AN ABSTRACT OF THE THESIS OF

Anatolia A. Mpunami for the degree of Master of Science in Botany and Plant Pathology presented on October 16, 1986

Title: Purification and Partial Characterization of a Virus Associated with Kongwe Leaf Spot Disease of Sisal

Abstract approved: Redacted for Privacy

Thomas C. Allen

Sisal (Agave sisalana Perrine), one of Tanzania's main cash crops, is severely affected by korogwe leaf spot disease (KLS). Infected plants show chocolate brown, concentric, scablike eruptions on both surfaces of mature leaves, and produce stained, low-quality fiber. A flexuous, rod-shaped virus was isolated from KLS-infected sisal leaves and studied at Oregon State University. This virus was partially characterized by mechanical transmission, host range, cytopathological effects, electron microscopy, serology and determination of some biophysical properties.

The virus was sap-transmissible with difficulty to young sisal and common bean. The sisal plants have, however, not shown KLS symptoms to date. Symptoms induced in beans included severe mosaic, leaf distortion and chlorosis. Occasionally, wilting and death occurred. Virus particles were very flexuous, with a helical substructure and about 12.7 nm diameter. They were consistently associated with KLS infection. Tendency for breakage in negative
stains, aggregation and adsorption onto plant tissue obscured determination of particle modal length. Lengths ranged between 100 nm and 4,000 nm with four modes at 950, 1075, 1450 and 1650 nm.

Cells of infected sisal and bean leaves contained aggregates of virus particles, and characteristic closterovirus inclusions in phloem tissue. None were found in cells of healthy plants.

The virus was best purified by gentle grinding with a mortar and pestle, precipitation with Polyethylene glycol (PEG), and rate-zonal centrifugation in sucrose/Cs₂SO₄ cushion gradients. Two bands were formed in these gradients; one at 5.5 cm from the top contained mainly fragmented particles, and the other at 8.0 cm contained intact particles, as well as the aggregated ones.

The purified virus had a typical closterovirus UV-absorption spectrum, with A_{260/280} ratio of 1.61, and A_{max/min} ratio of 1.21. In Cs₂SO₄ equilibrium density gradients, the fraction with intact virus particles had a buoyant density of 1.26 g/cc, while the fraction with predominantly particle fragments had a density of 1.23 g/cc.

Antiserum prepared against partially purified virus reacted homologously in microprecipitin tests, immunospecific electron microscopy (ISEM) and ELISA. It also reacted to citrus tristeza virus (CTV). Similarly, antisera to CTV produced in Switzerland and Florida reacted strongly to KLS-associated virus. The two viruses were however serologically distinguishable in reciprocal ISEM tests. The virus was not serologically related to a virus infecting white lupines at Oregon State University.
In microprecipitin tests, antisera to purified virus reacted homologously. However, antiserum to the fraction containing virus fragments did not react or reacted weakly with preparations containing unbroken particles. Antiserum to the latter reacted with both virus fractions.

KLS-associated virus was detected by ISEM in some symptomless sisal plants. The produced antiserum should therefore enhance detection of latent field infections. Previously undetected virus-infected plants could be eliminated, thus reducing spread of the disease through propagation material. KLS-associated virus is probably a new Closterovirus. Further characterization of this virus is essential for verification.
Purification and Partial Characterization of a Virus Associated with Korogwe Leaf Spot Disease of Sisal

by

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Finally, my two sisters Angela and Leonalda who lovingly cared for my son during this long period of separation deserve more than words can say. Non-tiring encouragements from family and friends made the environment good to work in and are appreciated. I dedicate this work to my son, Bahati Wendo Mwapachu, and my family for their love, patience and endurance.
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Sisal fiber, a long, creamy, natural fiber of very high tensile strength used in the rope and twine industries, is derived from the sisal plant (*Agave sisalana* Perrine). The crop is perennial, with a productive cycle of 10-12 years (Lock, 1969). Leaf harvesting for fiber extraction begins at 1-1/2 - 4 years after planting when leaves have attained a length of 60 cm (2 ft), and is subsequently repeated at one year interval until the end of cropping cycle (Lock, 1969).

Sisal production is limited to tropical and sub-tropical areas (Keswani *et al.*, 1982). Tanzania is one of the major producing countries, and sisal is among its main exports (Acland, 1971). The two commercial cultivars grown in Tanzania are common sisal (*Agave sisalana*) and Agave hybrid 11648, with respective potential yields of 17.5 - 20.0 and 50.0 tons fresh leaves per cycle (Lock 1969).

Sisal is affected by few important diseases. Of these, Korogwe leaf spot (KLS), whose cause is not yet known, has recently become a potential threat particularly to the high-yielding hybrid 11648 (Keswani *et al.*, 1982). First reported in 1951 on only one plantation, KLS has now spread to most of the main growing regions in the country. This disease, however, has not been reported to occur in any other sisal growing country.
The pathogen attacks leaves and the disease is more severe on high yielding hybrid sisal. Spread of the disease has been difficult to control for lack of knowledge about the causal organism. The present recommendation is to reduce severity of infection by shortening the harvesting cycle to less than a year. This practice was approximated to cause a 10% reduction in the potential yield of hybrid 11648 (Mwenkalley, 1978).

The etiology of KLS has remained obscure for 35 years since its discovery. Present knowledge about the disease is discussed in detail by Keswani and Mwenkalley (1982). Previous studies excluded fungi, bacteria, insect damage and nutritional deficiency as possible causal agents (Anon 1957, 1974, 1976; Lock, 1969; Keswani et al., 1982).

In previous experiments (unpublished), I was able to reproduce disease symptoms on healthy hybrid 11648 plants inoculated mechanically with KLS-infected sap. In addition, an unidentified rod-shaped virus was detected on two different occasions in samples sent to colleagues for electron microscopic observation. During early attempts to recover virus particles from KLS infected tissue, I detected long and very flexuous virus particles in sap of sisal leaves with KLS symptoms. None were detected in the leaves of young plantlets (bulbils).

The objectives of this study were:

(i) To develop a method of purifying the detected virus from KLS infected tissue in relatively pure and large amounts for antiserum production and serology.
(ii) To describe characteristics of that virus.

(iii) To establish whether or not the detected virus is the causal pathogen for korogwe leaf spot disease.
Korogwe leaf spot disease

Korogwe leaf spot, a necrotic leaf disorder of sisal (*Agave sisalana*), is characterized by chocolate brown, concentric, scablike eruptions on both surfaces of mature leaves (Fig. 1). Spots range from 1-30 mm diameter, and in severe cases, coalesce to cover a large portion of the leaf surface (Lock, 1969; Fig. 1). The disease, which is more severe on high yielding hybrid sisal, causes brown staining of fiber and reduces tensile strength, subsequently lowering quality. Severely infected leaves are unsuitable for fiber extraction (Mwenkalley, 1978; Keswani *et al.*, 1982).

This disease has so far been reported to occur only in Tanzania. First noticed on *A. sisalana* at a plantation near Korogwe in 1951, it gradually spread to neighboring plantations, and has now covered most of the major sisal growing regions in the country (Keswani *et al.*, 1982). Lack of knowledge about the causal organism and expansion of acreage under the susceptible hybrid 11648 are probably the main factors contributing to the rapid spread of this disease.

A detailed study of symptom development (Anon, 1955; Lock, 1969; Keswani *et al.*, 1982) revealed that internally the disorder starts with necrosis of the cell at the base of the stoma. The necrosis later spreads to the guard cells and adjoining tissues of the leaf. A cambium forms in response to infection, and cells in the infected area become suberized. When suberization reaches the
leaf surface, the epidermis cracks and a tiny spot, the size of a pinhead, appears. The spot continues to expand with time as more cells become infected and suberized, eventually producing the characteristic leaf spot, with concentric, brown eruptions of scab appearance.

During the first 20 years after its discovery, various attempts were made by different researchers to isolate fungi or bacteria from KLS infected leaves (Ebbels and Allen, 1979; Keswani and Mwenkalley, 1982). However, no fungal isolate obtained could reproduce KLS symptoms in inoculated healthy sisal. It was therefore decided that fungi present at the infection site were saprophytes (Ebbels and Allen, 1979).

Damage by sucking insects was also suspected as a possible cause of the disorder. However, experiments conducted at Mlingano Research Station (Anon, 1957) and studies on the insects that inhabit the sisal plant, demonstrated no relationship between the disease and the insect fauna (Lock, 1969).

Korogwe leaf spot is normally severe on sisal raised on poor soils, particularly those deficient in potassium. Based on this observation, it was considered possible that KLS was a deficiency disorder (Lock, 1969). The adopted standard practice of applying muriate of potash (60% K₂O) at a rate of 50 Kg/ha did not ease the problem. Furthermore, experiments conducted to determine the effect of various levels of combinations of potassium, nitrogen, and phosphorus, indicated that disease occurred even in plots with high levels of various fertilizer combinations (Anon, 1968, 1974,
1976). This led to the conclusion that KLS is not a deficiency disorder.

Sisal is vegetatively propagated from suckers (miniature plants arising from rhizomes) and bulbils (tiny plantlets borne in flower axils on inflorescences). A characteristic feature of KLS is that plants raised from suckers generally develop disease symptoms much earlier in the field than those raised from bulbils (Lock, 1969). Symptoms are also more severe on mature plants (2-1/2 - 3 years) than on younger ones. Under subjective conditions, however, young plants can develop disease symptoms in the field at an earlier age. This was reported at Mlingano Research Station (Anon, 1965) when young, potted plants of hybrid 11648 developed KLS symptoms within six months when placed among severely infected mature plants in the field. Keswani and Mwenkalley (1982) also reported locating young plants of Agave hybrid 11648 in the field that had succumbed to the disease.

During sap inoculation experiments that I conducted in the greenhouse in 1978-1980 (unpublished), disease symptoms developed on leaves of inoculated healthy hybrid 11648 plants within eleven months (Fig. 2A). Some virus indicator plants were also inoculated with infected sap. Phaseolus vulgaris cvs. Black Turtle Soup 2 and Long Tom developed severe mosaic and leaf puckering one month after inoculation (Fig. 3A). Chenopodium quinoa, Cucumis sativus cv. National Pickling and Nicotiana tabacum cvs. Debneyi, Samsun, White Burley and N. clevelandii did not show any symptoms when inoculated.
FIG. 1 Korogwe leaf spot symptoms on mature sisal leaves. Spots on lower leaf surface and in different developmental stages. Large spots coalescing.
The Closteroviruses

Closteroviruses are a group of plant viruses with long, very flexuous, rod-shaped particles of about 12 ± 1 nm diameter. The group name which was approved in 1975 (Shepherd et al., 1976) bears special reference to this characteristic and was derived from the Greek word 'kloster' which means spindle thread (Matthews, 1982). The type member is beet yellows virus (BYV). Group members have diverse properties but they share similarities in particle morphology and biochemistry, as well as biological characteristics. A very characteristic open and flexible structure with helical symmetry of pitch 3.4 - 3.8 nm is a typical feature of virus particles in the group (Bar-Joseph and Murant, 1982; Francki et al., 1985).

Individual Closteroviruses have restricted or moderately wide host ranges, and generally the main symptoms induced are yellowing with necrotic spots. In some cases infection results in decline and death of affected plants. These symptoms are a reflection of these viruses' ability to infect phloem tissue causing necrosis, in addition to severe derangement of chloroplasts (Duffus, 1973; Lister and Bar-Joseph, 1981). For most Closteroviruses, infection is confined to the phloem and phloem parenchyma of their hosts. Virus particles often aggregate in characteristic patterns in the cytoplasm of infected cells, and are associated with accumulation of membranous vesicles (Bar-Joseph et al., 1979; Lister and Bar-Joseph, 1981; Bar-Joseph et al., 1982; Francki et al., 1985). Infection by
Closteroviruses also causes accumulation of phytoferritin and starch grains in the chloroplasts of infected cells (Esau, 1968; Yamashita et al., 1979).

Both monocots and dicots are hosts of Closteroviruses. None of the reported members, however, have been shown to infect both plant groups (Bar-Joseph et al., 1979). Distribution is restricted for most Closteroviruses, except for citrus tristeza virus (CTV), beet yellows (BYV), apple chlorotic leaf spot (ACLSV) and carnation necrotic fleck (CNFV), whose distributions are reported worldwide (Bar-Joseph et al., 1979). The majority of Closteroviruses are transmitted semipersistently by aphids, one is transmitted by mealybugs (Francki et al., 1985) and several by whiteflies (Yamashita et al., 1979; Francki et al., 1985). Mechanical transmission by various inoculation techniques is possible for many Closteroviruses but only with difficulty (Bar-Joseph and Smookler, 1976; Garnsey et al., 1977; Russell, 1970).

Thermal inactivation points (10 min) range between 40-63 °C, dilution end points between $10^{-3} - 10^{-5}$ and longevity in vitro at room temperature (20-25 °C) between one and four days. These physical properties are rather poorly defined for Closteroviruses because their determination is influenced by factors such as pH, extract composition, virus concentration and duration in the host plant, as well as their tendency to aggregate and apparent presence of inhibitors in their hosts (Lister and Bar-Joseph, 1981).

The particles contain a single molecule of ssRNA which constitutes 5% of the particle weight. This is reflected in the
ultraviolet absorbance curves with $A_{260/280}$ ratios of about 1.1-1.2 (Lister and Bar-Joseph, 1981; Bar-Joseph and Murant, 1982). The absorbance curves of some Closteroviruses, e.g. BYV, however, show anomalous ratios of 1.5 or more, suggesting nucleic content of more than 5%. This was shown to be due to a low content of aromatic amino acids in the coat protein subunits (Bar-Joseph and Hull, 1974).
Identification and adequate description of a new virus is a key factor in assigning it to a particular virus taxonomic group (Hamilton et al., 1981). Information that is considered essential for adequate identification and subsequent description of any virus (Hamilton et al., 1981) includes the use of the virus both in crude sap and purified preparations to reproduce the disease symptoms. A detailed description of hosts and symptoms, mode of transmission and the type(s) of virus particles observed in negatively stained preparations in the electron microscope are other factors. Use of the light and electron microscopes to study the cytopathological effects of the virus on infected tissue is also an important aspect of the characterization process. Finally, a description cannot be complete without producing highly purified preparations for determining the physical-chemical properties of the virus, its coat protein and nucleic acid components, as well as producing high quality antiserum to the virus.

