were resuspended overnight in 0.05 M phosphate buffer, pH 8. The dissolved virus pellet suspension was further purified by a second precipitation with PEG-NaCl followed by sucrose density-gradient centrifugation.

Purification by Sucrose Density-Gradient Centrifugation. Density gradient centrifugation has been widely employed as a method of purification of virus and other biological macromolecules since Brakke (1951) first

invented it. In the present study, essentially the method of Brakke (1955) was employed. A linear gradient between 100 mg and 400 mg per ml sucrose was prepared by using a gradient mixer in a cold room. The sucrose solutions were prepared in 0.05 M phosphate buffer pH 7. The same buffer was used on all occasions in the purification process. The gradient tubes were stored in the cold room at least for 6-8 hrs. before use. One ml of virus preparation was layered on each tube and centrifuged at about 130,000 \times g for 2 hrs. in a Spinco SW 27 rotor. The gradients were then analyzed and fractionated with an automatic fraction collector attached to the ultraviolet analyzer (254 nm). Most of the time virus zones were fractionated by using a syringe with the needle bent for insertion into the top of a gradient column, and a beam of light shining into the column (Brakke, 1967). Virus zones were collected, dialyzed for 2-3 days against deionized distilled water, and used to determine yield, infectivity, and for other studies.

Purification by Density-Gradient Centrifugation in CsCl. This method, also called equilibrium centrifugation, was primarily used for the purification of ZyV 58, and occasionally to compare homogeneity and buoyant-density of CaV 196. The equilibrium centrifugation involves centrifugation of a solution containing virus and a low molecular weight solute such as CsCl until both the macromolecular component and the salt have obtained an equilibrium distribution in the given centrifugal field. When equilibrium is obtained, the salt concentration will be lowest at the

miniscus and highest at the bottom, and a continuous gradient of density forms throughout the centrifuge tube. Virus sediments from the miniscus and collects in a band of Gaussian shape centered about the isopycnic position.

To prepare the gradient tubes, the method of Mosch, et al. (1973) was primarily used. Tubes of an SW 39 rotor were filled with 2.5 ml of CsCl solution (6.23 g CsCl/10 ml water), 0.75 ml water, 0.1 ml virus suspension and 2 ml paraffin oil.

The same method was used to compare the buoyant density of CaV 196 with the common strain of TMV. In this experiment tube #1 contained CaV 196, tube #2 contained TMV (U1 strain), and tube #3 contained a mixture of both. The tubes were generally spun for 18 hours at about 130,000 x g at 4°C. The virus bands were visualized by light scattering and their position was determined by measuring the distance from the miniscus to the middle of the band or from the middle of the band to the bottom of the tube.

Infectivity Assay

Virus preparations were diluted serially and inoculated to the appropriate local lesion host plant for infectivity. Nicotiana glutinosa was used as the assay plant for CaV 196 and Chenopodium quinoa for the ZyV 58 isolate. Infectivity was determined on the basis of local lesion number per half leaf of test plant.

Spectrophotometry

For quantitative and qualitative studies of all the purified virus, protein, and nucleic acid preparations, spectrophotometry was employed. A Shimadzu MPS-50L multipurpose recording spectrophotometer was used to record the absorption spectrum of the preparations. After the measurement of absorption, a correction for scattering was made.

Correction for Scattering

Most nucleoproteins show maximum UV absorption around 260 nm wave length. The absorbance at 260 nm is not a direct measure of virus or nucleoprotein concentration because of scattering of the light by all solution components. Physical theory (Doty and Steiner, 1950) indicates that, if the logarithm of the absorbance is plotted against the logarithm of the wave length, there should result an essentially straight line sloping downward, where there is no specific absorption. Based on this theory, and using the methods of Reichmann (1959), and Englander and Epstein (1957), the correction was made for each measurement. The contribution of scattering to the absorbance at 260 nm was calculated by extrapolating the straight line to that wave length (260 nm). This absorbance was subtracted from the measured absorbance at 260 nm to give the absorbance of the sample itself. To correct for scattering, the measurements at 320 to 410 nm were used to draw the straight line, and extrapolated to 260 nm.

Extinction Coefficient

After the final step of purification, the virus pellets were resuspended in water and a series of dilutions were made. The absorbance of each dilution was measured on the spectrophotometer. The concentrations of the solutions were obtained by weighing 2 ml portions into 10 ml tared volumetric flasks with glass stoppers and lyophilizing and drying them at 90° to 95°C in vacuo to constant weight. The absorbance at 260 nm was plotted against the concentration of virus. The absorbances were corrected for scattering. The $E_{260}^{10\%}$ was calculated using Beer's equation:

$$A = EC1 \quad [1]$$

where E is extinction coefficient, C is concentration g/liter, and l is light path (in cm).

Biophysical Study

Analytical Ultracentrifugation

Analytical ultracentrifugation was used to measure the homogeneity and the sedimentation coefficient of the purified virus and coat-protein subunits. During the sedimentation velocity experiment, the solute distribution along a radius in a special cell containing a solution is observed and recorded at selected times. Analytical ultracentrifugation offers a means of "seeing" immediately what the solution contains, and gives important information for purifying and characterizing viruses and viral components.

Sedimentation Coefficient. The sedimentation coefficient, s , is the velocity of sedimenting solute molecules divided by centrifugal field strength, and a parameter which is determined most frequently. Its main advantage is indicated by its use in the Svedberg equation along with diffusion coefficient for the determination of molecular weight. In the virus literature, components present in virus preparations are often designated by their sedimentation coefficients alone. This illustrates the great popularity of sedimentation velocity method as a means for initial characterization of purified viruses. In addition, sedimentation coefficient is a reflection of macromolecule mass, its molecular shape, and its density relative to that of the solvent.

The final virus preparation suspended in 0.05 M phosphate buffer pH 7 was dialyzed for 12 to 24 hrs. against 0.1 M KCl with 3 to 4 changes of dialysing solution. The dialysis against KCl was desirable to eliminate the charge effect which affects the movement of solute molecules during the ultracentrifugation (Bower, 1970; Trautman and Hamilton, 1972). About 0.6 to 0.8 ml of virus solution was used in a 12 mm, single-sector cell with quartz windows, and centrifuged at 4°C in a Beckman Spinco Model E ultracentrifuge housed in Dr. W. E. Hill's laboratory of the Department of Chemistry, University of Montana. The speed was maintained at 26,000 rpm for virus and 60,000 rpm for protein preparations with an AND rotor. The schlieren patterns were photographed at a phase plate angle of 75° using Kodak Metallographic plates. The pictures were taken at 4- and 16-minute intervals.

To determine accurate sedimentation coefficients, the distance from the inside of the outside reference hole to the maximum of the schlieren peak was measured using a Nikon Model 6C Profile Projector. The logarithms of the distance of the peak in the schlieren pattern from the center of rotation were plotted against time, and a straight line was obtained. The slope of the line was calculated using a Wang Programmed Calculator Model 600. The slope of this line is proportional to the sedimentation coefficient according to the equation:

$$S_{T,b} = \frac{\ln r_1 - \ln r_2}{w^2 (t_1 - t_2)} \quad [2]$$

where $S_{T,b}$ = sedimentation coefficient in buffer at temperature,
 T (seconds)

r_1, r_2 = distance of boundary from axis of rotation at time
 t_1, t_2 (cm)

t_1, t_2 = time interval from t_0 to time when peak was at r_1, r_2
(sec.)

w^2 = angular velocity in radians/second

The sedimentation coefficient is in Svedberg units, S , where $S = 10^{-13}$ sec.

It is customary to express the sedimentation coefficient in corrected form, $s_{20,w}$ which is the value of the coefficient when pure water at 20°C is used as solvent (assuming no alteration of solute molecules). The sedimentation coefficient values were corrected for the temperature and viscosity of water according to the equation:

$$s_{20,w} = s_{T,b} \frac{\eta_{T,b} (1 - \bar{v}\rho)_{20,w}}{\eta_{20,w} (1 - \bar{v}\rho)_{T,b}} \quad [3]$$

where $s_{20,w}$ = sedimentation coefficient in water at 20°C

$\eta_{T,b}$ = viscosity of solvent (buffer) at experimental temperature

$\eta_{20,w}$ = viscosity of water at 20°C temperature

\bar{v} = partial specific volumes

ρ = density

The term $\frac{\eta_{T,b}}{\eta_{20,w}}$ can generally be written as the product of two factors:

(1) the ratio of viscosity of the solvent to that of water at experimental temperature, and (2) the ratio of the viscosity of water at the experimental temperature to that of water at 20°C (Schachman, 1959) or

$$\frac{\eta_{T,b}}{\eta_{20,w}} = \frac{\eta_{T,b}}{\eta_{T,w}} \frac{\eta_{T,w}}{\eta_{20,w}} \quad [4]$$

The most important correction factor is the ratio of viscosities.

The other correction term in Equation [3] is generally smaller (Schachman, 1959). In the present study, correction was made only for the viscosity of water at the experimental temperature (4°C) to the viscosity of water at 20°C, assuming there is no significant difference in viscosity of water and buffer at 4°C. The ratio of $\frac{\eta_{T,w}}{\eta_{20,w}}$ was obtained from the Handbook of

Chemistry and Physics. Assuming that partial specific volumes would be the same in water and buffer, and that there would be no significant effect of the difference in the densities of the two systems, no correction was made for these terms.

The sedimentation coefficients were determined at infinite dilution making correction for the concentration of the samples. Several dilutions of purified virus and protein samples were prepared and the sedimentation coefficient was determined at each concentration, using identical conditions and maintaining a constant 4°C temperature. The concentrations were corrected for radial dilution effect using the equation

$$C_t = C_o \left(\frac{r_o}{r_t} \right)^2 \quad [5]$$

where C_t = corrected concentration

C_o = initial concentration

r_o = distance of miniscus from the center of rotation

r_t = distance of peak from the center of rotation

The results of these runs were plotted against the concentration to obtain a value of $s_{20,w}$ by extrapolating the resulting line to infinite dilution.

Partial Specific Volume and Density

The weight per unit volume (g/ml) of a solution is called its density, ρ . It is a function of the nature and concentration of solutes and

depends slightly on temperature (Trautman and Hamilton, 1970). The partial specific volume, \bar{V} (ml/g), is a measure of the increase in volume when a gram of dry protein (nucleoprotein in the present case) is dissolved in an infinite amount of solvent.

Different values for partial specific volume have been reported by different workers. The partial specific volume is very important in molecular weight determination. An effort was made to accurately determine \bar{V} .

The partial specific volume of highly purified virus was determined using the methods of Ortega and Hill (1973), and Hill, et al. (1969). The virus suspended in 0.05 M phosphate buffer pH 7 was dialyzed against 0.1 M KCl solution for 12-24 hrs. with 3-4 changes of dialyzing solution. The density of the dialysate and that of purified virus solutions were measured with a Digital Density Meter DMA 02C manufactured by Anton Paar (Graz, Austria) and housed in Dr. Hill's laboratory of the Department of Chemistry, University of Montana. The hollow glass oscillator tube of the instrument was carefully washed several times with potassium dichromate, KOH, distilled water, and absolute alcohol, followed by drying with clean air. Virus samples equilibrated to 4°C were carefully inserted into the hollow glass oscillator tube of the instrument to avoid generation of air bubbles. After equilibration in the density meter for 25-30 min., readings were taken and the density calculated. The temperature used in the experiment was $4 \pm .005^\circ\text{C}$. The concentrations of the solutions and dialysate were determined by weighing 3 ml portions into 10 ml tared stoppered volumetric flasks and

lyophilizing and drying them in a vacuum oven at 98°-100°C to a constant weight.

After the density and concentration data were obtained, the apparent specific volume (ϕ) for the virus was calculated using the following equation:

$$\phi = \frac{1}{C} (1 - \frac{\rho - \rho^o}{C}) \quad [6]$$

where ρ is the density of the solution

ρ^o is the density of the dialysate

C is the concentration of the solution in grams per milliliter

Since no significant variation of ϕ with concentration was observed, it was concluded that these values are equal, within experimental error to the partial specific volumes, \bar{v} , of virus (Hill, *et al.*, 1969; Kupke, 1973). Samples were analyzed by means of sedimentation velocity in the Beckman Model E ultracentrifuge, after densities were measured, to insure that no significant dissociation or association had occurred during the experiment.

Electron Microscopy

Electron microscopy was used for virus and protein preparations. The morphology and size of the virus particles were determined from purified as well as crude preparations.

Preparation of Grids

A support film was made on 200 mesh copper grids (Ernest F. Fullam, Inc.). A fine wire gauze loaded with grids was placed on the bottom of a clean petri dish with about 20 ml of sterile distilled water. One or two drops of polyvinyl formaldehyde (Formvar) 0.3 per cent solution prepared in ethylene dichloride (Ernest F. Fullam, Inc.) were applied on the surface of the distilled water in the petri dish. The wire gauze loaded with grids was carefully lifted so that polyvinyl formaldehyde film was coated on the grids. The filmed grids were dried in the dark at room temperature for 12-24 hrs. before use.

Application of Material to the Grids

The usual method of sample application to the grids is to spray particulate purified material with the help of an atomizer or nebulizer. In the present study, very fine micropipettes with 0.5 mm opening end were often used, unless otherwise mentioned. A small drop of sample material was applied on the filmed side of the grids. The excess fluid was drained off by capillary action. Sometimes it was drained off using a corner of filter paper. This method was very simple, but successful.

Negative Staining

A solution of 2 per cent phosphotungstic acid (PTA) was made in distilled water and the pH was adjusted to 7 using M KOH. A mixture of the negative stain (PTA) and virus material to be contrasted was placed on a support film using the method described above. The essence of the process

(and the derivation of its name) is that the stain does not react with the particle but merely infiltrates and surrounds it. Being electron-dense, it provides contrast to the virus particles.

Positive Staining

A 1 per cent aqueous solution of uranyl acetate was prepared and applied the same way as PTA. This stain reacts directly with the particles.

Shadow Casting

In this method the specimen to be shadowed is placed face up and horizontal in a vacuum and is coated with a thin layer of electron-dense material by evaporating from a source above and to one side. The evaporated atoms travel in approximately straight lines so they coat the presented surface of projections, but not areas "unseen" by the source; such areas in the image resemble shadows, and the resemblance is often enhanced by reversing the normal contrast in the final print so that these shadows appear dark.

In the present study, the method of Bradley, in Kay (1965), was followed with occasional modifications. A tungsten wire about 6-7 cm long and 0.1-1.0 mm thick was bent into a V and clamped between terminals in the vacuum chamber with the V pointing at the specimen. An approximately 5-mm long palladium wire was looped over the filament and the chamber was evacuated to about 10^{-5} mm Hg. The filament was slowly heated by passing through it a large current at low voltage until the metal charge melted

and evaporation occurred. The specimen grids were placed at a 30°-45° angle from the V. After this the specimens were observed in the electron microscope.

General Morphology

To determine their size and shape, all the preparations were scanned using a Siemens Elmiskop I microscope at the Rocky Mountain Laboratory, National Institutes of Health, Hamilton, Montana. Particles in each preparation were photographed at 20,000-30,000 x magnification.

Size Determination

The infected leaf materials were sent to Dr. D. Lesemann, West Germany, for confirmed size determination of virus particles from crude preparations. For a crude preparation, the infected leaves were cut into very small pieces in 0.5 ml of 2 per cent neutral PTA to which one drop of 0.1 per cent bovine serum albumine solution had been added. The resulting mixture, without leaf debris, was put into a nebulizer and sprayed on the grid. A parallel measurement was done with the common strain of TMV and normal lengths of the viruses in the present study were determined (Brandes and Wetter, 1959). The same model of electron microscope was used for size determination.

Protein Preparation

Acetic Acid Degradation Method

The cold acetic acid method (Fraenkel-Conrat, 1957) was used with

little modification to prepare the protein. Two to four volumes of 67 per cent cold acetic acid were gradually added to an ice cold aqueous solution of purified CaV 196 (10 mg/ml). The mixture was allowed to stand for 15-30 min. with occasional stirring. At low concentration it took more than one-half hour for degradation. After 30 min. to several hours, a turbid, gelatinous precipitate was observed. The insoluble denatured RNA was removed by centrifugation and the supernatant was dialyzed against water for 2-3 days with 3-4 changes of water. During the dialysis the protein precipitated as the pH of the dialysate reached the isoelectric point of the protein. The aggregation of protein was enhanced by adding a few drops of 3 M pH 4.8 sodium acetate buffer and then concentrated by centrifugation at 50,000 rpm for 90 min. in Ti 60 rotor and suspended in 0.05 M pH 7.6 phosphate buffer overnight. The protein solution was then centrifuged at 25,000 rpm for 1 hr. to remove any residual insoluble material (degraded protein and undigested virus). The water-clear supernatant solution contained CaV 196 protein which was stored frozen and used for serology, electron microscopy, sedimentation velocity, and reconstitution experiments (see Appendix 1).

Mild Alkali Degradation Method

To compare the reconstitution result on protein prepared by the acetic acid degradation method, it was necessary to prepare protein using the alkali degradation method of Schramm (1955), and Fraenkel-Conrat and Williams (1955).

Purified CaV 196 was dialyzed against 0.1 M pH 10.5 carbonate-bicarbonate buffer for 72 hrs. Undegraded virus was separated by cold centrifugation, and the supernatant was brought to 0.4 saturation with ammonium sulfate. The protein was obtained in the precipitate, leaving nucleic acid in the supernatant (checked by spectrophotometry). The protein precipitate was dissolved in 0.05 M phosphate buffer pH 7.5 and precipitated twice again with 0.3 per cent saturated ammonium sulfate. The final precipitate was concentrated by low speed centrifugation at 4°C and dissolved again in 0.05 M pH 7.5 phosphate buffer, then centrifuged under refrigeration at high speed for 30 min. to remove undegraded residual virus. The water-clear supernatant contained protein (see Appendix 2).

Preparation of Nucleic Acid

The nucleic acid was prepared from purified CaV 196 to be used in reconstitution experiments and for virus characterization. The acetic acid degradation method and the phenol extraction method of Gierer and Schramm (1956) were employed.

Acetic Acid Method

The pellet obtained after acetic acid treatment of the virus during protein preparation was washed with cold 67 per cent acetic acid and then several times with water, then redissolved in water adjusting the pH to 7.5 with a slow addition of NaOH and centrifuged to remove insoluble and gelatinous material. The supernatant was precipitated twice by two volumes of

95 per cent ethanol and a few drops of 3 M pH 5.0 sodium acetate buffer, centrifuged to concentrate the precipitate and redissolved in water by adjusting the pH to 7.5 with NaOH. This final solution was given a high speed cold centrifugation for 15-20 min. to remove undegraded virus. The RNA content of the supernatant was determined spectrophotometrically. The infectivity was tested on N. glutinosa and C. quinoa (see Appendix 3).

Phenol Extraction Method

To a purified 10 ml preparation of CaV 196 (10 mg/ml) was added 0.5 ml of 5 per cent sodium dodecyl sulfate (SDS) and stirred for 5-10 min. and placed in an ice bath. Subsequent steps were carried out in a cold room. The mixture of virus and SDS was then shaken with 10 ml of water-saturated and redistilled phenol and centrifuged at low speed to separate phenol and the aqueous layer. The RNA-containing aqueous layer was shaken twice more with phenol and centrifuged as before. The final aqueous layer was washed three times with cold ether to remove phenol and precipitated twice with two volumes of 95 per cent cold ethanol and centrifuged in the cold. The RNA pellet was dissolved in water and used for spectrophotometry and infectivity tests (see Appendix 4).

The Reconstitution of Cav 196

For reconstitution of the virus, the method of Fraenkel-Conrat and Williams (1955) was used. One ml of 0.1 per cent protein solution was mixed with 0.1 ml of 0.1 per cent nucleic acid and 0.01 ml of 0.1 M pH 7 phosphate buffer or 3 M pH 6 acetate buffer. The mixture was incubated

for 6-24 hrs. at 30°C. The active reconstituted virus was obtained after a high speed centrifugation (see Scheme 2).

Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate (SDS)

Gel electrophoresis in an SDS system was performed using essentially the techniques of Shapiro, et al. (1967) and Agrawal and Tremaine (1972) with some modifications. Gels (5 per cent, 9 cm long) were prepared in glass tubes 10.5 cm long and 5 mm in diameter from the following solutions:

Solution A: 10 g acrylamide and 0.3 g N, N'-methylenbisacrylamide (Bis) dissolved in 100 ml of distilled water

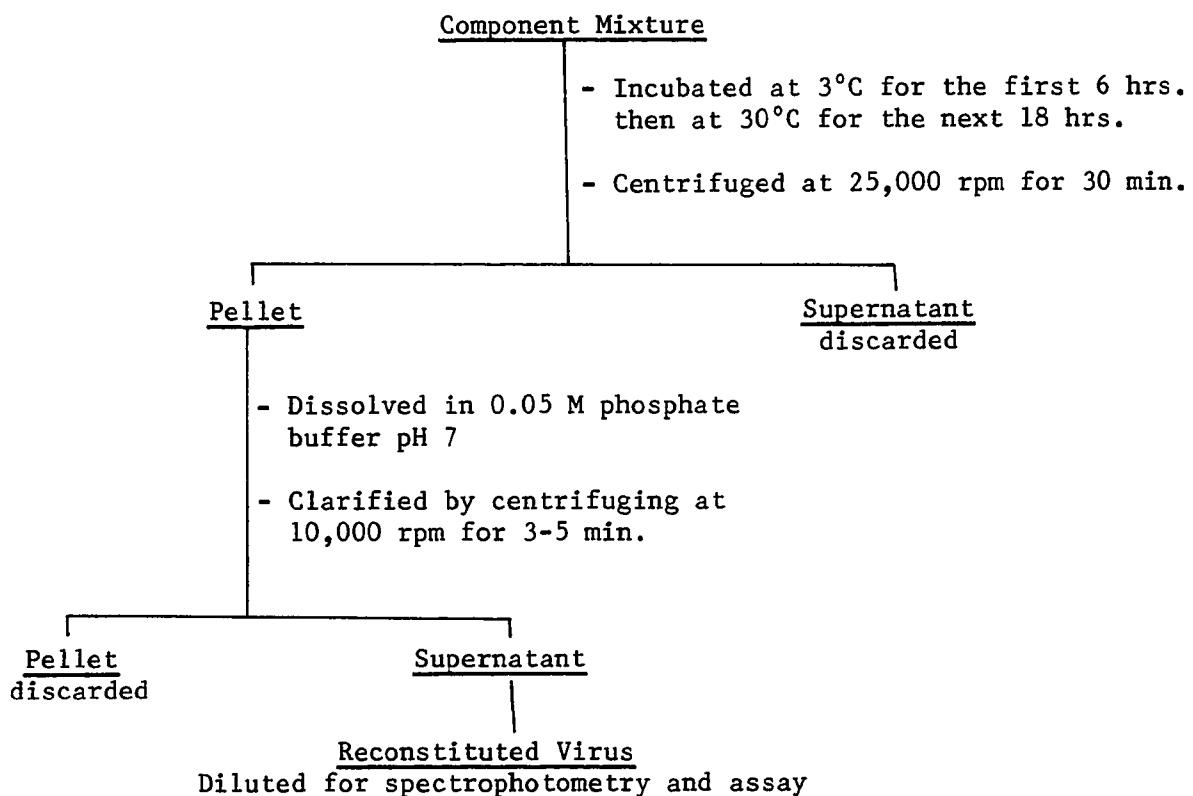
Solution B: 100 ml of 0.2 M sodium phosphate buffer pH 7.2 containing 0.2 g SDS and 0.2 ml N, N, N, N'-tetramethylethylenediamine (TEMED)

Solution C: 1 g ammonium persulfate in 100 ml 0.1 M sodium phosphate buffer pH 7.2

Ten milliliters of Solution A were mixed with 10 ml of Solution B, and then 1 ml of Solution C was added to the mixture. The gel solution was mixed well, gently poured into the glass tubes, overlaid with about 0.2 ml of distilled water, and allowed to polymerize in the presence of light at room temperature for 20-30 min. The tray buffer was 0.1 M sodium phosphate buffer pH 7.2 containing 0.1 per cent SDS and 0.1 per cent 2-mercaptoethanol.

