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# ETIOLOGY AND EPIDEMIOLOGY OF VIRUSES OF NATIVE CACTUS SPECIES IN ARIZONA

by

Gene McCoy Milbrath

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A Dissertation Submitted to the Faculty of the

DEPARTMENT OF PLANT PATHOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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GRADUATE COLLEGE

I hereby recommend that this dissertation prepared under my direction by Gene McCoy Milbrath

entitled ETIOLOGY AND EPIDEMIOLOGY OF VIRUSES OF NATIVE CACTUS SPECIES IN ARIZONA

be accepted as fulfilling the dissertation requirement of the degree of DOCTOR OF PHILOSOPHY

Merrill R Nelson — May 25, 1970

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| Milton Zaitlin | 5-26-70 |
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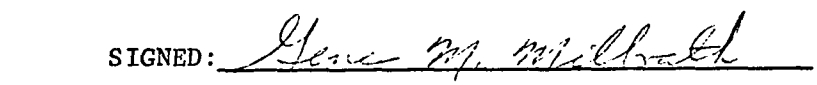
SIGNED: 

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ABSTRACT

The first virus found to infect the saguaro cactus has been isolated. This virus has been designated saguaro virus (SV) and is also the first isometric virus found to infect any of the cacti. It is 35 mμ in diameter and contains a single sedimenting component in purified preparations. The virus is widespread in saguaros. Approximately 40% of the saguaros assayed contained SV. It is unknown what long term effect SV may have on the native stands of saguaro.

Saguaro virus is easily detected in the floral parts of the saguaro, but it is difficult to detect the virus in the vegetative tissue. A convenient local lesion assay host for SV is Chenopodium amaranticolor. Another species of Chenopodium, C. capitatum, may be infected systemically with SV and eventually killed.

Saguaro virus has been characterized by a selected host range, sucrose density gradient centrifugation, analytical ultracentrifugation, and electron microscopy. It sediments as a single band in sucrose density gradient tubes and a single peak in the analytical ultracentrifuge. Calculated sedimentation coefficients $Sw,_{20}$ of 106, 107, and 112 have been determined from three individual runs. Electron micrographs of purified SV contain uniform isometric particles.

Saguaro virus was not found to be serologically related to cucumber mosaic virus, tobacco ringspot virus, rose mosaic virus, apple

mosaic virus, cherry necrotic ringspot virus, or plum line pattern virus. Further investigations are needed to establish the identity of SV.

A rod-shaped virus identified as Sammons' Opuntia virus (SOV) was found to occur in many of the Opuntia cacti. The symptoms on the cactus pads range from faint chlorotic markings to large concentric interlocking rings which sometimes cause depressions in the cactus pad. In addition, the infected cacti have paracrystalline inclusions which are visible in tissue sections in the light microscope. Another type of chlorotic ringspot, initially thought to be a viral symptom, was found to be induced by the Opuntia joint bug (Chelinidea vittiger Uhler).

Sammons' Opuntia virus was purified directly from infected pads by using either alternate high and low ultracentrifugation or polyethylene glycol precipitation. Purified virus was used for inoculation experiments, electron microscopy, analytical ultracentrifugation, and serology.

Electron micrographs of the purified material contained numerous particles with a normal length of 312 $\pm$ 6.96 mμ. A sedimentation co-efficient $Sw,_{20}$ of 183 was calculated from Schlieren patterns obtained in the analytical ultracentrifuge. Sammons' Opuntia virus was found serologically related to tobacco mosaic virus.

Native Opuntia are exposed to high temperatures (+50 C) for considerable periods of time in the desert climate. The temperature of the interior portion of the pad may range 6-8 C higher than the air

temperature. Despite these temperatures the SOV remains infective and is not inactivated. The formation of the paracrystalline inclusions may be helpful in protecting the virus.

# INTRODUCTION

Little is known about the cactus viruses in their native habitat although they have been studied extensively under cultivated conditions. This study is undertaken to determine which cactus viruses occur in the Sonoran desert and how they perform in their native habitat under the stresses encountered in a desert environment.

Cacti have long been the object of professional and amateur botanical collectors, because of unusual and varied structures and exceptionally colorful and beautiful blossoms. Such collections have been particularly popular for hundreds of years in Europe and have resulted in a number of sizeable public and private collections of cacti. Because of the intense interest in these plants, the first virus studies of cacti were primarily of these plants in Europe. As a result, the bulk of cactus virus literature has been published in German, Slavic, and Russian journals. Although unpublished observations of "virus-like" symptoms were made many years prior to 1962 in Arizona, this date roughly marks the point at which the presence of virus infection in native cactus plants was confirmed.

Cacti constitute a dominant flora in the desert biome of southern Arizona. Information on the diseases of these plants is important since they constitute an important natural resource. Factors which influence their potential for survival, such as disease, should be studied carefully. The viruses now known to be associated with

these cacti will be studied in this research. Because cacti are normally relatively long lived, an unusual opportunity is presented, that is, the chance to study the epidemiology of virus diseases in a stable population of plants in a hot, arid environment.

The review of literature which follows is extensive and exhaustive, constituting the first such review in English of papers written primarily in German. McWhorter, in his review on virus inclusions, has included some information involving cactus viruses (39), but few other reviews in English have mentioned them. This review covers the literature from 1885 to the present.

## LITERATURE REVIEW

In 1885, thirteen years before the classical work of Beijerinck (8) on tobacco mosaic virus, Molisch (53) reported finding "protein spindles of note-worthy importance" in cells of four species of Epiphyllum. These spindle-shaped inclusion bodies, which were thought for many years to be reserve food materials of the plant were subsequently shown to be graft transmissible by Mikosch in 1908 (40). It was not until 1951 (58) that definite experimental proof was presented to show that virus(es) actually are present in some cacti and that the protein spindles if not themselves composed of virus particles at least result from virus infection.

Further work of note on cactus viruses was done by Amelunxen in Germany in the late 1950's (2, 3, 4, 5) and at the same time and later by Milicic in Yugoslavia (42, 43, 44, 45, 46, 47, 48, 49, 50, 51). Cactus virus was reported in the United States in 1961 by Sammons and Chessin (59). Initially, viruses were reported only from domesticated cacti in the United States but Chessin found a virus in native Opuntia specimens from Arizona in 1965 (18).

### Symptoms

#### Inclusion Bodies (Internal)

Cactus protein spindles originally described as "reserve material" by Molisch are now known to have been the first viral

inclusion bodies described. Their discovery preceded the initial report of tobacco mosaic virus (TMV) inclusion bodies (in tobacco in 1903) by eighteen years (34). The inclusion bodies found in cacti may be amorphous, paracrystalline, crystalline, or a combination of these.

Numerous reports have been published about protein spindles in different genera and species of cacti. The reports are for the most part similar with respect to morphology and development of the protein spindles. The cacti in which the spindles occurred are tabulated in Table 1.

The most common virus inclusion bodies in cacti are the spindle-shaped bodies which are either straight or crescent shaped. The spindles can be striated in a longitudinal direction and in some cases cross striations are easily visible in the light microscope; while at other times the spindles appear homogeneous. They are usually oriented toward the longitudinal axis but slightly off the vertical axis of the cell. In addition to the protein spindles, such structures as loops, rings, figure 8's, rope braids, and whips can occur. X-bodies, which are amorphous globular material with no crystalline structure, have been described in cactus. It has also been noted that protein spindles can be included in the X-body. Weber (69) reported the presence of polyhedral crystals in Pereskiopsis pititache.

"Stachelkugeln," another form of inclusion body, have been described occurring in Opuntia monacantha f. variegata (2, 47, 76). They are refractive in polarized light and Milicic (46) believes the

Table 1. Cacti from which protein spindles have been reported.

| Cactus Host | Citation |
|---|---|
| Austrocylindropuntia cylindrica | 27 |
| A. subulata | 73 |
| Cereus peruvianus | 28 |
| C. bauplandii | 28 |
| Echinocereus procumbens | 28 |
| Echinopsis nigerrima | 28 |
| Epiphyllum altensteinii | 53 |
| E. bridgesii | 61 |
| E. hookeri | 53 |
| E. oxypetalum | 28 |
| E. russelianum | 53 |
| E. truncatum (Zygocactus) | 5, 10, 21, 53, 57, 62 |
| Opuntia brasiliensis | 20, 42 |
| O. camanchica | 27 |
| O. curassavica | 5 |
| O. engelmannii | 19, 27, 41 |
| O. filipendula | 27 |
| O. fragilis | 27 |
| O. grandis | 27 |
| O. haematocarpa | 27 |
| O. herrfeldtii | 2 |
| O. inermisDC (=O. stricta Haw) | 42, 45, 46 |
| O. lemaireana | 5 |
| O. leucotricha | 5 |
| O. lindheimeri | 5 |
| O. macrocentra | 18 |
| O. maxima | 27 |
| O. microdasys | 27 |
| O. monacantha | 5, 27, 35, 46 |
| O. monacantha f. variegata | 2, 5, 35, 46, 59, 72, 76 |
| O. nana (O. humifusa) (=O. vulgaris) | 44 |
| O. phaeacantha | 18 |
| O. rafinesquii | 27 |
| O. robusta | 27 |
| O. spirocentra | 27 |
| O. stricta | 44, 46 |
| O. subulata | 42, 73, 74 |
| O. tomentosa | 46 |
| O. vulgaris | 27 |
| Pereskia aculeata | 40, 68 |
| Pereskiopsis pititache | 2, 5, 68, 69 |
| P. spathulata | 52 |
| Rhipsalis cereuscula | 2, 73 |
| Schlumbergera bridgesii | 39, 53, 61 |

"stachelkugeln" may be similar to the sedimentary proteins (X-bodies) of Epiphyllum.

Dense inclusion bodies were found by Weber (66) in Epiphyllum and Rhipsalis. These structures were observed only in tissues fixed with $KI \cdot I_2$. It was found they were contracted protein spindles which resulted from the fixation process and are now considered artifacts.

Nonviral inclusion bodies that occur in cacti include calcium oxalate crystals, light refractive hexagonal protein crystals, and rhombic crystals (46, 47, 74). Rhombic plates were found in young noninfected cactus seedlings by Milicic (46). McWhorter (39) found virus disease increased lipid spherules in the cells and should not be confused with viral inclusion bodies. Insect stings or other damage have been mentioned briefly as other initiators of spindle formation. The viral protein spindles range in size from small inclusion bodies to very large robust crystals which fill an entire cell. It has been postulated the protein spindles are curved because of lack of space in the cells (77).

There has been a suggestion that the fibers of the spindles are held together by an interfibrillar cement or mortar. Weber and Kenda (77) showed large amorphous areas between the bands of laterally aggregated protein. Smaller granular areas have been shown to occur in other protein spindles. Flower petals of Schlumbergera were used by McWhorter to follow the development of protein spindles in which he thought he observed the formation of spindles along the protoplasmic strands. The strands serve as channels where protoplasmic streaming was observed in crystals of the developing spindles.

The distribution and localization of the protein spindles have been the subject of many reports in the literature. The reports generally relate to the presence or absence of protein spindles in various cells and organelles and the frequency of their occurrence. They are most numerous in the epidermal and subepidermal cells (21, 27, 53), and have been reported from cytoplasm (39, 42), nuclei (42), vacuoles (45), guard cells (45), and trichomes (45). They have not been found in root cells (21). With minor exceptions, the viral inclusion bodies when subjected to various chemical tests have tested positively for protein (2, 20, 21, 27, 39, 53). Treatment of the protein spindle with trypsin and pepsin completely disrupts it (5).

Mikosch grafted a scion of Pereskia aculeata containing protein spindles onto a spindle-free P. aculeata; after a period of time, protein spindles developed in the previously spindle-free stock (40). Gicklhorn (27) found protein spindles in O. monacantha f. variegata. Weingart (79) demonstrated, by grafting, the infectious nature of the mottling of O. monacantha f. variegata. He grafted a scion of a mottled plant onto a green one and the following summer a white flecking occurred on the previously all green O. monacantha giving it the appearance of O. monacantha f. variegata. The same results were obtained thirty years later by Kenda (35). Grafting, homogenate injection and transplanting of spindle containing tissue of Epiphyllum and Pereskia (58) resulted in protein spindle development in previously spindle-free pads. Spindle-free Epiphyllum bridgesii and E. truncatum were grafted onto spindle-containing P. aculeata. The reciprocal experiment of a spindle-free stock and a spindle-containing scion was

also performed. Protein spindles always developed in the spindle-free partners. About three weeks after grafting, spindles were found in both directions from the graft. If the stock and scion are separated from each other, the spindles remained in their respective pads (58). A homogenate from spindle containing cacti was passed through a Berkefeld filter, injected into spindle-free E. bridgesii and E. truncatum and after three months protein spindles appeared. The protein spindles initially developed around the point of injection. Controls included grafting of spindle-free cacti to spindle-free cacti, injection of water into pads, and injection of spindle-containing extract boiled in water. Protein spindles did not form in any control plants. On the basis of her results Rosenzopf believed the protein spindles were virus inclusions because infectivity could be demonstrated. Work by others using other combinations of cactus species confirmed the work of Weingart and Rosenzopf (3, 5, 49, 67, 73, 76).

Morphological Effects (External)

Some information about viral monstrosity forms has been published. Uschdraweit (63) reported that a virus might be involved with O. tuna monstrosa. The normal O. tuna looks like an Opuntia, while the monstrose form developed many small stems giving the plant a "witches broom" appearance. Van der Meer (64) has reported a similar condition occurring in O. exaltata. Graser [quoted in Uschdraweit (64)] grafted O. tuna monstrosa into other cacti. Various degrees of expression of the monstrosity form developed depending upon the scion and stock combination.

Stomata "twinning" and abnormal cell division have been suggested to result from virus infection (42, 46, 72). Others believe the etiology of these abnormalities has not been established experimentally (5).

Some workers pointed out that one difficulty in working with the cactus viruses has been the lack of external symptoms (5, 19, 47, 59). They maintained that detection of the virus is completely dependent upon the presence of the protein spindles or inclusion bodies or detection of virus particles by electron microscopy. Reports of external symptoms have been suggested or reported in some earlier works which apparently were overlooked by these workers. Chessin, Solberg and Fischer (20) working on the newly discovered cactus virus in North America could not find any external symptoms in Opuntia growing in the wild. Rooted pads of O. brasiliensis (or O. bahiensis) growing in greenhouses injected with spindle-containing sap developed a chlorotic flecking two years after inoculation. Cigar-shaped spindle bodies were also observed in the cells of O. brasiliensis. A more generalized mosaic was produced on systemically infected pads. The long incubation period required for symptom development may have been the reason for not observing external symptoms before 1963. The question of symptoms on Epiphyllum is still not answered.

In 1965 Chessin reported finding external virus symptoms in wild cacti in Arizona (18). Pads of O. engelmannii, O. phaeacantha, and O. macrocentra collected by Alcorn and Nelson in 1963 and sent to Chessin showed bright yellow chlorotic ringspotting of the pads. A greater proportion (56%) of the plants of the three species with

chlorotic markings showed inclusions than did those without the markings (19%) (21). The correlation between internal and external symptoms was obviously not absolute. The significance of the chlorotic rings in relation to infection is unclear especially in view of the insect induced chlorotic ringspots observed by Alcorn and Boulton of the University of Arizona (unpublished observations).

## Insect Transmission

The only report about insect vectors of cacti is the work of Blattny and Vukolov who were working with Epiphyllum mosaic (10). Scale insects, Orthezia insignis Dougl., were used to transfer virus from Epiphyllum showing mosaic to healthy Epiphyllum. These results have not been confirmed.

## Seed Transmission

Seeds obtained from a severely mottled plant of Phyllocactus gaertneri 'mackoyanus' were grown and observed by Pape (54). No mottled plants were detected. Seeds were collected and grown from E. bridgesii and E. truncatum which contained protein spindles. The seedlings were examined and found free of protein spindles (58). From these data it would appear that the virus was not seed transmitted.