Characterization and hence classification of Closteroviruses have been evolving gradually as more information on the physical and biological properties of the individual members is obtained. Properties used on most occasions included particle size, coat protein subunit molecular weight, coat protein composition, vector transmissibility and production of characteristic intracellular inclusions.
Bar-Joseph et al. (1979) subdivided Closteroviruses into two groups, A and B, based on the molecular weight of coat protein subunits and vector transmission. Subgroup A consisted of ten members (Table 1) which are aphid transmitted and have a coat protein of 23-25 x 10^3 daltons. This group also induces characteristic cytoplasmic vesicles in infected cells. It was designated typical Closteroviruses. Subgroup B, which was designated as atypical Closteroviruses, included three viruses (Table 1) with coat protein subunits of 27 x 10^3 daltons. These do not induce formation of membranous vesicles and vector transmission for them is unknown.

Approaching the subject from the viewpoint of comparative diagnosis, Lister and Bar-Joseph (1981) based their classification on particle length. They also came up with two subgroups, I and II. Subgroup I contained ten viruses (Table 1) with particle modal lengths above 1,000 nm, while subgroup II contained four viruses (Table 1) with modal lengths of less than 1,000 nm. Except for lilac chlorotic leaf spot virus, which is not aphid transmitted, all viruses in Subgroup I are aphid-borne, are associated with intracellular inclusions, including characteristic vesicles, and have coat protein subunits of about 23-25 x 10^3 daltons. Subgroup II members, on the other hand, are not aphid borne, are not associated with formation of characteristic vesicles and have coat protein subunits of about 27 x 10^3 daltons. The exception to this group is apple chlorotic leafspot virus, which has a coat protein subunit of 23.5 x 10^3 daltons. These two classification approaches
### TABLE 1 Viruses included in the Closterovirus group by Bar-Joseph et al. 1979.

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<td>Chenopodiaceae</td>
<td>Aphids</td>
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<td>Umbelliferae</td>
<td>Aphids</td>
<td>23.5</td>
<td>2.2</td>
</tr>
<tr>
<td>Apple chlorotic leaf spot (ACLSV)</td>
<td>720 x 12</td>
<td>Rosaceae</td>
<td>*</td>
<td>23.5</td>
<td>2.3</td>
</tr>
<tr>
<td>Festuca necrosis (FNV)</td>
<td>1725 x 18</td>
<td>Gramineae</td>
<td>Aphids</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Subgroup B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*Lilac chlorotic leaf spot (LCLV)</td>
<td>15-1600 x 12</td>
<td>Oleaceae</td>
<td>*</td>
<td>27.0</td>
<td></td>
</tr>
<tr>
<td>Apple stem grooving (ASGV)</td>
<td>620 x 12</td>
<td>Rosaceae</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potato virus T (PVT)</td>
<td>640 x 12</td>
<td>Solanaceae</td>
<td>*</td>
<td>27.0</td>
<td>2.2</td>
</tr>
</tbody>
</table>

*Lister and BarJoseph (1981) re-grouped the Closteroviruses into subgroups I and II basing on particle modal length. Subgroup I replaced A and II replaced B. Viruses prefixed b were shifted to subgroup II. The one prefixed a was moved to subgroup I. A new addition (1981) to the list is prefixed c.

* - The vector is not known.
ended up with basically the same members in the same groupings except for a few that were interchanged (Table 1).

With more information on similarities in the physical and biological properties, Bar-Joseph and Murant (1982) classified Closteroviruses into three subgroups, A, B, and C (Table 2). Particle length, sedimentation coefficients and molecular weight of RNA molecule were the classification criteria. They indicated that the differences in particle lengths between subgroups are reflected in genome sizes, and that the virus particles have similar ratios of RNA mass to modal length among Closteroviruses.

Subgroup A members have modal lengths of about 730 nm, sedimentation coefficients ca 96 S and contain a single molecule of ssRNA of molecular weight ca $2.5 \times 10^6$ daltons (Table 2). Subgroup B contained three members characterized by modal lengths of 1,250-1,450 nm, sedimentation coefficients of ca 110 S and ssRNA molecule of about $4.7 \times 10^6$ daltons. Assigned to subgroup C were viruses with modal lengths of 1,650-2,000 nm, sedimentation coefficients of ca 140 S and ssRNA molecule of $6.5 \times 10^6$ daltons. For all three subgroups, the RNA molecule constitutes 5% of particle weight.

Francki et al. (1985) have recently re-classified Closteroviruses into three subgroups, I, II and III (Table 2). Their basis for characterization of subgroups is basically the same as that used by Bar-Joseph et al. (1979). Subgroups I and II are the subgroup A of 1979 divided rather arbitrarily into those with long and short particles; while former subgroup B forms the new
subgroup III. In addition to the characteristics previously used, Francki et al. (1985) considered information available on serological relationships between subgroup members. For members of subgroup I (Table 2) a strong serological relationship has been detected between carnation necrotic fleck virus (CNFV) and beet yellows virus (BYV), while CNFV and wheat yellow leaf virus (WYLV) are distantly related. None of the other members are serologically related.

Members of subgroup II have similar characteristics to those in subgroup I except that the modal lengths of the former are shorter. Except for heracleum latent virus which is aphid transmitted in the presence of a helper virus, no vector has been found for members of subgroup II. Also, members of subgroup II are not serologically related. Another difference between members of subgroups I and II is in their cytopathological effects. Virus particles of subgroup I Closteroviruses accumulate in the phloem and phloem parenchyma in a characteristic manner, being aligned in ranks to give cross-banded inclusions visible in both the light and electron microscopes. This characteristic is not apparent for members of subgroup II. Furthermore, many subgroup I Closteroviruses induce in host cells what appear in the light microscope as amorphous cytoplasmic inclusions. In thin sections these are seen to consist mainly of membranous vesicles together with virus particles and normal components of the cytoplasm. These vesicles which were designated 'BYV-type' have not been demonstrated in tissue infected with subgroup II Closteroviruses, except for two potential group members,
grapevine leafroll associated virus (GLR-AV) and cucumber yellows virus (CuYV).

Subgroup III Closteroviruses are the same as those of subgroup B of Bar-Joseph et al. (1979). These do not induce BYV-type vesicles and their particle fine structure is not the typical open type. Cytopathological effects of lilac chlorotic leafspot virus (LCLV) include proliferation of the endoplasmic reticulum. The other two members have not been investigated. Only two members, apple stem grooving virus (ASGV) and potato virus T (PVT) are serologically interrelated. It was suggested by Bar-Joseph et al. (1979) that members of subgroup III are different from other Closteroviruses and may eventually form a basis for a separate new virus group.
TABLE 2  Viruses included in the Closterovirus group* according to Francki et al. 1985.

<table>
<thead>
<tr>
<th>Virus and acronym</th>
<th>Modal Length (nm)</th>
<th>Pitch of helix (nm)</th>
<th>Location in tissues</th>
<th>Type of vesicles induced</th>
<th>Vectors</th>
<th>Coat protein (MW x 10^3)</th>
<th>Nucleic acid (MW x 10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Subgroup I</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Members</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*Beet yellows (BYV)</td>
<td>1250-1450</td>
<td>3.0-3.4</td>
<td>Phloem (epidermis and parenchyma in local lesions)</td>
<td>BYV-type</td>
<td>Aphids</td>
<td>22.5-23.5</td>
<td>4.0-4.7</td>
</tr>
<tr>
<td>*Carnation necrotic fleck (CNFV)</td>
<td>1100-1250, 1400-1500</td>
<td>3.4</td>
<td>Phloem, epidermis, parenchyma, xylem</td>
<td>BYV-type</td>
<td>Aphids</td>
<td>23.5</td>
<td>3.95</td>
</tr>
<tr>
<td>Citrus tristeza (CTV)</td>
<td>- 2000</td>
<td>3.7</td>
<td>Phloem</td>
<td>BYV-type</td>
<td>Aphids</td>
<td>25.0</td>
<td>6.3-6.9</td>
</tr>
<tr>
<td>*Beet yellow stunt (BYSV)</td>
<td>- 1400</td>
<td>ND</td>
<td>Phloem, phloem-parenchyma</td>
<td>BYV-type</td>
<td>Aphids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Burdock yellows (BDYV)</td>
<td>1600</td>
<td>3.6</td>
<td>Phloem</td>
<td>BYV-type</td>
<td>Aphids</td>
<td></td>
<td></td>
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<tr>
<td>Carrot yellow leaf (CTLV)</td>
<td>1600</td>
<td>3.7</td>
<td>Phloem</td>
<td>BYV-type</td>
<td>Aphids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clover yellows</td>
<td>1700-1800</td>
<td>3.7</td>
<td>Phloem</td>
<td>BYV-type</td>
<td>Aphids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wheat yellow leaf (WYLV)</td>
<td>1600-1850</td>
<td>3.4</td>
<td>Phloem</td>
<td>BYV-type</td>
<td>Aphids</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Possible Members</strong></td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Alligatorweed stunting (AWSV)</td>
<td>1717</td>
<td></td>
<td>Phloem</td>
<td>BYV-type</td>
<td>Aphids</td>
<td></td>
<td></td>
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<tr>
<td>Dendrobium vein necrosis (DVNV)</td>
<td>1865</td>
<td></td>
<td>Mesophyll</td>
<td>Mitochondrial vesicles</td>
<td>Aphids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Festuca necrosis (FNV)</td>
<td>1725</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Heracleum virus 6 (HV6)</td>
<td>1600</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td><strong>Subgroup II</strong></td>
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<tr>
<td><strong>Members</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apple chlorotic leaf spot (ACLSV)</td>
<td>720</td>
<td>3.8</td>
<td>Parenchyma</td>
<td>None found</td>
<td>ND</td>
<td>23.5</td>
<td>2.15-2.48</td>
</tr>
<tr>
<td>Grapevine virus A (GVA)</td>
<td>800</td>
<td>3.6-4.0</td>
<td>Parenchyma</td>
<td>None found</td>
<td>Mealy-bug</td>
<td>22.0</td>
<td>2.55</td>
</tr>
<tr>
<td>Heracleum latent (HLV)</td>
<td>730</td>
<td>3.8</td>
<td>Phloem, phloem-parenchyma</td>
<td>ND</td>
<td>Aphids</td>
<td>23.5</td>
<td>2.15-2.52</td>
</tr>
</tbody>
</table>

*(continued)*

(continued)
### TABLE 2 (continued)

<table>
<thead>
<tr>
<th>Possible Members</th>
<th></th>
<th>1000</th>
<th>ND</th>
<th>ND</th>
<th>Phloem</th>
<th>ND</th>
<th>BYV-type</th>
<th>ND</th>
<th>Whitefly</th>
<th>ND</th>
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<tbody>
<tr>
<td>Cucumber yellows (CuYV)</td>
<td></td>
<td>650</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td>ND</td>
<td></td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Citrus tatter leaf (CiTLV)</td>
<td></td>
<td>~1000</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td>ND</td>
<td></td>
<td>ND</td>
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<tr>
<td>Grapevine leafroll associated virus (GLR-AV)</td>
<td></td>
<td>755</td>
<td>3.8</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td>ND</td>
<td></td>
<td>ND</td>
<td></td>
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<tr>
<td>Grapevine virus B (GVB)</td>
<td></td>
<td>ND</td>
<td>3.9</td>
<td>ND</td>
<td>Phloem</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>None found</td>
<td>ND</td>
</tr>
<tr>
<td>Nandina stem pitting (NSPV)</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td>ND</td>
<td></td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

**Subgroup III**

<table>
<thead>
<tr>
<th>Lilac chlorotic leaf spot (LCLV)</th>
<th>~1540</th>
<th>3.7</th>
<th>Phloem-parenchyma, mesophyll</th>
<th>Dilated endoplasmic reticulum</th>
<th>ND</th>
<th>27.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apple stem grooving</td>
<td>600-700</td>
<td>3.4-3.8</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>Potato virus T (PVT)</td>
<td>610-640</td>
<td>3.4</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td>ND</td>
</tr>
</tbody>
</table>

| ND - Not determined |

*BarJoseph and Murrant (1982) used separate criteria to classify Closteroviruses, but also produced three subgroups. Viruses they placed into subgroup A forms subgroup III of this table. Subgroups B and C make up subgroup I. Former subgroup B are marked with an asterisk.*
Purification of Closteroviruses

The purification and concentration of most Closteroviruses was previously reported to be difficult (Price, 1966; Bar-Joseph et al., 1970, 1972, 1974; Lister and Hadidi, 1971; Polak, 1971; Smookler, et al., 1974) because purified preparations usually contained virus particles that were damaged and were not pure enough for biophysical and biochemical characterization. Reasons given for those difficulties include a relatively low content of some of these viruses in infected plants, likelihood of the particles to break and shear under mechanical stresses during extraction and purification, and tendency of particles to aggregate as well as adsorb onto host contaminants. Some viruses are unstable in unfixed form to CsCl. Others, particularly citrus tristeza virus (CTV), are restricted to the phloem cells, which necessitates the use of large quantities of tissue containing phloem for purification. A number of recommendations have subsequently been made regarding points to consider when purifying Closteroviruses (Bar-Joseph and Hull, 1974; Lister and Bar-Joseph, 1981; Hamilton et al., 1981; Bar-Joseph et al., 1979). These include:

(1) Avoiding shearing of virus particles during extraction by gently grinding tissue with a mortar and pestle, as opposed to homogenization in a blender. Bar-Joseph et al. (1970) reported that mechanical homogenization caused so much breakage of CTV particles that only 5% were in the normal range of 1900-2000 nm. More than 60% of the particles were shorter than 500 nm. They
showed that gentle grinding was the only way to preserve these long particles.