Viral proteins were prepared by boiling the virus for 1 min. in 0.1 M sodium phosphate buffer, pH 7.2, containing 1 per cent SDS and 1 per cent 2-mercaptoethanol. Solutions of marker proteins were prepared in the same manner. The protein concentrations were adjusted to approximately 0.5

<u>Components</u>	<u>Quantity</u>	<u>Remarks</u>
A. Protein (1 mg/ml)	1.0 ml	
RNA (1 mg/ml)	0.1 ml	Opalescence appeared
Phosphate buffer 0.1 M pH 7	0.01 ml	after 6 hrs.
B. Protein (1 mg/ml)	1.0 ml	Opalescence appeared
RNA (1 mg/ml)	0.1 ml	after 6 hrs.
Acetate buffer 3 M pH 6	0.01 ml	



Scheme 2. Diagram of Isolation of Reconstituted CaV 196

mg per ml and made 20 per cent in glycerol. A volume of 0.1 ml was applied to the tube and lectrophoresis was performed at 6 MA per gel for about 3 hrs.

The gels were stained 4 hrs. to overnight in a .25 per cent solution of Coomassie brilliant blue in 91 ml of water, 91 ml methanol and 18.2 ml of glacial acetic acid, then soaked for several hours in a 1:1 solution of methanol and 7 per cent glacial acetic acid.

Serology

Preparation of Antisera

Albino rabbits previously bled for normal serum were injected with purified virus or purified protein. Four intramuscular injections of 1 or 2 ml each containing an equal quantity of Difco incomplete Freund's adjuvant were given one week apart, followed by an intravenous injection. Rabbits were trial bled 1 or 2 weeks after the last injection. If necessary, a booster injection was given intravenously. One or two weeks after the booster injection, rabbits were bled at one-week intervals.

Serological Techniques

Titers were checked using microprecipitin and complement fixation test using the methods described by Bercks, Koenig, and Querfurth, in Kado and Agrawal (1972). An attempt was made to demonstrate the relationships of virus with other members of the designate group using the Ouchterlony agar double diffusion test (1962). To enhance the diffusion of the reactants, high pH ammonia agar was used (Langenberg and Ball, 1972). Proper

controls in each experiment were invariably included to assure that the positive reactions were between virus and its antibody and that they did not involve any other constituents.

Complement Fixation Test

For this study the complement, hemolysin, and sheep red blood cells (Colorado Serum Company) were kindly supplied by Stella Duncan Research Institute staffs. Before the complement fixation test, the two components, complement and hemolysin, were standardized.

Standardization of Hemolysin. To standardize hemolysin the complement diluted 1:10 was held constant. A serial dilution of hemolysin was prepared in physiological saline with 0.01 per cent $MgSO_4$. Approximately 0.3 ml of 1:10 guinea pig complement was added to each homolysin dilution tube followed by the addition of 1.7 ml of Mag saline and .5 ml of a 2 per cent suspension of sheep red blood cells (SRBC). The contents of all the tubes were mixed thoroughly and incubated at 37°C for 30 min. The highest dilution of hemolysin giving complete hemolysis was considered one unit. The two units of hemolysin were equal to one-half the highest dilution showing complete hemolysis. In this test two units of hemolysin were equal to 1:2000 dilution. Proper controls were maintained to ensure the reliability of the experiment.

Standardization of Complement. To standardize the complement, the hemolysin was held constant. A serial dilution of the complement was pre-

pared in physiological saline with 0.01 per cent MgSO_4 . To each dilution tube, 0.5 ml of 2 unit hemolysin and 0.5 ml of 2 per cent suspension of sheep red blood cells were added and final volume was brought to 2 ml by adding the saline solution. The contents of the tubes were mixed thoroughly and incubated at 37°C for 30 min. The highest dilution of the complement giving complete lysis was considered the exact unit. The two units of the complement were equal to one-half the above dilution. In this experiment one unit was equal to a 1:32 dilution and the two units were equal to 1:16 dilution.

Determination of Antigenic Dose. Two-fold dilutions of purified CaV 196 and ZyV 58 were made. A 1:4 dilution of inactivated test serum (0.2 ml) was added to each dilution (0.5 ml) of the antigen. One ml of 2 units of the complement was added to each tube. Tubes were incubated overnight at 4°C . After incubation 0.5 ml of 2 unit hemolysin and 0.5 ml of a 2 per cent SRBC was added to each tube, and incubated at 37°C for 30 min. The largest amount giving 4+ fixation of the complement was considered to be one unit of the antigen dose and 2 units were equal to the next largest amount giving 4+ fixation. Three controls, serum control, antigen control, and hemolytic control, were prepared. CaV 196 showed 1:32, and ZyV 58 1:4 as two units.

Titration of Anti-CaV 196 Serum and Anti-ZyV 58 Serum. A serial dilution of inactivated test sera was prepared in physiological saline with 0.1 per cent MgSO_4 , and 0.2 ml of two units antigen solutions were

added to each dilution tube followed by the addition of 1 ml of two unit complement and incubated overnight at 4°C. The next morning 0.5 ml of two unit hemolysin and 0.5 ml of 2 per cent SRBC were added and incubated at 37°C for 30 min. The highest dilution of the test antiserum giving 4+ or 3+ fixation was the titer of the antiserum. Summarized results are presented in Tables XII, XIII, and XIV in the Results section.

CHAPTER III

RESULTS

Host Reaction

Of the several plant species inoculated with the two viruses (see Table I), Atriplex hastata, Chenopodium quinoa, C. gigantium, C. capitatum, C. hybridum, C. polyspermum, C. album, C. foliosum, C. urbicium, Datura stramonium, Gomphrena globosa, Hablitzia tamnoides, Nicotiana glutinosa, N. tabacum var. Turkish NN and systemic; var. White-Burley, var. Xanthi and var. Haranova, N. sylvestris, N. rustica, and Phaseolus vulgaris were infected with isolate CaV 196. Atriplex hastata, C. quinoa, C. gigantium, C. polyspermum, C. album, C. urbicium and H. Tamnoides were the only species which were infected with the isolate ZyV 58.

With few exceptions, most of the symptoms produced by CaV 196 on Solanaceous plant species were similar to those produced by the common strain of TMV. It produced local lesions on N. glutinosa, N. tabacum var. Turkish (NN) and N. tabacum var. Xanthi; local and systemic effects on N. rustica and N. sylvestris and only systemic effects on N. tabacum var. Turkish (systemic). The symptoms on Turkish systemic tobacco were severe mosaic, distortion of leaves, and blister formation. Extensive shoestring formation was noted very often on all inoculated plants. The distortion and blister effects were also observed on White Burley tobacco plants. The blister and shoestring effects were produced in all the environmental conditions.

TABLE I

HOST RANGE AND SYMPTOMS OF CaV 196 AND ZyV 58

Plant Species	CaV 196	ZyV 58
1. <u>Amaranthus caudatus</u> L.		SLC
2. <u>A. tricolor</u> L. Cv. Splendens		
3. <u>Atriplex hastata</u>	Chlorotic LL on inoculated leaves	Systemic mottling
4. <u>Chenopodium quinoa</u> Wild	Chlorotic LL followed by necrosis and leaf dropping	Necrotic LL on inoculated leaves, followed by severe syst. mosaic
5. <u>C. gigantium</u> D. Don	White-brown necrot- ic LL. No syst. effect	White-brown necrotic LL. No syst. effect
6. <u>C. capitatum</u> (1) Asch	Chlorotic diffused LL	
7. <u>C. hybridum</u> L.	Chlorotic LL. No syst. effects	
8. <u>C. polyspermum</u> L.	Necrotic LL. Ring spotting in high temperature, necrotic diffused white patches	Systemic mottling
9. <u>C. album</u> L.		Circular chlorotic LL. No syst. ef- fect

(continued on next page)

TABLE I (continued)

Plant Species	CaV 196	ZyV 58
10. <u>C. foliosum</u> L.	Chlorotic LL. No syst. effect	
11. <u>C. urbicum</u> L.	Chlorotic LL. No syst. effect	Circular chlorotic LL
12. <u>D. stramonium</u> L.	Necrotic local lesions (LL)	
13. <u>G. globosa</u> L.	Chlorotic LL followed by necrosis and syst. mottling and veinal necrosis of uninoculated young leaves	
14. <u>H. tamnoides</u> M. Biel	Chlorotic LL	Chlorotic LL green flecking in yellow background
15. <u>N. tacacum</u> var. Turk. NN	Necrotic LL	
16. <u>N. tabacum</u> var. Turk. systemic	Syst. mosaic vein clearing followed by syst. necrosis and blister and shoestring formation and distortion of young leaves	
17. <u>N. tabacum</u> L. var. White Burley	Necrotic LL. Syst. mottling and vein clearing, distortion of leaves	

(continued on next page)

TABLE I (continued)

Plant Species	CaV 196	ZyV 58
18. <u>N. tabacum</u> L. var. Haranova	Necrotic patches, etching and yellow flecking on inocu- lated leaves, blis- tering and deforma- tion of uninoculated leaves	
19. <u>N. tabacum</u> L. var. Xanthi	Necrotic dark LL on inoculated leaves	
20. <u>N. glutinosa</u> L.	Necrotic LL.	
21. <u>N. rustica</u>	Chlorotic LL followed by necrotic patches and syst. necrosis and puckering. NIB.	
22. <u>N. sylvestris</u>	Necrotic LL. Showing water-soaked appear- ance, syst. necrosis and complete death of plants. Younger leaves deformed and produced with massive glandular hairs. Blister forma- tion	
23. <u>P. vulgaris</u> L. var. Kidney bean	Minute necrotic LL	

Note.--SLC = symptomless carrier; LL = local lesions; syst. = systemic;
NIB = not inoculated back.

The initial symptoms on young Turkish systemic tobacco consisted of a mottling and distortion of the young leaves. Complete suppression of the lamina frequently occurred so that the leaves consisted mainly of long thin threads; sometimes leaves with half laminae only were observed. Very severe symptoms were observed on N. sylvestris. Water-soaked local lesions were produced on inoculated plant leaves about 3-4 days after the inoculation, followed by a systemic necrosis of uninoculated younger leaves in approximately one week. Younger leaves were deformed and produced massive glandular hair and blisters. Eventually all the inoculated plants were killed (Appendix, Plate I). Almost all the Chenopodium species used in the test showed local effects after being inoculated with CaV 196. It reacted on D. Stramonium in less severe form than the common TMV. On G. globosa it produced local lesions as well as systemic mottling. It did not apparently infect Amaranthus caudatus, A. tricolor, and C. album. No attempts were made to recover virus from these hosts.

ZvY 58 infected A. hastata, C. quinoa, C. gigantium, C. polyspermum, C. album, and C. urbicum. It did not infect D. stramonium, G. globosa, N. glutinosa, N. rustica, N. sylvestris, or P. vulgaris. Unlike CaV 196, ZyV 58 was found to be restricted to the family Chenopodiaceae. It produced necrotic local lesions on inoculated leaves of C. quinoa, followed by systemic mottling and severe mosaic of uninoculated leaves (Appendix, Plate VII). Small axillary leaves with severe mosaic symptoms were extensively produced.

Cytology

Paradermal sections of Zygocactus sp. infected with ZyV 58 were examined for the presence of inclusion bodies. Spindle-shaped inclusions were observed in the cells of virus-infected plants, similar to those described by Bercks (1971) in the cells of Amaranthus hybridus infected with cactus virus X (Appendix, Plate VII).

Physical Properties in Plant Sap

Thermal Inactivation Point

CaV 196 showed a thermal inactivation temperature of 85°-90°C for 10 min. when assayed on C. quinoa and N. glutinosa, and ZyV 58 showed a thermal inactivation temperature of 75°-80°C for 10 min. on C. quinoa (Table II).

Dilution End Point

The dilution end point fell between 10^{-8} and 10^{-9} for CaV 196 and 10^{-5} and 10^{-6} for ZyV 58 when assayed on N. glutinosa and C. quinoa, respectively (Table III).

Aging in Vitro

Sap from tobacco leaves infected with CaV 196 and diluted 1:10 was found to be infective after more than 15 days at room temperature (23°-27°C). ZyV 58 showed a longevity of 6-7 days at 30°-34°C when assayed on C. quinoa (Table IV).

TABLE II

THERMAL INACTIVATION POINTS OF CaV 196 AND ZyV 58.
 AVERAGE LOCAL LESIONS PER HALF LEAF
OF N. GLUTINOSA AND C. QUINOA, RESPECTIVELY

Temperature (°C)	CaV 196	ZyV 58
55 ± .5	89.5	58.00
60 "	90.0	55.25
65 "	85.25	53.56
70 "	80.50	39.75
75 "	70.00	11.00
80 "	60.00	4.00
85 "	35.00	0
90 "	0	0
95 "	0	0
100 "	0	0
Control	97.5	72.5

TABLE III

THE DILUTION END POINTS OF CaV 196 AND ZyV 58.
 AVERAGE LOCAL LESIONS PER HALF LEAF
 OF N. GLUTINOSA AND C. QUINOA, RESPECTIVELY

Dilutions	CaV 196	ZyV 58
Control	99.5	74.00
10^{-1}	85.0	42.00
10^{-2}	64.50	31.00
10^{-3}	42.50	20.50
10^{-4}	29.50	12.65
10^{-5}	21.25	7.50
10^{-6}	15.75	2.25
10^{-7}	12.00	0
10^{-8}	10.25	0
10^{-9}	9.5	0
10^{-10}	0	0

TABLE IV

LONGEVITY IN VITRO OF CaV 196 AND ZyV 58.
 AVERAGE LOCAL LESIONS PER HALF LEAF
 OF N. GLUTINOSA AND C. QUINOA, RESPECTIVELY

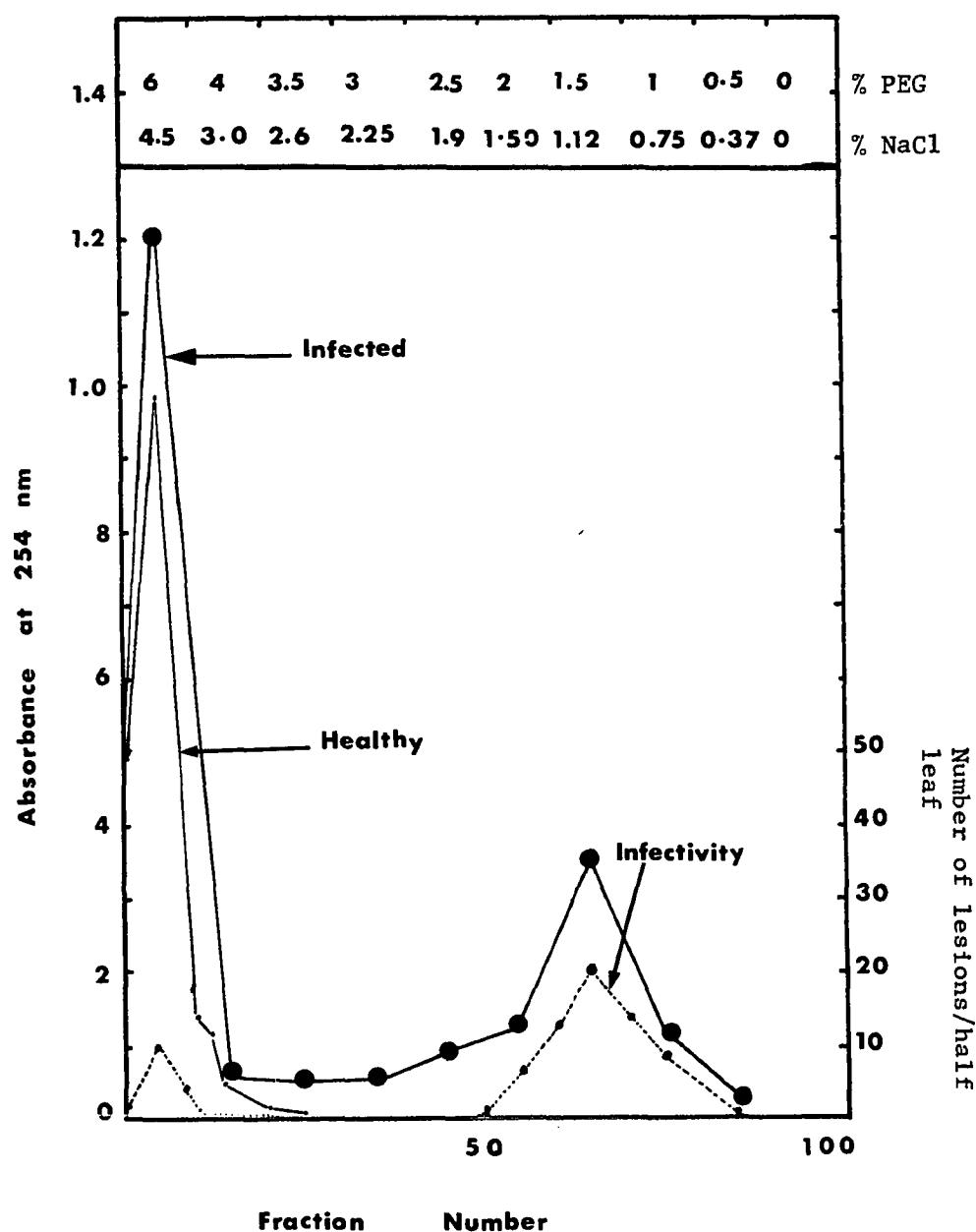
No. of Days	CaV 196	ZyV 58
1	116.00	65.75
2	104.00	58.75
3	102.00	50.5
4	92.50	40.00
5	90.25	25.00
6	87.75	21.00
7	84.50	11.50
8	75.00	0
9	71.25	0
10	67.50	0
11	60.00	0
12	65.00	0
13	58.00	0
14	50.00	0
15	51.00	0

PurificationPreparation of Virus

Differential Centrifugation. This method of purification yielded moderate amounts of virus. The methods of clarification, solvents, and types of buffer used, had little effect on the yield of virus. Final pellets were slightly yellow and gelatinous. About 300-400 mg/kg of CaV 196 and 100-200 mg/kg of ZyV 58 were obtained by differential centrifugation. Normally the color of purified CaV 196 pellet was milky-white but, when over-ultracentrifuged at high speed, it turned yellow. The virus preparations were highly infective.

Purification by Adsorption Chromatography. The results of this method were similar to the results of Venekamp and Mosch (1963). The purification with a cellulose column and PEG in NaCl showed a clear difference between the two preparations from diseased and healthy plants. A strongly UV absorbing host component was eluted by 6 per cent PEG in 4.5 per cent NaCl, both from diseased and healthy extracts. In most of the experiments it was noted by spectrophotometry that only about 50 per cent of the virus was adsorbed on the column. Only 1.2 per cent PEG in 1.15 per cent NaCl eluted a UV absorbing substance, possibly virus, from the column containing extracts from diseased plants (Figure 1). This substance was widely distributed from 2.5 per cent PEG in 1.9 per cent NaCl to 1 per cent PEG in 0.75 per cent NaCl. But the fraction eluted by 1.5 per cent

Figure 1. Fractionation of CaV 196 and other UV absorbing material from C. quinoa approximately 5 ml of clarified infected sap added to a cellulose column. Column: 5 g dry weight, 2 cm diameter, 5 cm in height. Absorbance recorded on ISCO absorption recorder. Fraction's volume approximately 2 ml.



in 1.12 per cent NaCl showed higher infectivity. This method of purification yielded small amounts of purified virus. Therefore it was not used for the isolate ZyV 58.

Purification by Precipitation with Polyethylene Glycol (PEG).

This method of purification produced high yields of CaV 196 but low yields of the isolate ZyV 58. Approximately 900-1000 mg/kg of virus was obtained by this method. The purified virus preparations were found very infective on N. glutinosa and C. quinoa. When precipitates obtained after PEG precipitation were dissolved in 0.1 M phosphate buffer pH 7.4-8, a very interesting result was obtained. The suspended material had a fibrous appearance. A smear from the suspension was made and observed in a light microscope. Long, slender, fibrous needles were observed at pH 5 (Appendix, Plate IV). When the pH of the suspension was raised to 7.5-8 the crystals disappeared. These needles were not observed in preparation from healthy plants.

After density gradient centrifugation at 25,000 rpm in a Beckman SW 27 rotor, two major zones were observed in each tube. The top zone was about 7 mm from the miniscus and 6-7 mm wide consisting of 3-4 diffused bands. An effort to separate these bands by recentrifugation in density gradient tubes failed. The fractions obtained from the top zones showed low UV absorbancy and infectivity. When top zone fractions were analyzed by means of sedimentation velocity experiment in a Model E analytical ultracentrifuge, three components were observed which will

be discussed in a separate section.

The second zone (bottom) was milky-white and about 1 cm above the bottom of the tube. The bottom zone showed higher UV absorbancy and infectivity (Figure 2). This method of purification was used for CaV 196 throughout the study.

An attempt was made to purify ZyV 58 using this method. However, no distinct zones were observed after PEG precipitation and sucrose density gradient centrifugation in the SW 27 rotor at 25,000 rpm. During fractionation with UV monitor, it was found that virus was distributed linearly from the bottom to the top of the tubes. The infectivity was higher in the fractions collected from the top of the tubes.

Purification of ZyV 58 by Density Gradient Centrifugation in CsCl.

When a partially purified virus preparation was centrifuged in cesium chloride density gradient tubes for 12-18 hrs. at 35,000 rpm a single pellicle type band was obtained. This band was observed by absorption at 254 nm 1 cm above the bottom of the tube. The fractions from this band were highly infectious (Figure 3).

Preparation of Protein and Nucleic Acid

Protein was prepared from highly purified CaV 196 by 67 per cent acetic acid treatment and alkali degradation methods. At virus concentrations of less than 10 mg/ml it took more than two hours by acetic acid treatment to degrade the virus and produce a precipitate but, at a concentration of 30 mg/ml, it took 40 min. to obtain complete degradation. At

Figure 2. Fractionation of CaV 196 after precipitation with 5 per cent PEG, dissolved in 0.1 M phosphate buffer pH 8.0 and centrifuged twice in SW 27 rotor at 25,000 rpm for 90 min. The curves show absorbance at 254 and infectivity on N. glutinosa.