Milicic (46) observed O. inermis from two locations in Yugoslavia and found spindle-containing and spindle-free specimens from both. On the basis of the presence and the absence of the protein spindles in the plants from the various locations, he concluded that the virus was not seed transmitted because of the large number of spindle-free seedlings. The spindle-containing specimens were

propagated by cuttings while the spindle-free plants probably started from seed and subsequently grew into mature spindle-free plants. Seed was obtained from spindle-containing O. inermis and forty young seedlings were grown. After two years they were examined and no spindles were found in the epidermal cells of the seedlings. It was concluded these plants were healthy and did not contain virus.

## Distribution of Cactus Viruses

As far as has been reported, there are four rod-shaped viruses occurring in cacti. They are Sammons' Opuntia virus (SOV) (15 x 317 mμ) (14, 59), cactus virus X (CaXV) (13 x 515 mμ) (13), zygocactus virus (ZV) (15 x 568 mμ) (17), and cactus virus 2 (CV2) (13 x 650 mμ) (13). Cactus virus X was previously designated as cactus virus 1. Since four viruses have been reported in cacti, it is undesirable to use "the cactus virus" as has been done for a number of years.

The elongated cactus viruses can be characterized better using serology and electron microscopy data. The inclusion bodies with their multiplicity of forms make it difficult to identify the viruses from these characteristics since more than one virus can occur in the same plant.

Until 1956 all of the published work on cactus viruses had been on material from European greenhouses and botanical gardens. Domesticated Opuntias, O. humifusa, O. ficus-indica, O. inermis, O. microdasys, O. monacantha, and O. brasiliensis growing in gardens and hothouses were most often infected. According to published information, cactus

viruses have been found in Austria, Czechoslovakia, France, Germany, Italy, Mexico, Poland, the Soviet Union, Spain, the United States, and Yugoslavia (1, 3, 20, 29, 47, 51, 59).

Sammons and Chessin were the first to report the occurrence of CaXV in cultivated cacti in the United States (59). They reported spindle bodies in O. monacantha f. variegata which had been reported approximately forty years earlier in Europe (79). They observed four other kinds of flat-padded Opuntia cultivated in Montana and California. One field sample of O. lindheimeri examined in Texas shortly after collection contained no viral inclusions and no spindles were observed in pads of twenty cactus species examined from field collections in Arizona, California, and Montana.

## Noncactus Hosts of Cactus Viruses

Representatives from the families Leguminosae, Solanaceae, Chenopodiaceae, Cruciferae, Tropaeolaceae, and Cucurbitaceae inoculated with extracts from O. monacantha f. variegata showed no external symptoms (55). In the same year (1961) Milicic and Udjbinac independently reported the first successful transmission of CaXV to a noncactus host using a spindle-containing homogenate from O. monacantha f. variegata (51). The inoculated leaves of Chenopodium amaranticolor and C. album developed both chlorotic and necrotic local lesions (1-3 mm diameter) 20 days after inoculation. Protein spindles were found in the local lesions of Chenopodium, but were not detected in the leaf away from the lesion. The inoculated leaves became chlorotic sooner than noninoculated leaves and dropped prematurely from the

plant. Inoculations from spindle-free Schlumbergera bridgesii and O. stricta did not produce local lesions or protein spindles on inoculated leaves of C. amaranticolor.

An infectious extract from O. monacantha was rubbed onto leaves of Beta vulgaris. Spindle-shaped inclusion bodies were found only in the inoculated leaf blade, but not the petiole or noninoculated leaves. The first inclusion bodies were observed nine days after inoculation and tests were successful in transmitting the virus from B. vulgaris back to C. amaranticolor (45). No other symptoms were observed on the beets and similar results were obtained with Agrostemma githago.

A suitable noncactus host for CaXV multiplication is C. quinoa which in addition to local lesion production becomes systemically infected. The inoculated leaves of C. quinoa reacted with chlorosis which induced premature abscission of the leaves. High concentrations of the virus were obtained in C. quinoa as determined by a positive serological test at a dilution of 1:2000 with the crude sap. The C. quinoa is suitable for increasing the CaXV for morphological and serological investigation, but it is not a definitive host for characteristic symptom expression (13).

The symptomatology for CaXV on C. quinoa includes yellow-brown necrotic lesions with the plants showing chlorotic symptoms after 14 days. The local lesions are surrounded by a yellow-green chlorotic ring, which in transmitted light is very distinct from the rest of the leaf. C. amaranticolor and C. quinoa react in a similar manner except that necrosis first appears at the outer edge of the lesion in

C. amaranticolor (55). The CaXV has been transmitted from Chenopodium back to Schlumbergera bridgesii (47).

While all of the infected cacti are systemically infected with cactus viruses, the herbaceous hosts are infected locally except in the cases of C. quinoa, Celosia cristata, and Amaranthus caudatus. The latter has been found useful for quantitative work with strains of CaXV which react in a necrotic manner on this host. Those strains reacting with chlorotic lesions are harder to detect on the green leaves, but can be easily seen as the leaves become chlorotic (56). Over forty species of plants have been reported to be infected by CaXV (56), including representatives of the following families: Amaranthaceae including six species of Amaranthus, and three species of Celosia; Caryophyllaceae, including Gomphrena globosa and Melandrum rubrum; Chenopodiaceae represented by eleven species of Chenopodium and Beta vulgaris and Labiatae represented by Ocium basilicum (56). A Zygocactus x Schlumbergera hybrid which had no symptoms was found infected with a virus which systemically infected C. quinoa, N. clevelandii, and N. glutinosa (17). The common name of zygocactus virus (ZV) is suggested (17).

## Physical Properties

The physical properties of cactus viruses have only been studied for CaXV. Rosenzopf found she could heat the extract of E. bridgesii and P. aculeata at 70 C for one hour without destroying infectivity. Boiling the extract in water eliminated infectivity (58). Reports using extracts from hosts including Epiphyllum, C. amaranticolor, and

C. quinoa indicate the thermal inactivation point lies between 80 - 82 C (13, 55, 56). Inoculum prepared from inoculated leaves of C. quinoa indicates the dilution endpoint lies between $1 \times 10^{-5}$ and $1 \times 10^{-6}$ (13, 55, 56). Longevity in vitro studies indicate the virus is infectious up to eighteen days in crude extracts of cactus (5, 51, 56). It has been shown that CaXV was unstable at pH 5.0 - 5.3 but was stable at pH 9.6. It is not known how long the virus is stable at this high pH value since it was kept at this level for only short periods of time. Virus particle aggregation was reduced when the virus was stored in the presence of 0.001 M $NH_4^+$, pH 9.6 for 25 days.

## Virus Composition

The only cactus virus analyzed for its chemical composition is a strain of CaXV (4). The virus particles are composed of 95% protein and 5% nucleic acid. The nucleic acid was characterized by ultraviolet absorption and paper chromatography and found to be a ribonucleic acid. The protein, analyzed by paper chromatography, contained: aspartic acid, glutamic acid, serine, glycine, threonine, alanine, leucine, isoleucine, proline, arginine, lysine, cysteine, tryptophan, and histidine.

## Purification

The CaXV was purified by Amelunxen from O. monacantha by first soaking the epidermal portion of the pad in water for 24 hours to facilitate the removal of the mucilage. This homogenate was clarified with chloroform and the virus purified further by repeated precipitation

with ammonium sulfate. The purified preparation showed only a single moving peak when subjected to electrophoretic techniques. A virus concentration of 170 mg of virus per kilogram of sap was determined by measuring the nitrogen content by the Kjeldahl method. Further quantitative data were not possible because no local lesion host was known for CaXV.

Chenopodium quinoa proved to be a better host for purification of CaXV, both because of it being an easily grown herbaceous plant and because of high concentration of the virus. The protein spindles also developed in infected C. quinoa plants (13, 55, 56, 79).

Spindle-free O. herrfeldtii was used by Amelunxen (5) as a host to test infectivity of the purified virus. Material obtained from a single homogeneous electrophoretic peak was injected into a spindle-free pad eighteen days after purification. Protein spindles were observed in the cells at the site of treatment fifty-one days after injection. Some pads that were injected did not have protein spindles in the cells after 38 or 51 days; but when they were examined after 108 days, all the injected pads had developed protein spindles.

## Serology

Serological investigations have been carried out by European workers (13, 50, 55, 56). The serological studies dealt with the 515 mμ virus particles of CaXV, and the 317 mμ particle of SOV (80). The serological relationships of the 650 mμ virus particle of CV2 has not been studied.

The CaXV antigen was tested against antisera to potato virus X (PVX), potato virus Y (PVY), white clover mosaic virus (WCMV), passion fruit virus (PV), bean virus 2 (BV2), hydrangea ringspot virus (HyRV), tomato blackring virus (TBV), and snapdragon virus (SV). Cactus virus X was found serologically related to WCMV, HyRV and PVX but the full extent of these relationships were not ascertained (13). An antisera prepared by Plese (55) to the strain of CaXV she studied reacted only weakly with the CaXV isolate used by Bercks. In two other reports concerned with serology of CaXV, six different isolates from various hosts were used to determine if different strains of CaXV could be detected serologically (50, 55). The tests were carried out at Braunschweig by Bercks and at Zagreb by Milicic. Their results showed that on the basis of serological reactions, four isolates were very similar, one isolate was intermediate in reaction and the other isolate was serologically different. Even though there are some differences in host reactions to the six isolates, on the basis of serological reactions these isolates were deemed similar enough to be considered strains of the same virus, CaXV (50).

Zygocactus virus, recently isolated by Casper and Brandes, was tested against antisera prepared to potato aucuba virus (PAV), PVX, and CaXV. No positive serological reactions were obtained with any of the tested antisera (17).

Sammons' Opuntia virus has been shown to be related to TMV. On the basis of serological reactions it was concluded that SOV is an independent member of a group of wild strains of TMV (80). It is

an example in the TMV group of serologically related viruses of similar structure that can differ in their normal length (12).

Although the protein spindles have been assumed to be aggregates of virus particles, little proof has been reported to confirm this idea, except for some serological tests utilizing fluorescent antibodies. Stained thin slices of spindle-containing tissue of Schlumbergera and E. bridgesii fluoresced a bright-yellow green in the damaged cells and some fluorescence was observed in the nucleus. The results of experiments showed a positive reaction had taken place and suggested that the protein spindles are composed of virus particles (61).

## Strains of Cactus Viruses

The SOV produces only local lesions on C. quinoa (14). Chlorotic local lesions which first appear eight days after inoculation become necrotic four days later. Isolations from uninoculated leaves were unsuccessful. No special studies of host range were conducted but this virus could not be transmitted to N. tabacum 'Samsun' or N. glutinosa. Seven isolates of CaXV have been studied by European investigators. Isolate K11, isolated from S. bridgesii, produced both chlorotic and necrotic local lesions on inoculated leaves of C. amaranticolor. Single necrotic lesions were used to increase this isolate which subsequently produced only necrotic local lesions. Local lesions developed four to five days after inoculation on C. quinoa and on Amaranthus caudatus. In addition to the local lesions, it also produced a veinal necrosis. Three isolates from O. microdasys ' albispina,' O. microdasys, and O. monacantha extracts produced chlorotic symptoms

in most hosts; but occasionally necrosis was observed on A. caudatus. Isolate B1, isolated from Zygocactus, was similar to the above three isolates, but it produced chlorotic lesions on A. caudatus. In spite of differences in host reaction, the isolates were similar serologically except the K11 isolate which seemed to be different.

## Electron Microscopy

The elongated virus particles of a virus from Epiphyllum were first observed in the electron microscope by Suhov and Nikiforova (62) and more recently by Casper and Brandes (17). Amelunxen reported similar structures the following year from Opuntia (2, 3) and since then numerous other reports have appeared reporting flexuous virus particles (5, 13, 18, 47, 52, 61). Unpurified homogenates, dip preparations or purified material either negatively stained or shadowed contained flexuous virus particles. Tissue containing numerous protein spindles has been embedded in acrylic plastic and sectioned with an ultramicrotome.

The thin sections contained filamentous elements which probably represent parts of virus particles (5). Brandes and Bercks, using Zygocactus as a virus source, embedded tissue with numerous inclusion bodies in acrylic plastic and sectioned it. They also found filamentous elements resembling virus particles. They rubbed C. quinoa with an extract of fresh Zygocactus which subsequently induced small pinpoint lesions on the inoculated leaves as well as systemic infection.

## Classification of Viruses

On the basis of morphological and serological data, CaXV and ZV have been placed as independent virus types in the PVX taxonomic group as proposed by Brandes (12) with WCMV, HyRV, and PVX. Cactus virus 2 can be placed in the potato virus S group as proposed by Brandes and SOV can be placed as an independent virus in the TMV group. Further investigations can now be undertaken with the above information to obtain a general idea of the distribution of virus, epidemiological relationships, and separation of the reported cactus viruses into strains (13).

## MATERIALS AND METHODS

### Saguaro Virus

#### Source and Distribution of Specimens

The saguaro cactus (Carnegiea gigantea) (Engelmann) Britton and Rose samples were collected at random within the boundaries of the east and west annexes of the Saguaro National Monument, Reddington Pass, and Soldier's Trail which are all in the general Tucson area. Samples from approximately 130 saguaros were assayed through the summer and fall of 1967 (Table 2).

#### Assay Procedure

Chenopodium amaranticolor Coste & Reyn., C. capitatum (L.) Asch., C. quinoa Willd. and Gomphrena globosa L. were used as assay hosts. C. amaranticolor was used in most instances to determine if virus was present. C. quinoa and C. capitatum were used as systemic hosts to increase the virus for purification. The leaves of all plants used in routine assays were dusted with Carborundum prior to inoculation. From saguaro, in addition to stems, fruits, and flowers, a mixture of pollen and nectar and pollen-free nectar were checked for the presence of virus.

Table 2. Identity and source of cactus plants collected one or more times during the course of this work

| Scientific name | Location | No. plants checked | Symptoms | Presence of protein spindles | Virus infected based on infectivity studies |
|---|---|---|---|---|---|
| Carnegiea gigantea | Saguaro Nat'l Monument | 130 | No | - | + |
| | Soldier's Trail | 9 | No | - | + |
| Cylindropuntia arbuscula | Saguaro Nat'l Monument | 2 | No | - | - |
| C. bigeloviii (flowers) | Organ Pipe Nat'l Monument | 2 | No | - | - |
| C. fulgida mamillata | Saguaro Nat'l Monument | 1 | No | - | - |
| C. leptocaulis | Saguaro Nat'l Monument | 1 | No | - | - |
| C. versicolor | Saguaro Nat'l Monument | 10 | No | - | - |
| Echinocereus robustus | Soldier's Trail | 1 | No | - | - |
| Ferocactus acanthodes | Desert Bot. Garden | 1 | No | 0 | 0 |
| F. wislizenii | Saguaro Nat'l Monument | 3 | No | - | - |
| Lemaireocereus thurberi | Organ Pipe Nat'l Monument | 6 | No | - | - |
| Lophocereus schottii | Organ Pipe Nat'l Monument | 6 | No | - | - |
| Mammillaria microcarpa | Saguaro Nat'l Monument | 1 | No | - | - |
| Opuntia basilaris | Palm Canyon, Ariz. | 6 | Yes | - | - |
| O. chlorotica | Santa Catalina Mts. | 2 | Yes | + | + |
| | Desert Bot. Garden | 1 | Yes | + | + |
| O. engelmannii | Saguaro Nat'l Monument | 3 | Yes | + | + |
| O. flavescens | Desert Bot. Garden | 1 | No | 0 | 0 |
| O. glomerata | Desert Bot. Garden | 1 | No | 0 | 0 |
| O. gosseliniana | Desert Bot. Garden | 1 | Yes | 0 | 0 |
| O. macrocentra | Organ Pipe Nat'l Monument | 3 | No | - | - |
| O. microdasys 'rufida' | Desert Bot. Garden | 1 | No | + | 0 |
| O. monacantha f. variegata | Private Garden, Tucson | 1 | Yes | + | + |
| O. phaeacantha | Saguaro Nat'l Monument | 2 | Yes | + | + |

+ = presence of protein spindles or presence of virus as shown in infectivity studies
- = absence of protein spindles or absence of virus as shown in infectivity studies
0 = not examined

Seed Transmission

Seeds were collected from both infected and healthy saguaro plants. The seeds were sown in pots with greenhouse soil and covered with Vermiculite. All seedlings when they were one month old were homogenized in their entirety and assayed on C. amaranticolor leaves.