(ii) Extraction in relatively high ratios of buffer to leaf weight helps to extract more virus from the tissue.

(iii) Ultracentrifugation which exposes virus particles to prolonged high centrifugal forces thereby causing breakage and aggregation is avoided by precipitating the virus with Polyethylene glycol (PEG).

(iv) Tendency for adsorption onto host components, another main cause of virus loss, is reduced by use of high molarity buffer which helps to break up bonds between the particles and host material.

(v) Use of chelating agents, like EDTA (Ethylene diamine tetracetate), or detergents, such as Triton X-100 in the extraction buffer helps reduce hydrophobic bonds between virus particles, hence reducing aggregation.

(vi) Though not essential, reducing agents like Na$_2$SO$_3$ are useful in reducing colored oxidation products from the preparation.

(vii) Clarification is considered a key step for good virus yields, and 'mild' reagents like bentonite are recommended for use in clarifying the sap to remove plant material.

(viii) Provision of an environment for maintaining particle integrity and restricting RNase activity on particle RNA is another important factor. This is achieved by maintaining high pH of extraction and resuspension buffers, and in some cases including divalent cations (Mg$^{++}$) which are essential for bonding subunits of virus coat protein.
For viruses like CTV and BYV which are sensitive to CsCl, the virus should be fixed with formaldehyde before loading onto CsCl gradients, or the milder Cs$_2$SO$_4$ are used instead in density gradients.

Finally, those viruses which tend to aggregate when density gradients are overloaded, are preserved by loading only small volumes of the virus preparation onto the gradients.

Despite all these considerations, yields for Closteroviruses are generally low, averaging about 0.1-10 mg/100 g of leaf tissue (Lister and Bar-Joseph, 1981). This is due to other factors such as the host plant from which the virus is purified, growth conditions for that host, time of harvest and the type of tissue harvested which may influence virus yields (Bar-Joseph and Loebenstein, 1973). Therefore, there is no generalized purification procedure for all Closteroviruses, or even for members of one subgroup. However, reasonably good results are possible if the above named principles are considered when developing a purification scheme. Procedures have already been developed for various Closteroviruses which provide adequate amounts and purity for physical, chemical and serological studies (Bar-Joseph and Hull, 1974; Kassanis et al., 1977; Gonsalves et al., 1978; Tsuchizaki et al., 1978; Bar-Joseph et al., 1985; Inouye, 1976; Lister and Hadidi, 1971; Salazar and Harrison, 1978; Brunt, 1978; Bem and Murant, 1979; DeSequeira and Lister, 1969).
Diagnosis of Closterovirus Infections

Symptom expression in natural and propagation hosts is a good diagnostic tool for most virus infections. This method, however, presents special problems for Closteroviruses since these viruses are not readily sap transmissible from their natural hosts, the content of particles may be low, and often the natural hosts contain inhibitors or protein-precipitating substances such as tannins and oxidizable polyphenols that hinder sap transmission (Lister and Bar-Joseph, 1981). Another factor is the perennial nature of most hosts for Closteroviruses in which symptom production is normally slow. Furthermore, Closterovirus host ranges are fairly restricted and symptoms are often not very distinctive in their natural hosts or in the test plants available. Under these circumstances, light and electron microscopy of virus particles and their associated subcellular inclusions and characteristic vesicles offer a better diagnostic tool.

As indicated earlier (Section 3), ultrastructural peculiarities characterize only viruses in subgroup I. Viruses in this subgroup induce formation of amorphous cytoplasmic inclusions which are visible by light microscopy as compact structures with occasional aleoration. By electron microscopy, these are seen as accumulations of membranous vesicles containing a network of fibrillar material together with varying amounts of viral particles, ribosomes and flaky or granular electron-dense material (Esau, 1968; Martelli and Russo, 1977). Other inclusions which appear to be fibrous or banded
were identified by electron microscopy as respectively aggregates of virus particles without a precise order, and those having a periodicity due to orderly arrangement of virions in stacked layers. Inclusions of this kind are absent in tissues infected by viruses in subgroups II and III.

Serological testing is another useful tool for Closterovirus diagnosis, especially when the tests can be applied directly to the natural hosts, thus circumventing the need for propagating the virus in a culture host. A range of serological procedures including tube precipitin and microprecipitin tests (Russell, 1970), latex flocculation (DeSequeira and Lister, 1969), SDS-immunodiffusion (Gonsalves et al., 1978), fluorescent antibody (Tsuchizaki et al., 1978) and ELISA (Bar-Joseph et al., 1979a) have been used for routine diagnostic testing of Closteroviruses. Due to its specificity and accuracy, and requirement for small volumes of testing material, ELISA was singled out as the most promising technique for large scale routine diagnosis of Closteroviruses (Lister and Bar-Joseph, 1981). They also pointed out that although Closteroviruses are moderately immunogenic in rabbits, complications may arise in the use of antisera because these antisera are most likely mixtures of antibodies. This situation arises from dissociation of virus particles both during virus purification and after introduction into the rabbits, leading to production of antibodies that recognize whole, undissociated particles, as well as those that recognize only various quarternary associations of protein subunits. Such possibilities may affect the ability of
different antisera to detect their homologous viruses, and also influence interpretations of indications of heterologous relationships. It was therefore emphasized that caution and careful standardization is essential in the use of Closterovirus antisera, particularly for studying serological relationships.
MATERIALS AND METHODS

Source and propagation of virus

Symptomatic leaves from field-infected 3-year-old hybrid sisal (No. 11648) and 17 bulbils (plantlets) of the same cultivar were provided by Agricultural Research Station, Mlingano, Tanzania. Random samples of KLS-infected leaves, and leaves from three randomly selected bulbils were used to prepare leaf dip preparations for observation in the electron microscope. Other samples were fixed for sectioning and electron microscopic studies. The rest of KLS-infected leaves were immediately frozen at -20 °C until used for virus extraction.

The bulbils were potted in equal parts soil, sand and vermiculite, and maintained in growth chambers. The growth chambers were regulated for a 27:21 °C temperature regime with a 12-hr photoperiod of 2,000-3,000 lux. Plants were watered once or twice a week and fertilized with RAPID'GRO Plant Food (23-19-17% NPK) as necessary. Three months after planting all sisal plants were again checked for presence of virus by inoculating sap from each onto unifoliate leaves of three bean plants (cv. Black Turtle Soup 2). After this assay, four sisal plants were selected at random and inoculated with KLS-infected sap extracted from frozen leaves. Another batch of two sisal plants were inoculated one month later with a partially purified virus preparation.

Sap for inoculation was first prepared by grinding KLS-infected tissue with a mortar and pestle in two volumes (w/v) of 0.1 M
phosphate buffer, pH 7.0. The extract was filtered through cheesecloth and used directly for inoculation. Since percentage infection was very low with this method of preparing sap, it was modified. KLS-infected tissue was ground to powder in liquid nitrogen with a mortar and pestle. The powder was thawed with two volumes (w/v) of 0.1 M phosphate buffer pH 7.0, containing 1% nicotine sulfate. The extract was expressed through cheesecloth, and the pulp extracted again with the same volume of buffer. The two filtrates were pooled and centrifuged at 12,100 g for 15 min. The supernatant was decanted and used for inoculation.

Four expanded leaves on each of the selected six disease-free sisal plants and unifoliate leaves of ten healthy 'Black Turtle Soup 2' beans were dusted with 400-mesh carborundum powder. These leaves were then rubbed with cotton swabs soaked with the inoculum, and excess carborundum was rinsed off. Control plants were mock inoculated with buffer. Inoculated sisal plants were returned to the growth chambers and maintained at the conditions described above. The beans were kept on a greenhouse bench and maintained at 24:21 °C with a 12-hr photoperiod.

**Host range**

Several herbaceous test plants were inoculated with sap from KLS-infected sisal leaves. They include: *Chenopodium quinoa* Willd., *C. amaranticolor* Coste and Reyn., *C. foliosum*, *Gomphrena globosa* L., *Nicotiana clevelandii* Gray, *N. debneyi* Domin., *N. tabacum* x *N. glutinosa* cv. samsun NN., *Datura stramonium* L., *Cucumis sativus* L.
cv. 'National Pickling' and *Phaseolus vulgaris* cvs. 'Bountiful' and 'Black Turtle Soup 2.' After inoculation all were kept in the greenhouse and maintained at 24:21 °C temperature regime with 12-hr photoperiod. Only *C. quinoa*, *D. stramonium* and the bean cultivars were later checked by electron microscopy and microprecipitin tests for presence of virus particles.

**Purification**

Virus purification was attempted from both symptomatic mature leaves of Agave hybrid 11648 and 'Black Turtle Soup 2' beans. Mechanical homogenization in a blender was compared with gentle grinding of tissue with a mortar and pestle in the presence of liquid nitrogen for its effect on particle integrity. Additives were also tested for their effectiveness in improving virus extraction and sap clarification. Nicotine sulfate and sodium sulfite were used for their capacity to complex polyphenolic compounds and subsequently prevent oxidation and inhibition of the virus (Francki, 1972). Chloroform, n-butanol and bentonite were used to remove chloroplasts and other host components (Francki, 1972; Dunn and Hitchborn, 1965). Magnesium ions (Mg^{2+}) in combination with other additives were also tested for their effect on clarification and particle integrity. The effects of the various substances and methods were assessed by electron microscopy; a preparation from each method was stained on a formvar-coated grid and virus particles were counted in ten randomly selected grid openings.
A number of purification procedures were tried, including those by Hiebert and MacDonald (1973) for purification of Potyviruses, Goodell (1983) for purification of a lentil strain of pea seedborne mosaic virus, and Price (1966), Bar-Joseph et al. (1970), Gonsalves et al. (1978), and Bar-Joseph et al. (1985) for purification of CTV. Others were Bar-Joseph et al. (1974) for purification of beet yellows virus, and Hampton (personal communications, June 1986) for purification of pea leafroll virus. Outlines for these procedures are provided in the Appendix. In some cases, however, the original procedures were modified to suit the requirements of the KLS-associated virus, and the outlines are presented in the modified form. Procedures which gave satisfactory partially purified preparations for virus characterization and production of antiserum are shown in Tables 3 to 5.
TABLE 3 Partial purification procedure for the KLS-associated virus by ultracentrifugation

1. Grind KLS-infected leaves to powder in liquid nitrogen with a mortar and pestle.
2. Thaw the powder with 3X (w/v) 0.05 M Tris (tris hydroxymethyl aminomethane)-HCl buffer, pH 7.4. Strain through cheesecloth. Save filtrate.
3. Extract the pulp twice with the same buffer volume, and combine all three filtrate portions.

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>4a. Centrifuge the filtrate 20 min at 12,100 g. Save supernatant.</td>
<td>4b. Centrifuge filtrate 10 min at 4,000 g. Save supernatant.</td>
</tr>
<tr>
<td>5a. Centrifuge supernatant at 29,000 rpm in 50.2 Ti rotor for 1.30 hrs. Discard supernatant.</td>
<td>5b. Centrifuge 5 min at 8,000 g. Save supernatant.</td>
</tr>
<tr>
<td>6a. Dissolve pellet in 1/5 volume 0.01 M Potassium phosphate buffer pH 7.0. Stir at least 2 hrs at 4 °C.</td>
<td>6b. To every 100 ml of supernatant, add 4 g PEG (8,000 MW) and 0.85 g NaCl. Stir at 4 °C for 1 hr, then centrifuge 15 min at 16,000 g. Discard supernatant.</td>
</tr>
<tr>
<td>7a. Centrifuge suspension 15 min at 12,000 g. Save supernatant.</td>
<td>7b. Resuspend pellet in 1/5 volume of 0.04 M sodium phosphate buffer, pH 8.2. Stir at least 2 hr at 4 °C.</td>
</tr>
<tr>
<td>8a. Repeat steps 5, 6, and 7, but dissolve the pellet in 1 ml of double distilled water.</td>
<td>8b. Centrifuge 10 min at 5,000 g. Save supernatant.</td>
</tr>
<tr>
<td></td>
<td>9b. Centrifuge 29,000 rpm in 50.2 Ti rotor for 1.30 hr. Discard supernatant.</td>
</tr>
<tr>
<td></td>
<td>10b. Resuspend pellet in 1 ml 0.04 M sodium buffer pH 8.2. Stir at least 2 hr at 4 °C, and centrifuge 10 min at 12,100 g. Save supernatant.</td>
</tr>
</tbody>
</table>

*Method A according to Price, 1966.