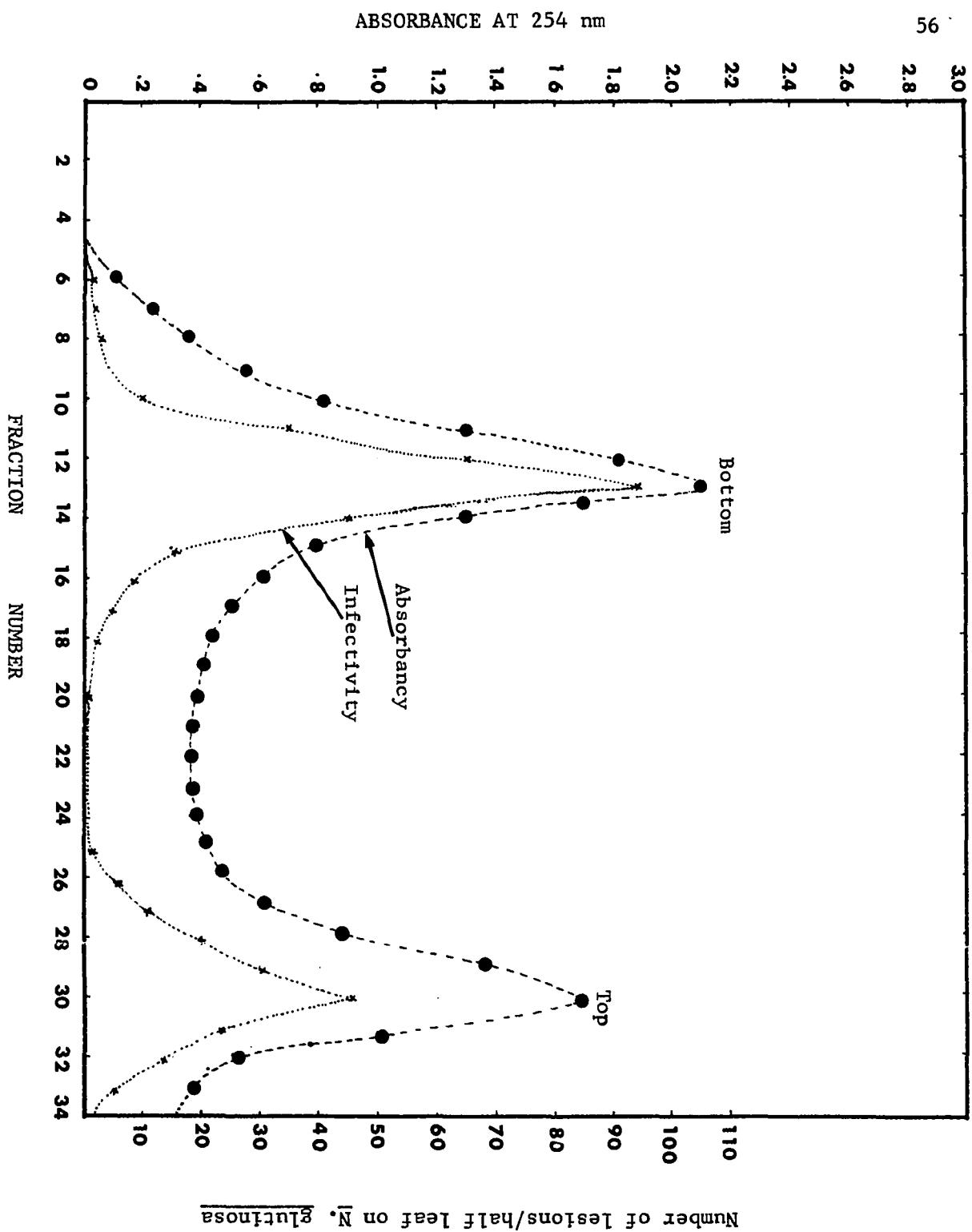
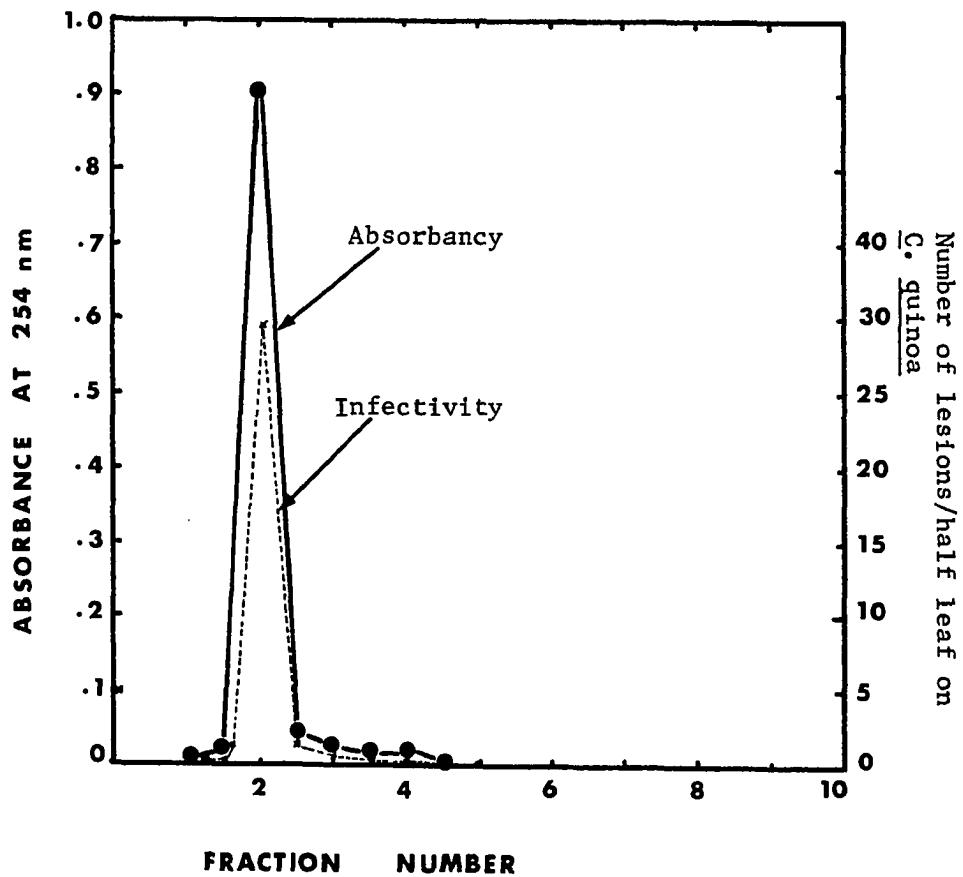


Figure 3. Purification of ZyV 58 in CsCl density gradient. The density gradient tubes were centrifuged for 18 hrs. at 35,000 rpm in SW 39 rotor. Fractions collected with an automatic fraction collector attached with UV analyzer. Fraction's volume 0.5 ml. Absorption recorded at 254 nm. Infectivity on C. quinoa.



this concentration, approximately 9-10 mg/ml protein was obtained using the acetic acid degradation method whereas a considerably lower yield of protein was obtained by the alkali degradation method.

Nucleic acid (RNA) was prepared from the precipitate obtained after acetic acid treatment of purified virus (see Appendix) and by phenol extraction method. The phenol extraction method yielded more purified nucleic acid than did the acetic acid method, and infectivity on N. glutinosa was obtained only by phenol extraction.

Physico-Chemical Characterization of Virus and Viral Components

Ultraviolet Absorption

Virus. Both isolates CaV 196 and ZyV 58, when purified, gave ultraviolet absorption spectra typical of nucleoprotein, with a minimum at 244-246 nm and maxima around 260-262 nm (Figures 4 and 5). The corrected ratio of the maximum:minimum absorption for CaV 196 was 1.13 and 1.2 for ZyV 58 and the ratios of 260:280 were 1.19 and 1.2, respectively. These values agree within experimental error with the values reported by previous workers for the common strain of TMV and for most of the members of the potato virus X group (Knight, 1962; Reichmann, 1958; Bercks, 1970, 1971).

As mentioned earlier, a correction for scattering was made from the measurements at 320-410 nm. When the logarithm of absorbance was plotted against the logarithm of wave length, a straight line was obtained

Figure 4. Corrected UV absorption spectrum of CaV 196. Purified by polyethylene glycol (PEG) precipitation and sucrose density gradient centrifugation. Final virus preparation suspended in 0.05 M, pH 7 phosphate buffer.

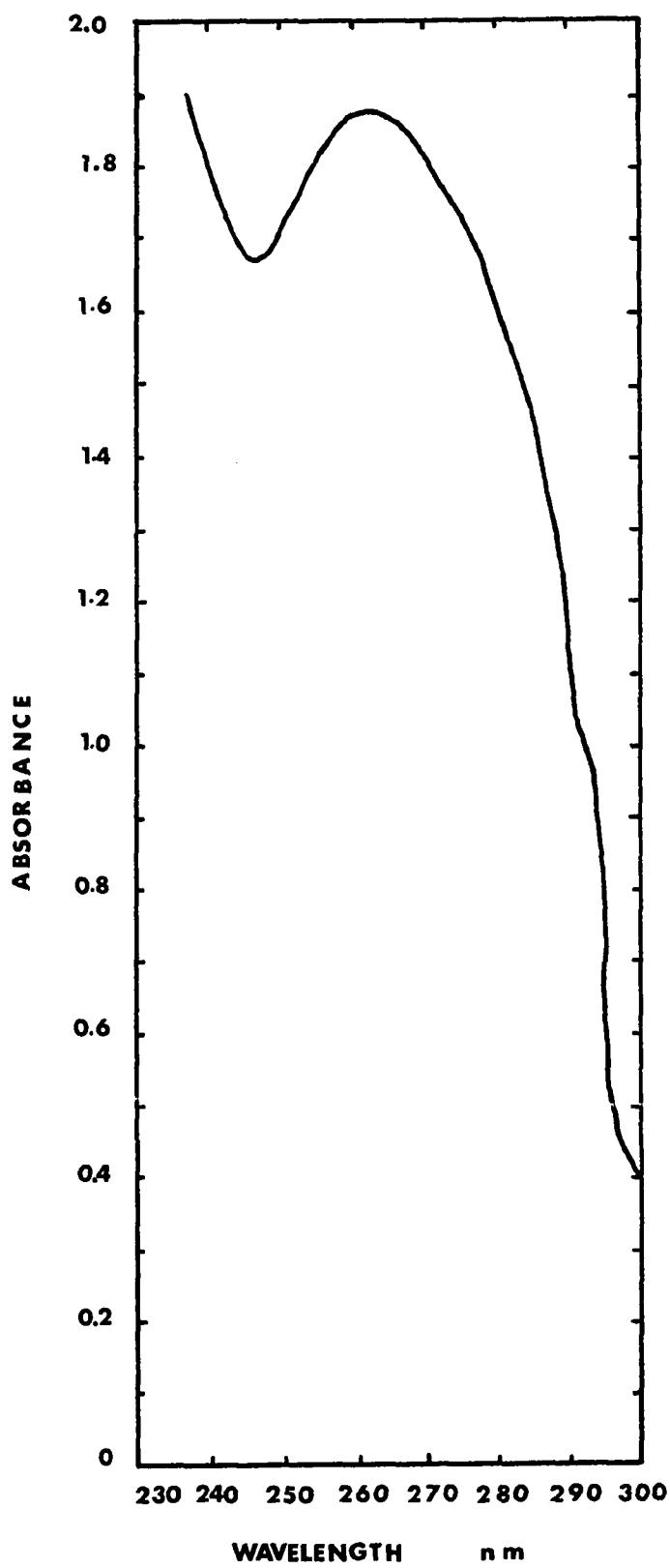
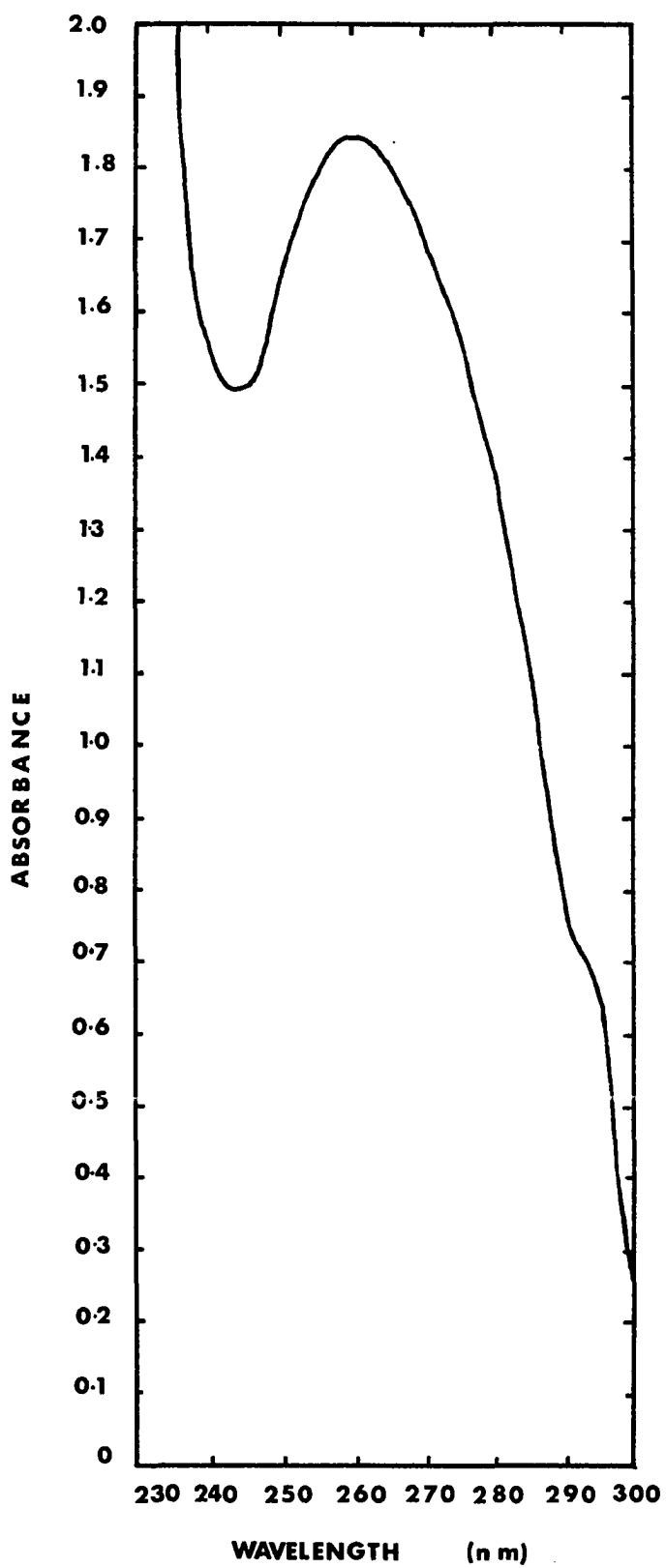


Figure 5. Corrected UV absorption spectrum of ZyV 58 purified by differential centrifugation and density gradient centrifugation. Final virus preparation suspended in 0.05 M, pH 7 phosphate buffer.



which was extrapolated to 260 nm using the least squares method (Figure 6).

The extinction coefficient of purified CaV 196 was determined by plotting the absorbance against the dry weights of the purified virus as shown in Figure 7. The extinction coefficient at 1 mg/ml concentration was found to be 3.10 at 260 nm wave length. Data for the experiment are presented in Table V.

Due to the insufficient amount of purified preparation of ZyV 58 in the present study, it was not possible to determine its extinction coefficient. For quantitative studies the extinction coefficient value of $E_{260}^{1 \text{ mg/ml}} = 2.9$, reported for potato virus X by previous workers, was used (Bercks, 1970).

Protein and Nucleic Acid (RNA). The protein preparations gave a typical ultraviolet absorption spectrum for protein with the minimum at 250 nm and the maximum at 282 nm. The maximum:minimum ratio was found to be 2.33. The maximum:minimum ratio for the common strain of TMV and the HR strain reported previously are 2.4-2.6 and 2.6-3.0, respectively (Fraenkel-Conrat, 1957). In the present study the extinction coefficient of 1.27 reported by Fraenkel-Conrat (1957) was used in most of the quantitative studies.

The nucleic acid preparation also gave a typical UV absorption spectrum with the minimum near 230 nm and the maximum at 260 nm. The maximum:minimum ratio was 2.12, whereas the previously reported ratio for

Figure 6. Correction for scattering in UV absorption of purified virus preparation. Logarithms of wave lengths plotted against logarithm of absorbance and extrapolated to 260 nm wave length.

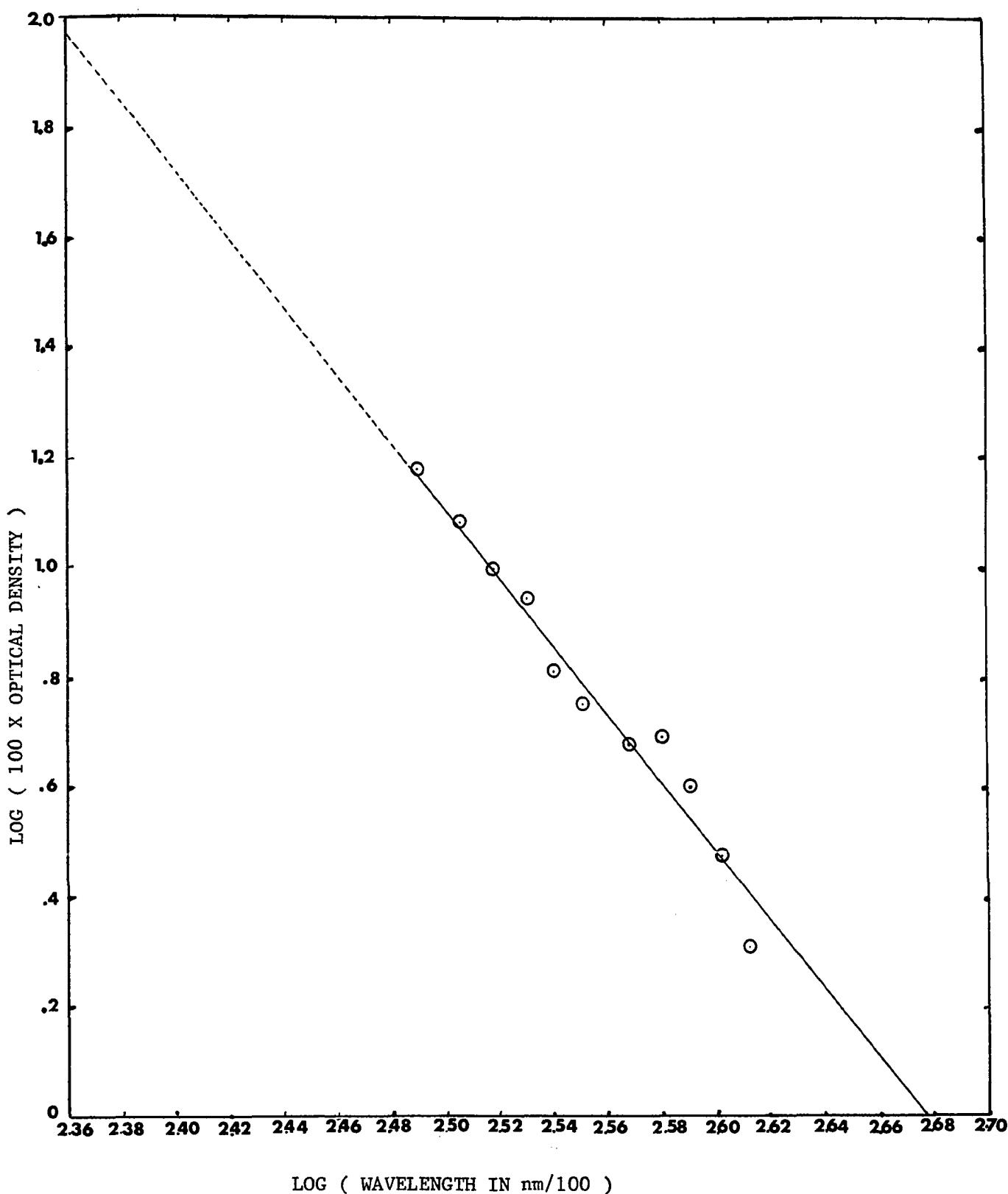


Figure 7. Extinction coefficient of CaV 196, purified by 5 per cent PEG precipitation and sucrose density gradient centrifugation and dialyzed against deionized water. The absorbance of each dilution was measured at 260 nm. The concentrations of the solutions were obtained by weighing 2 ml fractions into 10 ml tared volumetric flasks, lyophilizing and drying them at 90°-95° in vacuo to a constant weight.

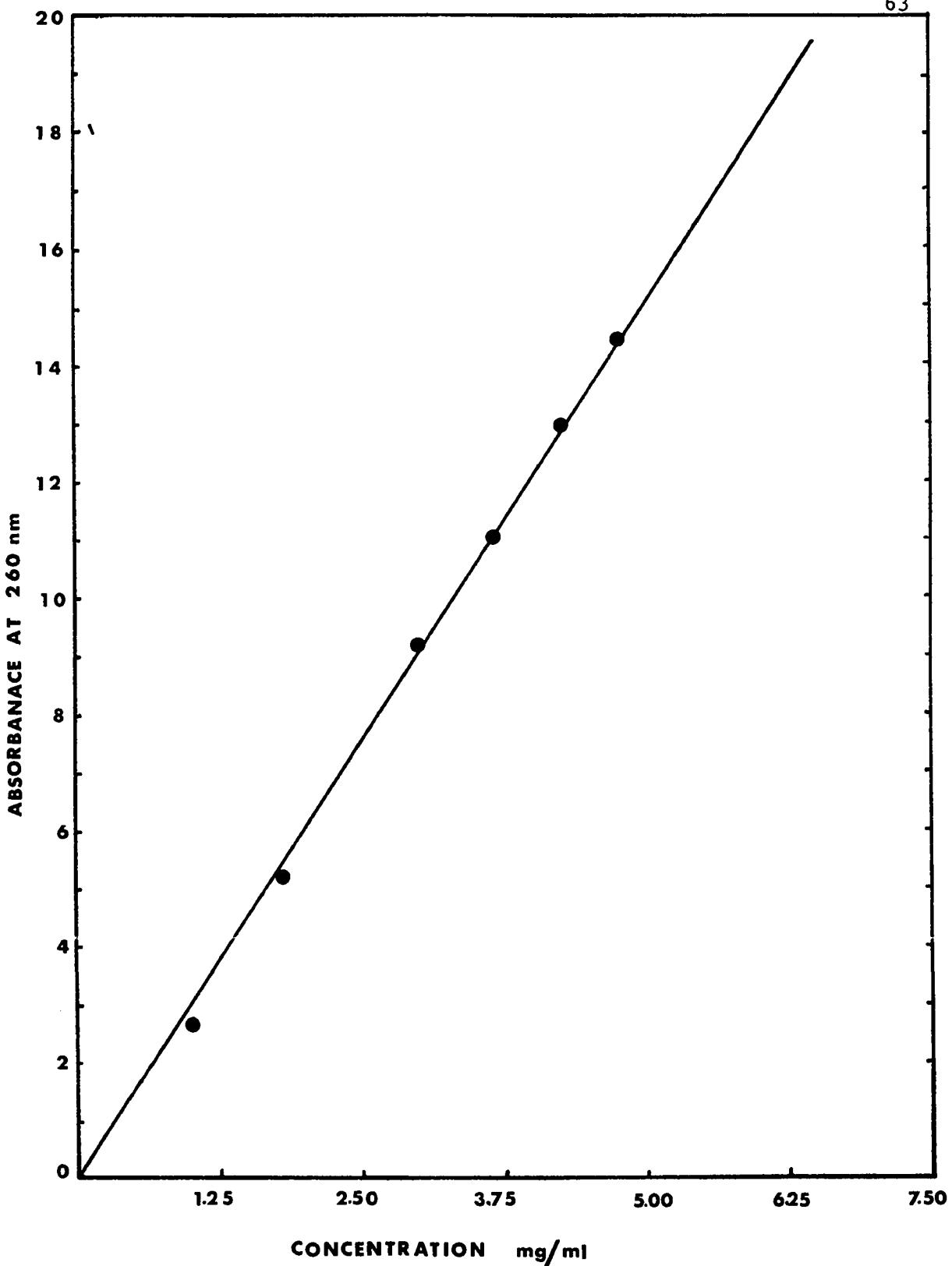


TABLE V

ABSORBANCE OF PURIFIED CaV 196
AT DIFFERENT CONCENTRATIONS AT 260 NM WAVE LENGTH

S. No.	Virus (mg/ml) Concentration	Absorbance at 260 nm
1	0.95	2.8
2	1.75	5.2
3	2.90	9.1
4	3.65	11.0
5	4.25	12.9
6	4.95	15.1

the RNA of common TMV is 3.0 (Fraenkel-Conrat, 1955). The nucleic acid prepared by acetic acid treatment showed a minimum around 235 and a maximum at 265 nm with a maximum:minimum absorption ratio of 1.4. The results indicate that the preparations contained considerable amounts of protein, which was also confirmed by electron microscopy. The absorption spectrum of protein and nucleic acid are shown in the figures of reconstitution experiments.