Inoculation of Saguaro Seedlings

Saguaro seedlings were started by sowing seeds directly in pots with greenhouse soil and covering them with Vermiculite. Other saguaro seedlings were started by placing seeds on moistened filter paper to germinate and then transplanting them to plastic pots. The seedlings were inoculated with SV when the cotyledons were fully expanded and the seedlings were three months old.

A sample of tissue from a single 3-inch tall saguaro seedling was assayed for infectivity on C. amaranticolor and shown to be negative. This saguaro seedling was then injected with 0.02 ml of the same extract used to inoculate the three month old seedlings.

Host Range Studies

The following plants were inoculated with extracts from local lesions induced by SV on C. amaranticolor leaves: C. capitatum, C. quinoa, Carthamus tinctorius, Datura metale, Gomphrena globosa, Nicotiana glutinosa, N. rustica, N. sylvestris, N. tabacum 'Havana,' N. tabacum 'Burley,' N. tabacum 'Hicks,' N. tabacum 'Samsun,' N. tabacum 'Xanthi,' Phaseolus vulgaris 'Pinto,' Triticum vulgare and Vigna sinensis 'Blackeye.' The plants were observed for the development

of symptoms. After 30 days back inoculations were made to three leaves of C. amaranticolor from both inoculated and noninoculated leaves of the test plants.

Purification

The SV was initially purified using 15 grams infected (local lesions) C. amaranticolor and C. quinoa leaves. The local lesions were punched out with a gelatin capsule. Entire inoculated leaves were used for purification when the local lesion number was large. The leaf tissue adjacent to the local lesions was also used as a source of inoculum, even though fewer local lesions resulted. The infected leaf tissue was triturated with a mortar and pestle and diluted with two and one-half volumes of cold 0.05M $K_2HPO_4$-$NaH_2PO_4$ buffer, pH 7.0. The ground tissue was filtered through cheesecloth and centrifuged at 10,000 g in a refrigerated centrifuge for 15 minutes (step 1). The supernatant was decanted and centrifuged at 100,000 g for 90 minutes (step 2). The supernatant was discarded and the pellet was resuspended in 3.0 ml cold 0.05M phosphate buffer, pH 7.0 (step 3). Triton X-100 (alkyl phenoxy polyethoxyethanol, Rohm and Haas Co., Philadelphia, Pennsylvania) was added to make a 1% solution after the first high speed centrifugation to remove the green material in the pellet. The resuspended pellet was centrifuged at 10,000 g for 15 minutes to remove the insoluble material. The clarified material was then subjected to another cycle of high and low centrifugation (step 4). Healthy plant sap was subjected to the same procedure used for purifying the virus from infected plants (Figure 1). Virus source material also included

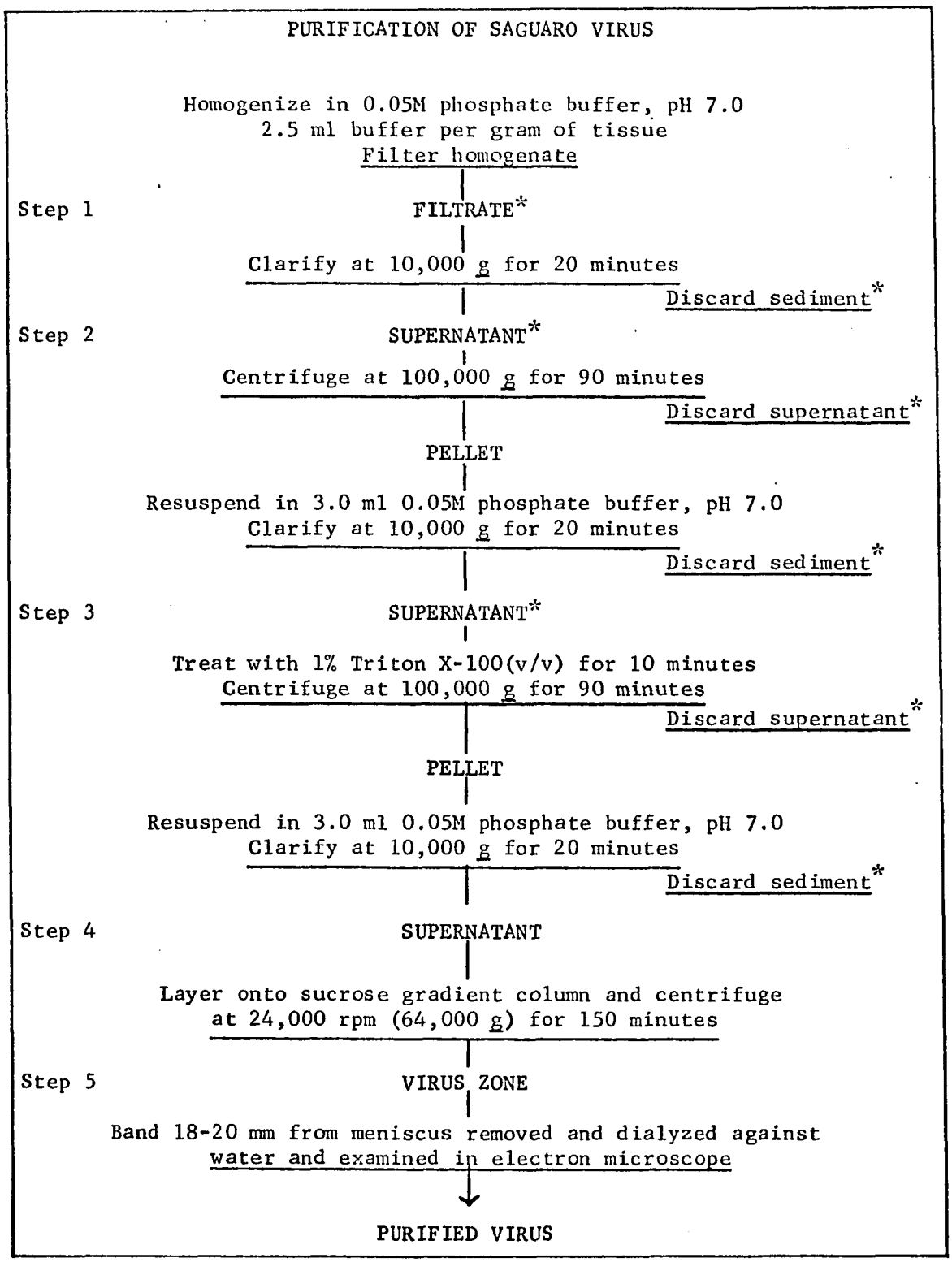


Figure 1. Flow diagram for purification of saguaro virus. Asterisk (*) indicates fractions assayed on C. amaranticolor.

systemically infected C. capitatum. Unless otherwise stated, a Beckman L-2 preparative ultracentrifuge was used for all centrifugation.

Purification attempts were also made using charcoal clarification as described by Corbett (22). Both systemically infected C. globosa and local lesions from C. amaranticolor were used as starting material. A 6 x 6 latin square whole leaf C. amaranticolor bioassay was used to check the various fractions during the purification process. The final virus pellet was resuspended in 10 ml of 0.05M phosphate buffer, pH 7.0 and centrifuged at 10,000 x g for 20 minutes to remove any insoluble material and stored at 5 C until required.

The final SV nucleoprotein and healthy protein concentration (mg/ml) were calculated on the same basis using a spectrophotometric method described by Layne (36). This method was used throughout to estimate the amount of material used in other tests.

Properties of the Nucleic Acid

The nucleic acid was extracted from purified SV preparations by phenol extraction (28). An equal volume of 80% redistilled phenol buffered with 1M SSC (0.15M sodium chloride - 0.015M sodium citrate) pH 7.0 with an addition of 50 mg/ml of washed bentonite was mixed with purified SV and shaken for 15 minutes. The aqueous phase was separated from the phenol by centrifugation at 5,000 x g for 5 minutes in a refrigerated centrifuge and was re-extracted with 5.0 ml phenol containing 50 mg/ml bentonite. The aqueous phase was separated as before from the phenol and extracted twice with anhydrous ether. The nucleic acid was precipitated twice with cold 95% ethanol, dissolved in water,

and then it was centrifuged for 2 hours at 40,000 rpm in the #40 rotor of the Spinco centrifuge, Model L. The supernatant was withdrawn carefully with a pipette and the ultraviolet absorbancy between 220 and 300 mμ at 5 mu increments was determined in the spectrophotometer (Figure 4C).

Infectious extracts of nucleic acid were prepared by degradation of the purified virus using lithium chloride as described by Francki and McLean (24). Purified SV in 0.05M phosphate buffer, pH 7.0, was mixed with an equal volume of 4M lithium chloride and frozen overnight. The mixture was thawed and centrifuged at 3,000 x g for 10 minutes to sediment the nucleic acid. The nucleic acid was resuspended in 5.0 ml 0.05M phosphate buffer, pH 7.0, and precipitated with 15 ml of cold 95% ethanol and centrifuged at 3,000 rpm for 10 minutes. The pellet was resuspended in 5.0 ml 0.05M phosphate buffer, pH 7.0. The resuspended nucleic acid was then centrifuged for 2 hours at 40,000 rpm in the #40 rotor of the Spinco centrifuge, Model L. The supernatant was withdrawn carefully with a pipette and the ultraviolet absorbancy between 220 and 300 mμ at 5 mμ increments was determined in the spectrophotometer.

The purified intact SV, phenol extracted SV, and lithium chloride degraded SV were tested by the diphenylamine method (16) for the presence of DNA and by the orcinol test for the presence of RNA. Deoxyadenosine was used as a standard for the diphenylamine test. Purified TMV-RNA was used as a standard for the orcinol test.

Density Gradient Centrifugation

Sucrose density gradient centrifugation was used to purify SV further (11). Gradient columns for rate zonal centrifugation were prepared by layering 0.5 ml of solutions containing 100, 150, 200, 250, 300, 350, 400, and 500 g sucrose per liter dissolved in 0.05M phosphate buffer, pH 7.0 in 1/2 x 2-inch nitrocellulose centrifuge tubes one day before they were to be used. After a 0.4 ml layer of virus solution had been layered on top of each tube, they were centrifuged at 40,000 rpm (130,000 x g) in the SW 50 rotor. When the 1 x 3-inch centrifuge tube was used, the gradient columns were made by layering 4, 7, 7, 7 ml of solutions containing, respectively, 100, 200, 300, and 400 g sucrose per liter one day before use. After a 2.5 ml layer of virus solution had been floated onto each of the gradient columns, they were centrifuged at 24,000 rpm (64,000 x g) in the SW 25.1 rotor for 2.5 hours. The fractions were analyzed after centrifugation in the ISCO density gradient fractionator and flow densitometer which gives an absorbance reading at 254 mu (step 5, Figure 1).

Analytical Ultracentrifugation

Purified SV from C. capitatum suspended in 0.05M phosphate was analyzed in a Beckman Model E analytical ultracentrifuge. The purified SV and healthy tissue extract were centrifuged in an AnD rotor and the sedimentation pattern observed with Schlieren optics. The pictures were taken at four minute intervals after reaching operating speed and corrected to standard conditions (38).

Electron Microscopy

Epidermal dips of saguaro virus infected C. capitatum were prepared by dipping a freshly stripped piece of cuticle onto a drop of uranyl acetate on a copper grid. The excess stain was removed by touching filter paper to the edge of the drop. The specimen was allowed to air dry and then examined in the electron microscope.

Purified virus preparations and fractions from density gradient centrifugation were examined in the electron microscope. The purified virus preparations were stained with 2% phosphotungstic acid, pH 7.0 (15), and 10% uranyl acetate, pH 4.0 (30). The preparations were examined immediately in a Philips EM-200 or Hitachi HS-7 electron microscope.

Serology

A sample of normal serum was removed from a rabbit by cardiac puncture prior to injection with the SV preparation. A rabbit was immunized by injecting 2 ml of purified SV in 0.05M phosphate buffer at weekly intervals for three weeks. Seven days after the last injection the rabbit was bled by cardiac puncture, the blood was allowed to clot before the serum was removed by centrifugation and tested for antibodies specific for SV. The antiserum was diluted 1:4 with physiological saline and tested for antibodies to SV against its homologous antigen and noninoculated plant sap in Ouchterlony gel diffusion tests. The plates were incubated 24 hours in a moist chamber before they were read (6). The plates were also examined after 48 and 72 hours.

Antisera to cherry necrotic ringspot virus (NRSV), apple mosaic virus (AMV), rose mosaic virus (RMV), and plum line pattern virus (WLPV) were obtained from Dr. R. W. Fulton, University of Wisconsin. The cucumber mosaic virus antiserum was prepared against an isolate from cantaloupe. The tobacco ringspot virus antiserum was obtained from Dr. M. C. Rush, North Carolina State University.

## Opuntia Viruses

### Source and Distribution of Specimens

Pads of prickly pear cacti (*Opuntia* sp.) with and without visible viral symptoms were collected from several areas of Arizona. Most of the material was collected from the Saguaro National Monument near Tucson, Arizona. Material was collected from native cacti in a remote area of the Organ Pipe National Monument, Ajo, Arizona. Specimens of *O. basilaris* were collected in Palm Canyon near Quartzsite, Arizona. The Desert Botanical Garden, Tempe, Arizona was a source of many of the virus-infected samples. A severely infected *O. chlorotica* from the botanical garden was an important source of material used in this study (Table 2).

Two specimens of *O. engelmannii* with distinct external symptom types were selected for extensive examination. One specimen had spreading yellow concentric interlocking rings which caused depressions in the pad, while the other specimen had small surface chlorotic rings. The latter will be shown later to be damage caused by insect feedings. These source plants were selected initially for use because they exhibited the separate symptoms and not a combination of the two types.

Assay of Infectivity

Tissue from the stems, fruits and flowers was sampled in the case of Opuntia, Cholla (Cylindropuntia), and Ferocactus to determine if the plants were infected with virus. Ten free-hand sections were examined with the aid of a light microscope to look for the presence of spindle inclusion bodies. Tissue from the cacti was used as a source of inoculum for leaves of C. amaranticolor. Carborundum was used as an abrasive and a stiff bristled brush was used as the applicator. Development of local lesions on the inoculated leaves indicated virus infected cacti.

Insect Damage

The Opuntia joint bug, Chelinidea vittiger Uhler, a hemiptera, was found by Boulton and Alcorn (unpublished results) and Dodd (23) to be active feeders on Opuntia spp. During the experiment with O. engelmannii the adults attached their eggs to the spines. The young nymphs hatched after eight days and started to feed immediately. A single nymph was removed from the rearing cage and placed on a prickly pear and its feeding habits were observed for two days.

Inoculation of Opuntia Seedlings

Opuntia seedlings used in these tests were grown from seeds which were scarified with a triangular file and then soaked in water three days before planting. These Opuntia seeds germinated in 3-4 weeks and could be used after the cotyledons were fully expanded. When used for inoculations, the inoculum was either injected with a hypodermic needle or brushed on the cotyledons with a stiff bristled brush.

Young Opuntia were collected from the desert to serve as virus free sources. These plants were checked for symptoms and five 1 $cm^2$ tissue slices were examined in the light microscope for crystalline inclusions as evidence for the presence of virus.

Temperature Measurements

Internal temperatures were taken of cactus pads and compared with external temperatures. The temperatures were checked during two summer days at one hour intervals in pads with their flat surfaces oriented in an east-west direction. A dial thermometer was inserted into the center of the pad and a glass thermometer was placed next to the shaded external surface to record the air temperature.

Purification

The virus was purified from virus infected Opuntia material. Opuntia pads were cut into 3 $cm^2$ pieces, placed in a large Waring Blendor (Model CB-4) and homogenized in 0.05M, pH 7.0 phosphate buffer (10 ml buffer per g tissue) for five minutes. The homogenate was clarified at 10,000 g for 20 minutes and the supernatant was decanted. If the supernatant was still viscous, it was homogenized again in the Waring Blendor for two minutes and reclarified at 10,000 g for 10 minutes. The supernatant was then centrifuged at 78,000 g for 90 minutes. The supernatant was decanted and the pellets were resuspended in 5.0 ml 0.05M, pH 7.0 phosphate buffer. The resuspended pellets were clarified at 10,000 g for 10 minutes. The high and low speed centrifugation was repeated for two additional cycles. The final low

speed supernatant was checked for infectivity on C. amaranticolor and examined in the electron microscope for virus particles.