TABLE 4 Purification of the KLS-associated virus with minimal ultracentrifugation

1. Grind KLS-infected tissue to powder in liquid nitrogen with a mortar and pestle.
2. Thaw the powder with 2X (w/v) 0.1 M Tris-HCl buffer, pH 7.4, containing 2% nicotine sulfate. Strain through four layers of cheesecloth and one layer of Kimwipes. Save filtrate.
3. Extract the pulp twice with the same buffer. Combine all three filtrate portions.
4. Centrifuge 10 min at 4,000 g, then 5 min at 8,000 g, saving the supernatant in each case.
5. To the supernatant add bentonite suspension (in 0.05 M Tris-HCl buffer pH 7.0 containing 0.0001 M MgCl₂) to make a final concentration of 1.6 mg/ml bentonite. Mix, then centrifuge 15 min at 6,000 g. Save supernatant.
6. To every 100 ml supernatant add 5 g PEG (8,000 MW) and 0.8 g NaCl while stirring. Continue to stir at 4 °C for 1 hr; then centrifuge 15 min at 11,000 g. Discard supernatant.
7. Resuspend pellet in 1/5 volume 0.5 M Tris-HCl buffer, pH 8.0. Add Triton X-100 up to 0.3% and stir 2 hrs at 4 °C.
8. Centrifuge 10 min at 3,000 g. Save supernatant.
9. Layer the supernatant over a 2 ml 30% sucrose cushion (dissolved in 0.05 M Tris-HCl buffer, pH 8.0) in Beckman 65 rotor tubes. Centrifuge 1 hr at 32,000 rpm. Discard supernatant.
10. Resuspend pellet in 1 ml 0.05 M Tris-HCl buffer pH 8.0 and stir 2 hr or overnight at 4 °C. Centrifuge 10 min at 5,900 g. Save supernatant. The preparation is now partially purified.
11. Further purification is done on Cs₂SO₄ gradients. In Beckman SW 40 tubes put 6.5 ml of 53%(w/v) Cs₂SO₄ solution in 0.05 M Tris-HCl buffer, pH 8.0. Over each solution layer a mixture of 1.5 ml virus preparation, 1.5 ml buffer and 3 ml 53% Cs₂SO₄. Centrifuge 17 hrs at 26,000 rpm.
12. Scan the gradients for ultraviolet light-scattering zones with an ISCO-UA-4 monitor and collect 0.6-ml fractions with the ISCO 640 density gradient fractionator. Pool the lower fractions of the gradient with peak absorbance, and dialyze twice at 1 hr interval then overnight against 0.05 M Tris-HCl buffer pH 8.0. Store the purified virus frozen at -20 °C if not used immediately.
TABLE 5  Simplified procedures* for purification of KLS-associated virus with Cs$_2$SO$_4$ cushion gradients

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Grind 60 g KLS-infected tissue to powder in liquid nitrogen with a mortar and pestle.</td>
<td>1. Grind 30 g KLS-infected tissue to powder in liquid nitrogen with a mortar and pestle.</td>
</tr>
<tr>
<td>2. Thaw in 5X (w/v) 0.1 M Tris-HCl buffer, pH 7.8 containing 0.01 M Na$_2$SO$_4$.</td>
<td>2. Thaw in 5X (w/v) 0.1 M sodium citrate, 0.01 M EDTA containing 1% monothioglycerol. Grind thoroughly to mix after thawing.</td>
</tr>
<tr>
<td>3. Immediately on thawing, add Triton X-100 up to 1% of buffer volume and a drop of Antifoam B. Grind thoroughly until fully thawed.</td>
<td>3. Add Celluclast (enzyme) up to 2% buffer volume, and shake at 17,000 rpm at room temperature for 4 hrs.</td>
</tr>
<tr>
<td>4. Strain through four layers of cheesecloth and one layer Kimwipe. Save the filtrate.</td>
<td>4. Add Triton X-100 to 1% of buffer volume, and stir at room temperature for 30 min.</td>
</tr>
<tr>
<td>5. Extract the pulp with the same buffer made up 1% with Triton X-100. Pool both filtrates.</td>
<td>5. Strain through four layers of cheesecloth and one layer of Kimwipes. Save the filtrate.</td>
</tr>
</tbody>
</table>

6. Centrifuge the filtrate 10 min at 4,000 g, then 5 min at 8,000 g. Save the supernatant each time.

7. To every 100 ml of supernatant add 20 ml of 30% PEG (8,000 MW) dissolved in 0.6 N NaCl, and an additional 2 ml of 20% NaCl solution. Mix and let stand 1 hr at 4 °C.

8. Centrifuge 15 min at 16,000 g. Discard supernatant.

9. Resuspend the pellet in 16 ml 0.04 M sodium phosphate buffer pH 8.2. Stir gently for 1 hr at 4 °C.

10. Centrifuge 10 min at 5,900 g. Save supernatant. The preparation is partially purified.

11. Prepare 10% sucrose solution in 0.04 M sodium phosphate buffer, pH 8.2; then prepare Cs$_2$SO$_4$ step gradients by layering 1 ml each of 0, 15, 22.5, 30, and 40% (w/v) Cs$_2$SO$_4$ dissolved in this buffer (in Beckman SW 40 rotor tubes).

12. Layer 8.0 ml of the partially purified virus preparation over each gradient and centrifuge 2.30 hr at 37,500 rpm (175,000 g).

13. With a syringe and bent needle, draw out the sharp, light scattering bands from the gradient columns. One very sharp band forms at 5.5 cm from the top of the gradient, and another less conspicuous one at just below the green material (about 1.0 cm from the bottom of the gradient). Pool separately the upper and bottom bands.
TABLE 5 (continued)

14. Dilute each fraction with an equal volume of 0.04 M sodium phosphate buffer, pH 8.2.

15. Clarify the bottom fraction by centrifuging 10 min at 5,900 g. Save supernatant, and layer it on a second step gradient prepared as in (11), and centrifuge as in (12).

16. Scan the gradient for ultraviolet light-scattering zones with an ISCO-UA-4 monitor and collect 0.6 ml fractions with the ISCO 640 density gradient fractionator. Pool the lower fractions with peak absorbance and dialyze against 0.05 M Tris-HCl buffer pH 8.0 (Twice at 1 hr interval, then overnight, at 4 °C). Store the purified virus frozen at -20 °C.

17a. The upper, very sharply banding fractions from step 13 will be still greenish if method A was used at the beginning. To remove the green material, layer the preparation over 2 ml of 30% sucrose in Beckman 65 rotor tubes. Centrifuge 1 hr at 32,000 rpm, and discard supernatant. Resuspend the pellet in 1 ml 0.04 M sodium phosphate buffer pH 8.2 by gentle stir at 4 °C for at least 2 hr. This preparation can then be layered on a second step gradient prepared as in step (11) and the gradient scanned as for the other gradient (step 16).

b. If the tissue was digested with an enzyme (method B) the sharp band is normally clear and can be directly layered on the second step gradient.

Electron Microscopy

Negatively stained leaf dip preparations were examined with a Philips EM 300 electron microscope at 60 KV. The initial procedure, in which leaf pieces were diced in 2% aqueous sodium phosphate tungstate (PTA) pH 6.9, on formvar-coated grids, was not satisfactory. For better results leaf tissue was chopped in 5 volumes (w/v) of 0.01 M phosphate buffer pH 7.0. A drop of this sap was adsorbed onto a 400-mesh formvar-coated grid for 2 min. The grid was then blotted with filter paper and stained with a drop of either 2% neutral PTA, 1% ammonium molybdate pH 6.5, 1% uranyl formate pH 7.2 or 2% aqueous uranyl acetate. After 1-2 min the grid was blotted and examined in the microscope. The microscope was calibrated with a diffraction-grating replica with a linear spacing of 2160 lines/mm, and polyesterene latex particles of 0.234 μ diameter. Partially purified and purified virus preparations (Tables 3-5) were diluted 20-100X in buffer before being adsorbed onto grids.

The concentration of virus particles in tissue was generally low and often none could be detected in KLS-infected tissue. Therefore, immunologically specific electron microscopy (Derrick, 1973; Milne and Luisoni, 1977) was regularly used after antiserum to partially purified virus was produced. Most grids for particle length measurements were prepared by this procedure. Formvar-coated grids were backed with a carbon film; then 3 or 4 grids were floated (coated side down) over a 40 μl drop of antiserum in a plastic petri
plate. The antiserum had been diluted 1:500 in 0.06 M phosphate buffer pH 6.5. The plate was incubated 60 min in a moist chamber at 37 °C. Grids were then washed twice by floating them on buffer at 15 min intervals. Grids were floated on a drop of extracted sap (or purified virus) diluted 1:40 in Sorenson's phosphate buffer pH 6.5. They were again incubated in a moist chamber at 37 °C for 60 min, and washed as before. The virus was then fixed by floating the grids on a drop of 5% glutaraldehyde and incubating 30 min at 37 °C. They were washed again, and stained. For staining, each grid was held with tweezers and a drop of stain was placed on the coated side. This was immediately blotted away with filter paper. The process was repeated 5 times, and grids were observed in the electron microscope.

For decorating the virus with its antiserum, grids were floated on another drop of antiserum (diluted 1:500 in buffer) just before fixation in glutaraldehyde; incubated 60 min at 37 °C and washed as usual.

Tissues were prepared for thin sectioning by the procedure of Soeldner (personal communication, May 1985). Fresh tissue was diced into 0.5 mm³ blocks and fixed for 2-4 hrs under vacuum in a 3% glutaraldehyde solution in 0.2 M cacodylate buffer, pH 7.2. They were washed three times for 30 min each in 0.2 M cacodylate buffer pH 7.2. They were then postfixed for 30 min in equal parts 4% aqueous osmium tetroxide and 0.2 M cacodylate buffer pH 7.2. Next, they were passed through a graded acetone dehydration series in which they were stained with 1% uranyl acetate. The series was
successively 50%, 70% + uranyl acetate, 100, 100 and 100% acetone, at 10 min per solution. Infiltration with Spurrs plastic was done gradually, first for 2 hrs in 2 parts acetone: 1 part plastic, then overnight in 1 part acetone and 2 parts plastic. Embedding was also gradually done in Spurrs plastic under vacuum for 9 hrs at room temperature followed by overnight at 37 °C.

Embedded pieces were sectioned either longitudinally or transversally with a diamond knife, poststained for two min in lead citrate solution, dried and examined under the microscope.

Negatives taken at approximately 13,000 X or more were projected at a magnification of 2.5X, and images traced with thread for length measurements. The lengths of the thread tracings were measured with a standard ruler.

**Light microscopy**

Sisal and 'Black Turtle Soup 2' bean leaf tissues for light microscopic examination were prepared by the procedure described by Christie and Edwardson (1977, 1986). Two stains, Azure A and Orange-green, a combination of calcomine orange 2 RS and Luxol brilliant green (E.I. du Pont de Nemours and Co., Inc., Wilmington, DE, 19898) were used. Stains were prepared just before use from the stock solutions. Azure A was made by mixing the stock solution with 0.2 M dibasic sodium phosphate (Na$_2$HPO$_4$) in a ratio of 9:1. Orange-Green was prepared by mixing the green dye, orange dye and water in a ratio of 8:1:1.
Epidermal strips from bean leaves, prepared by inserting sharp-pointed tweezers under the epidermis of a vein on the lower surface were floated, torn side down onto Azure A or Orange-Green stain in a watchglass. Strips were stained for 10 min and excess stain removed by floating in 95% ethanol for 15 sec. They were then mounted over a drop of Euparal (Carolina Biological Supply, Burlington, NC 27215) on a glass slide. Strips stained in Azure A were mounted in regular Euparal, and those stained in Orange-green in Euparal 'Vert'. After covering with cover slips, excess medium was removed by gentle blotting, and examined with a light microscope (oil immersion).

For sisal tissue which is covered with a waxy cuticle, epidermal strips were not practical. The cuticle was peeled off, and thin longitudinal or transverse sections made with a razor blade. Sections were immediately soaked in 2-methoxyethanol for 30 min to remove chlorophyll and other pigments. They were then stained and mounted in the same way as the epidermal strips.

Preparation of antiserum to partially purified virus

Antiserum to partially purified virus was produced in three New Zealand White rabbits. One of these was injected with a preparation purified by method B (Table 3) while the other two were injected with a preparation of method A (Table 3). Before the first injection, each rabbit was bled to collect preimmune serum. Three injections were then administered intramuscularly at weekly intervals. For the first week, 0.75 ml of the virus preparation was
emulsified 1:1 with Freund's complete adjuvant, and 1 ml of the emulsion injected in the animal's hind thigh. In subsequent injections, incomplete adjuvant was used to emulsify the virus preparation.

Antiserum was collected over a four-week period beginning the fifth week after the first injection. The method used to separate serum from blood and purify immunogammaglobulin (IgG) from whole antiserum is described in Appendix B. Antiserum titer was determined by the microprecipitin test (Ball, 1974). Serial dilutions of the antiserum, KLS-infected sap and healthy sisal sap were made in phosphate-buffered saline (PBS, pH 7.4). Twenty μl drops each of the antigens and antiserum were mixed in squares drawn with wax pencil over sterile, disposable plastic petri-plates. Incubation of the plates was in a moisture chamber at 37 °C for 2 hrs, then overnight at 4 °C. No oil was used to cover the drops. Checking the drops for formation of precipitate was done twice with a binocular microscope in a room with subdued light. A light source was at an angle from the side of the plate and black paper was placed below the plate to improve visibility.

Preparation of antiserum to purified KLS-associated virus

A slightly different procedure was used to immunize rabbits to purified KLS-associated virus. Four New Zealand White rabbits were used. Two of them were injected with the heavy, lower fraction and the other two with the lighter upper fraction from sucrose/Cs₂SO₄ step gradients (Table 5).
For the first injection, 0.6 ml of virus preparation was emulsified 1:1 with Freund's complete adjuvant. Emulsification in plastic tubes resulted in loss of emulsion sticking to the sides, so that only 0.8 ml of emulsion was recovered for injection. In subsequent injections, 0.75 ml of the preparation was emulsified to provide 1 ml of emulsion.

The first injection was given intradermally, after the rabbits were shaved on the back. Fractions of emulsion ranging from 0.05-0.075 ml were injected per site. The two rabbits injected with the lower fraction virus were injected at respectively 16 and 12 sites; while for their counterparts it was 14 and 12 sites. The second injection was done intramuscularly in the hind thighs with virus preparations emulsified 1:1 with Freund's complete adjuvant. Incomplete adjuvant was used to emulsify virus in subsequent injections.

Collection of primary antiserum was started after three injections on two rabbits (one of each group) which showed large granulomas in response to the first intradermal injection. The other two were given another injection before antiserum collection was started. Antiserum collection was continued at weekly intervals, and booster injections were administered when the titer began to decline.