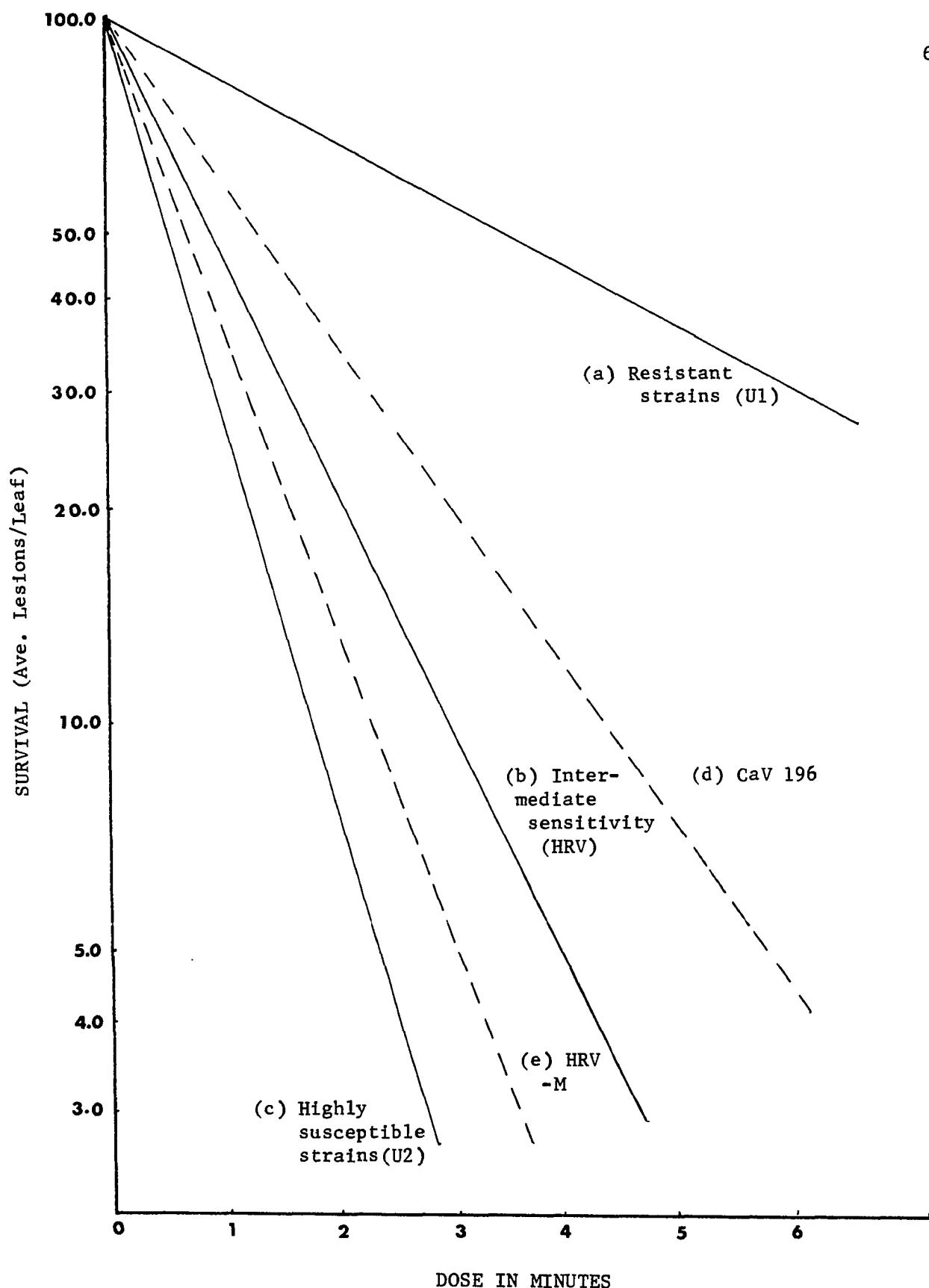
Ultraviolet Inactivation of Virus Infectivity

This part of the study was done by a fellow graduate student, Mrs. Jacqueline F. Perryman. She compared CaV 196 along with HRV-M (Holmes Rib-Grass-Montana) to the eight strains used by Siegel and Wildman (1954). In Figure 8 three lines represent three groups of viruses: (a) those resistant to inactivation including the U1 strain (common TMV); (b) those intermediate in susceptibility to inactivation, including U8 strain; and (c) those highly susceptible to inactivation including U2 strain of TMV. The dotted lines represent the CaV 196 (d) and HRV-M (e). CaV 196 was found more resistant than any of the other strains except U1 (Perryman, 1972).

Analytical Ultracentrifugation Studies

Analytical ultracentrifugation runs were made for all the purified virus and protein preparations. Their homogeneity and sedimentation coefficients were determined.

Figure 8. A comparison of the susceptibilities to UV inactivation of CaV 196 and HRV-M with several other TMV strains studied by Siegel and Wildman, by survival-dose curve (Perryman, 1972).



Sedimentation Coefficient Determination of Virus. Sedimentation coefficients corrected for water at 20°C ($s_{20,w}$) were determined for purified viruses. The top zone of CaV 196 preparations showed three components with $s_{20,w}$ of 86.82, 123.81, and 151.86S. These values are not corrected to infinite dilution (Appendix, Plate II). The $s_{20,w}$ of the bottom zone was determined using a series of dilutions and corrected to infinite dilution. The bottom zone also showed two components, 183 and 200S. The second component (200S) is possibly aggregated material, dimers. The value 183S agrees within experimental error with the monomeric value of the common strain of TMV, 187S, as previously reported by Lauffer (1944). The results of the present study are shown in Table VI.

The sedimentation coefficient ($s_{20,w}$) of CaV 196 depended on the concentration of virus (Figure 9).

The sedimentation coefficient of purified ZyV 58 was determined. Two values were often obtained for ZyV 58, $s_{20,w} = 200-213S$ and 120-123S. It is possible that 200-213S component was an aggregated virus material and 123S monomer. The sedimentation coefficients for the members of potato virus X group, to which ZyV 58 possibly belongs, have been reported to be from 110-125S (Varma, et al., 1970).

The $s_{20,w}$ of ZyV 58 was not extrapolated to infinite dilution.

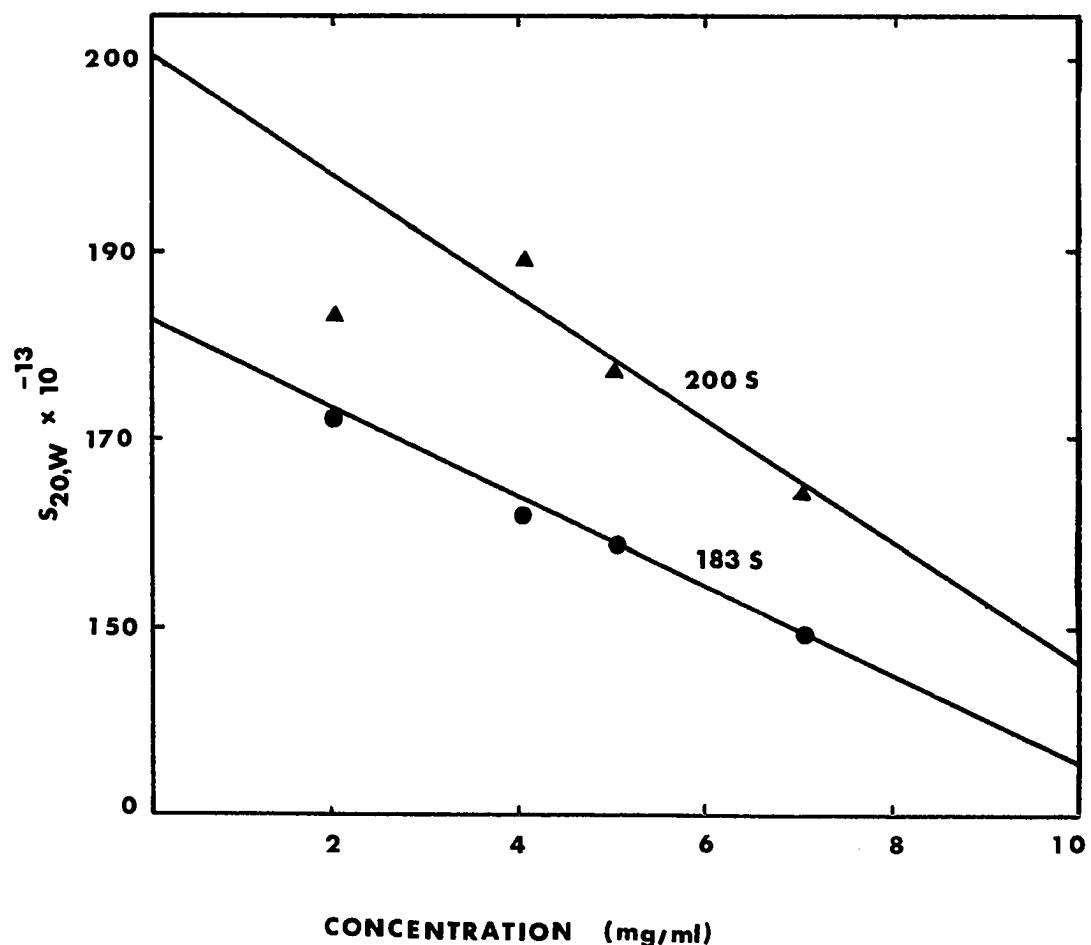
Sedimentation Coefficient of Protein. The sedimentation coefficients of purified coat protein of CaV 196 were determined and extrapolated to infinite dilution by the least square method. As is evident

TABLE VI

SEDIMENTATION COEFFICIENTS OF CaV 196 AT DIFFERENT DILUTIONS

Concentration in mg/ml (corrected for radial dilu- tion)	$s_{20,w}$ component I (s)	$s_{20,w}$ component II (s)
2.5	172.0	182.9
4.0	161.8	189.7
5.5	158.8	176.9
7.0	149.4	163.6

Figure 9. Concentration dependence of the sedimentation coefficients of purified CaV 196, extrapolated to infinite dilution. Straight line drawn by least square method. Speed of centrifugation 26,000 at 4°C. The extrapolated sedimentation coefficients ($s_{20,w}$) are 200S and 183S.



CONCENTRATION (mg/ml)

from Table VII, contrary to Table VI, $s_{20,w}$ increases with increasing concentration (Figure 10).

It is also evident from the Table and schlieren diagram (Appendix, Plate V), that two components with $s_{20,w}$ 3.487S and 14.962S were observed. The second component sedimenting at 14.960S could be a polymerized form of protein. Whether the component sedimenting at 3.48S is a dimer or trimer will be discussed later.

Electrophoretic Analysis of Coat Protein

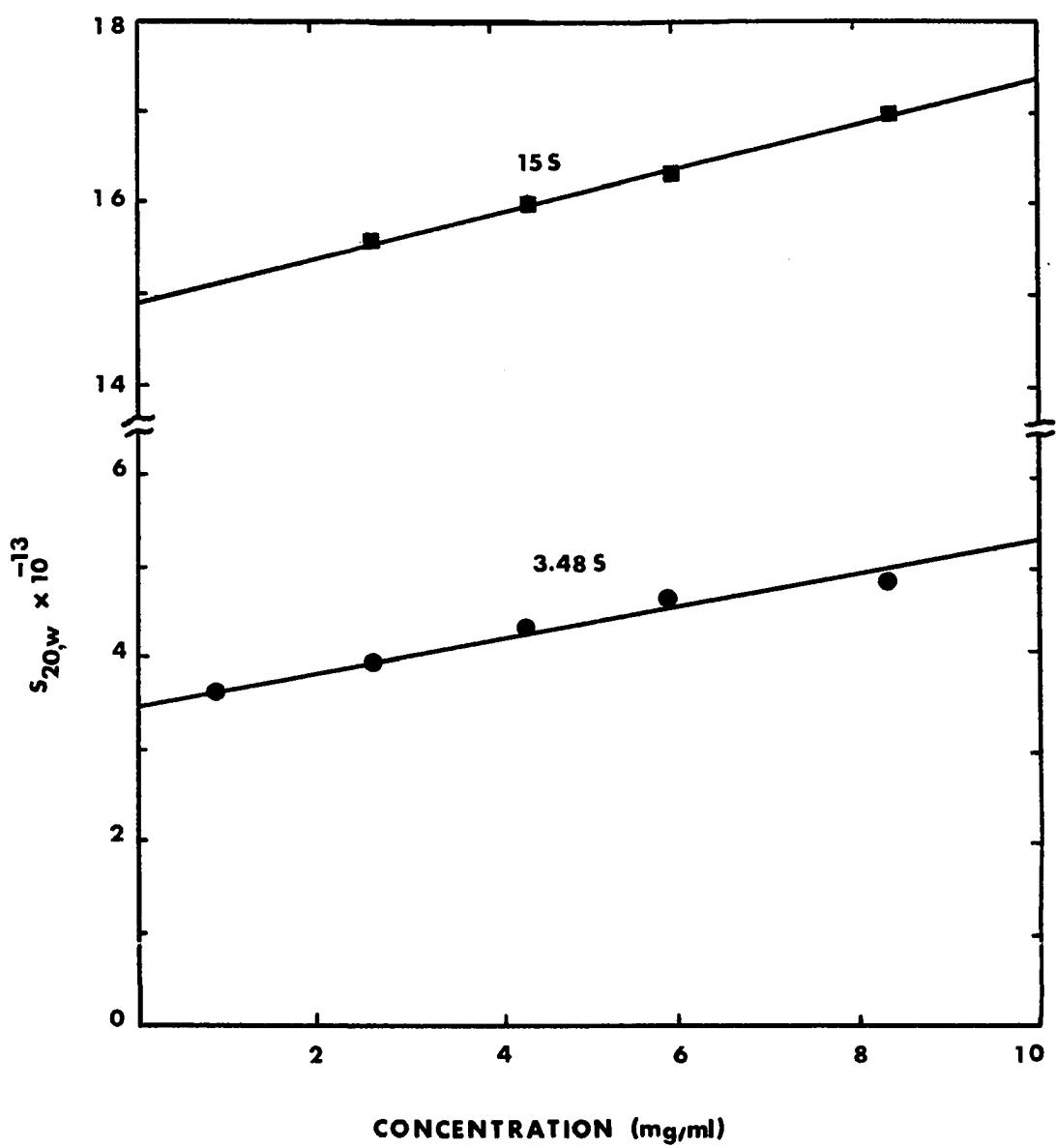
Polyacrylamide Gel Electrophoresis with SDS. An attempt was made to analyze CaV 196 A-protein electrophoretically and compare it with the protein of common TMV. Before electrophoresis purified CaV 196, TMV and A-protein (CaV 196) were treated by boiling with 1 per cent SDS and 1 per cent 2-mercaptoethanol for 1 min. After electrophoresis and staining, unusual results were found. Both protein preparations of CaV 196 appeared with additional minor bands which were not distinctly separated from the major bands (Appendix, Plate V). All gels with CaV 196 A-protein and TMV protein showed very thick broad bands which could possibly be due to high concentration of sample proteins. Sometimes host contaminants, such as ribosomes and host proteins, form thick bandings (Wolf and Casper, 1971) but in the present study there is little possibility for host contaminants. The virus and protein preparations were checked for homogeneity and purity by analytical ultracentrifugation, and were found to be highly purified except for the aggregated and polymerized components.

TABLE VII

SEDIMENTATION COEFFICIENT ($s_{20,w}$) OF PURIFIED COAT PROTEIN
OF CaV 196 AT DIFFERENT PROTEIN CONCENTRATIONS

Concentration of protein (mg/ml) corrected for radial dilution	$s_{20,w}$ of component I (S)	$s_{20,w}$ of component II (S)
0.833	3.5	Not visible
2.588	3.96	15.62
4.313	4.21	15.99
5.965	4.54	16.33
8.301	4.80	17.00

Figure 10. Sedimentation coefficient ($s_{20,w}$) of CaV 196 coat protein subunits. Protein prepared by 67 per cent acetic acid treatment, suspended in 0.1 M phosphate buffer pH 7.72. Speed of centrifugation 60,000 rpm at 4°C. Line extrapolated to infinite dilution by least square method.



The possible implications of the additional bands in A-protein of CaV 196 are discussed later.

Partial Specific Volume and Density of the Virus

The partial specific volume (\bar{v}) of highly purified CaV 196 was determined using digital densitometer. For density, readings were taken until a constant value was obtained. A linear relationship was obtained when density was plotted against concentration using the least square method (Figure 11). The dry weights of all the samples were obtained by heating under vacuum at temperatures between 98° and 100°C until a constant weight was obtained. After the concentrations and densities were measured, the partial specific volume values were calculated using the formula described earlier (Table VIII). No attempt was made to determine the partial specific volume of coat protein of ZyV 58 isolate.

Buoyant Density

An attempt was made to compare the buoyant density of CaV 196 with the common strain of TMV by equilibrium centrifugation in CsCl. Both virus preparations reached equilibrium in one narrow opalescent band indicating that preparations were homogenous with respect to density. CaV 196 and TMV reached equilibrium at 32.50 and 32.00 mm, respectively, from the miniscus of the tube. There was a difference of only 0.5 mm in distance of the two virus bands which could be attributed to measurement error. There was no significant difference in buoyant density of CaV 196 and TMV (Appendix, Plate III).

Figure 11. Density measure of purified CaV 196. Purified by 5 per cent PEG precipitation and two sucrose density gradient centrifugations and dialyzed against dionized distilled water and KCL₂ (0.1 M). The concentration of the solutions and dialyzate determined by weighing 3 ml portions into 10 ml tared volumetric flasks, lyophylizing and drying them in vacuo at 98°-100°C to a constant weight. A straight line was drawn using least square method. Experimental temperature 40°C.

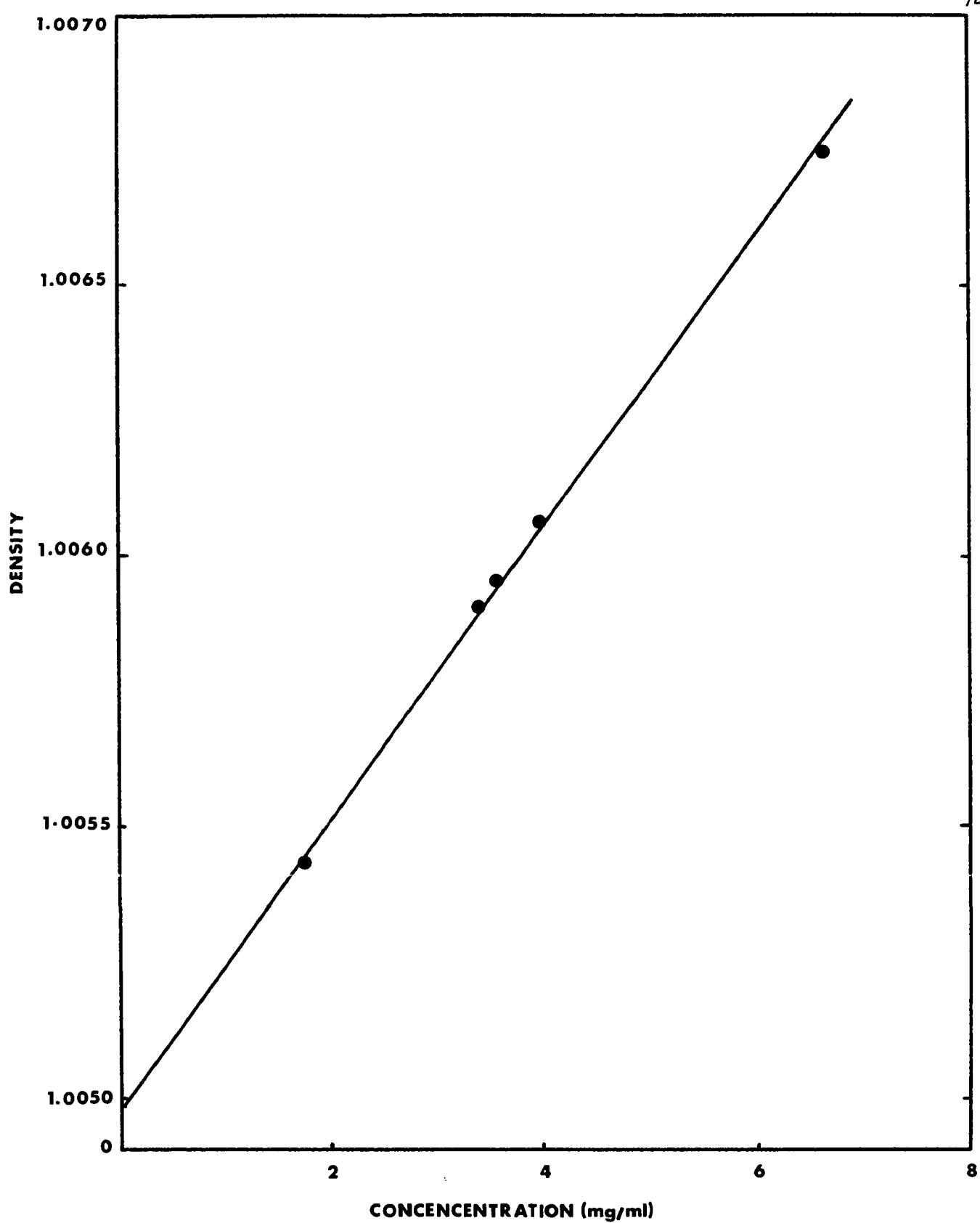


TABLE VIII

DENSITY AND PARTIAL SPECIFIC VOLUME MEASUREMENT
OF PURIFIED CaV 196

S. No.	Concentration in mg/ml	in g/ml	(ml/g) at 4°C
1	Buffer	1.00498	-
2	6.59	1.00674	0.730
3	3.98	1.00606	0.725
4	3.53	1.00595	0.721
5	1.69	1.00543	0.730

Electron Microscopy

Electron microscopy of the purified preparations of the virus and protein was done. For particle size determination of the viruses, crude preparations from infected leaves were used. Negative staining and shadow casting techniques were generally employed, but occasionally positive staining was also employed. For shadow casting, particles on grids were shadowed with a palladium at an angle of 30°-40°.

Morphology of CaV 196 Virus

Virus particles were found in purified and crude preparations. They were elongated, rigid rods. Sometimes slightly bent particles were also observed. This might possibly be due to end-to-end aggregation of two particles. PTA had penetrated in the central core of the particles. No significant difference was found in the particle size between common strain of TMV and CaV 196. The normal length was determined to be 302 ± 5 nm. The results of the particle size measurements are given in Table 9 and Figure 12). The particles of CaV 196 were compared with the particles of the common strain of TMV, using grids prepared from a mixture of both. The purified preparation showed aggregation of particles (Appendix, Plate II).