An alternate method included the use of polyethylene glycol (Carbowax 6000) precipitation in the purification schedule (31). Approximately 140 g of pad tissue (O. engelmannii) was homogenized for two minutes in 520 ml cold 0.05M phosphate buffer, pH 7.0 in a Waring Blendor. The homogenate was passed through cheesecloth and clarified at 10,000 g for twenty minutes. The supernatant was decanted through cheesecloth; polyethylene glycol was added during stirring to make a concentration of 2%; and enough sodium chloride was added to give a 0.1M salt concentration. After the Carbowax and sodium chloride were dissolved, the mixture was placed in the refrigerator at 10 C for twenty minutes and then centrifuged at 10,000 g for 10 minutes. The supernatant was decanted and the pellet resuspended in 0.05M phosphate buffer, pH 7.0. The resuspended pellet was clarified at 10,000 g for 10 minutes. The resulting supernatant was used for density gradient centrifugation. The concentration of Carbowax and sodium chloride was added to the supernatant (from the first 10,000 g centrifugation) to bring the concentration up to 4% Carbowax and 0.2M sodium chloride to precipitate any remaining virus.

Density Gradient Centrifugation

The density gradient tubes were prepared and run in the same manner as described for SV. Just prior to centrifugation 0.4 ml of purified virus was layered on top of the tubes.

Analytical Ultracentrifugation

Purified virus from Opuntia suspended in 0.05M phosphate was analyzed in a Beckman Model E analytical ultracentrifuge. The virus preparations were treated in the same manner as SV.

Electron Microscopy

Purified virus preparations from O. engelmannii, O. phaeacantha, and O. chlorotica were examined in the electron microscope. The preparations were treated as described for SV.

A freshly cut surface of an O. chlorotica pad was touched to a drop of distilled water and then removed. The preparation was allowed to air dry before being shadowed with chromium at tan $\theta = 17^{o}$ and examined in the electron microscope. The microscope was calibrated using a Ladd diffraction grating of 54,864 lines per inch.

Serology

A sample of normal serum was removed from a rabbit by cardiac puncture before the animal was injected with a purified Opuntia virus preparation (1.0 mg/ml). A rabbit was immunized by injecting 2 ml of purified Opuntia virus preparation in 0.05M phosphate buffer at weekly intervals for four weeks. Seven days after the last injection the rabbit was bled by cardiac puncture and the blood was allowed to clot several hours before the serum was clarified by centrifugation. The antiserum was diluted 1:4 with physiological saline and tested against its homologous antigen and inoculated plant sap in Ouchterlony gel diffusion tests. The plates were read after 24, 48, and 72 hours of incubation in a moist chamber.

The antiserum was absorbed against extracts from healthy Opuntia by adding 0.2 ml aliquots of Opuntia extract to 5.0 ml of antiserum; this was then incubated at 37 C for 24 hours. Two drops of chloroform were added to each tube as an antibacterial agent. The antiserum was placed in centrifuge tubes of a Spinco Model L centrifuge and centrifuged at 20,000 g for 20 minutes. The supernatant was removed with a pipette and tested for the presence of normal plant proteins in a gel diffusion plate. The process was repeated until no further reaction could be detected.

Antiserum was prepared to TMV-U1 and a sample of TMV antiserum was obtained from Dr. M. C. Rush, North Carolina State University. Both of these sera were absorbed with extracts from healthy tobacco to remove the normal plant proteins as described for Opuntia.

# RESULTS

## Saguaro Virus

### Source and Distribution of Infected Specimens

Saguaro flowers brought into the department during May, 1967 in connection with bacterial studies were assayed out of curiosity to see if they contained any of the prickly pear viruses. The results of inoculations of leaves of C. amaranticolor with triturates of saguaro floral parts demonstrated there was an infective, mechanically transmissible entity present. During the remainder of this spring, additional flowers were collected in the general Tucson area to confirm this finding and extend the knowledge of this entities' distribution.

When C. amaranticolor leaves were inoculated with homogenates from flowers and fruits of saguaro, as well as from the trunk of the saguaro, numerous red-bordered local lesions on the inoculated leaves appeared within four to five days (Figure 2). In some cases the lesion spread a short distance along the leaf veins. The results of the inoculations from the flower parts indicated that 40% (52 of 131) of those plants assayed were infected. Samples of saguaro nectar with and without pollen, obtained from Dr. S. M. Alcorn, were shown to induce large numbers of local lesions (15-50) upon inoculation to C. amaranticolor leaves. In sharp contrast to the floral tissue, was the tissue obtained from the vegetative portion, such as trunk and arms, which

A B

Figure 2. Chenopodium amaranticolor leaves 10 days after inoculation with saguaro extracts

A) Uninoculated leaf

B) Inoculated leaf

when used as a source of inoculum on C. amaranticolor resulted in only one or two local lesions on the inoculated leaves. Either the virus existed in small quantities or an inhibitor was present in these tissues.

The virus isolated from the saguaro was found in most areas around Tucson where collections were made. The largest number of virus infected plants (48 of 107 or 45%) occurred in the eastern section of the Saguaro National Monument. Saguaros sampled along Soldier's Trail were 33% (3 of 9) infected. This was followed by the western section of Saguaro National Monument with 8% (1 of 13) infected. Two samples taken from the Reddington Pass area showed no infection.

The saguaro does not appear to show any visible symptoms of viral infection. Some saguaros have chlorotic spots of a very uniform size, localized on the ribs. These spots have a hard core in the center of the chlorotic area, and because of their uniform size and similarity to the chlorotic spots found on Opuntia, these were considered to be damage by the insects rather than by the virus.

Seed Transmission

No virus was found when 100 seedlings from fruits of noninfected saguaros were assayed on C. amaranticolor leaves; no virus was found in 40 seedlings obtained from the fruits of infected saguaros and 20 seedlings tested from a random seed collection of noninfected and infected fruits when inoculated to leaves of C. amaranticolor.

Inoculation of Saguaro Seedlings

Fifteen three-month-old saguaro seedlings were inoculated with highly infectious virus from density gradients. The saguaro plants were homogenized after 30, 120, and 180 days and assayed for infectivity on C. amaranticolor and tested serologically. No local lesions were induced on the inoculated leaves. Either the virus was quickly inactivated and could not multiply or else the incubation period was not sufficient to detect the virus in the small seedlings. The single 3-inch tall saguaro was injected with 0.02 ml of the same extract used to inoculate the three-month-old seedlings. This seedling was tested after 30, 120, and 180 days in the area of the site of injection. No virus was found by assay on C. amaranticolor or serological test. A final test for infectivity was made thirteen months after the initial injection with SV inoculum. A homogenate from the cactus induced local lesions on the inoculated leaves of C. amaranticolor. A C. capitatum inoculated with the same extract became systemically infected.

Host Range Studies

Of the plants inoculated only G. globosa and all the Chenopodium species developed symptoms. The positive results are tabulated in Table 3. In the case of G. globosa both the inoculated and noninoculated leaves developed small local lesions. In the case of the Chenopodium species it was discovered that C. capitatum did not produce local lesions, but was infected systemically with the virus. The infected plants showed definite vein clearing and a downward curling of the leaves. The plants were stunted and eventually killed (Figure 3).

With the development of the local lesions in both C. amaranticolor and C. quinoa there was a yellowing and cupping of the leaves followed by a premature leaf drop.

Table 3. Plant susceptible to the saguaro virus[a]

| Species | Symptoms |
|---|---|
| Chenopodiaceae | |
| Chenopodium amaranticolor | necrotic local lesions |
| C. capitatum | vein clearing, systemic necrosis |
| C. quinoa | vein clearing, necrotic local lesions |
| Amaranthaceae | |
| Gomphrena globosa | local lesions and systemic infection |

[a]Leaves of C. amaranticolor with local lesions used as inoculum

Storage of the Saguaro Virus

Two vials of C. amaranticolor leaves with local lesions were lyophilized for storage. After five months the vials were tested for infectivity on C. amaranticolor. The virus was recovered from the lyophilized leaf tissue. The virus can apparently be stored for long periods of time without the loss of infectivity in lyophilized tissue.

Purification

When extracts of 15 grams leaves of C. amaranticolor (inoculated with the SV) were subjected to alternate high and low centrifugations,

Figure 3. Chenopodium amaranticolor and Chenopodium capitatum inoculated with saguaro extracts.

A) Healthy C. amaranticolor leaf.

B) C. amaranticolor leaf 10 days after inoculation with SV.

C) Healthy plant of C. capitatum.

D) C. capitatum 30 days after inoculation with SV.

Figure 3. C. amaranticolor and C. capitatum inoculated with saguaro virus

the final high speed pellet, resuspended in five ml of 0.05M phosphate buffer, pH 7.0 induced 4-5 local lesions per leaf of C. amaranticolor.

Charcoal clarification of plant extracts, which has been used successfully for some elongated viruses such as PVX, was attempted with sap from inoculated leaves of C. amaranticolor. The technique has been successfully used with some viruses to remove green host material resulting in a clear infectious extract of virus. A complete loss of infectivity occurred after charcoal filtration. The same results also occurred with this method when systemically infected G. globosa was used for purification of the virus. Corbett (22) proposed that the charcoal pad in the Buchner funnel acted as an ion exchange and the pad as a chromatographic column. The charcoal pad appears to remove the virus as well as the plant components in this case.

Because C. capitatum could be infected systemically with the SV, this host was used as a source of infected tissue for virus purification. Healthy and virus infected tissue were prepared as outlined in Figure 1 in the Materials and Methods. Fractions in the initial purification steps were tested for infectivity and are tabulated in Figure 1.

SV-infected leaves (16 grams) were homogenized in 0.05M phosphate buffer, pH 7.0 (2.5 ml per gram fresh weight of leaf tissue) (step 1, Figure 1). The homogenate was filtered through cheesecloth and centrifuged at 10,000 g for 20 minutes (step 2, Figure 1). The resulting supernatant contained a majority of the infectivity as determined by local lesions produced on the inoculated leaves of C. amaranticolor. The 10,000 g supernatant obtained after resuspension of the 100,000 g pellets in a total of 6.0 ml phosphate buffer contained about one-fourth

the infectivity of the 10,000 g pellet (step 3, Figure 1). Another cycle of high and low centrifugation produced a 3.0 ml purified SV preparation that was used for sucrose density gradient centrifugation (step 4, Figure 1).

The final purified preparations from healthy and infected C. capitatum leaves showed a typical nucleoprotein absorption spectrum between 220 and 300 mμ at 2 mμ intervals with a minimum at 240 mμ and a maximum at 260 mμ as shown in Figure 4B. Using a spectrophotometric method described by Layne (36), the final concentration of SV was approximately 0.6 mg/ml nucleoprotein and 0.2 mg/ml protein from the healthy material calculated on the same basis. The final pellet of the healthy and infected C. capitatum was used for sucrose density gradient centrifugation. A heavy band was observed 3-5 mm below the meniscus after sucrose density gradient centrifugation of partially purified virus (Figure 4A). The fraction was collected and dialyzed against distilled water overnight.

The undiluted crude juice and the 10,000 g supernatant fraction of infected C. capitatum showed a marked increase in infectivity when a 1:10 dilution was made (Table 4). An inhibitor whose effectiveness was reduced with dilution seemed to be present.

Inhibitory substances have been isolated from several higher plants including carnation, pokeweed, cucumber, and tobacco which all act in a similar manner as a competitive inhibitor with the virus for the infectible site (7, 32, 65, 81). The effectiveness of this type of inhibitor is reduced by dilution. It has also been shown that by

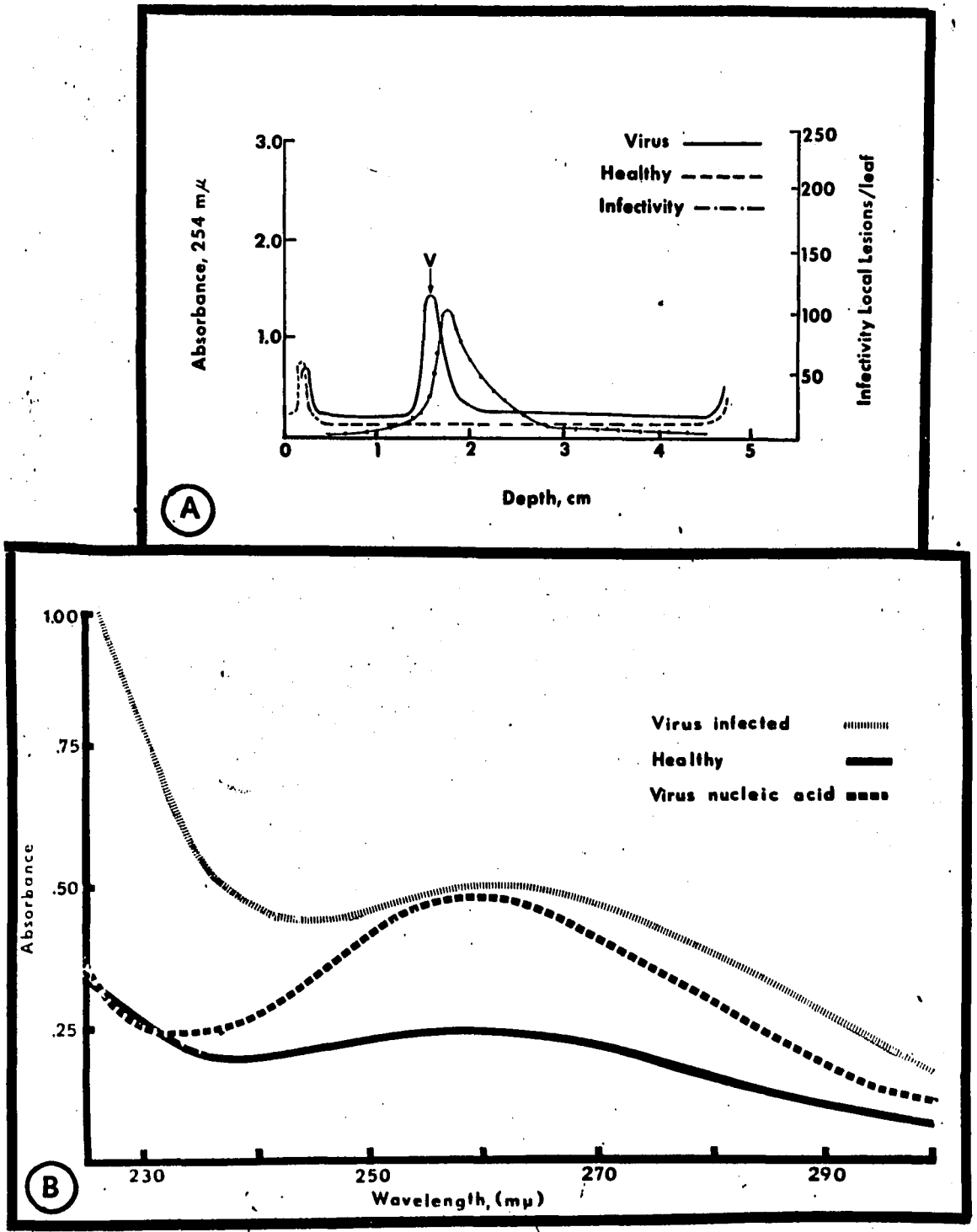


Figure 4. ISCO trace of sucrose density gradient tube and absorption spectrum of SV.

using sucrose density gradient centrifugation that the inhibitors may be separated from the virus (9).