Separation of serum and determination of serum titer were done as described above. Purification of IgG was, however, done by a modified procedure of Clark and Adams, 1977, outlined in Appendix B (Hampton, personal communication).
Serological tests

Sensitivity of the antiserum for detecting their homologous antigens and other related viruses was tested by both ELISA and immunologically specific electron microscopy (ISEM). ELISA tests were conducted by the double antibody sandwich procedure described by Clark and Adams (1977), in flat bottom Dynatech Immulon 2 microplates (Dynatech Lab. Inc.). Plates were washed by flooding wells with buffer using a squirt bottle. Buffers used are described in Appendix B.

Serial dilutions (1:100-1:1000) of purified IgG at 1 mg/ml were made in ELISA 'coating buffer,' pH 9.6. Diluted IgG (250 μl) was added to the appropriate well of the microplate as shown in Fig. 4. Plates were incubated at 37 °C for 4 hrs to allow the IgG to adhere to the polysterene walls of the wells. Wells were then washed with PBS containing 0.05% Tween (PBS-Tween); three times with a 3-min soak between each wash. Leaf samples, ground with a mortar and pestle or blender in ELISA 'sample buffer,' pH 7.4 and diluted 1:5 - 1:100, were added to the respective wells (Fig. 4) at 230 μl per well. Plates were incubated 4 hrs at 37 °C or overnight at 4 °C, and washed as described above. Next, the conjugated IgG, also serially diluted in ELISA 'sample buffer,' pH 7.4, was added to the appropriate wells (Fig. 4) at 200 μl per well. Plates were incubated 4 hrs at 37 °C, and again washed as before. After washing, 200 μl of alkaline phosphatase substrate (p-nitrophenyl phosphate, Sigma #104) dissolved in ELISA 'substrate buffer,' pH 9.8
were added to each well and the plate incubated at room temperature. After about 30-60 min when wells that were coated with virus-containing samples started to change color from clear to yellow, the plates were read for light absorbance at 405 nm wavelength. Absorbance readings were taken with an automatic ELISA microplate reader (Bio-Tek Instruments, Burlington, Vermont). This was done twice at 1 hr interval, and again after overnight incubation at room temperature if color developed very slowly.

The results were considered positive when the absorbance at 405 nm ($A_{405}$) of the test sample was greater than $A_{405}$ of the healthy control sample in the plate by a factor of 2 or more. The combination of IgG, leaf sample and conjugated IgG dilutions which gave the best positive value (detecting virus) were then adopted as standard dilutions for routine ELISA tests.

ISEM was done by a modified procedure of Derrick, 1973 (Allen, personal communication); or according to Milne and Luisoni (1977). The procedure has been described above (sub-section on Electron microscopy). The procedure of Milne and Luisoni, however, differed slightly from the one previously described. IgG was diluted 1:100 in 0.1 M phosphate buffer, pH 7.0, and the virus sample, 1:10 in the same buffer. Incubation times were also reduced to 15 min each for both IgG and virus sample. Instead of floating grids on buffer, washing was done with 20 consecutive drops of water. The degree of reaction in ISEM was determined by recording the number of particles observed in 10 randomly selected grid openings.
Serological relationship was studied between KLS-associated virus, citrus tristeza virus, and a virus infecting white lupines.

**Determination of physical-chemical properties**

The ultraviolet absorption spectrum of 1 ml portions of purified virus (suspended in 0.05 M Tris-HCl buffer, pH 8.0) was measured in a Beckman Model 25 Spectrophotometer. The concentration of virus particles in the preparation was estimated using the extinction coefficient of BYV of 2.0 cm$^2$ mg$^{-1}$ (Bar-Joseph and Hull, 1974). Calculations were based on the relationship between optical density (i.e. extinction or absorbance) and virus concentration as shown by the formula, $E = abc$ (Bos, 1983, Manning, 1972) where:

- $E =$ absorbance at 260 nm wavelength
- $a =$ absorptivity (= extinction coefficient)
- $b =$ light pathlength of 1 cm.
- $c =$ concentration of virus particles in the suspension (mg/ml)

For determination of extinction coefficient, purified virus preparations were lyophilized. The powder was then weighed, and suspensions of virus were prepared in double distilled water at 25 °C at a concentration of 1 mg/ml. The ultraviolet absorption spectrum for each suspension was measured with a Beckman Model 25 spectrophotometer. After adjusting for light scattering in the visible range (300-340 nm wavelength), the extinction coefficient was calculated as the mean optical density value of the preparations at 260 nm.
The nucleic acid content of the virus was estimated from the same curves as the ratio of the absorbances of 260 nm \(A_{260}\) and 280 nm \(A_{280}\) which are attributed respectively to nucleic acid and virus protein.

The buoyant density in Cs\(_2\)SO\(_4\) was determined by equilibrium centrifugation in a Beckman SW 40 rotor in an L8M ultracentrifuge. Two SW 40 rotor tubes were each filled with 6.5 ml of 53\% (w/v) Cs\(_2\)SO\(_4\) solution in 0.05 M Tris-HCl buffer pH 8.0 (\(\rho = 1.384\) g/cc). Over this dense solution was layered 6.5 ml of virus preparation, obtained from the second Cs\(_2\)SO\(_4\) cushion gradients (Table 5) and diluted 1:1 with 0.05 M Tris-HCl buffer pH 8.0. Gradients were immediately centrifuged at 26,000 rpm for 14.5 hrs at 8 °C. At the end of the run, gradient columns were fractioned by an ISCO 640 density gradient fractionator at a flow rate of 0.3 ml/min. Each column was monitored at 254 nm by an ISCO-UA-4 monitor during fractionation. The refractive indices of the various fractions were determined by ATAGO Type 500 hand sugar refractometer at 25 °C and converted into density values. The buoyant density was taken to be the mean of density values for the fractions which were associated with peak UV-light absorption at 260 nm in the spectrophotometer, after the fractions were dialyzed.
RESULTS

Host range and symptoms

Characteristic KLS symptoms on field-infected mature leaves of hybrid sisal No. 11648 are shown in Fig. 1. Fig. 2A shows KLS symptoms on leaves of sap-inoculated young sisal grown in the greenhouse. Plants that were raised in the growth chamber (Fig. 2B) and inoculated with KLS-infected sap have not developed any symptoms to date.

Of the sap inoculated herbaceous test plants, only 'Black Turtle Soup 2' beans showed symptoms. Infection in beans was systemic, with symptoms appearing on the trifoliate leaves, as early as 12 days after inoculation in some cases. The inoculated unifoliate leaves remained symptomless, except for occasional brown specks on the margins. Affected leaves showed a variety of symptoms including severe mosaic, distortion and chlorosis (Figs. 3B, C). Mosaic symptoms were similar to what was previously observed in greenhouse experiments in Tanzania (Fig. 3A). Severely affected plants wilted and died within 2 weeks of inoculation. This reaction was, however, less frequent. It was observed in only two experiments out of six, and affected respectively 8.0 and 22.0% of total inoculated plants in the experiments it occurred in. Buffer inoculated plants remained healthy (Fig. 3D).

While in Tanzania symptoms appeared on trifoliate leaves of bean plants three weeks after inoculation and continued to appear on most of the younger trifoliates, under Oregon conditions symptoms...
occurred as early as 12 days after inoculation. In addition, 
symptoms were observed on only the first 4-7 trifoliates; the rest 
appeared normal.

Another difference observed under Oregon conditions is that 
development of symptoms in beans was influenced by light and 
temperature conditions during the first week after inoculation. 
Symptoms developed only if inoculated plants were subjected to a 
24-hr photoperiod for at least 4 days after inoculation; and during 
this period temperature was set at 24-27 °C every 12 hrs followed by 
21 °C for 12 hrs.

Percentage infection was between 25-30% both in Tanzania and 
Oregon. Plants that showed severe mosaic were stunted and set very 
few or no pods. Inoculation with partially purified virus 
preparations did not increase the percentage of plants infected.

Despite lack of symptoms on sap-inoculated hybrid sisal in the 
growth chamber, virus particles were isolated 5 months after 
inoculation from 2/4 of the first inoculated batch (Jan. 1986). Sap 
from these plants caused the typical mosaic symptoms when inoculated 
on bean plants, and virus particles were re-isolated from those 
beans. The concentration of virus particles in these plants was, 
however, low and could be detected only by immunologically specific 
electron microscopy (ISEM).

During another ISEM assay of all the sisal plants, conducted at 
12 months from planting (April 1986), virus was detected in one 
noninoculated plant.
FIG. 2 Transmission of KLS in sap.
A. Symptoms on leaf of sap-inoculated sisal in the greenhouse (Tanzania).
B. Sap-inoculated sisal in the growth chamber with no symptoms but contain virus particles.
C. Healthy sisal in the field.
FIG. 3 Symptoms induced by KLS-associated virus in beans. 
A. Severe mosaic in the greenhouse with temperature not regulated (Tanzania). 
B. Mosaic and distortion under regulated greenhouse temperature (Oregon). 
C. Leaf chlorosis, temperature regulated (Oregon). 
D. Healthy leaf.
FIG. 4 ELISA test plate arrangement for evaluation of antiserum.*

<table>
<thead>
<tr>
<th>Test sample dilutions (horizontal direction)</th>
<th>Dilutions of Coating IgG (vertically running)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer only</td>
<td>1:100</td>
</tr>
<tr>
<td>1:5</td>
<td>1:200</td>
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<td></td>
<td>5</td>
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<td></td>
<td>buffer</td>
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</tbody>
</table>

Conjugated IgG Dilutions (x100)  
(vertically running)

* A second test plate similarly arranged but containing coating IgG at dilutions 1:500, 1:800, and 1:1000.

*1 Numbers 1–12 across the top, and letters A–H running vertically correspond to those printed on the 'Dynatech Immulon' microelisa plate. Border wells were filled with buffer for each incubation step.
Purification procedures

Purification procedures outlined in Tables 3-5 and in Appendix A were all attempted for purification of the KLS-associated virus with variable results. Those outlined in the appendix were generally unsuccessful.

Initial attempts to extract the virus from sisal leaves with a blender, followed by clarification with either butanol (Hiebert and MacDonald, 1973) or chloroform (Goodell, 1983), caused respectively loss and severe breakage of the particles. Figures 5A and B show electron micrographs of preparations partially purified by these procedures. In preparations clarified with butanol, disintegrated particles were associated with elongate, open-structure, tubular bodies of unknown identity (Fig. 5A). These are probably inclusions arising from virus activity in host cells. No virus was recovered by either of the two procedures because attempts to band the preparations in sucrose gradients were unsuccessful, and precipitation occurred as soon as the suspension was mixed with CsCl.

Grinding the tissue with a mortar and pestle was effective in reducing particle breakage when compared to mechanical homogenization (Fig. 6A). The advantage of gentle grinding was, however, lost when the extract was purified by the differential centrifugation procedure of Price, 1966 (Table 3, method A). This procedure yielded relatively pure but highly fragmented particles (Fig. 5D) after one cycle of differential centrifugation. If the
preparation was passed through a second cycle of differential centrifugation total loss of virus occurred. Consequently, all preparations used for production of antiserum were purified through only one cycle of differential centrifugation. Preparations from this procedure also contained elongate, less flexuous, tubular structures that appeared fuzzy in 2% neutral PTA (Fig. 5C). They were about 22.5 nm wide with modal lengths of 1,600 and 1,740 nm. They are possibly virus inclusions.

Precipitating the virus with PEG (8,000 MW) before the extract was passed through one cycle of differential centrifugation (Table 3, method B) slightly reduced particle breakage, and yielded preparations with a few intact particles (Fig. 6B). This procedure was, however, not reliable for preparation of virus particles for size characterization.

A modified procedure of Gonsalves et al., 1978 (Table 4) which involved pelleting the preparation through a 30% sucrose cushion was found to be a better alternative to reducing particle breakage. Tris-HCl buffer (0.05 M) was preferred to 0.01 M potassium phosphate as a resuspension buffer because the latter tended to promote particle aggregation. Although the intact particles from this procedure were relatively free of aggregation (Fig. 7), it was observed that the shorter particle fragments tended to adsorb onto the open-structure, tubular bodies that were always present in the preparation (Fig. 8B).

Virus preparations pelleted through a 30% sucrose cushion were infective to 'Black Turtle Soup 2' beans. Symptoms produced were,
however, milder, mainly chlorosis; and virus yield was low (0.125 mg/100 g tissue) when purification from the symptomatic plants was attempted.

Despite the fact that the improved procedure considerably reduced particle breakage (Fig. 7), difficulty was still experienced in banding the virus preparations onto either sucrose or Cs\(_2\)SO\(_4\) gradients. No visible band was formed in 10-40% preformed sucrose gradients after centrifuging for 2.5 hrs at 32,000 rpm in a Beckman SW 40 rotor. Assay of fractions from the gradient column indicated that most of the virus was contained in a pellet formed at the bottom of the gradients.

The Cs\(_2\)SO\(_4\) gradient described in the procedure (Table 4) was first centrifuged at 23,000 rpm for 17 hrs in a Beckman SW 40 rotor. No visible virus band formed. Fig. 9A shows a UV-scan at 254 nm of a sample gradient fractionated into 0.6 ml fractions after centrifuging for 17 hrs at 23,000 rpm. Electron microscopic assay of all the fractions showed that virus particles were still in the top three fractions of the gradient. Increasing the centrifugation time to 18 hrs improved particle separation and a weak, light-scattering band was formed at 4 cm, while plant material sedimeted as a flocculate precipitate at 6 cm from the top of the gradient. A UV-scan of the fractionated gradient showed two UV-absorbing peaks in respectively the 15-16\(^{th}\) and 19-20\(^{th}\) fractions (Fig. 9B). Virus particles were detected in each of these fractions.
FIG. 5 Effect of mechanical grinding and sap clarification with solvents on virus purification.
A. Structures of unknown identity in extract ground mechanically and clarified with n-butanol.
B. Excessive particle breakage in extract ground mechanically and clarified with chloroform.
C. Fuzzy-staining, virus-like structures in preparations purified in ultracentrifugation.
D. Particle breakage caused by ultracentrifugation.
FIG. 6  Electron micrograph of gently extracted KLS-associated virus after precipitation with PEG (A), followed by ultracentrifugation (B).
FIG. 7  Electron micrograph of KLS-associated virus, partially purified by pelleting through 30% sucrose cushion, and stained in 2% PTA, pH 6.9.
FIG. 8 Problems associated with particle breakage.
A. Severe breakage in leaf dip preparations stained in 2% PTA, pH 6.9 without prior fixation with glutaraldehyde.
B. Adsorption onto tubular, open-structure inclusions by broken particles during purification.
Cs₂SO₄ gradients at concentrations other than those described in the procedure (Table 4) were loaded with virus suspensions and centrifuged for 18 hrs at 23,000 rpm to compare their effectiveness in banding the virus:

(i) A 10-40% Cs₂SO₄ step gradient prepared before use in 0.05 M Tris-HCl buffer, pH 8.0, and 1.5 ml of virus suspension layered on top.