Morphology of ZyV 58

As mentioned earlier, the electron microscopy of ZyV 58 was done using purified as well as crude sap preparations. Negative staining and

TABLE IX

SIZE DISTRIBUTION OF CaV 196 PARTICLES IN CRUDE INFECTED SAP

Number of Particles	Average Length of Particle (nm)
60	281.83
60	288.10
230	294.36
330	300.62
340	306.88
100	313.15

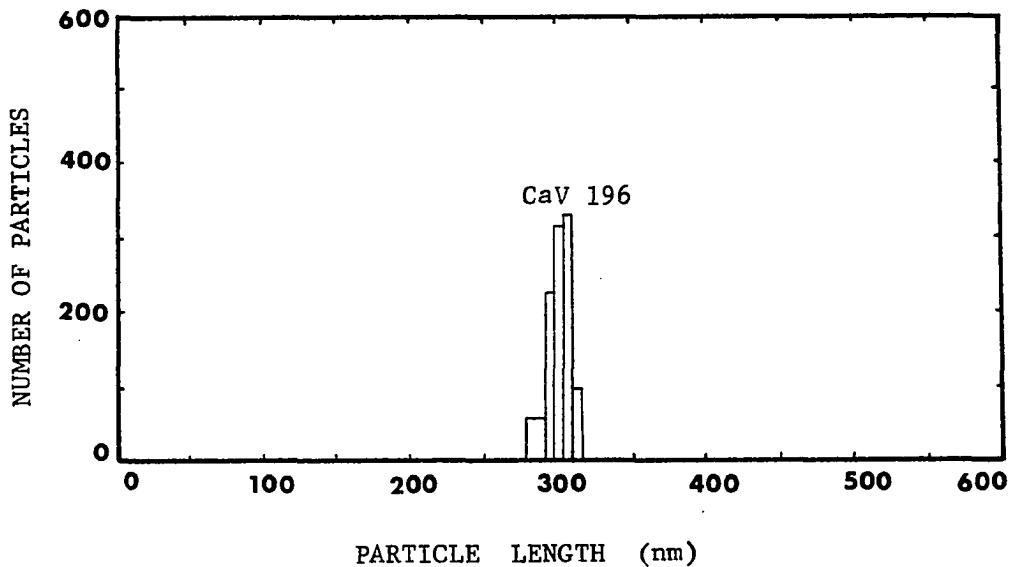


Figure 12. Particle measurement and size determination of CaV 196. The electron microscope grids were prepared from crude sap of infected leaves in 2 per cent phosphotungstic acid pH 7.

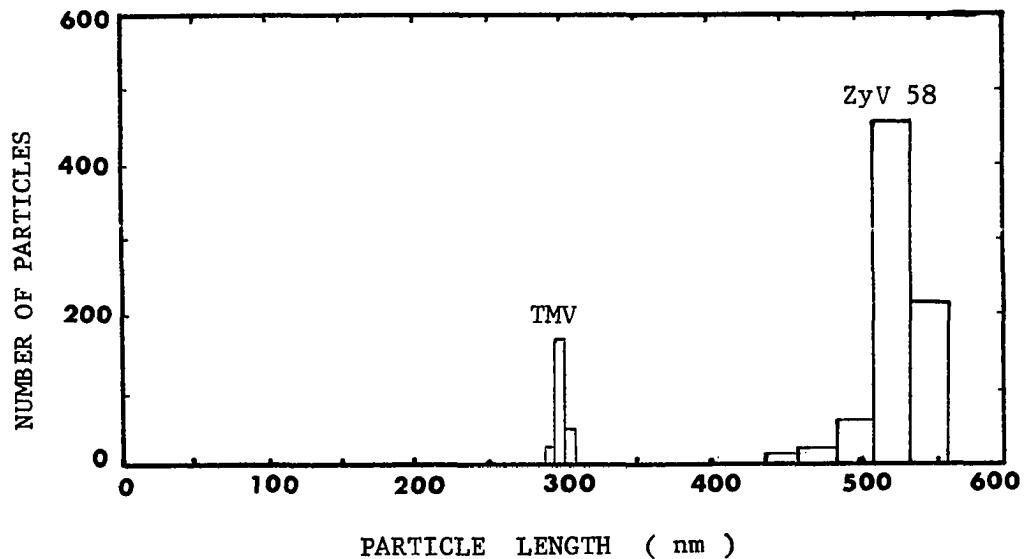


Figure 13. Particle measurement and size determination of ZyV 58. The electron microscope grids were prepared from crude sap of infected leaves in 2 per cent PTA pH 7. Particles of common TMV were used as standard.

shadow casting were both employed. Particles were shadowed at an angle of 45°. The particles appeared to be elongated, slightly flexible rods, with 519 ± 10 nm normal length (Appendix, Plate VIII). Due to flexibility, the particles were heavily aggregated in the purified preparation depending partially upon types of buffer used. Different types of buffer, but of the same molarity and pH, were used to overcome the aggregation problem. Although no significant results were achieved, particles were least aggregated in .05 M citrate buffer pH 7. Phosphate buffer, acetate buffer, and distilled water showed heavily aggregated particles. The results of the particle size measurements are given in Table X and Figure 13. The size determinations for both of the isolates were done by Dr. D. Lesemann at the Virus Research Institute, Braunschweig, West Germany. In an earlier measurement (Giri and Chessin, 1972) a normal length of 464 nm was determined. The possible reasons for the discrepancy in two measurements are discussed later.

Reconstitution of Active CaV 196

From Nucleic Acid and Protein Components

Reconstitution of TMV protein and RNA into virus particles possessing full infectivity was reported by Fraenkel-Conrat and Williams (1955). The goal of the reconstitution study at present was to see whether this non-infective nucleic acid prepared by acetic acid treatment was still capable of reassociating with protein subunits and restoring virus infectivity.

TABLE X

SIZE DISTRIBUTION OF ZyV 58 PARTICLES
IN CRUDE INFECTED SAP

Number of particles	Particle size (nm)
50	435-455
120	460-485
120	485-510
460	510-535
220	535-560

For the reconstitution of active CaV 196, 1 ml of approximately 0.1 per cent protein solution was mixed with 0.1 ml of 0.1 per cent RNA solution. 0.01 ml of two different buffers were used in two different sets of tubes. The mixture with phosphate buffer 0.1 M, pH 7 showed opalescence a few minutes after the addition, but a mixture with 3 M acetate buffer pH 6 showed opalescence 6-12 hrs. after incubation at 30°C.

Pelleted and clarified reconstituted virus preparations were inoculated on half leaves of N. glutinosa and their infectivity was compared with native CaV 196, protein, and nucleic acid preparations inoculated on N. glutinosa at the same time. Native virus preparation showed a high degree of infectivity, but protein and RNA prepared by acetic acid method did not show any infectivity. These results indicated that protein solutions did not have any undegraded virus particles.

In contrast to that prepared by the acetic acid method, RNA prepared by the phenol extraction method, showed a low degree of infectivity. The reconstitution mixture of protein and RNA prepared by acetic acid treatment did not show any lesions on inoculated leaves. The results of infectivity experiments are given in Table XI.

It is evident from the Table that when protein prepared by acetic acid was mixed with RNA prepared by acetic acid, it did not produce any lesions but, when the same protein preparation was mixed and incubated with RNA prepared by phenol treatment, it produced a small number of lesions.

The reconstituted virus which produced lesions also showed a UV

TABLE XI

INFECTIVITY ASSAY OF NATIVE VIRUS,
PROTEIN, RNA, AND RECONSTITUTED VIRUS ON N. GLUTINOSA

Material Tested	Lesions/half leaf
1. Purified CaV 196	120
2. Protein: (a) Prepared by acetic acid treatment	0
(b) Prepared by alkali degradation	0
3. Nucleic acid (RNA):	
(a) Prepared by acetic acid degradation	0
(b) Prepared by phenol extraction method	22.5
4. Reconstituted virus:	
(a) Protein and RNA prepared by acetic acid treatment	0
(b) Protein prepared by alkali degradation and RNA prepared by phenol extraction method	15
(c) Protein prepared by acetic acid treatment and RNA by phenol extraction method	12.5

absorption spectrum typical of nucleoprotein with a maximum near 265 nm and minimum near 248 nm and maximum:minimum ratio 1.2 (Figure 14). The reconstitution mixture of protein and RNA by acetic acid treatment also showed a UV absorption spectrum with maximum near 260 nm and minimum at 248 nm with maximum:minimum ratio 1.09, but no infectivity, as mentioned earlier (Figure 15).

Electron microscopy of the reconstituted virus was also done. The reconstituted virus which appeared infective on N. glutinosa showed elongated rigid rods typical of native CaV 196. Some of them were just small fragments with few identical to native particles. No attempt was made to do size measurements of reconstituted virus particles (Appendix, Plate VI).

Serological Properties

An attempt was made to establish the serological relationship of the two isolates with other members of the designate groups and to determine the antigenicity of the isolates themselves. Highly purified virus preparations were injected into albino rabbits, as mentioned earlier. The titer of the antisera were determined by tube precipitin and complement fixation tests. The complement fixation tests showed higher sensitivity than did the tube precipitin tests (Ring test). The results of titer determinations are shown in Tables XII, XIII, and XIV. Considering the amount of antigen injected into the rabbit for preparation of antiserum against CaV 196, the titer of the antiserum was somewhat low. This isolate

Figure 14. UV absorption spectrum of nucleic acid (RNA) prepared by phenol extraction method, protein prepared by alkali degradation method, reconstituted CaV 196, and native CaV 196.

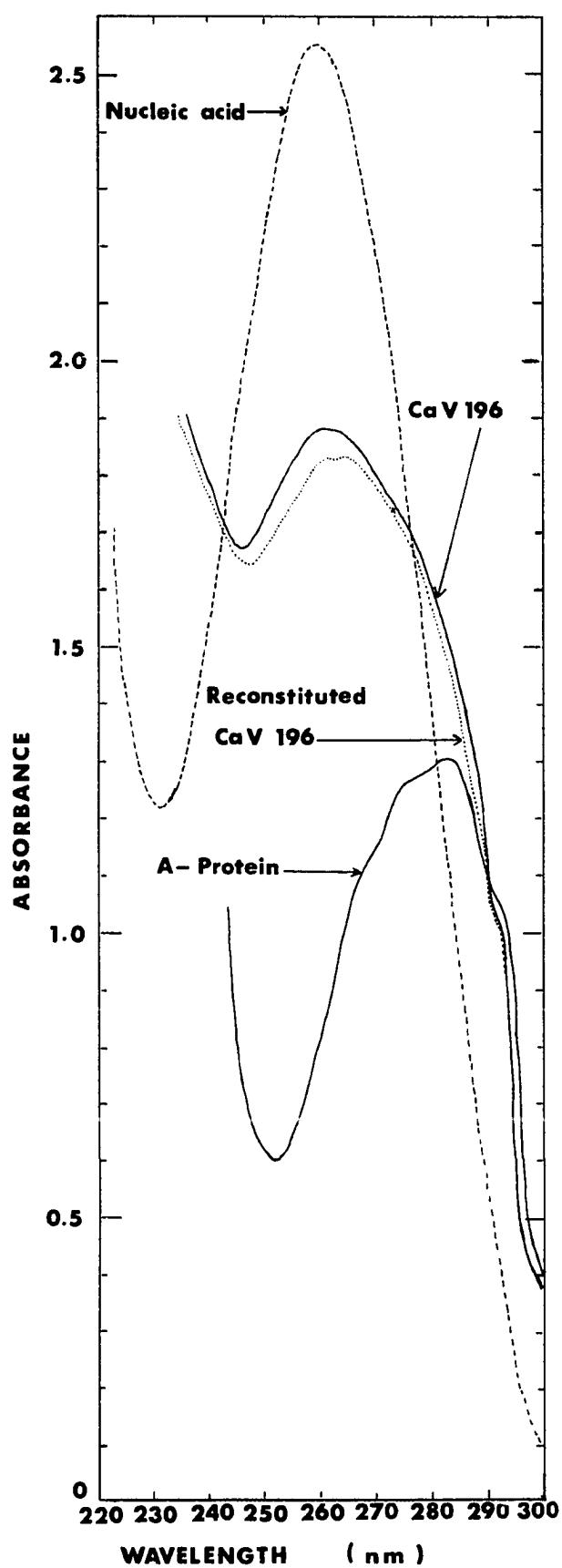


Figure 15. UV absorption spectrum of nucleic acid (RNA) and protein prepared by acetic acid method, reconstituted virus (?) and native CaV 196.

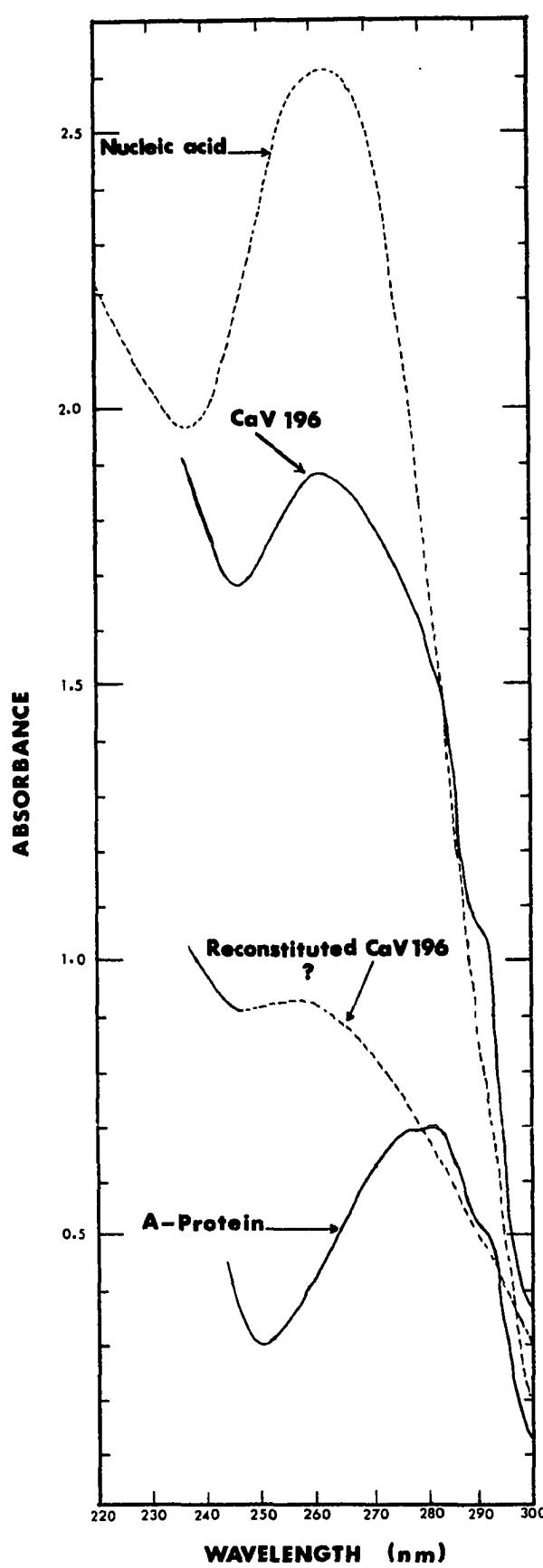


TABLE XII
TITER DETERMINATION OF ANTISERA BY COMPLEMENT FIXATION TEST
USING HOMOLOGOUS ANTIGEN (2 UNITS) SOLUTION

Antigen	Dilutions of Homologous Antisera						
	1	1:2	1:4	1:8	1:16	1:32	1:64
CaV 196	+++	+++	+++	+++	+++	+++	+++
ZyV 58	+++	+++	+++	+++	+++	+++	+++

TABLE XIII

TITER DETERMINATION OF ANTISERA BY TUBE PRECIPITIN TEST
USING HOMOLOGOUS ANTIGEN SOLUTION

Antigen	Dilutions of Homologous Antisera						
	1	1:2	1:4	1:8	1:16	1:32	1:64
CaV 196	++++	++++	++++	++++	++++	++++	+++
ZyV 58	++++	++++	++++	++++	+++	++	+

TABLE XIV

TITER OF THE ANTISERA DETERMINED BY TUBE PRECIPITIN TEST
AND COMPLEMENT FIXATION TEST

Antiserum	Titer determined by tube precipitin test	Titer determined by complement fixation
CaV 196	512	1024
ZyV 58	256	512

appears to be moderately immunogenic. Isolate ZyV 58 was found less immunogenic than other members of the PVX group.

CaV 196

The Ouchterlony double diffusion plate method was employed using high pH ammonia agar. The isolate was found to be related to the common strain of TMV. The antigen of CaV 196 was reacted with the antiserum of TMV and vice versa. The precipitin lines obtained with TMV antiserum or antigen were weaker than those obtained in homologous reactions (Appendix, Plate IV). No attempt was made to determine its relationship with other strains of TMV. No relationship was found between CaV 196 and ZyV 58. Antiserum against CaV 196 absorbed with TMV antigen still reacted weakly with homologous antigen.

ZyV 58

Antisera against seven members of the group, namely cactus virus X (CaVX); potato virus X (PVX); Casper's zygocactus virus (ZyV); white clover mosaic virus (WCMV); clover yellow mosaic virus (CYMV); Narcissus mosaic virus (NMV); and potato aucuba mosaic virus (PMV) were used in double diffusion tests. Only CaVX and PVX antisera gave positive reactions with the antigen ZyV 58. This indicates that ZyV 58 is related to cactus virus X and potato virus X. The antigen ZyV 58 failed several times to diffuse through the agar gel in several double diffusion plates. The micro-double diffusion on microscope slide and tube precipitin tests were found successful. The results of the serological relationship study are shown in Table XV.

TABLE XV

SEROLOGICAL RELATIONSHIP OF ZyV 58
TO SEVEN MEMBERS OF THE POTATO VIRUS X GROUP

Antisera prepared against	Reciprocal antiserum titer		
	Homologous antigen	ZyV 58 antigen	
	a	b	
1. Potato virus X	528	64	32
2. Cactus virus X	2560	256	64
3. Casper's ZyV	128	-	-
4. Potato aucuba mosaic virus	1280	-	-
5. White clover mosaic virus	1024	256	-
6. Clover yellow mosaic virus	512	-	-
7. Narcissus mosaic virus	512	-	-

Note.--a = precipitin test; b = Ouchterlony Double Diffusion Test; - = no reaction.

Antisera 1-4 were obtained from Dr. R. Casper, Braunschweig, West Germany

Antisera 5-7 were obtained from Dr. R. Stace-Smith, Canada Department of Agriculture, Research Station, Vancouver, B. C., Canada

CHAPTER IV

DISCUSSION

Cactus Virus 196

Host Range

As mentioned earlier, the objective of the study of this isolate was to see whether it was the same virus (SOV) which was reported by Sammons and Chessin (1961) or the common TMV (*Vulgare* on U1), or some strain of TMV. It is evident from Table XVI that reaction of CaV 196 is different from all other strains on *N. sylvestris*. The U1 strain has often been described as producing chlorotic lesions on inoculated leaves followed by systemic mottling (Kunkel, 1934; Bawden and Pirie, 1936; Kado, et al., 1968), whereas in the present study the plants infected with CaV 196 showed water-soaked necrotic primary lesions on inoculated leaves followed by a systemic necrosis. This systemic necrosis was accompanied by blistering and puckering of young leaves. The eventual consequence was the complete death of whole plants. Knight (1947) has described a strain named J 14 D1 as producing necrotic primary lesions on inoculated leaves followed by systemic necrosis and death of *N. tabacum* var. Turkish. These results are similar to the symptoms produced by CaV 196 on *N. sylvestris*. The comparison in Table XVI indicates that none of the strains of TMV causes this type of effect on *N. sylvestris*. Earlier workers have very often used *N. sylvestris* and *N. tabacum* var. Turkish to distinguish the strains of TMV (Siegel and Wildman, 1954; Wang and Knight, 1967; Kado, et al., 1968; Kunkel, 1934; Knight, et al., 1962). In the present study, the major

TABLE XVI

HOST REACTION OF SELECTED STRAINS OF TMV
ON N. SYLVESTRIS AND N. TABACUM VAR. TURKISH

Strain	<u>N. sylvestris</u>	<u>N. tabacum</u>
1. <i>Vulgare</i> (common or U1)	Chlorotic primary lesions, systemic clearing of vein ¹ Typical mosaic, some distortion ²	Typical mosaic, much distortion ²
2. U2	Local lesions ³	Mild mosaic ³
3. U3	Systemic mosaic	Dark green vein banding ³
4. U4	Systemic	None
5. U5	Local lesions	Rugose leaves
6. U6	Local lesions	Yellow mosaic
7. U7	Systemic	Mild mosaic
8. U8 (Holmes Rib-Grass)	Local lesions	Necrotic spots and band on leaves ³
9. Aucuba mosaic	Local lesions ^{4,5}	Necrotic local lesions ⁴
10. <u>Lychnis</u> strain	Local lesions on inoculated leaves ²	Fine mosaic, little or no distortion ²
11. <u>Odontoglossum</u> strain (ORSV)	None ⁶	None ⁶
12. Y-TAMV	Necrotic local lesions (large) ⁷	Mild green mottling ⁷

(continued on next page)

TABLE XVI (continued)

Strain	<u>N. sylvestris</u>	<u>N. tabacum</u>
13. G-TAMV	Nectorit local lesions (small)	Mild green mottling
14. VC 60	Local lesions ⁸	Chlorosis and etching
15. VC 61	Local lesions	Green mosaic and some necrotic spots ⁸
16. AC 9	Local lesions	Necrotic mottle pronounced at leaf tips and mild green mosaic farther back on leaves ⁸
17. HD	Local lesions	Mild green mosaic
18. SJ	Local lesions	Mild green mosaic
19. Ventura	Local lesions	Pronounced green mosaic ⁸
20. Australian II	Local lesions	Mild green mosaic
21. Dutch I	Local lesions	Green flecking irregular green rings
22. YLGP	Local lesions	Severe chlorosis
23. PTA	Local lesions	Mild mosaic
24. PTV	Local lesions	Mild mosaic
25. SAF	Local lesions	Mild green mosaic, late necrotic spots

(continued on next page)

TABLE XVI (continued)

Strain	<u>N. sylvestris</u>	<u>N. tabacum</u>
26. K1	Local lesions	Chlorotic spots and chlorotic concentric rings
27. SOV	None ⁹	None ⁹
28. Orchid strain 01	Necrotic lesions ¹⁰	Mild mosaic ¹⁰
29. Orchid strain 02	None	None
30. Orchid strain 03	Necrotic lesions	Mild mosaic
31. Orchid strain 04	Necrotic lesions	Systemic etching
32. Orchid strain 05	Necrotic lesions	None
33. Orchid strain 06	Necrotic lesions	Yellow mosaic
34. Orchid strain 07	None	None
35. Cucumber virus 3	None ¹¹	None ¹¹
36. Cucumber virus 4	None	None
37. CaV 196 ¹²	Water-soaked necrotic local lesions, systemic necrosis, blistering, puckering and complete death of plants	Severe mosaic, systemic necrosis, blistering, distortion of leaves and shoestrong formation

(continued on next page)

TABLE XVI (continued)

Strain	<u>N. sylvestris</u>	<u>N. tabacum</u>
38. J 14 D1 ¹³	No information ¹³	Necrotic local ¹³ lesion on inocu- lated leaves fol- lowed by systemic necrosis and death of plant

¹Kunkel (1933)
²Chessin, et al. (1967)
³Siegel and Wildman (1954)
⁴Kunkel (1934)
⁵Smith (1967)
⁶Jensen and Gold (1951)
⁷Knight, et al. (1962)
⁸Wang and Knight (1967)
⁹Wetter and Paul (1967)
¹⁰Kado, et al. (1968)
¹¹Kado, et al. (1970)
¹²Present study
¹³Knight (1947)

distinction was made between the reaction of isolate CaV 196 with that of U1 strain on these two hosts.