Table 4. Number of local lesions/single leaf on C. amaranticolor inoculated with extracts diluted and undiluted, from C. capitatum.

| Treatment | Local lesions |
|---|---|
| A. Healthy crude C. capitatum | 0 |
| B. Virus infected crude C. capitatum[a] | 7 |
| C. Virus infected crude C. capitatum 1:10 dilution | 204 |

[a]16 grams of leaf tissue inoculated with SV was homogenized in 40 ml of buffer

To test whether an inhibitor existed in C. capitatum, the fraction 0-3 mm in depth was removed from a sucrose density gradient tube and tested for inhibition with TMV. When 0.01 mg/ml TMV-U1 was inoculated to N. tabacum 'Xanthi,' an average of 133 local lesions per half leaf was induced. When 0.2 ml of TMV-U1 (0.01 mg/ml) was mixed with 1.8 ml of the undiluted fraction from C. capitatum, an average of 82 local lesions per half leaf was induced on the 'Xanthi' inoculated leaves. When the same amount of TMV-U1 was mixed with a 1:10 dilution of the fraction, then an average of 130 local lesions per half leaf was obtained. This number of local lesions is comparable to the untreated control (130 vs 133). The effect of the inhibitor was eliminated or reduced as has been found in other cases with similar substances.

Properties of the Nucleic Acid

The intact SV, phenol extracted nucleic acid, and lithium chloride extracted nucleic acid were tested for deoxyribonucleic acid (DNA) by Burton's modification of the diphenylamine test with deoxyadenosine as a reference standard (16). The intact virus and nucleic acid extracts gave a negative reaction for DNA. The same preparations were tested for ribonucleic acid (RNA) with orcinol using purified TMV-RNA as a reference standard. The intact virus and nucleic acid extracts gave a positive reaction for RNA.

Undiluted viral nucleic acid with 50 mg/ml washed bentonite added was rubbed onto leaves of C. amaranticolor. After five days the inoculated leaves developed local lesions indicating the preparation was biologically active.

The absorption spectrum of the saguaro virus nucleic acid suspended in 0.05M phosphate buffer had a 260:230 ratio of 2.31 and a 260:280 ratio of 2.1 indicating the presence of relatively little protein.

Density Gradient Centrifugation

The results of the rate zonal centrifugation showed a single faint band in the tube from the infected C. amaranticolor. A much more distinct band was observed in the same area when infected C. capitatum tissue was used as the source of virus. When the tube was fractionated with an ISCO Model D fractionator, no peak was detected in the healthy material which corresponded to the peak from infected tissue (Figure 4A). After centrifugation the tubes were marked and 1.0 ml fractions were collected by puncturing the side of the tube with a hypodermic needle

and bioassaying 0.5 ml on C. amaranticolor. The results are tabulated in Table 5.

The three tubes from a sucrose density gradient were fractionated into four major fractions. The meniscus (0-4 mm) area has been reported to contain an inhibitory substance (9) and the viral band had a maximum at 260 mμ. The major portion of the infectivity was associated with a band (18-20 mm) below the meniscus. The infectivity found in the other fractions probably resulted from inaccurate fractionation procedures. Fractions of the gradient from tubes 1 and 3 in Table 5 show good separation while the fractions from tube 2 do not show as sharp a distinction. Subsequent sucrose density gradient tubes were scanned and 10-drop fractions were collected using an ISCO density gradient fractionator. These tubes, each containing 10-drop fractions, collected in the region of the viral band induced local lesions when inoculated to C. amaranticolor leaves. The majority of infectivity was found in the contents of 3 or 4 tubes.

The scanning patterns of centrifuged density gradient columns showed a single infectious sedimenting component. There were no shoulders or secondary peaks to indicate that a second component was present (Figure 4A). To confirm the results further, the virus was subjected to analytical ultracentrifugation.

## Analytical Ultracentrifugation

Three analytical ultracentrifuge runs of purified extracts from healthy and SV-inoculated C. capitatum were carried out at 22,000 rpm. Healthy material (0.15 mg/ml) and SV material (0.6 mg/ml) were

Table 5. Number of local lesions on a single C. amaranticolor leaf inoculated with 0.5 ml undiluted fractions of density gradient tube.

| Fraction | Local Lesions | | |
|---|---|---|---|
| | Tube 1 | Tube 2 | Tube 3 |
| Meniscus[a] | 43 | 35 | 11 |
| Above zone[b] | 48 | 54 | 47 |
| Zone[c] | 124 | 78 | 111 |
| Below zone[d] | 30 | 80 | 4 |
| Bottom of tube[e] | 44 | 28 | 1 |

[a]Sample removed 0-4 mm ± 1 mm below meniscus of the tube

[b]Sample removed 4-17 mm ± 1 mm below meniscus of the tube

[c]Sample removed 18-20 mm ± 1 mm below meniscus of the tube

[d]Sample removed 20-45 mm ± 1 mm below meniscus of the tube

[e]Small pellet resuspended in 0.5 ml of distilled water

centrifuged during the same sedimentation runs. A single symmetrical peak was observed in the virus preparation which had corrected sedimentation coefficients ($S_{w,20}$) of 106, 107, and 112 from the three individual runs. Figure 5 shows the result of an individual run with the virus preparation. No comparable peak was observed in a similar preparation of healthy material.

The results obtained from the Model E analytical ultracentrifugation confirmed the density gradient experiments in which a single sedimenting component was found in the purified virus material.

Electron Microscopy

When negatively stained, infective material was examined from purified preparations and sucrose density gradients, uniform icosahedral particles were observed (Figure 6). Seventy-five individual particles were measured of the stained material. The individual particles were measured directly from the glass negative with the aid of an ocular with a micron scale. An average particle diameter of 35 mu was found in the preparations. The preparations were very uniform and no ribosome- or fraction 1-like particles were detected. The epidermal dip preparations of SV-infected C. capitatum contained numerous icosahedral particles similar in size to those particles found in the purified preparations. Epidermal strips from noninoculated C. capitatum did not have comparable particles present. The results of the electron micrographs are consistent with density gradient and analytical ultracentrifugation data that indicate the presence of one type of particle.

Figure 5. Sedimentation patterns of purified saguaro virus.

Figure 6. Photograph of gel diffusion plates of saguaro virus, and electron micrograph of saguaro virus.

A) Left, center well contains undiluted saguaro virus antiserum. Peripheral well 1 contains healthy sap extract, wells 2, 3, and 4 contain 0.6, 0.06, and 0.006 mg/ml respectively.

Right, center well contains 1.0 mg/ml saguaro virus, wells 1, 3, 5, and 7 contain saguaro virus antiserum; wells 2, 4, 6, and 8 contain cherry necrotic ringspot virus, apple mosaic virus, rose mosaic virus, and plum line pattern virus respectively.

B) Saguaro virus negatively stained with uranyl acetate (50,000X).

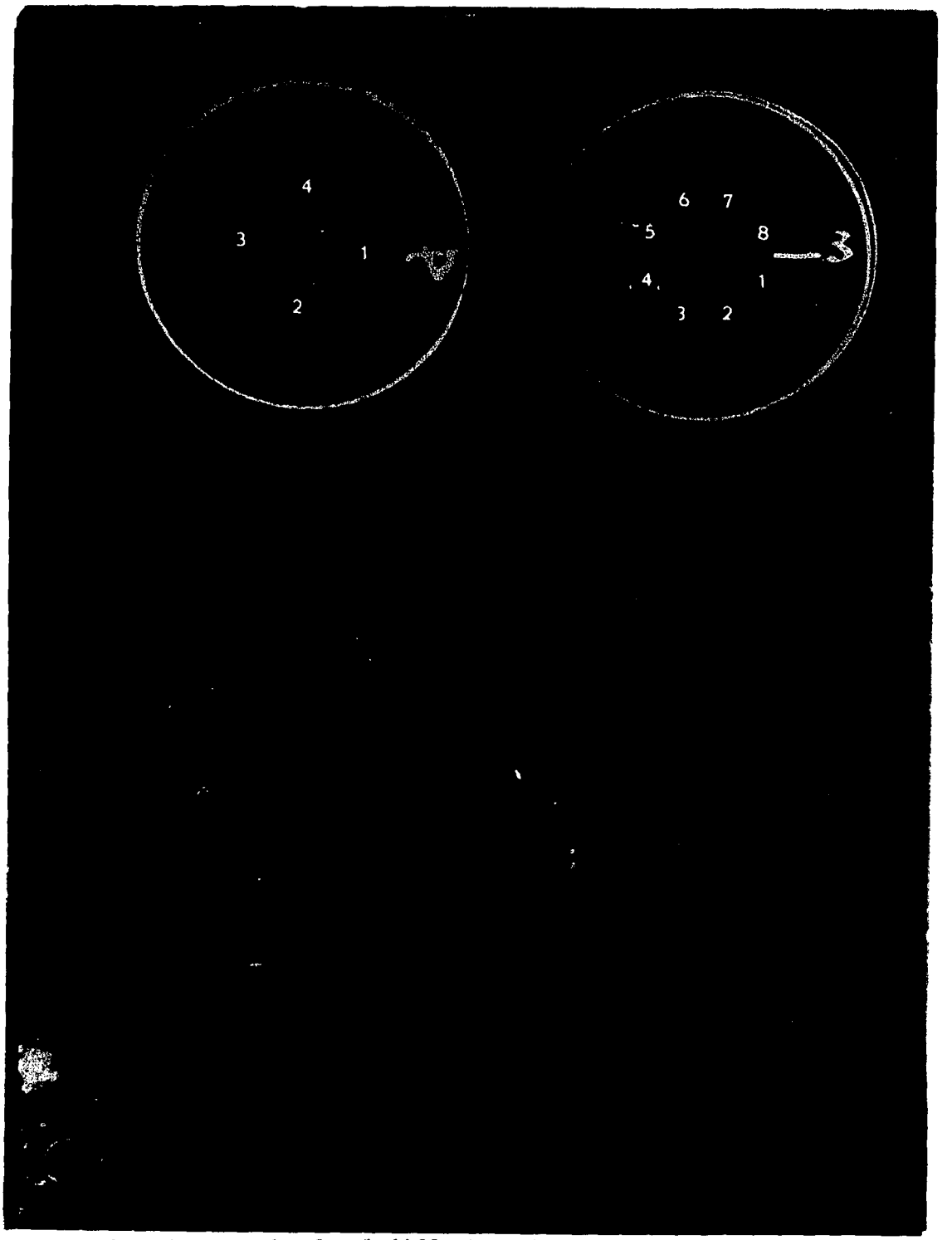


Figure 6. Photograph of gel diffusion plates of saguaro virus and electron micrograph of saguaro virus.

Serology

The antiserum to SV was tested for the presence of antibodies to normal Chenopodium antigens by double diffusion experiments. No reactions were observed against healthy Chenopodium leaf antigens. No precipitation zones occurred in agar gel diffusion tests when the normal serum was tested with the antiserum. In agar diffusion tests, SV formed a single band arced around the antigen well in tests against its homologous antiserum (Figure 6A).

Leaf tissue extracts of C. amaranticolor with local lesions did not react with the antiserum, whereas sap from systemically infected C. capitatum did so readily. This was probably because of low virus concentration generally associated with local lesion plants.

A tissue extract from a young saguaro injected 13 months previously with SV gave a positive serological test for the virus in an agar diffusion test. A single heavy band arced around the antigen well containing the cactus extract.

The antiserum of SV did not react with tobacco ringspot or cucumber mosaic viruses. The antigen did not react with the antisera to cucumber mosaic virus, tobacco ringspot virus, rose mosaic virus, apple mosaic virus, cherry necrotic ringspot virus, or plum line pattern virus (Figure 6A).

## Opuntia Viruses

Source and Distribution of Infected Specimens

The ringspot symptoms of Opuntia observed for some years in Southern Arizona were shown in 1965 to contain virus particles (19).

The sizes of the particles are in close agreement with those described by Brandes and Chessin (14) from an O. engelmannii source plant for SOV obtained from the Desert Botanical Garden, Tempe, Arizona. The rigid rod particles (317 mμ in length) have been designated as SOV. It is likely the virus purified from symptom bearing O. engelmannii with a normal length of 312 ± 6.96 mμ is also SOV.

While a clump of prickly pear (usually a single plant) may be infected by SOV, symptoms may still show in only certain of the pads. Many localized areas of SOV infection exist in prickly pears in the area around Tucson.

In contrast, the Organ Pipe National Monument southwest of Tucson contains very few infected Opuntia. They are not as prevalent as they are near Tucson, but those observed were located around the Park Headquarters, and at Quitobaquito, an old Indian settlement, in the extreme southwestern corner of the park. Vegetative tissue from senita (Lophocereus schottii (Engelmann) Britton & Rose), organ pipe (Lemaireocereus thurberi (Engelmann) Britton & Rose), and Echinocereus sp. were not infected with SOV as determined by the absence of protein inclusion bodies in sections examined in the light microscope or induction of symptoms (local lesions or systemic infection) in C. amaranticolor.

Two types of chlorotic markings are commonly observed on the Opuntia pads. One, which is caused by the Opuntia joint bug, C. vittiger Uhler (Boulton and Alcorn, unpublished data), has been observed on Opuntia sp., Cholla sp., Ferocactus sp., and C. gigantea. The

symptom commonly observed is a chlorotic spot of 5-10 mm in diameter which occurs only in the epidermal and subepidermal tissues (Figure 7C).

A young nymph of this insect, was able to cause a virus like chlorotic ring on a previously unmarked pad after feeding for only 15 minutes. A typical feeding mark is shown in Figure 7B. The nymph frequently changed feeding areas and each time a new feeding mark was observed. In 48 hours a single nymph was observed to have caused 36 distinct feeding rings. In an enlargement of a feeding area caused by an adult, a plug can be seen which resulted from the hardening of the mucilage after feeding (Figure 7D). Such symptoms were observed on Opuntia spp., Cholla spp., Ferocactus spp., and C. gigantea. The insects are heavy feeders and a natural pest of Opuntia and can cause considerable damage including in extreme cases the complete collapse of the pads after sustained feeding (23).

The other chlorotic marking consist of various sized chlorotic interlocking rings (Figure 8B, C). At times this symptom might be mild causing only a chlorotic flecking on the pad. At other times, the pad is depressed where the chlorotic rings occur. The chlorotic rings are cylindrical structural entities that extend through the pad. Where this occurs, the areoles protrude above the surface of the pad giving it a very rough and uneven appearance.

The most severe manifestation of virus infection is exhibited by an O. chlorotica specimen at the Desert Botanical Garden in Tempe (Figure 8D). This entire plant is chlorotic with a variety of symptoms. The pads are very compressed and the cells of the pads are filled with many of the typical cigar-shaped paracrystalline bodies. The apparent

Figure 7. Opuntia engelmannii with insect induced chlorotic spots.

A) Healthy O. engelmannii pad.

B) Chlorotic spots caused by sucking insect. Arrow shows nymph actively feeding.

C) Chlorotic spots caused by adult Chelinidea.

D) Enlargement of feeding site. Note the exudate plug in the center of the area.