(ii) Virus preparation (1.5 ml) mixed with 3.0 ml buffer, then thoroughly mixed with 4.5 ml of 53% Cs₂SO₄ and layered over 3.0 ml of 53% Cs₂SO₄.

In each of these gradients, a weak, light-scattering band was formed at 6.0 cm, and the plant material sedimented to 8.0 cm from the top of the gradient. Therefore, they were not better than the former gradient in suspending the virus preparations, and were not used in further tests.

When the centrifugal force was increased from 65,000 g (23,000 rpm) to 85,000 g (26,000 rpm) and time was maintained at 17 hrs, a weak, light-scattering band was formed at 3.5 cm from the top of the gradient, and plant material sedimented 3 cm below this band. Results of a UV-scan of the fractionated gradient was similar to the one in Fig. 9B. Electron microscopic examination of the light-scattering bands revealed that they were composed mainly of the fragmented particles adsorbed onto the tubular structures as observed before samples were loaded on the gradients (Fig. 8B).

Virus yields from these Cs₂SO₄ gradients were generally low, ranging between 0 and 2.043 mg/100 g tissue. This problem was compounded
by loss of virus during dialysis due to adsorption onto the tubing. This was later overcome by pretreating the dialysis tubing with bovine serum albumin (tubing was soaked overnight in 1% bovine serum albumin solution).

A combination of gentle grinding, precipitation with PEG, and concentration of the virus in sucrose/Cs$_2$SO$_4$ step gradients (Table 5) provided a quick and efficient method of purifying the virus. It also produced preparations that were satisfactory for virus characterization and production of quality antiserum. The extract was readily banded on the step gradients, forming a sharp, light-scattering band at 5.5 cm from the top of the gradient. A second, less conspicuous band was immediately below the green plant tissue band at 8.0 cm from the top of the gradient. The two bands were separately drawn from the gradient with a bent needle. When diluted, clarified and loaded over a second gradient, the preparation that banded at 8.0 cm in the first gradient did not form a visible band. However, on fractionation, a sharp UV-absorbing peak was detected in the 16-18$^{th}$ fractions from the top, and another minor peak in the 20$^{th}$ fraction (Fig. 10A). These peaks were associated with long, flexuous nonaggregated and aggregated virus particles.

Preparations that banded sharply at 5.5 cm in the first step gradients still formed a sharp band at the same position when loaded on a second gradient. Three UV-absorbing peaks were detected when the gradient was scanned during fractionation, but virus particles were predominatly in the 10-13$^{th}$ fractions (Fig. 10B).
FIG. 9 UV absorbancy profiles for Cs₂SO₄ density gradients of partially purified KLS-associated virus centrifuged at 23,000 rpm in SW40 rotor for 17 hrs (A) and 18 hrs (B). Fractions were 0.6 ml.
FIG. 10 UV absorbancy profiles for second sucrose/Cs₂SO₄ cushion gradients of partially purified KLS⁻² associated virus, centrifuged 2.30 hr at 37,500 rpm in SW40 rotor.
(A) Fraction banding at 8 cm from top of first sucrose/Cs₂SO₄ gradient and (B) the one banding at 5.5 cm.
FIG. 10
Preparations from this purification procedure were reasonably clean, as indicated by the ultraviolet light spectrum of a sample virus preparation (Fig. 11); and yields were slightly higher than in previous procedures (0.602 to 5.56 mg/100 g tissue). Fractions banding lower in the gradients (at 8.0 cm) were characterized with peak UV-absorbance at 260 nm (Fig. 11A), suggesting that the particles were intact. In contrast, particles banding at 5.5 cm in the gradients showed ultraviolet absorption spectra with peak UV-absorbance at 266-270 nm, as shown in Fig. 11B. This suggests that the particles were either damaged or were predominantly empty protein shells.

**Effect of host on purification**

Leaves of Agave hybrid 11648 and 'Black Turtle Soup 2' beans were both used for purification of the virus. Purification from sisal was limited to the leaves sent from Tanzania. These were showing severe KLS symptoms and were harvested from 3-year old plants. The concentration of virus in mechanically inoculated young sisal was too low for them to be used for purification. There was, therefore, little opportunity to test whether virus yields could be influenced by factors such as age of host, stage of infection, etc. Yields ranged between 0 and 5.56 mg/100 g tissue depending on purification procedure.

Although beans have the advantage of rapid propagation, they were not a satisfactory purification host under conditions of this investigation. It was very difficult to induce severe symptoms in
the plant to allow for reasonable virus yields. Yields ranged between 0.125-0.405 mg/100 g tissue when purification was from plants with mild symptoms (chlorosis). However, when severely affected plants (severe mosaic, stunting, and wilting) were used, yield increased to 2.607 mg/100 g tissue. This shows that 'Black Turtle Soup 2' could be a suitable propagation host for purification if conditions were favorable for severe symptoms.

**Effect of additives on purification**

Chemical additives were tested as part of certain purification procedures and were not necessarily repeated in different methods, particularly when they were seen to be detrimental to the virus. Excessive particle breakage and/or disintegration was evident in extracts that were mechanically homogenized in a blender and clarified by either n-butanol or chloroform (Fig. 5A, B). It was, however, not established whether most damage was caused by mechanical grinding or was solvent-induced.

Stepwise low speed centrifugation (4000 g for 10 min, then 8000 g for 5 min) followed by bentonite at a concentration of 1.6 mg/ml was most effective in clarification of gently ground extracts (Table 6). The effectiveness of bentonite in clarification was, however, influenced by presence and concentration of Mg++ ions. In the absence of Mg++ ions, there was loss of virus (Table 6) due to adsorption by bentonite. When tested at concentrations of 0.0001 - 0.1 M Mg++, it was observed that at concentrations of 0.001 M and above, the clarified extract was very clear, but the preparation
TABLE 6 The effect of bentonite on clarification of KLS-infected sap

<table>
<thead>
<tr>
<th>Extraction buffer, 0.1 M Tris-Hcl, pH 7.8 either containing (a) or without (b) 2% nicotine sulfate</th>
<th>Color of clarified extract after precipitation with PEG</th>
<th>No. of virus particles per 10 grid openings. (2 replicates)</th>
<th>General appearance of preparations in the electron microscope</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.6 mg/ml Bentonite</td>
<td>0.0001 M Mg&lt;sup&gt;2+&lt;/sup&gt; ions</td>
<td>Greenish yellow</td>
<td>16</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>Yellow with green tinge</td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>Light yellow</td>
<td>40</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>Light yellow</td>
<td>40</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>Green</td>
<td>12</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>Yellow</td>
<td>10</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>Very light yellow</td>
<td>49</td>
</tr>
<tr>
<td>(b)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TABLE 7 Purification of KLS-associated virus with Cs₂SO₄ cushion gradients: effect of concentration of Triton X-100 and time of solubilizing tissue with Celluclast on virus yield.

<table>
<thead>
<tr>
<th>Method of extraction++</th>
<th>Color of extract after precipitation with PEG</th>
<th>Virus yield* (mg/100 g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Procedure A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No Triton X-100, + Nicotine sulfate</td>
<td>Greenish yellow</td>
<td>0.694</td>
</tr>
<tr>
<td>+ 0.5% Triton X-100</td>
<td>Green</td>
<td>0.194</td>
</tr>
<tr>
<td>+ 1.0% Triton X-100</td>
<td>Light green</td>
<td>3.093</td>
</tr>
<tr>
<td>+ 1.5% Triton X-100</td>
<td>Greenish yellow</td>
<td>1.027</td>
</tr>
<tr>
<td>Procedure B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 hr shaking with Celluclast</td>
<td>Light yellow</td>
<td>2.53</td>
</tr>
<tr>
<td>4 hr shaking with Celluclast</td>
<td>Light yellow</td>
<td>5.56</td>
</tr>
<tr>
<td>6 hr shaking with Celluclast</td>
<td>Light yellow</td>
<td>2.467</td>
</tr>
<tr>
<td>16 hr shaking with Celluclast</td>
<td>Light yellow</td>
<td>2.12</td>
</tr>
</tbody>
</table>

++ Method of extraction, A or B, as outlined in Table 5.

* Yields were determined after the first cushion gradients. There was a decrease of up to 30% when the preparations were further purified through a second gradient.
precipitated and no virus was recovered. Mg\(^{++}\) ion concentration of 
0.0001 M was most effective (Table 6). Sensitivity to Mg\(^{++}\) was also 
observed in the procedure for purification of BYV (Bar-Joseph et 
al., 1974). The extract precipitated in the resuspension buffer, 
which contained 0.01 M MgCl\(_2\), and no virus was recovered by that 
procedure.

Nicotine sulfate (1 or 2\%) was as effective as 0.01 M Na\(_2\)SO\(_3\) in 
reducing oxidation products in the extracts. The latter was 
therefore preferred because it is safer.

KLS-associated virus was precipitated by both 4\% and 5\% PEG in 
the presence of 0.8\% NaCl. Yields were higher with 5\% PEG. Virus 
loss was evident when precipitation with PEG was done twice.

The virus was very sensitive to CsCl. Extracts precipitated as 
soon as they came in contact with the salt. Equilibrium 
centrifugation was therefore done in Cs\(_2\)SO\(_4\).

The detergent Triton X-100 was useful in solubilizing 
chloroplasts and reasonably improved virus purification. Yields 
increased from less than 1 mg to between 1.027 - 3.09 mg/100 g 
tissue. Observation of sample preparations in the electron 
microscope showed that a concentration of 1.5\% caused slight damage 
to the particles, but no damage was seen when a 1\% concentration was 
used.

Celluclast, a cellulitic enzyme, used in combination with 1\% 
Triton X-100, produced the highest virus yields in this 
investigation. The level of contamination with plant material was 
also considerably reduced (Fig. 11A, B). The optimum time for
solubilizing the tissue was found to be 4 hrs, beyond which yields declined. Some particle damage occurred, but there were many which were intact for characterization purposes. The enzyme also helped separate the shorter virus fragments from the virus inclusions to which they were previously seen to adhere.

**Electron Microscopy**

Long and very flexuous, rod shaped particles were consistently found in negatively stained leaf extracts from Agave hybrid 11648 with KLS symptoms (Fig. 5B and D, 6, 7). Extracts from leaves of 'Black Turtle Soup 2' beans with severe mosaic, chlorosis or wilt symptoms, also contained similar particles (Fig. 8A). Virus particles were not found in extracts from healthy, 'mock' inoculated sisal and bean plants, or beans which did not show symptoms after they were inoculated with KLS-infected sisal sap. Sap of sisal bulbils contained no virus particles when tested before they were planted, although twelve months later particles were detected in one noninoculated, symptomless plant.

The virus particles showed a high tendency for aggregation and adsorption onto plant tissue (Fig. 6A). They were also very easily broken into fragments if the environment was unfavorable (Fig. 5B and D, 8A and B). These two characteristics caused difficulties in determination of particle modal length. Measurement of particles in leaf dips and partially purified preparations showed lengths between 100 nm and 4,000 nm. For 215 particles measured in leaf dip preparations (93 particles from sisal sap and 122 from bean sap)
stained in 2% PTA, pH 6.9, lengths were between 100 nm and 3,000 nm with a modal length of 250 nm (Fig. 12). Only 24 particles were more than 1,000 nm long. The excessive breakage was probably induced by the stain.

Particle breakage in negatively stained extracts was slightly reduced by fixing the preparations in 5% glutaraldehyde before staining. For 286 particles measured in clarified and fixed preparations from sisal and beans, lengths were between 200 and 4,000, but no specific modal length was found (Fig. 13). By the technique of Lacotelli and Allen (1979) in which the number of particles in each length category is summed with those in the preceding and succeeding groups, four modal lengths were detected respectively at 950, 1,075, 1,450 and 1,650 nm (Fig. 13). Particle diameter ranged between 9.09 nm and 18.0 nm, with a mode at 12.7 nm.

Of the four negative stains tried, 1% ammonium molybdate (pH 6.5) and 2% PTA (pH 6.9) stained the particles satisfactorily. Aqueous uranyl acetate (pH 4.2) tended to precipitate in the bottle and caused 'clumping' of virus particles with plant tissue on the grids. Uranyl formate (1%, pH 7.2) usually stained the particles too faintly for good resolution. Estimation of particle lengths was therefore made on particles stained in either ammonium molybdate or PTA. At pH 6.3, PTA could not stain the particles. It is possible that pH influences the effectiveness of the stain used.