The reaction of CaV 196 on N. tabacum var. Turkish was similar to U1 strain of TMV except that the shoestring production was very common in all environmental conditions. Shoestring production has been noticed also in the plants infected with U1 strain under specific environmental effect such as high temperature (Smith, 1957). Shoestrings were noticed generally during the early stage of infection. This result agrees with Tepfer and Chessin (1959). Shoestring formation in tobacco plants infected with CaV 196 was also preceded by distortion of leaves and blister effects on uninoculated leaves. Shoestring production in plants infected with the U1 strain of TMV has been attributed to the limited development of procambium and marginal meristems (Tepfer and Chessin, 1959). This finding indicates the anatomical changes caused by this isolate and U1 strain. Besides TMV strains among the mechanically transmitted viruses, only "datura shoestring virus" has been reported to induce shoestring-like leaf modifications in Datura metel and N. glutinosa (Giri and Agrawal, 1971). Besides U1 strain, the severe distortion effect was described in tobacco plants infected with cow pea virus (Lister and Thresh, 1955) and Lychnis strain (Chessin, et al., 1967), both a strain of TMV.

As indicated in Table XVI, U1, U3, U4, and U7 strains of TMV have been reported to induce systemic mottling on N. sylvestris (Siegel and Wildman, 1954) and several other strains such as tomato, aucuba; U2, U5, U8, and orchid strains have been reported to produce only local lesions on

inoculated leaves of N. sylvestris (Knight, et al., 1962; Wang and Knight, 1967; Kado, et al., 1968). In the present study the production of systemic necrosis and complete death of plants preceded by large water-soaked necrotic primary lesions on N. sylvestris, suggests that this isolate is different from other strains, but it may be similar to J 14 D1 which produces similar symptoms on Turkish tobacco.

Sammons' *Opuntia* virus (Sammons and Chessin, 1961), the only TMV-like virus reported so far from cactus, has been found to infect plants of only the family Chenopodiaceae outside of its native host Opuntia sp. (Wetter and Paul, 1967). SOV has been found to show chlorotic, concentric rings on infected pad of Opuntia sp. (Milbrath and Nelson, 1973). The cactus plant from which the isolate CaV 196 was isolated did not show any marked symptoms. SOV did not infect N. tabacum and N. glutinosa (Brandes and Chessin, 1965), whereas CaV 196 has successfully been found to infect these two host plants. This excludes the possibility of its being SOV or identical to SOV.

Physico-Chemical Properties of Virus and Viral Components

Physical Properties in Plant Sap. The TMV and its strains have long been known to possess high stability to high temperature and to aging in vitro. The thermal inactivation point of CaV 196, when assayed on C. quinoa and N. glutinosa, was found to fall between 85° and 90°C at 10 min. The relationship between dilution and infectivity was rarely a linear one, but the range over which the decrease in the number of les-

ions was most nearly proportional to the dilution was from 10^{-3} to 10^{-8} .

The 1:10 dilution of infected sap was found still infective after more than 15 days when room temperature was 23°-27°C. The physical properties of CaV 196 in plant sap have been compared with those of U1 described by Bawden and Pirie (1936), in Table XVII.

Some variations are expected as a consequence of various circumstances, even though conditions of the tests are made as constant as possible. According to McKinney (1927), higher dilutions of virus may lower the thermal death point several degrees. Johnson and Grant (1932) determined the thermal inactivation point of common TMV in undiluted extracts from various Solanaceous plants in which virus occurred systemically. They concluded that in the species tested the thermal inactivation point for TMV was just below 90°C, but they also concluded that certain host species used in the experiment might vary it as much as 5°C.

The Components of CaV 196. After sucrose density gradient centrifugation, often two zones were observed (Figure 2). Infectivity was always higher in the bottom zone. Top zone also showed infectivity but of low order. Sometimes this top zone consisted of three diffused bands. An attempt to separate these bands on CsCl gradient failed. When the top zone was studied in an analytical ultracentrifuge, three sedimenting components with $s_{20,w}$ of 86.8, 123.8, and 151.8S were observed. It is possible that these components could be host ribosomes, end-to-end aggregated particles, and some fragmented particles of CaV 196.

TABLE XVII

A COMPARISON OF PHYSICAL PROPERTIES
OF CaV 196 AND U1 STRAIN IN PLANT SAP

Properties	CaV 196	Common TMV
1. Thermal inactivation point at 10 min.	85°-90°C	90°-95°C*
2. Dilution end point	10^{-8} - 10^{-9}	10^{-8} - 10^{-11} *
3. Resistance to aging	More than 15 days	Several months

Spectral Studies

Purified Virus.--Viruses being nucleoprotein have characteristic absorption spectra. The UV maximum and minimum absorption spectrum of most of the purified TMV strains varies between 260-270 and 244-250 nm, respectively, depending upon the purity of preparations. The spectral properties have been used as one of the taxonomic criteria to differentiate the strains of TMV (Wetter and Paul, 1967). In Table XVIII the spectral properties of CaV 196 have been compared with selected strains of TMV.

CaV 196 showed $E^{1\%}$ of 31.0 which is in agreement with the values reported₂₆₀ for the strains of TMV which vary from 27-35 (ISCOTABLES). It is evident from the Table that CaV 196 has greater similarity to U1 strain of TMV than SOV and ORSV.

Protein and Nucleic Acid.--No difference was found in the spectral properties of protein prepared by acetic acid treatment and alkali degradation. The results agree with those of Fraenkel-Conrat (1957), and Schramm, et al. (1955). The spectral values have been compared with U1 strain in Table XIX. There was a marked difference in the UV spectrum of nucleic acid prepared by the acetic acid treatment and phenol extraction methods. The RNA (phenol) preparation showed a typical spectrum of pure nucleic acid whereas a little shift toward the protein side in the spectrum of RNA (acetic acid) was observed. This shift in the spectrum could be due to the presence of some protein. When RNA (acetic acid) preparation was observed in an electron microscope, a trace of A-protein was found.

TABLE XVIII

A COMPARISON OF SPECTRAL PROPERTIES
OF PURIFIED VIRUS (CaV 196) AND SELECTED STRAINS OF TMV

Virus	E280/ E260	Emax/ Emin	Maximum at nm	Minimum at nm	Source of information
SOV	0.95	1.33	269	247	Wetter and Paul (1967)
ORSV	1.28	1.56	277	250	Wetter and Paul (1967)
TMV	0.86	1.27	266	247	Wetter and Paul (1967)
CaV 196	0.86	1.13	262	246	Present study

TABLE XIX

UV ABSORPTION SPECTRUM OF PROTEIN AND RNA
OF CaV 196 AND COMMON TMV

Component	E _{max} /E _{min}	Maximum at nm	Minimum at nm	Source of Information
TMV:				
Protein	2.4	282	250	Fraenkel-Conrat (1957)
RNA	3.0	260	230	Fraenkel-Conrat (1957)
CaV 196:				
Protein	2.30	282	250	Present study
RNA ^a	2.12	260	230	Present study
RNA ^b	1.40	265	235	Present study

^a = prepared by phenol extraction method^b = prepared by acetic acid treatment

These results do not agree with Fraenkel-Conrat (1957). His preparation showed infectivity of low order and was equally pure to previously reported infective nucleic acid preparation (Fraenkel-Conrat and Singer, 1957) as indicated by phosphorous content and UV studies. The RNA (acetic acid) preparation in the present study was not infectious on N. glutinosa, but RNA (phenol) preparation showed infectivity of low order. No attempt was made to do chemical analysis of the preparation.

Susceptibility of CaV 196 and Its Components to Acetic Acid.

Fraenkel-Conrat (1957) used the acetic acid degradation method to prepare protein from U1 strain exposing the purified virus to cold 67 per cent acetic acid for 15 min. He also mentioned that for Holmes Rib-Grass strain a longer time (about 60 min.) is required. This indicates the different susceptibility of strains of TMV. In the present study, an effort to prepare protein by short exposure to cold acetic acid failed. A successful preparation of A-protein was obtained exposing purified virus (10 mg/ml) to cold acetic acid for 40-50 min. and for 2 hrs. at lower concentration (7 mg/ml). Fraenkel-Conrat, at the same time, reported that the RNA preparation obtained from acetic acid treatment was infective, whereas in the present study no infectivity was observed from a similar preparation.

This isolate has therefore exhibited two characteristics: (1) longer time required for complete degradation than that of U1, and (2) increased susceptibility of nucleic acid to acetic acid treatment resulting in an eventual loss of infectivity. Acetic acid is believed to

block ionization of the carboxyl groups of proteins and displaces any hydrogen, eventually dissociating protein from the RNA. At the same time it precipitates and degrades the RNA while keeping the protein in solution (Tsugita and Hirashima, 1972). In light of this theory, a difference in protein at tertiary and quaternary structure levels can be predicted.

Reconstitution of Active Virus from Protein and Nucleic Acid of CaV 196.

The mixture of A-protein and RNA (acetic acid treated) after incubation at 30°C showed UV absorption spectrum similar to nucleoprotein (Figure 15), but it did not show any infectivity when assayed on N. glutinosa. When it was examined in the electron microscope, very few particles smaller than a normal particle were observed. Whether they were residual particles or reconstituted is still uncertain. When an infective RNA preparation, prepared by the phenol extraction method, was used as a second component, active virus particles were reconstituted which showed infectivity of low order. Particles similar to native particles were observed when such preparations were examined in the electron microscope. The latter results agree with the earlier work of Fraenkel-Conrat and Singer (1957) and Fraenkel-Conrat and Williams (1955). It has already been established by several workers (Fraenkel-Conrat and Singer, 1957; Holoubek, 1962; Kado and Knight, 1970), that only RNA carries the genetic information for infection. It is possible that the RNA of this isolate is more sensitive to longer exposure of acetic acid than that of common TMVs. Because of its increased sensitivity, it loses its infectivity plus

efficiency to reconstitute an active virus particle. Homologous reconstitution does not have taxonomic significance as such, but it indicates the differential susceptibility of TMV strains to acetic acid treatment.

Crystallization of CaV 196 With Polyethylene Glycol. PEG has very often been used to purify plant and animal viruses. So far there is no published evidence about the chemical implication of PEG upon the virus. In the present study, when virus was precipitated by PEG and suspended in 0.05 M phosphate buffer pH 7.4-8, long, slender needles were observed when examined in the light microscope at pH 5. These needles were slightly longer than the crystals obtained by Stanley (1935) after ammonium sulfate precipitation of TMV. When the pH of the solution was brought up to pH 7.5 the crystals disappeared and no needles were observed in the EM microscope.

Analytical Ultracentrifugation Studies--The Sedimentation Coefficients.

Intact Rods.--When $s_{20,w}$ values were plotted against concentration and extrapolated to infinite dilution, a concentration dependency of the sedimentation coefficient was observed. It may appear from Figure 9 that the sedimentation coefficient decreases markedly as the concentration is increased. This result agrees with the results and theories propounded by Lauffer (1944), Schachman (1959), Hill (1969), and van Holde (1971). Lauffer (1944), while studying the sedimentation rate of TMV, also reported that the reciprocal of the sedimentation coefficient is

a linear function of concentration. Van Holde (1971) has described that any interaction between the sedimenting molecules will alter the sedimentation behavior. In the most usual cases, molecules are thought of as interfering with each other so as to make the frictional coefficient increase with concentration. When the solute molecules of any component have a higher frictional coefficient (f), their sedimentation coefficients are low. A highly elongated molecule like CaV 196 particle or random coil will have a larger value of f and, hence, a lower value of sedimentation coefficient (s), than a compact molecule of the same weight. The concentration dependency of solute molecules can be described by the equation:

$$s = \frac{s^0}{1 + kC} \quad [7]$$

where s = sedimentation coefficient as a function of concentration

s^0 = sedimentation constant at infinite concentration

k = frictional constant

C = concentration

The rods of intact infectious virus of all the strains of TMV have been shown to have approximately the same range of sedimentation coefficient (Paul, et al., 1965; Wetter and Paul, 1967). However, the sedimentation coefficient ($s_{20,w}$) of CaV 196 is comparable to some of the selected strains of TMV in Table XX.

In the present study two components, $s_{20,w}$, 183 and 200S were observed (Appendix, Plate II). The component with $s_{20,w}$ of 200S could

TABLE XX

PARTICLE SIZE AND $s_{20,w}$ COMPARISON
OF CaV 196 WITH SELECTED STRAINS OF TMV

Strain	Natural Host	Particle Length nm	$s_{20,w}$ (S)
U1	Tobacco	299	201 ^a 187 ^b
ORSV	Orchids	296	212 ^a
SOV	Cactus (<u>Opuntia</u>)	317	196 ^c
CP	Leguminous plants	298	No informa- tion
CaV 196	Cactus (<u>Opuntia</u>)	302	183 ^d

a = Paul, et al. (1965)

b = Lauffer (1944)

c = Wetter and Paul (1967)

d = Present study

possibly be an aggregated component. The purified virus of TMV strains have different tendencies to aggregate. Ul strain and SOV form aggregated component with $s_{20,w}$ of 240S, and OSRV strain seldom tends to aggregate; rather, they break into small pieces, mainly 119S. In the present study, CaV 196 formed aggregated component of only 200S which is smaller than the Ul and SOV (Paul, *et al.*, 1965; Wetter and Paul, 1967). This behavior points to the differences in quaternary and tertiary structures of their proteins which are responsible for the rod formation and the stability (Wittman, 1972).

Coat Protein Subunits (A-Protein).--When the $s_{20,w}$ values of the protein were plotted against the concentration and extrapolated to infinite dilution, a result opposite to that of intact virus was found, confirming the results of Ansevin and Lauffer (1959). It is evident from Figure 10 that $s_{20,w}$ increases with increasing concentration.

The increase in $s_{20,w}$ with increasing concentration is presumed to be the result of the shift in the equilibrium toward the higher aggregates as the concentration increases (Schachman, 1959; van Holde, 1971). It is also evident from Figure 10 that the two components with $s_{20,w}$ of 3.48S, corresponding to a degradation product (dimer or trimer), and 15S, corresponding to an aggregation product, were found when measurement was made at 4°C. Lauffer (1958) reported that, at a concentration of 1 mg/ml and above, TMV A-protein in 0.1 M phosphate buffer at pH 6.5 exhibits sedimentation coefficients (corrected to water) in the neighborhood of 4S when measurements are made at 5°C. Although the smallest well-defined

aggregate of TMV A-protein is the 4-4.7S trimer, it is very difficult to characterize it because of its tendency to aggregate into larger aggregates. The hydrogen ion concentration, temperature, and protein concentration play important independent roles in aggregation or polymerization of protein. Lauffer, et al. (1958) showed that protein prepared by alkali degradation method and placed in a 0.1 M phosphate buffer pH 6.5 becomes polymerized at room temperature and depolymerized in the cold. At pH 5, the TMV protein was only in a polymerized state at temperatures 8°, 23.8°, and 29.5°C and only in a depolymerized state at pH 7.7 and temperatures ranging from 5°-29°C.

Contrary to the results of Lauffer, et al. (1958), an aggregated component of $s_{20,w}$ of 15S, nearly at the same pH range (pH 7.72), has been found in the present study at 4°C. Casper (1963) predicted on theoretical grounds that the most stable aggregates which can be formed from TMV monomer ($s_{20,w}$ 1.85-2) would be the dimer 3.1-3.4S, the cyclic trimer 4.2-4.6S, the cyclic heptamer 7.6-8.2S, the two-turn disc 18-19S, the three-turn helical segment 28-30S, and higher multiples of the two-turn disc aggregates, e.g., a dimer 33-35S or trimer 44-47S.

McCarthy (1968), with a similar protein preparation, but in 0.04 M tris + glycine buffer pH 8, separated different stable A-protein aggregates of TMV electrophoretically, corresponding to 3-4S and 8-9S at 0°C, and 2,3, and 4S at 17.8°C. McCarthy was hesitant to call his 3S component a dimer. Lonchapt, et al. (1972) found two components with $s_{20,w}$ of 5 and 13S at 10 mg/ml concentration in 0.06 M triphosphate buffer pH 7.2 at 20°C.

There is no clear evidence so far for an intermediate with $s_{20,w}$ of 15-16S.

In the present study two electrophoretic components corresponding to the two sedimenting components 3.48 and 15S have been found. They are not distinctly separated from each other, and their stability after being treated with 1 per cent SDS and 1 per cent 2-mercaptoethanol before electrophoresis is questionable.

The electron microscopy of the CaV 196 A-protein preparation showed the presence of disc aggregates and much amorphous material of uniform size (Appendix, Plate VI). This result agrees with those of McCarthy (1968). The values 3.48 and 15S in the present study are closer to the value of a dimer and two-turn disc, respectively, than any other intermediate. Possibly a 15S component could be an incomplete two-turn disc. But to call 3.48S component a dimer instead of a trimer with a high degree of certainty, some more work would be desirable. It is also possible that, during the course of aggregation, an intermediate with $s_{20,w}$ of 13-15S is formed before a two-turn disc with $s_{20,w}$ of 18-20S is formed. Although protein polymerization has not been used as a taxonomic criterion, these results are different from those generally observed in TMV, and may have taxonomic significance.

Density and Partial Specific Volume (\bar{v}). Figure 11 shows that density of the purified virus is a linear function of the concentration, agreeing with the results of Ulrich, et al. (1964), and Ortega and Hill (1973) on ribonuclease and ribosomes, respectively. The apparent partial

specific volume (ϕ) was an independent function of the concentration and, since no variation of ϕ with concentration was apparent, it was concluded that this value is equal within experimental error to the partial specific volume, \bar{v} , of the virus rods (Hill, *et al.*, 1969; Kupe, 1973). Different \bar{v} values have been reported by different workers. A comparison of \bar{v} value of TMV and CaV 196 is presented in Table XXI.

The \bar{v} of CaV 196 is in agreement with the values reported by Bawden and Pirie (1936), and Lauffer (1944) for the TMV. All the strains of TMV should show the similar \bar{v} value. \bar{v} , for most viruses varies between 0.65 and 0.75. The values obtained in the present study are in the range described for TMV strains. Partial specific volume as such does not have taxonomic significance unless it is used in molecular weight and s determination. It is an intrinsic property of the molecules in the solution and adds to the physical properties of the viruses.

Isolate CaV 196 exhibited a buoyant density in CsCl equilibrium centrifugation similar to U1 strain. Mosch, *et al.* (1973) compared the buoyant density of 18 TMV isolates from tomato and found the similar buoyant density except U1 with little lower density. This property of CaV 196 brings it closer to U1 strain.

UV Inactivation. As is evident from Figure 8, CaV 196 has exhibited less resistance to UV light than that of U1 strain. But it is much less susceptible than other strains. Siegel and Wildman (1954) found that the strains of TMV differ greatly with respect to inactivation by UV light.

TABLE XXI

PARTIAL SPECIFIC VOLUMES FOR TMV COMMON STRAIN AND CaV 196

Strain		Source of Information
U1	0.73	Bawden and Pirie (1936)
U1	0.77	Stanley (1938)
U1	0.646	Eriksson-Quensel and Svedberg (1936)
U1	0.73	Lauffer (1944)
CaV 196	0.72-0.73	Present Study

Their results support the finding in the present study. On the basis of this finding, Perryman (1972) concluded that CaV 196 (then described as CaVX 196) is a different strain but closer to U1 strain.

Virus Morphology

It has often been noticed that even remotely related strains of TMV are quite similar in outer morphology of the virus particles, although differing clearly in coat protein primary structure and having very different host ranges. Thus rods of intact infectious particles of TMV strains have been shown to have nearly the same normal length of 300 nm except SOV with 317-320 nm (Brandes and Chessin, 1965; Rower and Ginoza, 1956). A normal length of 302 ± 5 nm was determined for CaV 196.

Serological Properties

As mentioned in the Results section, the isolate CaV 196 was found moderately immunogenic. It showed higher titer by the complement fixation test than that of the tube precipitin test confirming the results of Weaver, *et al.* (1952), Wright and Hardy (1961). As all other strains of TMV, CaV 196 exhibited a serological relationship to common strain of TMV. The antiserum against CaV 196 cross-absorbed with common TMV showed a very weak precipitin line with the homologous antigen. This result indicates that CaV 196 is serologically similar to common TMV but not identical to it. Each strain has a specific antigenic portion not present in any other strain. After absorption of an antiserum with a related heterologous antigen, an antibody fraction remains constantly in the serum which reacts only with

the homologous antigen (Hennig and Wittmann, 1972).

Zygocactus Virus 58

Host Reaction

This isolate was restricted in its inoculated host range to the family Chenopodiaceae. In Amaranthus caudatus it did not show any distinct symptoms but upon back inoculation to C. quinoa it produced mottling, indicating a latent presence of the virus in the former species. Brandes and Bercks (1962, 1963) isolated a slightly flexible elongated virus from Zygocactus sp. which was later found to be similar to the cactus virus X described by Amelunxen (1958). The isolate ZyV 58 is similar to the previously reported CaVX in its host reaction in C. quinoa but differs in A. caudatus and Gomphrena globosa. The present isolate (ZyV 58) does not infect G. globosa and remains in A. caudatus without producing any distinct symptom. The ordinary CaVX has been found to cause local lesion in G. globosa and A. amaranthus followed by systemic effect in latter (Bercks, 1971).

Casper's Zygocactus virus (Casper and Brandes, 1969) causes systemic infection in N. glutinosa, whereas ZyV 58 and ordinary CaVX do not cause any symptoms. A comparison of ZyV 58 with ordinary CaVX and Casper's ZyV is presented in Table XXII. It seems obvious that ZyV 58 is different from both ordinary CaVX and Casper's Zygocactus virus, but more like CaVX.

Physico-Chemical Properties of Virus

TABLE XXII

HOST REACTION COMPARISON OF ZyV 58
WITH ORDINARY CaVX AND CASPER'S ZYGOCACTUS VIRUS

Host Plant	ZyV 58	Ordinary CaVX	Casper's ZyV
<u>A. caudatus</u>	No symptoms but virus recovered upon back inoc- ulation to <u>C.</u> <u>quinoa</u>	Necrotic LL and systemic effect	Not known
<u>C. quinoa</u>	Necrotic primary LL on inoculated leaves followed by severe sys- temic mottling	Necrotic LL and systemic mottling	LL and sys- temic
<u>G. globosa</u>	None	Local lesions	None
<u>N. glutinosa</u>	None	None	Systemic in- fection

LL = local lesions

Physical Properties in Plant Sap. The thermal inactivation point of the members of the potato virus X group varies from 60°-75°C at 10 min. (Brandes and Wetter, 1959). The thermal inactivation point of ordinary CaVX has been reported to be 82°C and 10 min. (Bercks, 1971), whereas ZyV 58 has exhibited a thermal inactivation point of 75°-80°C at 10 min. The dilution end point and resistance to aging have been compared with other members of the group in Table XXIII.

It is evident from Table XXIII that ZyV 58 differs from other members of the group in its thermal inactivation temperature and resistance to aging but has a similar dilution end point. Most of the members of the group have a similar dilution end point.