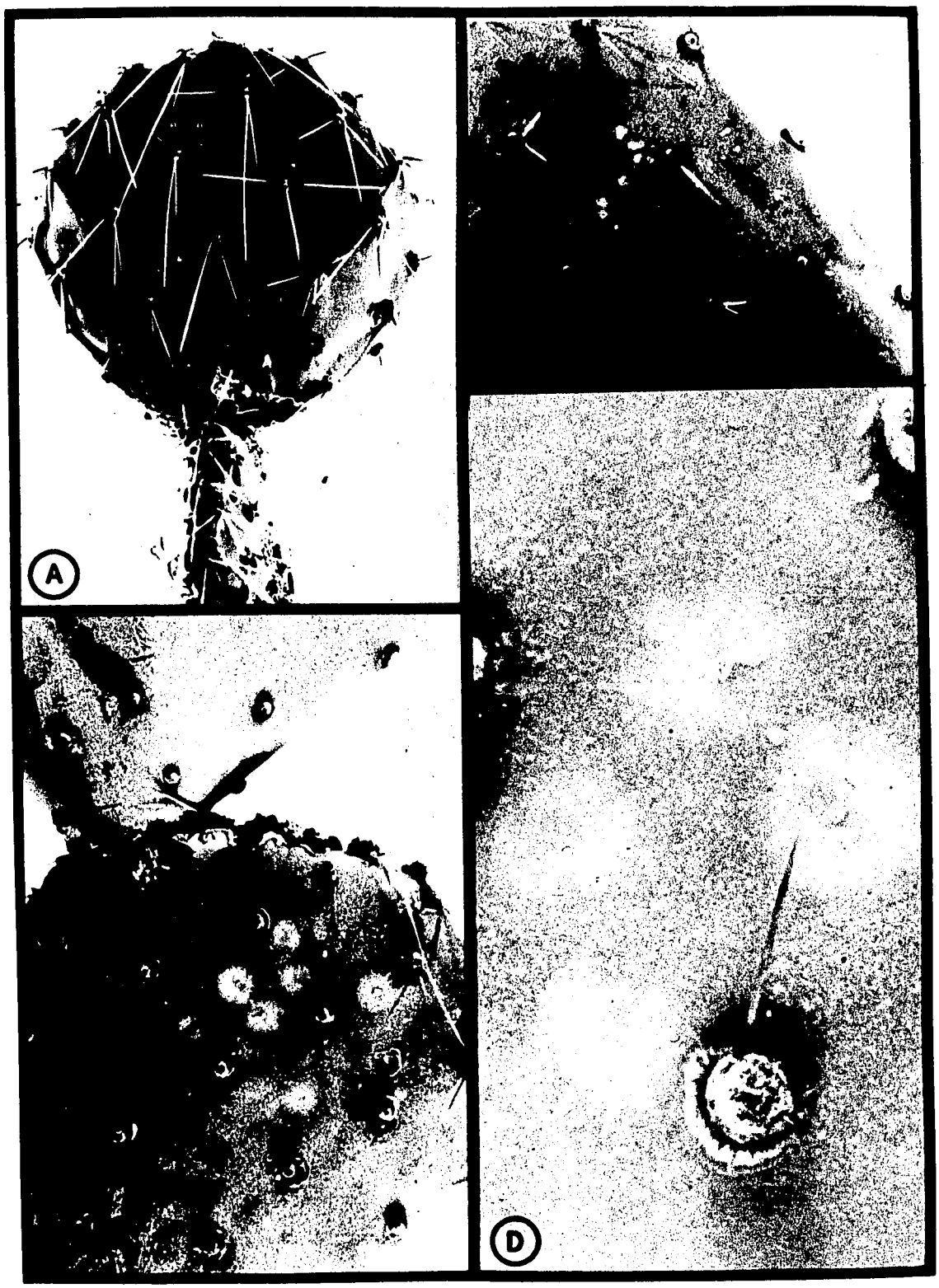


Figure 7. Opuntia engelmannii with insect induced chlorotic spots.

Figure 8. *Opuntia* species with viral symptoms.

A) Healthy *O. engelmannii*.

B) SOV-infected *O. engelmannii* with large interlocking chlorotic rings.

C) SOV-infected *O. engelmannii* with discrete chlorotic rings.

D) Virus infected *O. chlorotica* from Desert Botanical Garden.

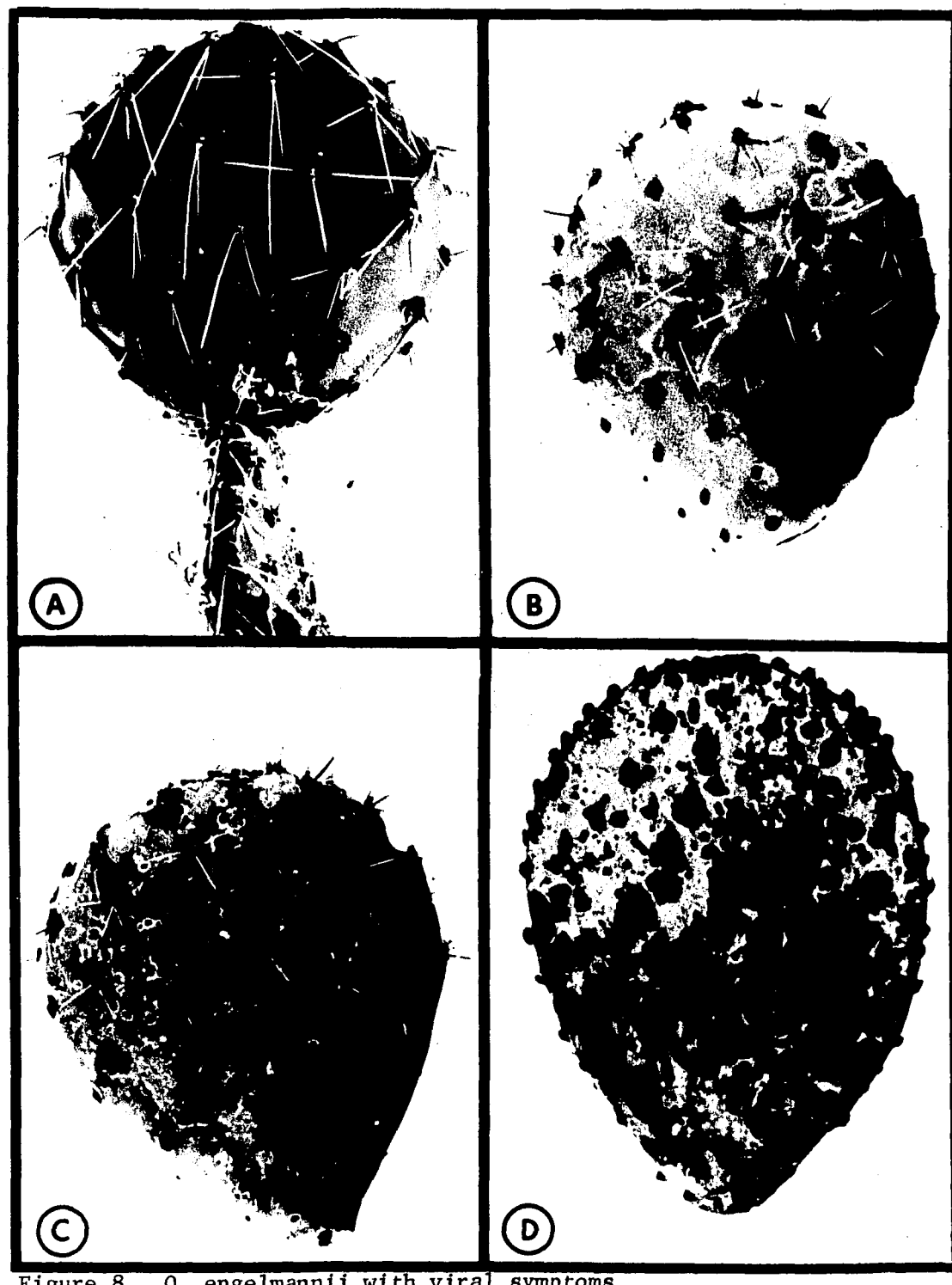


Figure 8. O. engelmannii with viral symptoms

increased severity of symptoms may be the result of two viruses infecting this plant. A rigid rod, probably SOV, and a flexuous rod have been observed in dip preparations in the electron microscope.

This plant was the only one of either O. engelmannii or other O. chlorotica in which two distinct types of virus particles were found. Only this particular plant has been found among native cactus species in Arizona to be infected with two viruses. The other infected plants had only SOV type particles present in the epidermal dip preparations or in purified material.

Inoculation of Opuntia Seedlings

Young O. engelmannii seedlings grown in the greenhouse were examined for protein spindles in the light microscope. Protein spindles were not observed. The seedlings were then either injected or rubbed with the homogenate of an O. engelmannii with SOV symptoms. Tissue slices of the inoculated seedlings were examined after 30 and 60 days in the light microscope. No protein spindles were found in the sections. Five seedlings were injected with the fraction from the main band in the sucrose density gradient. Twelve months after injection of the virus into the Opuntia seedlings, protein spindles were observed in many of the cells of one seedling indicating possible transmission of the virus to the young seedling.

Opuntia Pad Temperature

Since heat treatments have been used to eradicate viruses from living tissue, it was of interest to measure the temperature of the Opuntia pads to obtain an estimate of how hot the interior portion of

the pads become during a summer day. A pad temperature of 56.2 C was obtained by MacDougal and Working in 1921 (37).

Table 6 records temperature measurements of pads obtained from a private garden in Tucson at hourly intervals for two days. A maximum of 52 C was obtained in the interior of the pad and sustained for two hours. The internal minimum temperature of 20 C was recorded during the night. The internal temperature was 6-8 C higher than the external air temperature. These results are consistent with those published by Gates, Alderfer, and Taylor (25). The Opuntia is the only desert plant reported to date that demonstrates that the internal pad temperature exceeds the external air temperature.

Purification

The method outlined in the Materials and Methods was satisfactory for obtaining purified virus. The mucilage occurring in the pads caused some problems. The mucilage problem could be reduced by repeating the homogenization step in a Waring Blendor after clarification at 10,000 g. Attempts to reduce the mucilage with bacterial degradation as well as enzyme degradation were unsuccessful.

The alternate high and low centrifugation was used to purify SOV from O. engelmannii. The polyethylene glycol method of purification was also satisfactory but was not as convenient to use as centrifugation. Butanol or chloroform added to the homogenate did not help to remove the green plant material. If anything, the traces of residual butanol degraded some of the virus in the preparation as judged by sedimentation in the analytical ultracentrifuge. When the

Table 6. Air and internal temperatures of O. engelmannii pads

| | August 21 | | August 22 | |
|---|---|---|---|---|
| Time | Air | Internal | Air | Internal |
| 8:30 A.M. | 22[a] | 20 | 22 | 20 |
| 9:30 A.M. | 27 | 27 | 28 | 23 |
| 10:30 A.M. | 29 | 26 | 30 | 26 |
| 11:30 A.M. | 31 | 28 | 32 | 29 |
| 12:30 P.M. | 33 | 30 | 34 | 30 |
| 1:30 P.M. | 39 | 32 | 42 | 35 |
| 2:30 P.M. | 44 | 38 | 42 | 42 |
| 3:30 P.M. | 47 | 46 | 45 | 46 |
| 4:30 P.M. | 47 | 50 | 43 | 50 |
| 5:30 P.M. | 46 | 52 | 43 | 45 |
| 6:30 P.M. | 44 | 52 | 38 | 43 |
| 7:30 P.M. | 39 | 45 | 34 | 36 |
| 8:30 P.M. | 34 | 37 | - | - |
| 9:30 P.M. | - | 33 | - | - |
| 10:30 P.M. | - | 30 | - | - |

[a]Temperature Centigrade

SOV preparations prepared by polyethylene glycol were examined in the electron microscope. There were many fragments of incomplete virus particles.

The absorption spectrum of the purified SOV showed a maximum absorption at 275 mu and a minimum at 250 mu (Figure 9A). This is a definite shift from the 260 mu and 240 mu maximum and minimum that is characteristic for nucleoproteins such as TMV. This is roughly the maximum and minimum absorbance previously reported for SOV (80).

Density Gradient Centrifugation

Purified virus from O. engelmannii and O. chlorotica was subjected to density gradient centrifugation following purification. At the end of the run the tubes were examined visually and scanned optically for the presence of opalescent bands. Five ml of purified TMV-U1 (1.5 mg/ml) was used in one tube as a reference. The healthy Opuntia prepared in the same manner as infected material did not have any bands below the top of the tube. There was a light band near the meniscus which was probably fraction 1 protein or other small ultraviolet absorbing compounds.

The tube containing TMV had two closely associated bands 22 and 24 mm from the meniscus. The tube of purified SOV from O. engelmannii also had opalescent bands 22 and 24 mm from the meniscus. The band 24 mm from the meniscus sediments faster than the main band and could be aggregated virus particles.

The behavior of SOV in density gradient centrifugation indicates it has sedimentation properties similar to TMV-U1. The ISCO traces of

Figure 9. Absorption spectrum of purified Sammons' Opuntia virus and ISCO trace of density gradient tube.

A) Absorption spectrum of purified extract from healthy and SOV infected O. engelmannii.

B) Ultraviolet absorbance ( 254 mμ ) of purified healthy and SOV infected O. engelmannii after sucrose density gradient centrifugation at 130,000 x g for 60 minutes in SW 50 rotor.

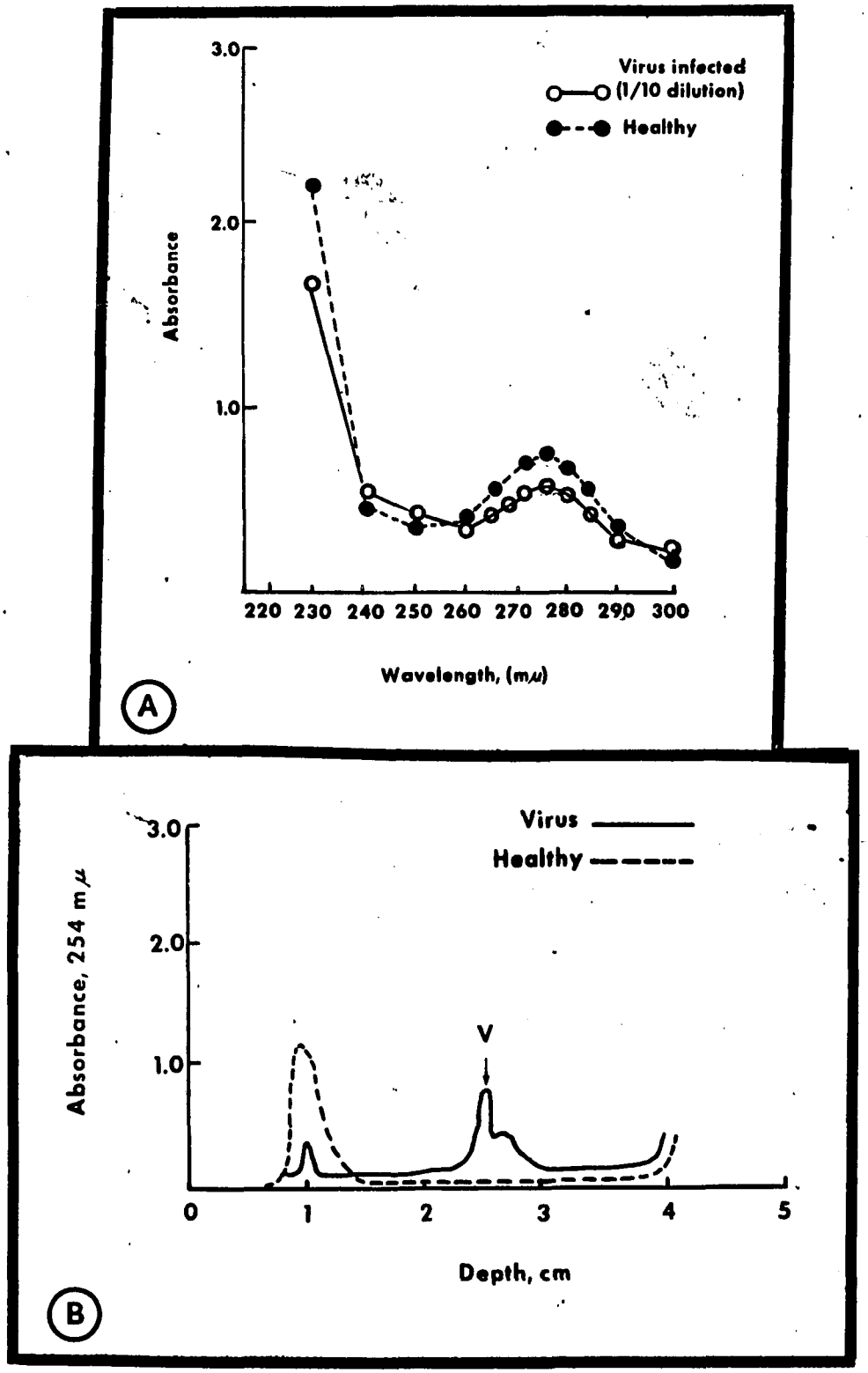


Figure 9. Absorption spectrum of purified Sammons' Opuntia virus and ISCO trace of density gradient tube.

the gradient tubes with the virus show peaks at approximately the same depth in the tubes (Figure 9B).