Virus particles were often resolved to show a helical substructure, and a hollow center (Fig. 6, 8A). No attempt was made to determine the pitch of the helix.
FIG. 11  UV absorption spectra of KLS-associated virus purified by sucrose/CaSO₄ cushion gradients. Spectrum of the fraction banding at 8.0 cm from the top of the gradient (A) differs slightly from that of the one banding at 5.5 cm (B).
FIG. 11
FIG. 12 Histogram of particle length distribution in leaf dip preparations from sisal and bean leaves, stained in 2% PTA, pH 6.9.
FIG. 13  Histogram of 25 nm categories (solid) and additions of three successive 25 nm categories (clear) of particle length measurements made from electron micrographs of clarified virus extracts. Particles were fixed with 5% glutaraldehyde before staining in 2% PTA or 1% Ammonium molybdate.
In thin sections of sisal leaf tissue expressing KLS symptoms, and bean leaves with severe mosaic (after inoculation with KLS-infected sisal sap), virus particles were found in the phloem. In the sieve elements virus particles occurred in a dispersed state (Fig. 16A). In some cases they occurred both in dispersed state and as compact aggregates (Fig. 16B). In the phloem parenchyma cells, and often in sieve elements, particles appeared as isolated, loose aggregates (Fig. 16C) in the cytoplasm. Particles were not observed in cell nuclei.

Extensively investigated was the phloem tissue. The epidermis and ground parenchyma were not searched for virus particles in detail. The particles observed to accumulate in infected phloem tissue closely resemble those described for beet yellows virus (Esau, 1968; Francki et al., 1985), carnation necrotic yellow fleck (Bar-Joseph et al., 1977), and citrus tristeza (Francki et al., 1985). Virus particles were not seen in sections of healthy sisal and bean leaves.

Clusters of membranous vesicles were observed in the cytoplasm of infected phloem cells (Fig. 15A and B). None were found in cells of healthy tissue. These vesicles also resemble those reported for Closteroviruses (Esau, 1968; Bar-Joseph et al., 1977; Franckie et al., 1985).

Chloroplasts in phloem parenchyma cells of healthy tissue showed a well organized thylakoid system with the grana and intergrana running along the long axis of the plastid (Fig. 14A). Osmiophilic globules in the stroma interspersed with the thylakoid
system were small and less electron dense. In KLS-infected phloem tissue, parenchyma cells contained chloroplasts with an irregularly arranged thylakoid system (Fig. 14B). The osmiophilic globules in these plastids were large and more electron dense (Fig. 14B). Other chloroplasts were swollen from accumulation of starch granules (Fig. 15C). Flaky, electron-opaque material was seen interspersed with ribosomes in the cytoplasm (Fig. 14B). Similar effects were reported in cells of Beta vulgaris infected by beet yellows virus (Esau, 1968).

Light microscopy

Phloem cells in strips of symptomatic bean leaves and thin sections of sisal leaves stained in Azure A, contained numerous phloem-limited amorphous inclusions with many small vacuoles (Fig. 17A). These inclusions stained reddish-violet in Azure A. Other cells contained brightly staining fibrous inclusions (Fig. 17B, C). Occasionally, long strips of phloem cells filled with virus inclusions stained dark blue and were easily detected in sections of infected tissue (Fig. 17D). No inclusions were seen in the epidermis and ground parenchyma of infected tissue, nor in phloem tissue of healthy plants. The observed inclusions were similar to those induced by citrus tristera virus in phloem cells of sweet orange (Christie and Edwardson, 1977). Sections stained in calcomine orange-luxol brilliant green stain did not show any viral inclusions.
FIG. 14 Chloroplasts in healthy and KLS-infected cells.
A. In healthy sisal bulbil leaf, chloroplasts in phloem cell show regular arrangement of lamella and grana interspersed with small osmiophilic globules.
B. Chloroplast in phloem cell of KLS-infected sisal leaf. Osmiophilic globules are enlarged and electron opaque. Flaky osmiophilic granules are interspersed with ribosomes.
FIG. 15 Effect of KLS infection on subcellular structures. A, B. Phloem cells of bean leaf with membranous vesicles accumulating in the cytoplasm. C. Chloroplast in infected phloem cell of bean leaf swollen from accumulation of starch granules.
FIG. 16 Thin sections of phloem tissue from KLS-infected sisal and bean leaves.
A. Virus particles dispersed in phloem sieve cell in sisal. Flaky osmiophilic granules are interspersed with ribosomes.
B. Virus particles aggregated and dispersed in cytoplasm of phloem sieve cell in beans.
C. Virus particle aggregate in bean phloem parenchyma cell.
FIG. 16
FIG. 17  Epidermal strip of bean leaf and sections of sisal leaf (infected with KLS), stained in Azure A.
A. Amorphous inclusion with many vacuoles in section of sisal leaf.
B,C. Fibrous inclusions in sisal leaf.
D. Phloem cells in bean leaf deeply staining at position of virus inclusion accumulation.
Serological tests with antiserum to partially purified virus

Microprecipitin tests were used to determine antiserum titer. Antisera produced in different rabbits showed different titers depending on the method of virus purification, the rabbit and time when antisera were collected (Table 8). The peak antiserum titer was attained 31 days after the first injection, irrespective of the rabbit or method of virus preparation. Antiserum produced in rabbits No. 7013 and 7015, which were injected with virus prepared by one cycle of differential centrifugation (Table 3, method A), had peak titers of 32 and 64 respectively. Antiserum from rabbit No. 7014, which was injected with virus prepared by PEG precipitation and ultracentrifugation (Table 3, method B), attained a peak titer of 512.

The dilution of virus preparation in which antigens could be detected by homologous antiserum also varied with the rabbit, method of virus preparation and time of antiserum collection (Table 8). Antiserum from rabbit No. 7014 could detect virus in preparations diluted 1024 times, 31 days after the first injection. For the other antisera, the virus titer was respectively 64 and 16 (Table 8).

Pre-immune serum was not precipitated by partially purified virus preparations. At high concentrations of both antigen and serum, however, the mixtures looked turbid.

The intensity of reaction between antiserum and antigen was assessed by visual evaluation of the quantity of precipitate formed.
The rating system was set between 0 and 4. Drops in which there was no precipitate were rated 0, and those with precipitate filling up about 1/2 or more of the drop were rated 4. Intermediate quantities were rated either 1, 2 or 3 accordingly. A reaction was considered positive even if the same dilution of antiserum and healthy sisal sap formed a precipitate, provided that the precipitate with healthy sap had a lower rating value than with virus. All antisera formed precipitates with healthy sap, but the amount of precipitate was less than that formed with virus preparations.

Both antiserum and antigen titer began to decline 38 days after the first injection, in tests with partially purified virus. However, there was no decline in antiserum titer when tests were made with crude KLS-infected sap. Healthy sisal sap also showed a high titer, except that the amount of precipitate was much less in comparison to tests with KLS-infected sap.

Antiserum collected 24 days after the first injection was totally insensitive to KLS-virus in ELISA tests. This is in agreement with what was reported by Clark and Adams (1977). Antiserum collected from day 31 onwards was sensitive to variable levels. In 83% of ELISA tests made (10/12 experiments) the reaction was masked by high background readings (the absorbance readings with healthy sap were more than half those for KLS-infected sap). Reactions were therefore considered non-specific. The antiserum was cross-absorbed with purified healthy sap to eliminate antibodies to
plant antigens, and hence the high background readings. Purified antiserum was mixed with healthy sap (partially purified by same procedure as the virus) in a 9:1 ratio and incubated overnight at 4 °C. The mixture was then centrifuged 1 hr at 67,000 g, and the pellet discarded. The supernatant was then used to coat ELISA plates as usual. The enzyme-conjugated IgG was also cross-absorbed by mixing with purified healthy sap at the same ratio, and incubating overnight at 4 °C prior to use. In one experiment using both cross-absorbed IgG and conjugated-IgG, the background absorbance was considerably reduced so that antiserum reliably detected its homologous antigen. Absorbance readings were 0.375 and 0.172 respectively for KLS-infected and healthy sap. The titer was 100 and 500 respectively for the IgG and conjugated IgG.

It was also observed in that experiment that the concentration of sap affected absorbance readings. On the same plate, readings for infected sap diluted 5, 10 and 50x were 0.049, 0.077 and 0.282 respectively. The corresponding reading for healthy sap diluted 5x was 0.07, a value higher than that for KLS-infected sap at the same dilution. These results suggest that there are inhibitors in the sisal sap which hinder antibody-antigen reactions when the sap is concentrated.

Another factor contributing to change of the trend in results was that in this test plant sap was ground by mechanical homogenization in a blender. These results suggest that homogenization, which was previously observed to cause particle breakage, helped to increase the sensitivity of the antiserum.
Probably the antiserum contained more antibodies that can recognize only fragments of the virus.

Tests for serological relationship with citrus tristeza virus by ELISA were also masked by the high background readings. Antisera to CTV showed higher absorbance readings with KLS-associated virus than the KLS virus with its homologous antibodies. Thus the results were also discarded as being non-specific reactions. KLS-associated virus was not serologically related to the unidentified virus infecting white lupines (in the greenhouse) at Oregon State University. Antiserum to the lupine virus had absorbance readings of more than 2.00 with its homolog, while corresponding readings with healthy lupines and KLS-infected sap were 0.046 and 0.069 respectively.

The antiserum to partially purified KLS-associated virus was very useful in detecting its homolog in ISEM tests. Grids that were pre-coated with antiserum always trapped more virus particles than the non-coated. In tissues where virus concentration was low and could not be detected on uncoated grids, ISEM detected the virus. It was therefore possible with this antiserum to detect virus particles even in the symptomless, sap-inoculated sisal plants, and in one noninoculated plant.

By counting the number of particles trapped on 10 randomly picked grid openings, a serological relationship was detected between KLS-associated and citrus tristeza viruses. In a reciprocal test, grids coated with antiserum to CTV trapped 49 CTV particles and 38 of KLS-associated virus, while those coated with antiserum to
TABLE 8 Antiserum to partially purified virus: schedule* for collection and titer in microprecipitin tests.

<table>
<thead>
<tr>
<th>Test with</th>
<th>Date of</th>
<th>Days after</th>
<th>Antiserum and antigen titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>partially</td>
<td>Collection</td>
<td>first injection</td>
<td>Rabbit No. 7013</td>
</tr>
<tr>
<td>purified</td>
<td>virus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>virus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oct. 24, '85</td>
<td>24</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Oct. 31, '85</td>
<td>31</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>Nov. 7, '85</td>
<td>38</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>Nov. 14, '85</td>
<td>45</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Test with</td>
<td>Oct. 24, 31, '85</td>
<td>24, 31</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Nov. 7, '85</td>
<td>38</td>
<td>1024</td>
</tr>
<tr>
<td></td>
<td>Nov. 14, '85</td>
<td>45</td>
<td>1024</td>
</tr>
<tr>
<td>crude KLS-</td>
<td>Test with</td>
<td>Oct. 24, '85</td>
<td>24</td>
</tr>
<tr>
<td>infected</td>
<td>healthy</td>
<td>Oct. 31, '85</td>
<td>31</td>
</tr>
<tr>
<td>sap</td>
<td>Nov. 7, '85</td>
<td>38</td>
<td>1024</td>
</tr>
<tr>
<td>Nov. 14, '85</td>
<td>45</td>
<td>1024</td>
<td>1024</td>
</tr>
</tbody>
</table>

*Injection of rabbits done intramuscularly on 30 Sept., 7 and 14 Oct., '85. Amount not determined.

ND - Not determined

No precipitate between pre-immune serum and partially purified virus, but with crude KLS-infected sap precipitation occurred; titer 8.0.
Table 9 Schedule for rabbit injection and antiserum collection, and titer in microprecipitin tests for antiserum to purified virus.

<table>
<thead>
<tr>
<th>Rabbit No. and antigen tested</th>
<th>Amount of virus (µg) and date injected</th>
<th>25 days</th>
<th>32 days</th>
<th>39 days</th>
<th>46 days</th>
<th>64 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7-24-86</td>
<td>7-31</td>
<td>8-7</td>
<td>8-14</td>
<td>9-11-86</td>
<td>As</td>
</tr>
<tr>
<td>2728 (A)</td>
<td>38.7</td>
<td>33.5</td>
<td>28.5</td>
<td>0</td>
<td>37.8</td>
<td>32</td>
</tr>
<tr>
<td>(B)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>64</td>
</tr>
<tr>
<td>2704 (A)</td>
<td>38.7</td>
<td>33.5</td>
<td>28.5</td>
<td>85</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>(B)</td>
<td></td>
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</tr>
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<td>2886 (A)</td>
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<td></td>
</tr>
<tr>
<td>(B)</td>
<td>44</td>
<td>72.5</td>
<td>53.8</td>
<td>0</td>
<td>51.5</td>
<td>32</td>
</tr>
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<td>2889 (A)</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>(B)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

As* - antiserum
Ag# - antigen
ND - Not determined
NR - No reaction

Antiserum titer with healthy sap, 8.0

Antigen type (A) is the virus fraction that contained mostly unbroken particles and (B) is the lighter fraction, composed mostly of fragmented particles.

Rabbits No. 2728 and 2704 were immunized with type (A) while 2886 and 2889 were immunized with type (B).
KLS-associated trapped 57 of its homolog and 26 CTV particles. Attempts to decorate the viruses with antiserum to KLS-associated virus were unsuccessful, due to the interference of plant debris on top of the particles.

Serological tests with antiserum to purified virus

Antiserum titer was also determined by the microprecipitin test. The antigen was the respective purified virus used to immunize rabbits and purified healthy sisal sap. Similar to results with antiserum to partially purified virus, the titer differed depending on type of virus fraction used for immunization, rabbit and time of antiserum collection. Results are presented in Table 9.

Unlike the previous antiserum, titer with healthy sap was very low (8.0). Peak titer was attained at different times depending on injection schedule and virus used for immunization. Rabbits No. 2728 and 2886, which received three injections prior to start of antiserum collection, produced antiserum with peak titer 39 and 25 days respectively after the first injection. Similarly, rabbits No. 2704 and 2889, which received four successive injections before antiserum collection began, produced peak titer antisera respectively at 39 and 32 days from first injection.