Properties of the Purified Virus. It showed poor yield when purified by adsorption chromatography (Venekamp and Mosch, 1963) and PEG precipitation (Hebert, 1963). As compared to the PEG precipitation method, the conventional differential centrifugation method gave a higher yield of ZyV 58. It gave a maximum UV absorption around 260 and minimum at 244 nm, exhibiting a maximum:minimum ratio of 1.2 and 260:280 ratio 1.19-1.2, agreeing with the values reported by Knight (1962), Reichmann (1958), and Bercks (1970, 1971) for potato virus X.

Ultracentrifugation Studies. The schlieren pattern of purified ZyV 58 often showed two components with sedimentation coefficient ($s_{20,w}$) of 120 and 213S. These two components were due to aggregation of the purified preparation. The $s_{20,w}$ of nonaggregated preparation was deter-

TABLE XXIII

PHYSICAL PROPERTIES OF ZYV 58 IN PLANT SAP
COMPARED WITH SELECTED MEMBERS OF THE DESIGNATE GROUP

Properties	ZyV 58	CaVx ^a	PVx ^b	Casper's XyV ^c	White clover mosaic virus
Thermal inactivation point at 10 min.	75°-80°C	82°C	68°-74°C	72°-74°C	60°C
Dilution end point	10 ⁻⁵ -10 ⁻⁶	10 ⁻⁵	10 ⁻⁵ -10 ⁻⁶	Not known	10 ⁻⁵ -10 ⁻⁶
Aging in vitro	7 days at room temperature	More than 6 weeks at room temperature	Several weeks at 20°C	Not known	10-99 days at room temperature

a = Bercks (1971)

b = Bercks (1970)

c = Casper (1969)

mined to be 120-123S. No attempt was made to extrapolate the $s_{20,w}$ values to infinite dilution. As is evident from Table XXIV, the $s_{20,w}$ of other members of the group varies from 110-125S (Varma, et al., 1950).

Virus Morphology

Slightly flexible elongated rods were observed in negatively stained and shadowed preparation of the purified virus (Appendix, Plate VIII).

As mentioned earlier, the electron microscope grids were prepared with crude infected sap and sent to Dr. D. Lesemann of Braunschweig, West Germany, and a normal length 464 nm was originally determined (Giri and Chessin, 1972). Recently, when we sent infected leaf material of ZyV 58 for remeasurement using their own grids, virus particles from the crude sap of the recultivated stock showed a different normal length than that previously reported. According to the new measurement the normal length is 519 ± 10 nm, which is similar to potato virus X and cactus virus X described by Brandes and Wetter (1959) and Bercks (1970, 1971). The discrepancy in two size determinations was possibly due to the difference in two grids from two places, one being used for calibration and the other used for the measurement. It is also possible that virus particles mounted in the EM grids change their size over the time taken between transportation and measurement.

Serological Properties

This isolate was found less immunogenic. Considering the amount of

TABLE XXIV

SEDIMENTATION COEFFICIENTS AND NORMAL LENGTHS
OF SELECTED MEMBERS OF POTATO VIRUS X GROUP

Virus	$s_{20,w}$ (S)	Normal length nm	Source of information
PVX	124	515	Lauffer and Cartwright (1952) Brandes (1964)
PVX	118	515	Reichmann (1959)
CaVX	Not known	520	Bercks (1971)
Casper's ZyV	Not known	580	Casper (1967)
WCMV	119	480	Varma, <u>et al.</u> (1970) Brandes (1964)
WCMV	112	480	Pratt and Reichmann (1961)
ZyV 58	120-123	519	Present work

antigen injected into the rabbits, the titer of antiserum was low. The antiserum showed homologous titer of 512 by the complement fixation test and 256 by the tube precipitin test. The complement fixation test has very seldom been used in plant virus serology because it is a time consuming process. This is a very sensitive method as compared to the tube precipitin test. The results found in the present study agree with the results of Wright and Stace-Smith (1966). When its serological relationship was tested with the antisera for seven members of the potato virus X group, it was found distantly serologically related to CaVX and PVX both by the tube precipitin test and the Ouchterlony double diffusion test (Appendix, Plate VII). It also showed a positive reaction with the antiserum against white clover mosaic virus (WCMV) in the tube precipitin test, but this was not reproduced in an Ouchterlony double diffusion test. Results of serological tests with seven members of the designate group is presented in Table XV. Since its relationship with WCMV could not be reproduced in the Ouchterlony diffusion test, its relationship to WCMV remains uncertain.

A distant serological relationship between cactus virus X, potato virus X, and white clover mosaic virus has been reported by previous workers (Brandes and Bercks, 1962, 1963). No attempt was made to do cross adsorption tests.

CHAPTER V

SUMMARY AND CONCLUSION

CaV 196

The isolate CaV 196 was isolated from a wild cactus Opuntia basilaris. Its host range, physical properties in sap, physico-chemical properties, morphology and serological properties have been studied.

The symptoms caused by CaV 196 on N. sylvestris differed from those caused by all other strains of TMV, although most were like those induced by J 14 D1 on Turkish tobacco.

CaV 196 exhibited thermal inactivation point 85°-90°C at 10 min. dilution end point 10^{-8} - 10^{-9} and aging in vitro of more than two weeks at room temperature.

The purified preparation of CaV 196 showed UV absorption spectrum typical of nucleoprotein with minimum at 244-246 and maximum around 260-262 nm. The extinction coefficient $E_{260}^{1 \text{ mg/ml}} = 3.10$ was determined.

In the sedimentation velocity experiment, CaV 196 exhibited rods with $s_{20,w}$ of 183 and 200S.

Electron microscopy of crude and purified material was done, using negative staining and shadow casting methods. CaV 196 exhibited elongated rigid rods with a normal length of 302 ± 5 nm.

CaV 196 showed a partial specific volume (\bar{v}) of 0.72-.73 ml/g and a concentration dependency in its density.

A-protein was prepared by 67 per cent cold acetic acid and alkali degradation methods. In 0.1 M phosphate buffer pH 7.72 at 4°C, A-protein

showed two components with $s_{20,w}$ of 3.48 and 15S. These two components are tentatively called dimer and two-turn disc. A shadowed preparation of A-protein showed disc aggregates and amorphous materials of uniform size in an electron microscope.

Antiserum against purified CaV 196 was prepared by injecting it into rabbits. Tube precipitin and complement fixation tests were used to determine the homologous titer. Serological relationship of CaV 196 with U1 strain was studied by the Ouchterlony double diffusion test. The isolate CaV 196 was found serologically similar to U1 strain but not identical to it.

ZyV 58

The isolate ZyV 58 was isolated from Christmas cactus, Zygocactus sp. Its host range, physical properties, morphology and serological properties have been studied.

The host range of ZyV 58 differed from ordinary cactus virus X, Casper's Zygocactus virus and potato virus X.

Isolate ZyV 58 showed thermal inactivation point 75°-80°C at 10 min., dilution end point 10^{-5} - 10^{-6} , and aging in vitro of 6-7 days at room temperature.

The purified preparation of ZyV 58 showed a UV absorption spectrum of typical nucleoprotein with minimum at 244 and a broad maximum around 260 nm.

In sedimentation velocity study, purified preparation of ZyV 58

showed particles with $s_{20,w}$ of 120-123S. No attempts were made to correct the sedimentation coefficient to infinite dilution.

In electron microscope study, crude and purified preparation of ZyV 58 exhibited slightly flexible elongated rods with a normal length of 519 nm.

Antiserum against purified ZyV 58 was prepared as mentioned before. Serological relationships were studied using antisera for seven other members of the potato virus X group by Ouchterlony double diffusion and tube precipitin tests. Isolate ZyV 58 was found distantly serologically related to potato virus X and cactus virus X.

Conclusion

Although there are several areas which need further investigation and more detailed study, on the basis of the reaction of CaV 196 in N. sylvestris, its physico-chemical properties and serological reactions, it is concluded that it is a new strain of TMV from wild cactus. It is closely related to U1 and J 14 D1 more than to any other strain.

The host reactions and serological properties of isolate ZyV 58 have excluded the possibility of its being identical or even similar to Casper's XyV and ordinary cactus virus X. But its morphological properties and reaction in C. quinoa determine it to be similar to ordinary CaVX. It is concluded that it is a new member of the PVX group and similar to the cactus virus X.

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A P P E N D I X

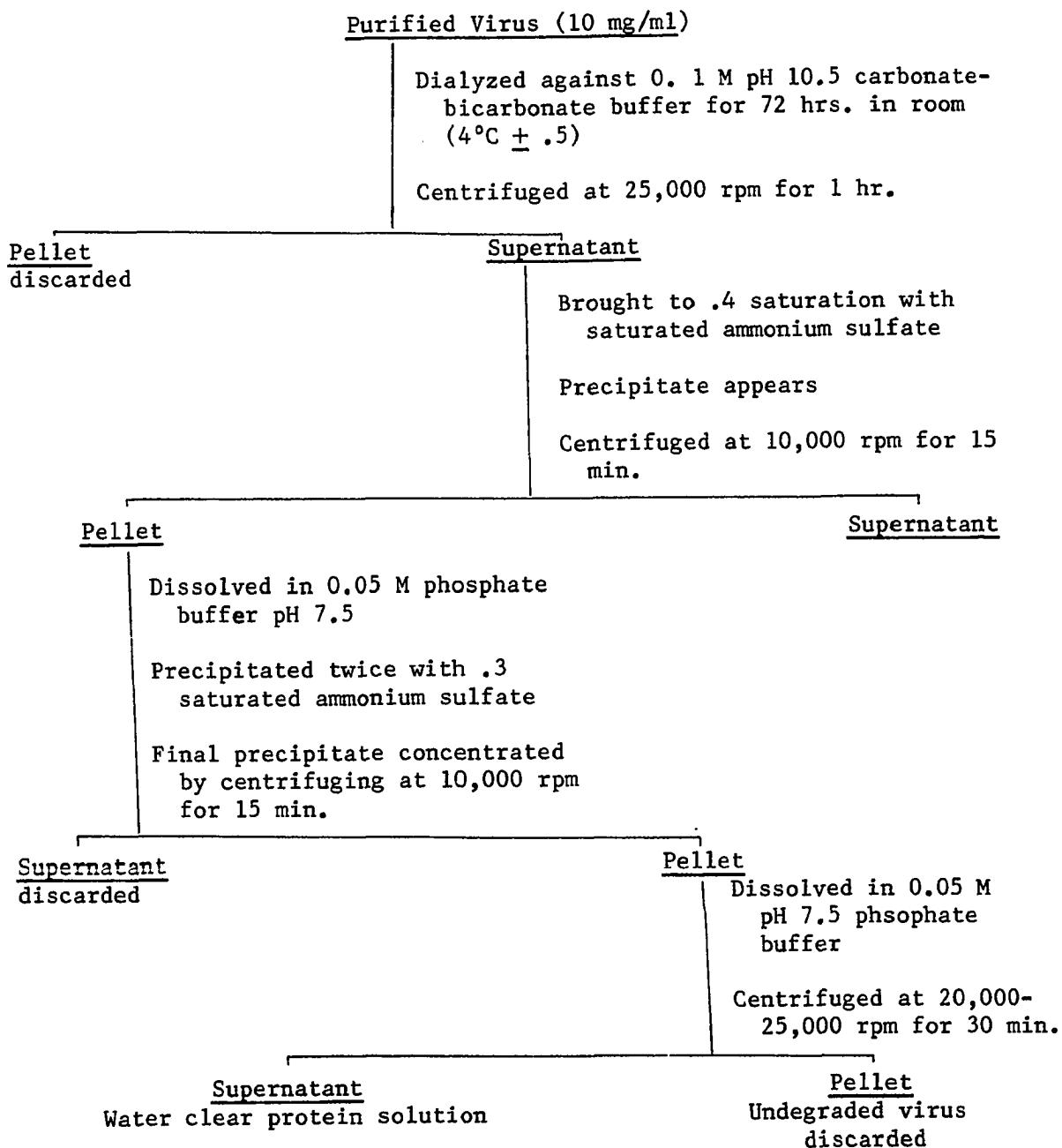
APPENDIX 1

DEGRADATION OF CaV 196 WITH ACETIC ACID

<u>5 ml Purified Virus (10 mg/ml)</u>	
	Added 2-4 volumes cold 67 per cent acetic acid
	Precipitate appears after several hours
	Centrifuged at 10,000 rpm for 10 min.
<u>Pellet #1</u>	<u>Supernatant (protein)</u>
	Dialyzed in water for 2-3 days
	Aggregation caused when protein reaches its I. P.
	Added few drops of 3 M pH 4.8 sodium acetate buffer
	Centrifuged at 40,000-50,000 rpm for 1 hr.
<u>Pellet #2</u>	<u>Supernatant discarded</u>
	Dissolved in 0.05 M pH 7.6 phosphate buffer. pH adjusted with NaOH
	Centrifuged at 25,000 rpm for 1 hr.
<u>Pellet #3 (undegraded virus) discarded</u>	<u>Supernatant Water clear protein</u>

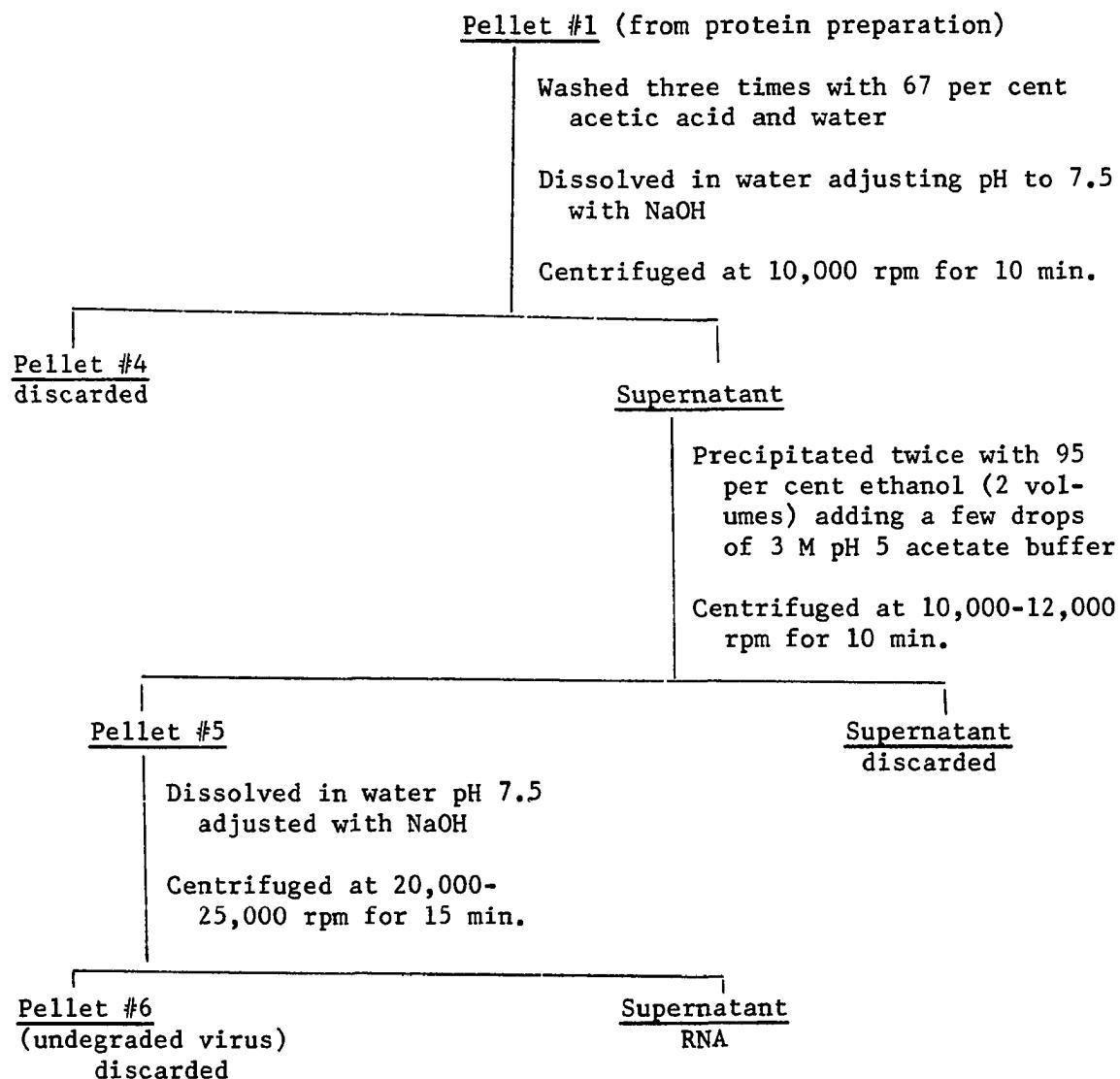
APPENDIX 2

PROTEIN PREPARATION BY ALKALI DEGRADATION OF CaV 196



APPENDIX 3

NUCLEIC ACID PREPARATION



APPENDIX 4

RNA PREPARATION FROM CaV 196 BY PHENOL EXTRACTION METHOD

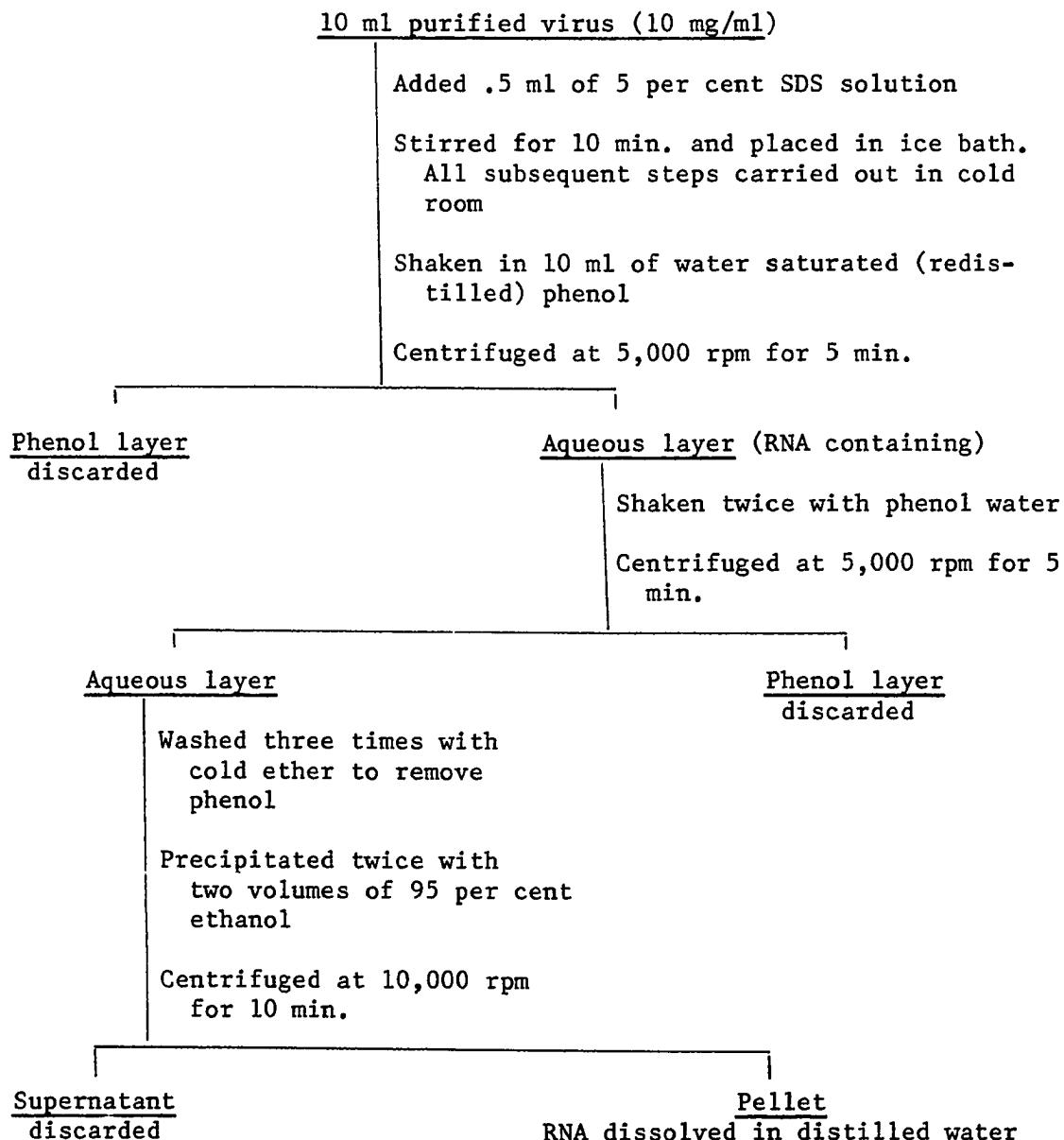


PLATE I

- Figure 1 : CaV 196 infected and healthy plants of N. sylvestris. Water-soaked necrotic primary lesions on inoculated leaf.
- Figure 2 : Systemic effect of CaV 196 on N. sylvestris. Systemic necrosis, blister formation and deformation of young leaves.
- Figure 3 : Severe systemic necrosis on N. sylvestris.
- Figure 4 : Complete death of infected N. sylvestris plants.
- Figures 5 & 6: CaV 196 infection on Turkish tobacco. Systemic necrosis and shoestring formation (5).