Purified extracts of O. chlorotica subjected to density gradient centrifugation showed two areas of opalescence. One band occurred 7-13 mm below the meniscus followed by a band 20-23 mm below the meniscus. The band 20-23 mm below the meniscus is in the same area found in purified preparations from O. engelmannii subjected to sucrose density gradient centrifugation.

Analytical Ultracentrifugation

The healthy Opuntia extracts contained only a single detectable band of slow sedimenting material which had a 19S sedimentation coefficient. This corresponds very closely to the values of fraction 1 protein. In the preparations of TMV-U1 and Opuntia there was no evidence of a 19S fraction and in both the O. engelmannii and O. chlorotica preparations are components which sediment very closely to TMV-U1. In the Schlieren patterns there is an indication that there are two peaks which probably correspond to nonaggregated virus particles and aggregated virus particles. The $S_{w,20}$ was calculated using the peak nearest the meniscus. The $S_{w,20}$ of the second peak was also measured and found to have a similar $S_{w,20}$. Both have sedimentation values ($S_{w,20}$) of 183 which are almost equivalent to TMV. When TMV-U1 and the extracts from the SOV infected O. engelmannii ring were centrifuged in the same cell the samples sedimented as a single peak.

Electron Microscopy

Preparations of purified SOV extracts from O. engelmannii with confluent ring symptoms were examined in the electron microscope and 300 particles were measured directly from the negatives with a Bausch & Lomb ocular with micron divisions. Particle lengths were measured between the lengths 200 mμ and 400 mμ. Fifty-four particles measured 320 mμ in length and an additional 26 particles measured 300 mμ (Figure 14). A normal length of 312 $\pm$ 6.96 mμ was calculated for the preparation. The preparations contained some fragments and aggregates and these were not measured (Figure 14A).

A purified virus preparation from O. phaeacantha with faint chlorotic rings was examined in the electron microscope. A total of 191 particles were measured between 200 mμ and 400 mμ. Sixty-six of the rigid particles had a length of 350 mμ and the width of the particles was 15 mμ; when a standard error was calculated, a normal length particle mean of 351 $\pm$ 1.0 mμ was obtained. The measurements are very close to those in O. engelmannii and are probably also SOV.

An epidermal dip preparation of the O. chlorotica from the Desert Botanical Garden had two types of particles. A rigid rod and a flexuous rod were observed (Figure 12A). A normal length mean of 314.5 $\pm$ 3.1 mμ was calculated for the rigid particles. The flexuous virus particle had a normal length mean of 600 $\pm$ 10.4 mμ. Fifty-eight of the particles measured 600 mμ; while the next most common was 570-580 mμ. The length of the flexuous virus particle is in the size range where it could be ZV, CaXV or CV2. The measurements of the rigid rod are

probably the most accurate because of the calibration with the diffraction grating for measurement of particles.

Serology

Antisera were prepared against purified virus extracts from O. chlorotica, O. engelmannii, and extracts from chlorotic spots caused by C. vittiger. Seven days after the last injection, the rabbits were bled by cardiac puncture and the sera tested in gel diffusion plates for homologous reactions. Peripheral wells containing sap from spindle-free O. engelmannii seedlings gave weak precipitation zones against the antisera; these were different from the zones resulting when the well contained the purified virus preparations (Figure 10A).

The antisera were absorbed as described in the Materials and Methods and upon retesting did not show any reaction to normal plant proteins. Only the specific virus antigens remained (Figure 10A).

A ring interface precipitation test utilizing normal and absorbed sera showed there was no nonspecific (spontaneous) precipitation when tested with a 1:4 dilution of healthy Opuntia extract. When the absorbed antisera were tested against sap from Opuntia with symptoms and sap from pads with only the chlorotic spots caused by insects, there was a positive reaction only with the antisera prepared against infected O. engelmannii and O. chlorotica extracts. No positive reaction was obtained with the antisera prepared against the insect induced chlorotic spot. The antisera were not tested with any other virus isolates.

Figure 10. Diagrams of gel diffusion plates for Opuntia viruses.

Plate 1. Center well contains healthy Opuntia engelmannii extract; wells 1, 3, and 5 contain unabsorbed antiserum; wells 2, 4, and 6 contain absorbed antiserum.

Plate 2. Center well contains O. macrocentra antiserum; wells 1, 3, and 5 contain healthy Opuntia extract, well 2 contains extract of SOV-infected O. engelmannii, well 4 contains TMV-U1, and well 6 contains extract of O. chlorotica.

Plate 3. Center well contains O. engelmannii antiserum, well 1 contains healthy Opuntia extract, well 2 contains extract of O. chlorotica, well 3 contains extract of O. engelmannii, well 4 contains purified TMV-U1, well 5 contains normal serum, well 6 contains extract from insect induced spots from O. engelmannii.

Plate 4. Center well contains TMV-U1 antiserum, well 1 contains purified TMV-U1, well 2 and 5 contain an extract from SOV-infected O. engelmannii, well 3 contains extract of O. chlorotica, well 4 contains an extract from O. monacantha f. variegata, well 6 contains normal serum.

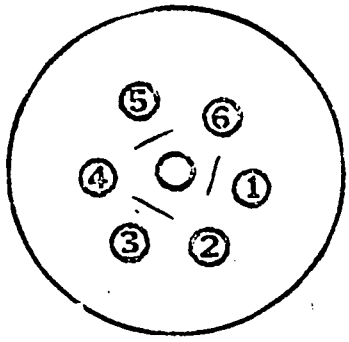


Plate 1

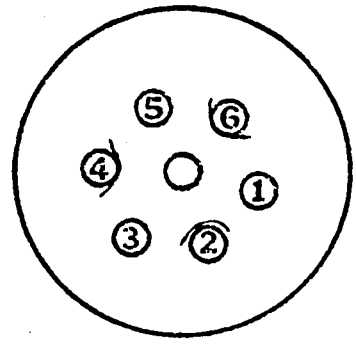


Plate 2

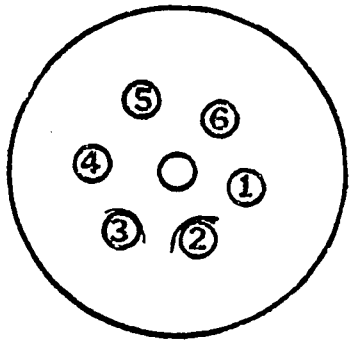


Plate 3

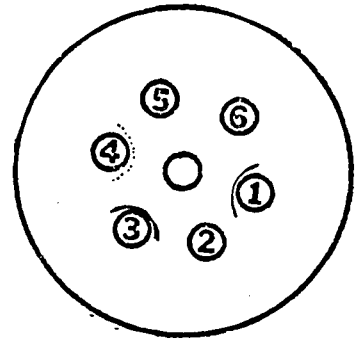


Plate 4

Figure 10. Diagrams of gel diffusion plates for Opuntia viruses.

Undiluted absorbed antiserum was used in gel diffusion tests. The antisera prepared against virus infected O. macrocentra extracts reacted with extracts from O. engelmannii with chlorotic ring symptoms, TMV-U1, and virus infected O. chlorotica. Antisera prepared using O. chlorotica extracts reacted only with its homologous antigen. Antisera to symptom bearing O. engelmannii reacted to its homologous antigen and in one case to the extracts of infected O. chlorotica (Figure 10C). Antisera prepared against TMV-U1 reacted strongly with extracts of infected O. chlorotica. A slight reaction resulted when TMV antisera was reacted with extracts of O. monacantha f. variegata (Figure 10D). The antisera were not tested against other viruses of similar size to establish serological relationships.

Figure 11. Electron micrographs of virus from O. engelmannii and O. chlorotica.

A) Purified extract from O. engelmannii (50,000X)

B) Purified extract from O. chlorotica (50,000X)

Figure 11. Micrographs of virus from O. engelmannii and O. chlorotica

Figure 12. Electron micrographs of virus from O. chlorotica.

A) Dip preparation of O. chlorotica from the Desert Botanical Garden (6000X).

B) Section of A showing both kinds of particles (50,000X).

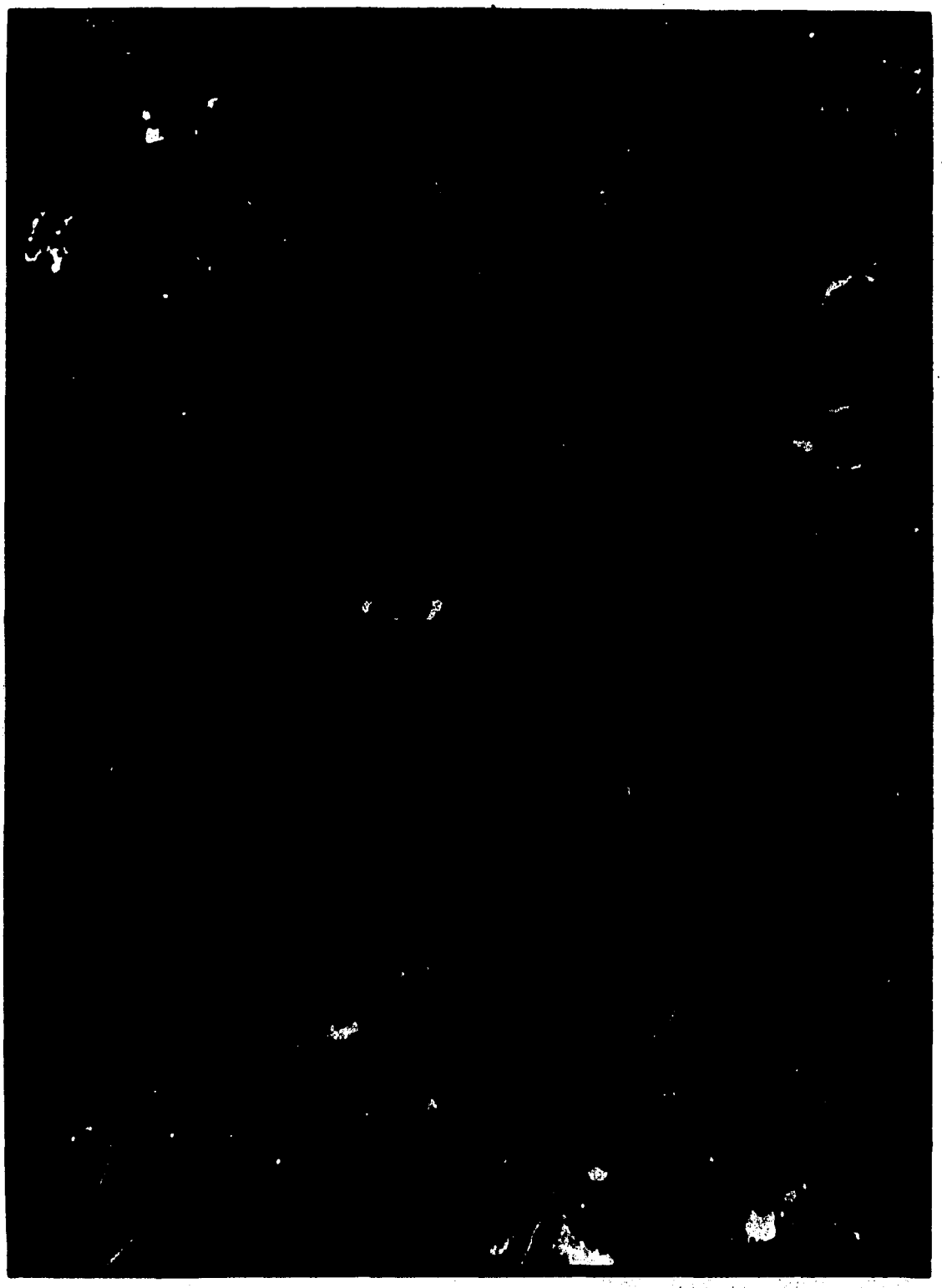

Figure 12. Electron micrographs of virus from *O. chlorotica*.

Figure 13. Electron micrograph of virus purified from O. chlorotica.

Total magnification (50,000X)

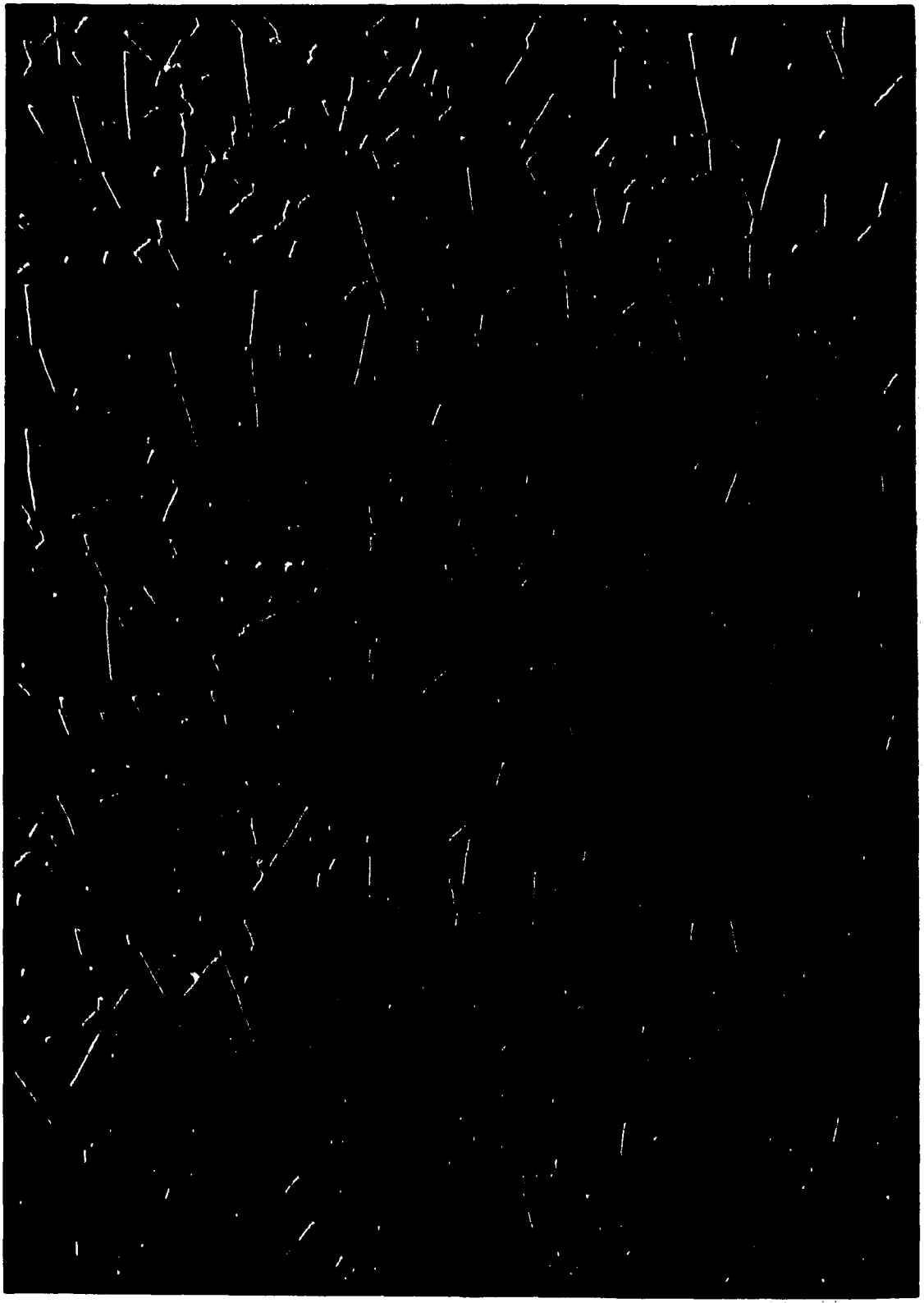

Figure 13. Electron micrograph of virus purified from O. chlorotica.

Figure 14. Bar graphs of particle distribution of Sammons' Opuntia virus.

A) Particle measurments of SOV particles from O. engelmannii.

B) Particle measurements of flexuous virus particles from O. chlorotica.