PLATE I

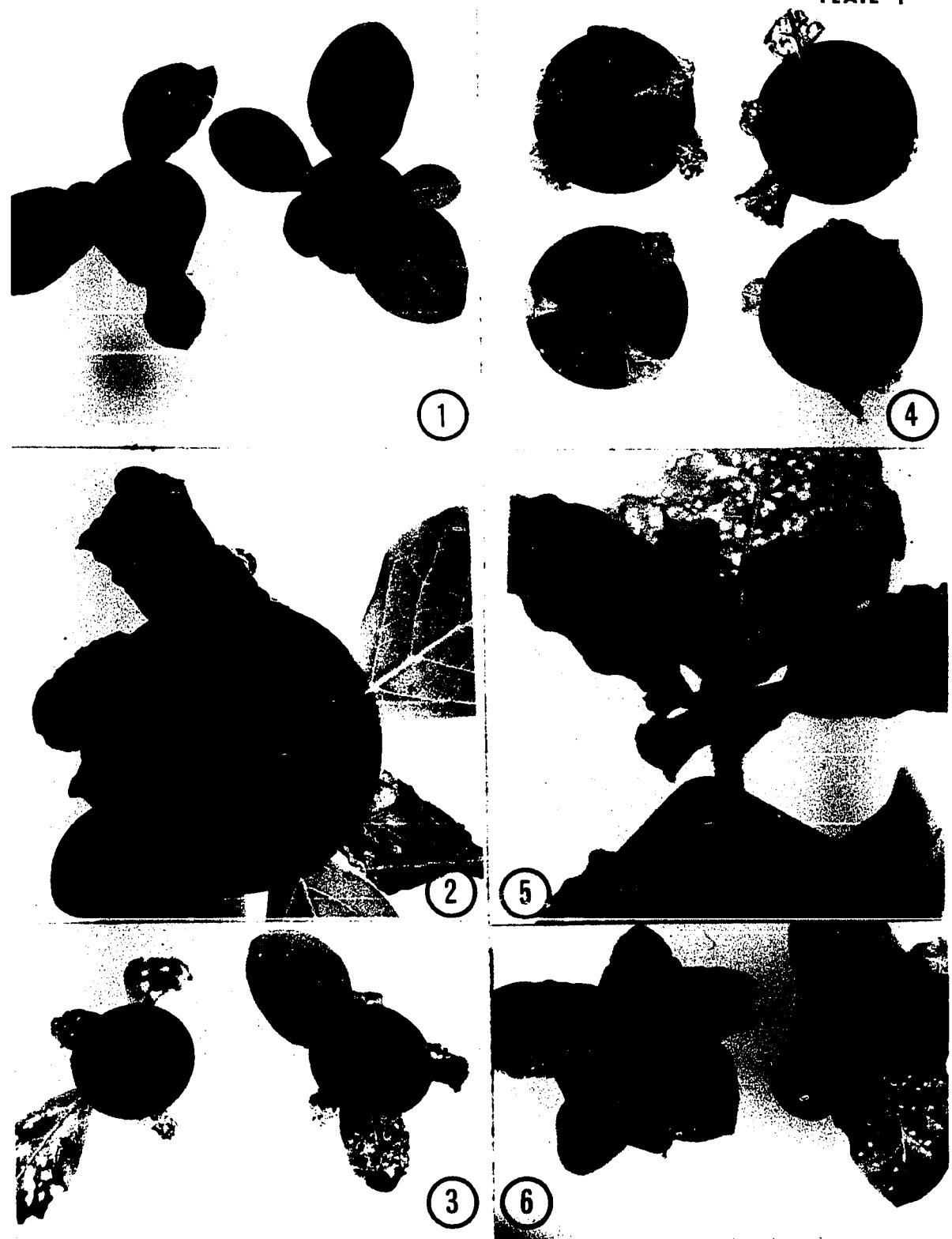


PLATE II

- Figure 1: Sucrose density gradient (10-14%) tube after centrifugation in SW 27 rotor at 27,000 rpm for 90 min. An unusual zone formation (continuation of bottom zone from the middle of the tube to the bottom). Arrows point at the top zone.
- Figure 2: Analytical ultracentrifugation schlieren pattern of top zone. Sedimentation from right to left. Pictures taken 10 and 20 min. after reaching 26,000 rpm speed.
- Figure 3: Analytical ultracentrifuge schlieren pattern of bottom zone. Sedimentation from right to left. Pictures taken 10 and 20 min. after reaching 26,000 rpm speed. Sedimentation coefficients 200 and 183S.
- Figure 4: Electron micrograph of purified CaV 196 stained in PTA, 2 per cent pH 7.2
- Figure 5: Electron microscope photograph of purified CaV 196. Shadowed with palladium.

PLATE II

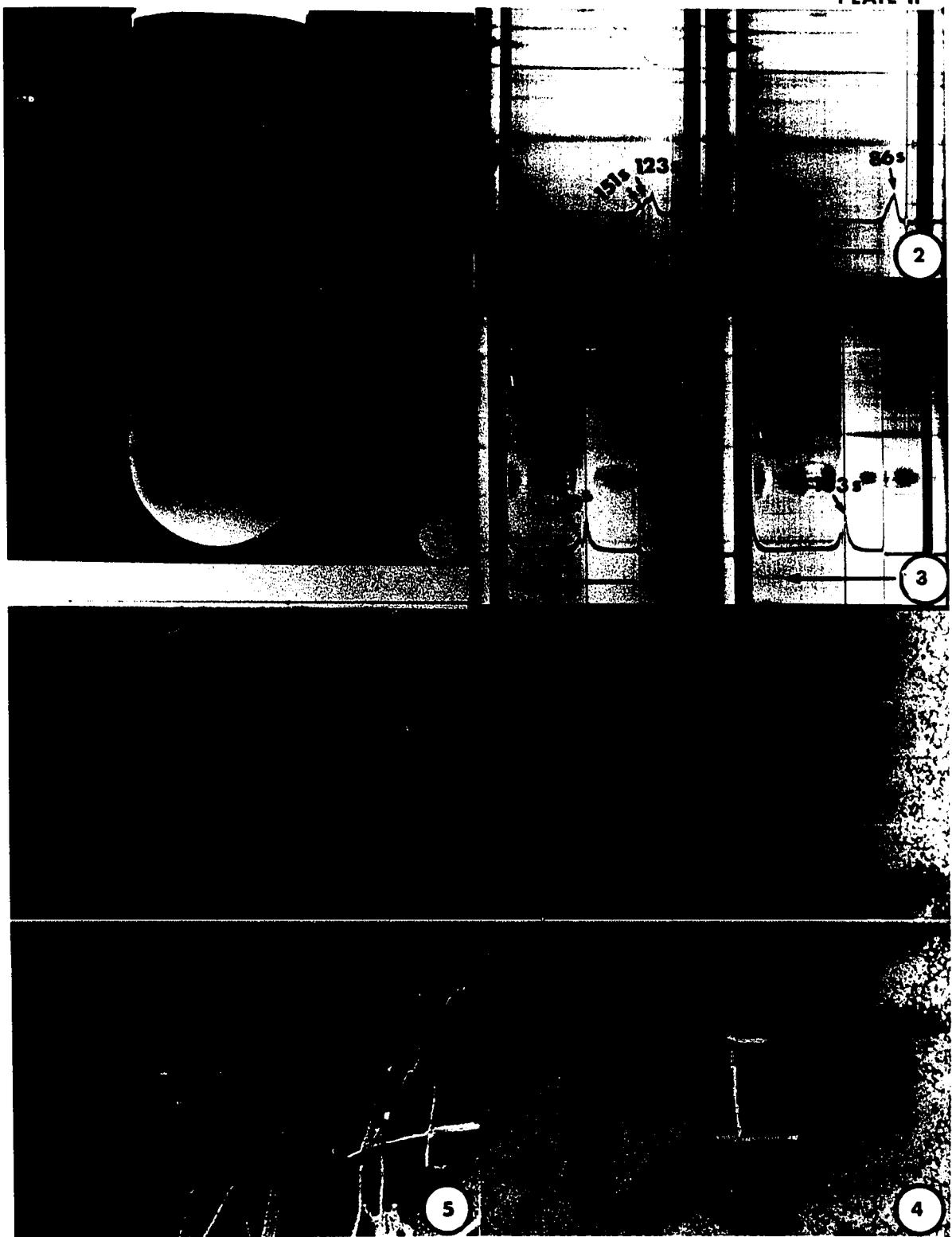
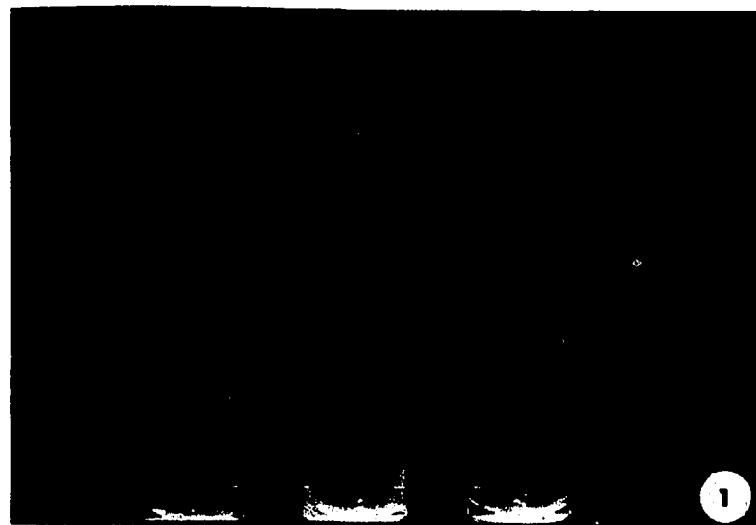


PLATE III

Figure 1: Comparison of buoyant density of CaV 196 with TMV in cesium chloride equilibrium density gradient centrifugation at 35,000 rpm for 18 hrs. in SW 39 rotor.

Figure 2: Analytical ultracentrifugation schlieren pattern of purified ZyV 58. Sedimentation right to left. Picture taken 15 min. after reaching 26,000 rpm speed.

PLATE III



1 2 3

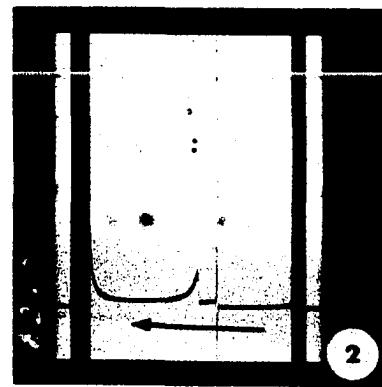


PLATE IV

Figure 1: Needles of CaV 196 after precipitation with PEG.

Figure 2: Ouchterlony double diffusion serological plate. Wells a, b and c = anti CaV 196 serum; wells d, e and f = anti TMV serum; center well = CaV 196 antigen.

Figure 3: Wells d, e and f = anti CaV 196 serum absorbed with TMV antigen; wells a, b and c = unabsorbed anti CaV 196 serum; center well = homologous antigen.

PLATE IV

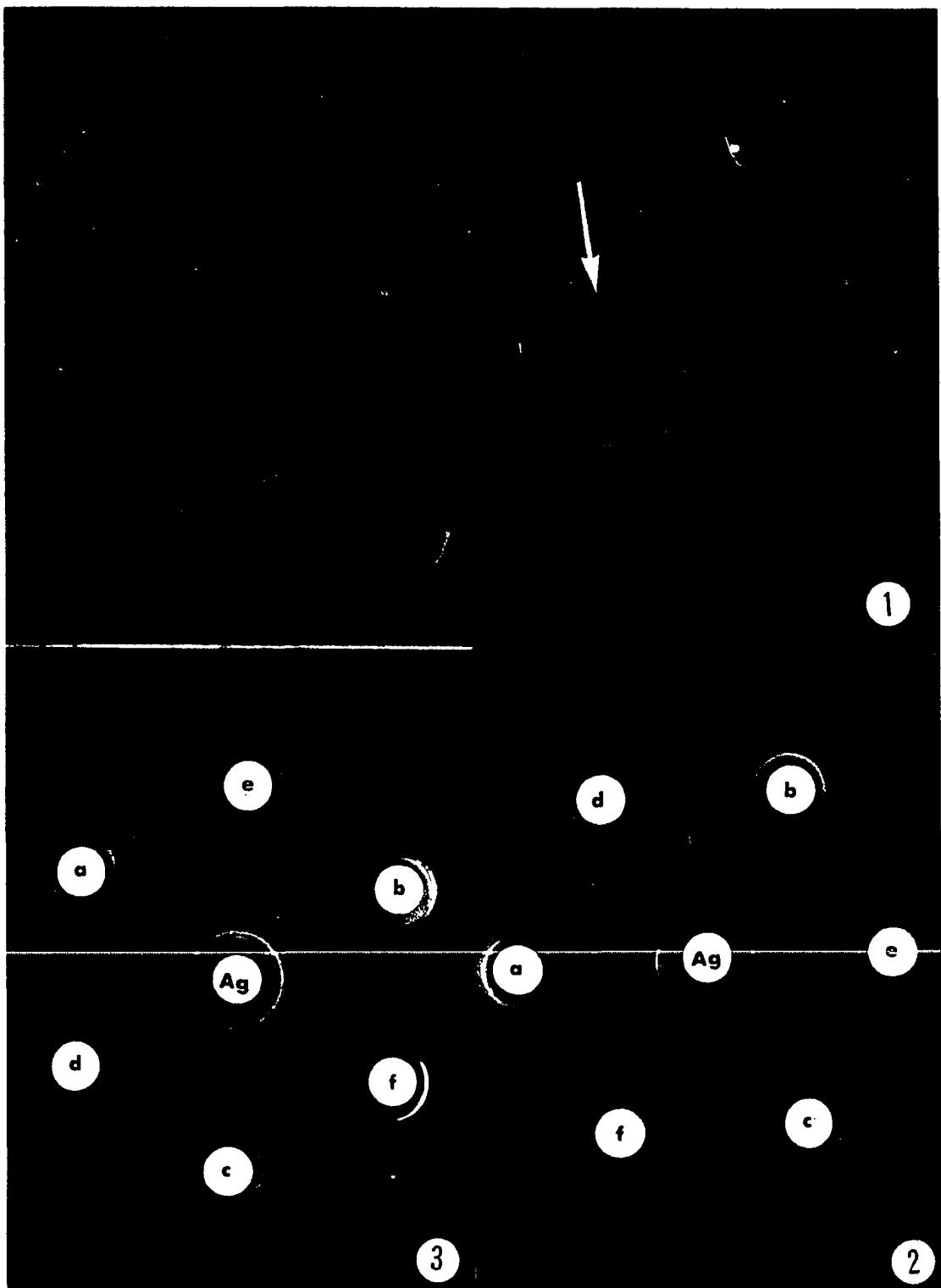


PLATE V

Figure 1: Analytical ultracentrifuge schlieren pattern of A-protein of CaV 196. Sedimentation from right to left. Pictures taken 15 and 31 min. after reaching 60,000 rpm speed.

Figure 2: Polyacrylamide gel electrophoresis gels of CaV 196 protein. Gels 1 and 2 contained A-protein and gel 3 contained protein prepared by boiling purified virus with 1 per cent SDS and 1 per cent mercaptoethanol. A-protein was also treated in a similar fashion.

PLATE V

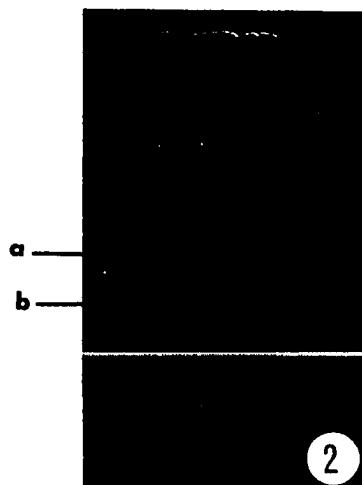
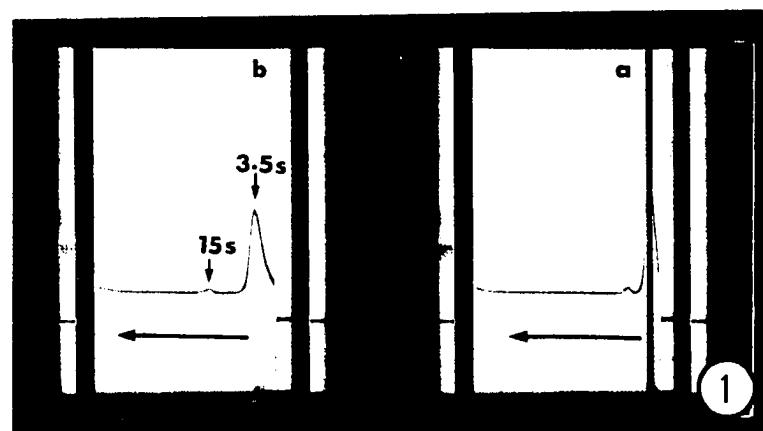


PLATE VI

Figure 1: Two-turn discs of CaV 196 A-protein.

Figure 2: Stacked two-turn discs of protein subunits.

Figure 3: Multiple aggregates of two-turn discs of protein subunits of virus.

Figure 4: Active particles of CaV 196 reconstituted from its nucleic acid and protein components.

PLATE VI

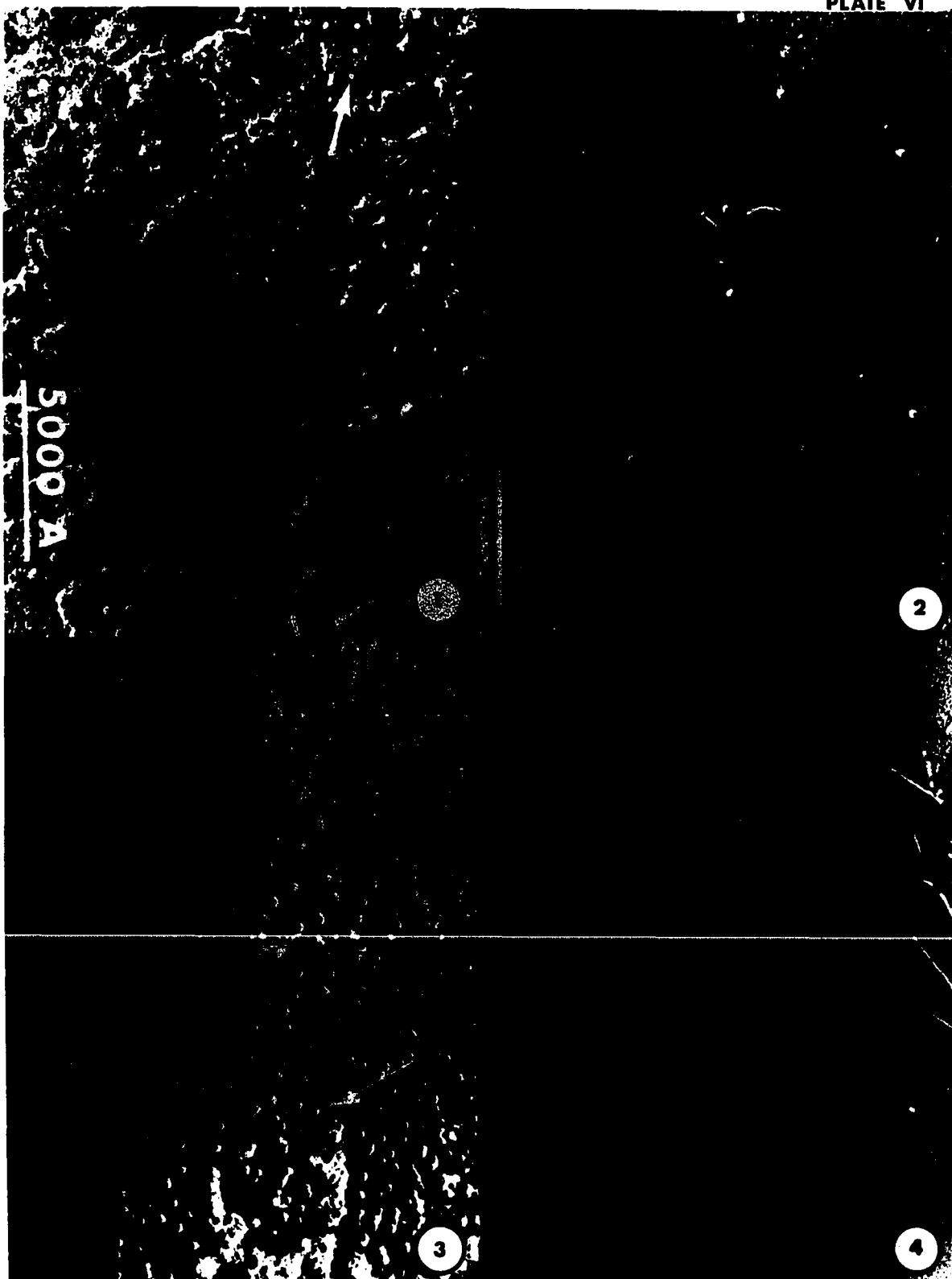


PLATE VII

- Figure 1: ZyV 58 infected and healthy leaves of C. quinoa. Systemic chlorotic local lesions.
- Figure 2: ZyV 58 infected and healthy leaves of C. album. Local lesions on inoculated leaf.
- Figure 3: ZyV 58 infected and healthy leaves of A. hastata. Mottling on leaf.
- Figure 4: Spindle-shaped inclusions in Zygocactus sp. (X 500).
- Figure 5: Ouchterlony double diffusion plate. Center well = ZyV 58 antigen; a = CaVX antiserum; b = PVX antiserum; c = CYNV antiserum; d = WCMV antiserum; e = PAMV antiserum; f = Casper's XyV antiserum. Arrow points at very weak precipitin line formed with CaVX and PVX antisera.
- Figure 6: Center well = ZyV antigen; wells a, b and c = homologous antiserum; d = NMV antiserum; e and f = normal serum.

PLATE VII

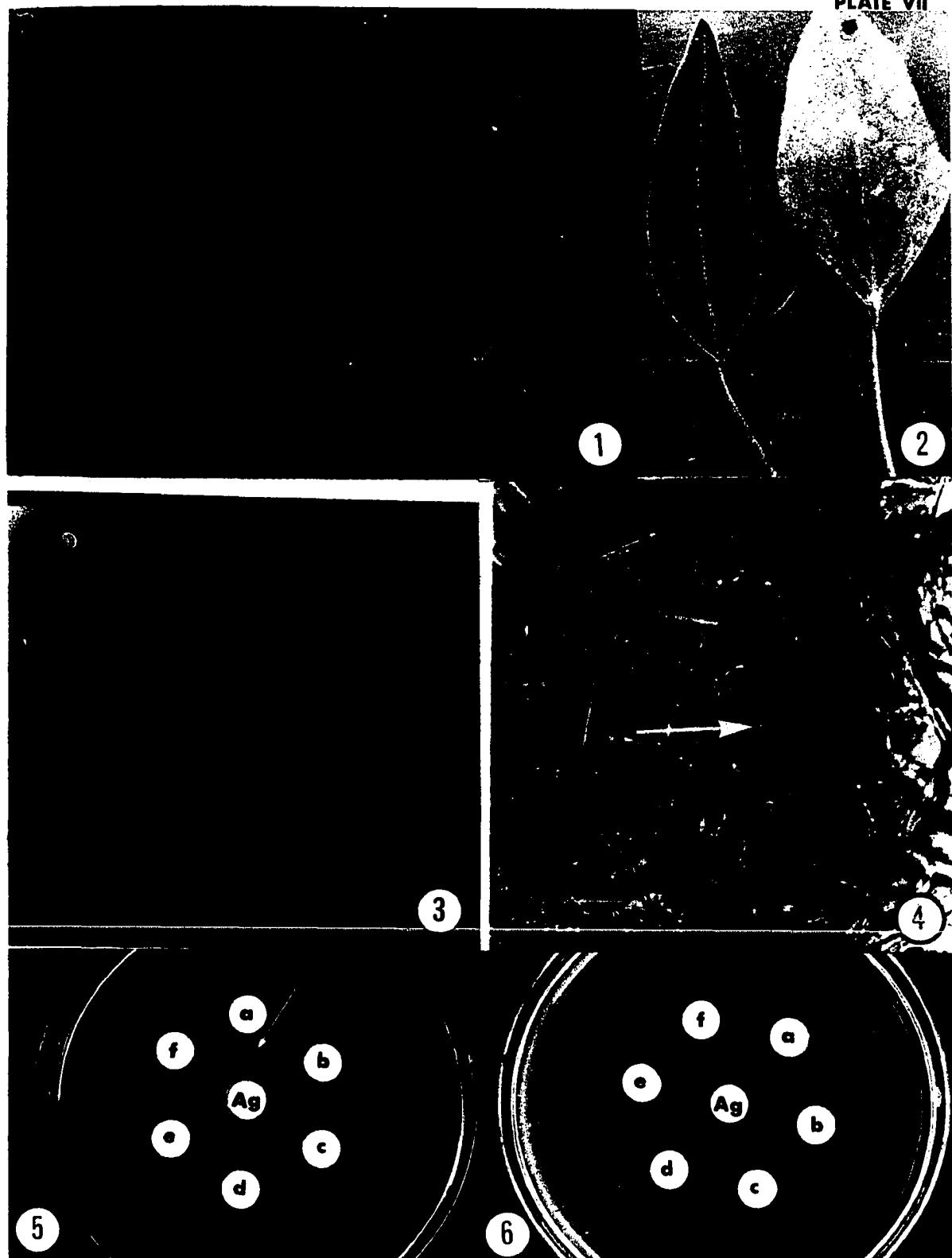


PLATE VIII

Electron micrograph of ZyW 56 showing slightly flexuous
particles of purified virus. Shadowed with palladium at 45°
angle.

PLATE VIII