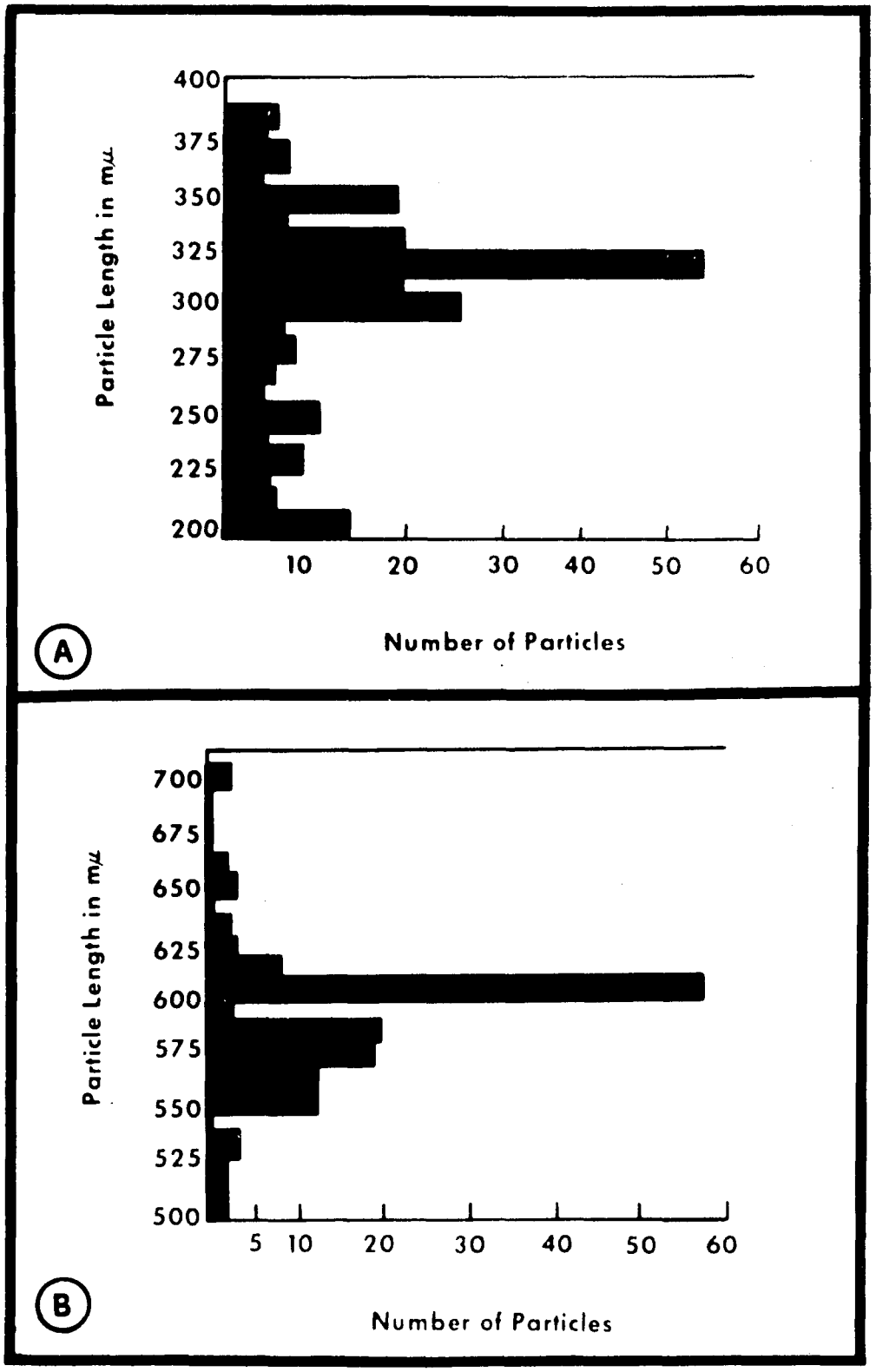
Particle Length in mμ
400
375
350
325
300
275
250
225
200
10
20
30
40
50
60
Number of Particles
A
Particle Length in mμ
700
675
650
625
600
575
550
525
500
5
10
20
30
40
50
60
Number of Particles
B

Figure 14.

## DISCUSSION

The intent of this study was to characterize and study the distribution of the viruses infecting cacti in Southern Arizona. Only recently has the presence of viruses in native cacti in this area been confirmed. Most of the previous work on virus diseases of cacti has been done in Europe. Since all previous work has revealed only rod-shaped viruses, mainly in Opuntia sp., these plants were the initial subject of the study.

### Saguaro Virus

During the course of the study an attempt was also made to isolate virus from saguaro flowers and fruits brought to the laboratory by Dr. S. M. Alcorn to see if any of the Opuntia viruses could be isolated from that plant. On the first attempt, local lesions developed on C. amaranticolor similar in appearance to those associated with several of the Opuntia rod-shaped viruses. Despite this similarity in symptomatology however, it soon became apparent that the virus isolated from saguaro was probably completely different than the rod-shaped viruses described in Opuntia.

Electron micrographs of leaf dips from C. capitatum and purified virus preparations from C. capitatum both contained uniform icosahedral particles. This virus represents the first icosahedral virus isolated from cacti and the first virus to be isolated from the saguaro

(C. gigantea). Further collections of tissue samples indicated the virus was widespread in areas around Tucson. Unfortunately, the discovery of virus in saguaro blossoms came near the end of the spring blooming season so collections were somewhat limited. The homogenized tissue (0.5 grams/5 milliliters) from the flowers and fruits induced large numbers of local lesions (60-65 per leaf) on the inoculated leaves of C. amaranticolor; in contrast, homogenized tissue from the main trunk and arms removed with an increment bore usually caused only a few local lesions (3-5 per leaf) on leaves of C. amaranticolor using the same amount of tissue as the floral parts. Both the cut surfaces of floral tissues and trunk tissue oxidize very rapidly when exposed to air; the presence of inhibitors in the older vegetative tissue could account for the difference in local lesion number.

The characteristics of SV tend to place it in a size range similar to cucumber mosaic virus and sowbane mosaic virus. Cucumber mosaic virus is known to be present in Arizona on a regular basis. Only further work with the virus will tell with precision its relationships if any to those viruses mentioned.

Because of the high concentrations of virus in reproductive structures, seed transmission was considered as a possible method of transmission. One hundred-sixty seedlings were assayed, grown from seed from plants known to be infected, but all results were negative. These results are probably inconclusive because thus far it has been very difficult to isolate the virus from the vegetative tissue of known infected plants. Also a larger sample should be tested before seed transmission is ruled out.

Thirteen months after injection a 3-inch tall saguaro seedling was found to be infected. A positive serological reaction was obtained in agar gel diffusion tests. Some of the same extract induced local lesions on inoculated leaves of C. amaranticolor and systemic infection in C. capitatum. The results of these experiments showed that in addition to natural infection the saguaro might be mechanically infected with the virus. It is possible that during this extended period between inoculation and the appearance of infection that some insect from an outside source could have transmitted the virus to the seedling.

Chenopodium amaranticolor was not a satisfactory virus source because of the low concentration of virus associated with local lesion infection. Saguaro virus was easily purified from systemically infected C. capitatum. The addition of 1% Triton X-100 was sufficient to solubilize most of the green material. The final virus pellet was relatively clear and free of host material. Chenopodiaceae contain inhibitory substances in their sap (32, 33), which were easily removed with sucrose density gradient centrifugation since it remains in the aqueous layer at the top of the tube as reported (9). Although the mechanism of inhibition was not investigated, it is suspected that the inhibitory substance acts in a similar way as other inhibitors isolated from higher plants (7, 33, 81). A ten-fold dilution was sufficient in most cases to reduce the effect of the inhibitor on the virus.

In some cases using equal quantities of healthy and SV-infected C. capitatum leaf tissue, a larger amount of material absorbing at 260 mu was obtained from the healthy tissue than the virus infected

tissue as determined by the absorption spectrum of equal quantities of healthy and SV-infected material. After sucrose density gradient centrifugation of healthy and SV-infected material there was no band in the tube containing the healthy tissue in the same region as the virus band. Whatever substance is present probably remained at the top of the sucrose gradient tube.

Saguaro virus is a good antigen. By serological means, the virus could be detected in C. capitatum and saguaro even when the symptoms were masked. Serological tests with cucumber mosaic virus and tobacco ringspot, however, failed to show any positive reactions.

## Opuntia Viruses

Rigid virus rods (15 x 312 $\pm$ 6.96 mμ) were found in electron micrographs prepared from epidermal dips and from purified virus obtained from O. engelmannii. A rigid rod particle (15 x 351 $\pm$ 1.0 mμ) was found in partially purified O. phaeacantha. In both cases the virus isolated is probably SOV.

Many of the native prickly pears (Opuntia) have varying degrees of symptoms ranging from very mild chlorotic flecks to severe confluent rings with severe pad chlorosis and pad compression. Infection is spread generally throughout Southern Arizona. Viral symptoms were observed in Opuntia at Quitobaquito, a remote Indian village near the extreme southwest corner of the Organ Pipe National Monument. The only Opuntia at the monument that had viral symptoms were those used for landscaping the headquarters' grounds. Other Opuntia, observed away from the headquarters and Indian settlement, were without visible

symptoms and inclusion bodies. Since some farming was done at one time at Quitobaquito, it is possible the virus may have spread to native cacti from some domestic source.

Virus infection is usually confined to an area of several acres or several square miles while other such areas may show no symptoms or virus infection at all. Such a distribution pattern of a virus in such a readily and rapidly vegetatively propagated plant as the Opuntia suggests a relatively recent introduction of the virus.

The virus infected cacti form large paracrystalline bodies within the cells. Although numerous reports of other forms of inclusion bodies have been found, only the paracrystalline spindle-shaped bodies were detected in the native Opuntia of Arizona.

It is significant that SOV occurs in the native cacti and probably has been there undetected for many years. Various European cytologists and virologists have had an interest in cacti because of the unique crystals associated with them. The virus investigated most frequently in Europe is CaXV which forms protein inclusion bodies but does not produce virus symptoms. This virus has been placed in the PVX group of elongated plant viruses (13). This virus has not been found in native Opuntia in Arizona but has been reported in cultivated cacti in the United States (1, 59).

Opuntia pads were used as a source of virus for purification studies. When the pads were homogenized, the homogenate was very viscous with mucilage. The mucilage was reduced most efficiently by homogenizing the extract a second time after the initial clarification

at 10,000 g. Repeated alternate high and low centrifugation produced good preparations of the virus.

Purified preparations of SOV were obtained from the pads by means of the polyethylene glycol (PEG) precipitation method (31). Addition of 4% PEG and 0.2M NaCl precipitated the virus which could be resuspended in buffer after centrifugation at 10,000 g. The preparations did have a large number of virus particle fragments.

When the partially purified virus suspension is examined in the spectrophotometer, the absorption spectrum has a maximum at 275 mμ with a minimum at 250 mμ. The shift is different than the normally expected 260 mμ maximum. Wetter and Paul (80) reported at 269 mμ maximum and a 247 mμ minimum for SOV; they reported a 277 mμ maximum and a 250 mμ minimum for Odontoglossum ringspot virus. The shift may result from host impurities that are still present in the preparation or attached to the virus, but it seems the shift would more likely represent amino acid changes in the protein and nucleic acid base composition to cause such a shift in the absorption spectrum.

When the purified virus was subjected to density gradient centrifugation, two bands were found close together at a depth where TMV-U1 would be found. When the preparations were dialyzed against water, shadowed, and examined in the electron microscope, rigid rods 312 ± 6.96 mμ were found. These are identical to those previously reported to occur in pads with chlorotic rings and intracellular inclusions (19).

Virus-free seedlings of O. engelmannii have been injected in the cotyledons with a preparation of virus from the virus band observed in the density gradient tubes, after five months there was no sign of

symptoms. After thirteen months, paracrystalline crystals were observed in the inoculated cacti, and epidermal dips examined in the electron microscope showed rigid rods. Although no symptoms have developed on the inoculated pads, it has been shown that SOV will cause the inclusion bodies.

Serological tests show that the extracts from symptom bearing O. macrocentra and O. engelmannii will react with each homologous antisera. In some cases, positive reactions will occur from those pads with undetectable or very faint symptoms. It was found that crude extract from O. chlorotica reacted to the antisera prepared against SOV. Wetter and Paul (80) have shown that SOV antiserum reacts very weakly with Holmes' rib grass strain of TMV and not at all with Odontoglossum ringspot virus. They concluded that SOV belongs to an independent group of wild strains of TMV.

The O. chlorotica, from the Desert Botanical Garden which has some of the most severe symptoms observed, is infected with a mixture of viruses. Epidermal dips contain some short rigid rods similar to SOV, as well as some flexuous rods in a 1:4 ratio. The purified preparations examined and measured in the electron microscope show a group of particles 15 x 600 $\pm$ 10.4 mμ whose relationship to other filamentous viruses has not been determined. No serological positive reaction was obtained with antisera to PVX from a commercial source. A new virus recently isolated from a Zygocactus hybrid has a flexuous rod 580 mμ long (17). The virus is different from CaXV (515 mμ). The diversity of viruses found in cacti, both cultivated hybrids and native cacti, lends support to the idea that distinct groups of viruses can exist within an arbitrarily devised group (12).

Further investigations of the cactus viruses from various sources for comparative purposes should further clarify the occurrence and distribution of cactus viruses in the southwestern United States.

## SUMMARY

Viruses in the native cacti in the southwestern desert have been studied. The viral infection of cacti is generally widespread in the Tucson basin area.

I. Saguaro

A. An isometric virus, designated SV, 35 mμ diameter has been isolated. The SV is the first virus isolated from the saguaro. In addition, the SV is larger than the other commonly found isometric viruses found in Arizona.

B. Saguaro virus contains a ribonucleic acid as determined by chemical tests.

C. Calculated sedimentation coefficient Sw,20 of 106, 107 and 112 have been determined from three individual runs.

D. The SV proved to be a good antigen. Antiserum prepared to it reacted strongly to its homologous antigen. The antiserum did not react with any other common isometric viruses, CMV and TRV, from Arizona.

E. Saguaro virus was isolated from the flowers, fruits, nectar, main trunk and arms of the saguaro cactus.

F. A survey showed that 40% (52 of 130) of the saguaros in the general Tucson area were infected with SV. The largest percentage of infection (45%) occurred in the Saguaro National Monument, East.

II. Prickly pears (Opuntia sp.)

A. The Opuntia are infected predominately with what appears to be SOV. This virus forms large inclusion bodies in the cells and causes ring symptoms on the pads. As a result of morphological changes due to rings, some pads are roughened and compressed.

B. The purified virus suspensions have an absorption spectrum shifted from the usual 240 mμ minimum and 260 mμ maximum for plant viruses to a 250 mμ minimum and a 275 mμ maximum. While this could result from impurities, it is suspected that it reflects at least in part, a difference in the chemical composition of the virus from the composition known for TMV.

C. A virus-like chlorotic ring on many of the Opuntia pads was caused by feeding punctures of C. vittiger. Both adults and nymphs can cause the ringspotting that occurs on many of the cacti. A feeding period of 10-15 minutes is sufficient to induce the symptom.

D. The viruses of prickly pear are able to withstand high temperatures (+50 C for considerable periods of time). Direct measurements of pad temperatures show the interior of the pads may be 6-8 C higher than air temperature. Temperatures up to 52 C were recorded in prickly pear tissue (34).

E. Opuntia chlorotica, collected in the Desert Botanical Garden, is infected with a mixture of viruses. Epidermal dips of cut surfaces of the pad revealed rigid rods similar to SOV (15 x 314 $\pm$ 3.1 mμ) as well as flexuous rods (15 x 600 $\pm$ 10.4 mμ). Electron micrographs had only the rigid rod in purified preparations.

F. Antiserum prepared to the rigid rod particle found in O. engelmannii reacts with the crude preparations of O. chlorotica indicating the presence of the shorter virus particle (probably SOV).

The introduction of virus infection in cacti must remain speculative at this time. The viruses have been present for a long time and only recently have detailed studies started. The infected cacti found in Europe may have been infected when they were imported to Europe and other areas for the cochineal dye industry. The cacti infected in North America may represent a natural spread of the cacti from their origin in the Caribbean Islands through South America and Mexico into the United States.

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