Antiserum from rabbits No. 2886 and 2889, which was produced to the lighter virus fraction (containing mainly fragmented particles), reached peak titer within a shorter period and as quickly began to decline (Table 9). The antiserum to intact virus particles produced in rabbits No. 2728 and 2704 reached peak titer more slowly and had
not begun to decline when a booster injection was applied. Although antiserum to intact particles could react with both virus fractions, antiserum to the lighter fraction could not recognize the fraction containing intact particles (Table 9). This difference in reactions between fractions occurred with antisera from all the rabbits before booster injections were administered. Two weeks after booster injection, however, antiserum from rabbit No. 2889 reacted weakly with the fraction containing intact particles.

Use of these two antisera in ELISA and ISEM is in progress, but will not be presented as part of this thesis.

**Physical-chemical properties**

Ultraviolet light absorption spectra for purified KLS-associated virus in 0.05 M Tris-HCl buffer, pH 8.0 were examined (Fig. 11). The absorbance characteristics were slightly different for the virus fraction containing intact particles (Fig. 11A), than the one that contained fragmented particles (Fig. 11B). While the spectrum for the former was typical of Closteroviruses, with peak absorbance at 260 nm wavelength, the latter showed peak absorbance at 266-270 nm, suggesting a higher protein content.

The $A_{260/280}$ ratio for purified virus containing intact particles (Fig. 11A) was 1.61 (after correction for light scattering). This value, however, fluctuated between 1.40 and 1.74, and was consistent irrespective of whether it was taken before or after dialysis. This ratio suggests a nucleic content of more than 5% (Bar-Joseph and Hull, 1974). The ratio for the fraction
containing broken particles was 1.23, and fluctuated between 1.20 and 1.45 in different purifications. The $A_{\text{max}/\text{min}}$ ratio was 1.21 and 1.20 respectively for the fractions with intact and broken particles. In partially purified preparations, the ratio was often higher, up to 1.85, but decreased to within the mentioned range on completion of purification.

The purified virus suspended in 0.05 M Tris-HCl buffer pH 8.0, did not resuspend in distilled water after it was lyophilized. This hindered determination of the extinction coefficient. Irreversible aggregation was also experienced during attempts to pellet the purified virus suspended in the same buffer.

Buoyant density was calculated from refractive index values of the virus-containing fractions after equilibrium centrifugation. For calibration, standard curves for Cs$_2$SO$_4$ solutions of concentrated 10-53% at 25 °C were prepared. One was used for refractive index vs concentration, and the other for concentration vs density. To determine the density of each solution, a formula developed by Ludlum and Warner (1965) was used:

$$\rho_{25} = 1.0047 + 0.28369 \, m - 0.017428 \, m^2$$

(0.5 ≤ $m$ ≤ 3.5, where $m$ is molarity)

By fitting refractive index values for the virus fractions onto standard Cs$_2$SO$_4$ curves, the density of particles was determined.

Virus bands in equilibrium gradients were diffuse. The gradient for the fraction with predominantly broken particles was more diffuse, spreading over 8, 0.3 ml fractions; which reflected the uneven size of particles. Density for 5, 0.3 ml fractions in
which the preparation with intact particles suspended was between 1.247 and 1.302, with a mean density $\rho = 1.26$ g/cc. Similarly, the preparation with broken particles suspended in 8, 0.3 ml fractions of density 1.191-1.255 and mean density $\rho = 1.23$ g/cc.
DISCUSSION AND CONCLUSIONS

Results strongly indicate that the flexuous virus particles isolated from KLS-infected sisal leaves are the infectious agent of KLS, because this virus could be transmitted to healthy sisal in sap-inoculation tests. In addition, this virus induced the same symptoms in successive generations of healthy beans and particles were re-isolated from symptomatic plants. Inability to reproduce symptoms on sisal in the growth chamber is attributed to environmental conditions, since it was possible before to reproduce symptoms under greenhouse conditions. Factors would be temperature, light intensity and period of illumination, which are known to influence symptom expression in viral infections (Gibbs and Harrison, 1976; Bos, 1973). Furthermore, Korogwe leaf spot disease is known to have a long incubation period (Lock, 1969; Keswani et al., 1982). In previous experiments, it was 11 months before symptoms appeared on sap-inoculated sisal in the greenhouse; thus, it is not surprising that symptoms did not develop on our inoculated plants.

Detection of virus particles in one noninoculated sisal plant is also not surprising, because sisal bulbils from which plants were raised are vegetative propagation units, borne in flower axils on inflorescences. Although caution was taken to harvest bulbils from nonsymptomatic mother plants, this study has demonstrated that lack of symptoms is no assurance for freedom from virus infection. The finding however, puts to doubt the results indicating
sap-transmission of virus particles and reproduction of KLS symptoms. There is no assurance that those sisal plants which gave positive results were not infected already from their source plants, because at very low concentration, virus particles could have escaped detection in the leaf dip preparations performed before planting. Furthermore, a random sample of 3 out of 17 is under these circumstances not adequate, and assay on the indicator host was probably not sensitive enough a test to detect virus at very low concentration. A confirmative test would require use of a very sensitive test such as ISEM to screen all young, healthy plantlets before inoculation with KLS-infected sap is undertaken.

The percentage natural infection is very low, since it was only one plant out of 9 (11%) noninoculated or 'mock' inoculated plants that contained particles. These results agree with speculations by Keswani et al. (1982) that if a virus is the pathogen for KLS, then it is unable to reach, or reaches with difficulty, the reproductive organs of the plant. Thus symptoms are delayed and low disease incidence occurs in the field on crops that are planted from bulbils as compared to those raised from suckers.

KLS-associated virus is distinct from the virus-like organisms associated with parallel streak, a disorder reported to occur naturally in sisal in Kenya (Pinkerton and Bock, 1969), in the area adjacent to the KLS-affected area in Tanzania. Unlike the organisms in parallel streak affected plants, which are spherical, KLS-associated virus has rod-shaped, long and very flexuous particles. Presence of these flexuous rods in leaf dip extracts and purified
preparations from symptomatic sisal leaves and sap-inoculated leaves, as well as particles in phloem cells of KLS-infected tissue, is strong evidence for relating the virus to the disease. It also confirms previous findings (Anon, 1974, 1976; Ebbels and Allen, 1979; Lock, 1969; Keswani et al., 1982) that KLS is not caused by either fungi, bacteria, insect damage or nutritional deficiency. Absolute proof of viral etiology for KLS will, however, require additional tests since detection of virus particles in a noninoculated plant have cast doubt on the results from previous sap-inoculation experiments.

Due to the long incubation period involved in sisal, faster results may be achieved using beans as an inoculum source. More sensitive tests such as ELISA or ISEM will also be available. They are faster and more reliable for screening plants for virus infection.

Many attributes of Closteroviruses (Bar-Joseph et al., 1979; Lister and Bar-Joseph, 1981; Francki et al., 1985), including restriction to phloem tissue, are shared by KLS-associated virus. As suggested for some of these Closteroviruses (Price, 1966; Bar-Joseph et al., 1979), this property may possibly explain the difficulty encountered in mechanical transmission and purification of this virus.

Host ranges for described Closteroviruses are restricted to either monocots or dicots alone (Lister and Bar-Joseph, 1981). KLS-associated virus, however, differs in that it can infect both sisal (monocot) and common bean (dicot). However, symptoms were
induced in beans under subjective greenhouse conditions, and not natural field conditions. Infection may, therefore, not occur under normal conditions. Consequently, field experiments will be required to establish whether or not this is only a greenhouse phenomenon.

Purification procedures gave yields of 0.0-5.56 mg/100g tissue. These are similar to amounts usually obtained in purification of Closteroviruses (Lister and Bar-Joseph, 1981). Among factors which influence virus yield are the type of host plant, host growth conditions, time of harvest, type of harvested tissue and purification procedure. This study investigated two factors: purification procedure and type of host plant. Both were shown to influence yield considerably. These factors were reported to influence yields of other Closteroviruses, including CTV (Bar-Joseph et al., 1973), BYV (Bar-Joseph et al., 1974) and ACLSV (Lister and Hadidi, 1971). Similar to Closteroviruses, KLS-associated virus was very sensitive to shear under stress during mechanical extraction and when sap was clarified with organic solvents. The tendency for shearing during extraction was probably the main reason for inability to band the virus in sucrose rate-zonal gradients. This property was the main obstacle in purification of CTV in sucrose gradients (Garnsey et al., 1981).

Adsorption of virus particle fragments onto light weight, open-structure, tubular bodies during purification is a unique property of KLS-associated virus. This property was most likely the cause for virus remaining at the top of Cs$_2$SO$_4$ density gradients
when relatively low centrifugal force (65,000 g) was applied for 17 hrs. It also explains the formation of two virus bands in sucrose/Cs$_2$SO$_4$ cushion gradients, with the shorter particles banding higher up in the gradient. These open-structure bodies most probably reduce the buoyant density of attached particles. Lister and Hadidi (1971) reported during purification of ACLSV that low-molecular weight virus degradation products remained at the top of gradients during isopycnic centrifugation. There is a possibility that the open-structure tubular bodies observed in this study are degradation products of KLS-associated virus. That could be a possible reason why the UV-absorption spectrum for the virus fraction that banded high up in sucrose/Cs$_2$SO$_4$ gradients showed maximum absorption at 266-270 nm, as opposed to the expected pattern which was obtained for intact particles.

KLS-associated virus was similar to CTV (Bar-Joseph et al., 1970), BYV (Bar-Joseph et al., 1974) and ACLSV (Lister and Hadidi, 1971) in not withstanding centrifugation in CsCl density gradients. Unlike BYV and ACLSV, KLS-associated virus did not need Mg$^{++}$ ions for particle integrity. However, a low concentration of these ions was essential when sap was clarified with bentonite to prevent complete adsorption of the virus by the clay. Concentrations of 0.001 M and above caused aggregation and subsequent precipitation. Lister and Hadidi (1971) reported that the importance of Mg$^{++}$ ions in particle integrity and higher yields of ACLSV was influenced by pH, while Chairez and Lister (1973) did not find Mg$^{++}$ ions essential when ACLSV was infecting pear rather than apple. pH may have
an influence on the precipitating behavior of KLS-associated virus in the presence of Mg$^{++}$ ions and is worthy of further investigation.

Attempts to concentrate KLS-associated virus by pelleting after overnight dialysis against 0.05 M Tris-HCl buffer pH 8.0, resulted in irreversible aggregation. The pellet could not resuspend in the same buffer. ACLSV has a similar property but it is also pH dependent. When dialysed in buffer of pH 7.5-7.6, ACLSV aggregates irreversibly, however, the pellet easily resuspends at pH 8.4. This might imply that pH influences are also involved with KLS-associated virus. Another characteristic shared with ACLSV is a tendency for loss during overnight dialysis of purified preparations. Whereas loss of ACLSV was attributed to particle degradation, loss of KLS-associated virus is more likely due to adsorption onto dialysis tubing, because it was reduced by prior coating of the tubing with bovine serum albumin.

The $A_{260/280}$ ratio of purified preparations of between 1.40 and 1.74 (corrected for light scattering) is unusually high for typical rod-shaped viruses whose values are normally 1.1-1.2 (Lister and Hadidi, 1971). This suggests a rather unusual RNA and/or protein content or composition. The ratio is close to values reported for HLV (Bem and Murant, 1979), CNFV (Bar-Joseph and Smookler, 1976), ACLSV (Lister and Hadidi, 1971), LCLV (Brunt, 1978), and BYV (Bar-Joseph and Hull, 1974), which are respectively 1.50, 1.53, 1.57, 1.69 and 1.73. For CNFV and BYV, a high $A_{260/280}$ ratio was associated with absence of tryptophan in the coat protein
Particle density in Cs₂SO₄ at equilibrium of 1.26 and 1.23 g/cc for respectively the intact and fragmented particles is quite normal for a rod-shaped virus, and is close to the value of 1.27 reported for BYV (Kassanis et al., 1977) and ACLSV (Bar-Joseph et al., 1974). This implies that the high A₂₆₀/₂₈₀ ratio may not be due to unusual proportions of RNA and protein. Thus, it probably reflects an unusual composition of protein or RNA.

Closteroviruses form a non-uniform group, which is arbitrarily subdivided according to molecular weight of coat protein and vector transmission (Bar-Joseph et al., 1979), or particle length (Lister et al., 1981). Francki et al. (1985) has recently used a combination of both classification criteria and cytopathological effects induced on the host to divide the group into three subgroups. Information provided by this study is still scanty for definite placement of KLS-associated virus into a specific subgroup. However, the cytopathological effects induced by this virus in the phloem cells of infected leaves suggest affiliation with subgroup I. The amorphous inclusions observed both in the light and electron microscopes, and fibrous inclusions seen in the light microscope are typical of what was described for subgroup I Closteroviruses (Esau, 1968; Christie et al., 1977; Bar-Joseph et al., 1979; Francki et al., 1985). KLS-associated virus is sap transmissible as are four members of subgroup I: CTV, CNFV and BYV. All members are naturally vectored by aphids in a semi-persistent manner. However, aphids have not been previously reported to feed on sisal. Recent reports
of transmission of some Closteroviruses by mealybugs (Francki et al., 1985) and whiteflies (Yamashita et al., 1979) suggest that insect populations associated with sisal should be examined for a possible vector.

Although determination of specific modal length of KLS-associated virus was obscured by preparation techniques, the four modal lengths at 950, 1075, 1450 and 1650 all favor affiliation with subgroup I Closteroviruses. It is well documented that because of their flexuous nature, Closteroviruses may assume different dimensions depending upon the stain (Bar-Joseph et al., 1979; Lister and Bar-Joseph, 1981; Francki et al., 1985). Also, PTA stain is not a particularly good stain for Closteroviruses because it induces extensive breakage (Hamilton et al., 1981; Lister and Hadidi, 1971). Use of PTA stain and the tendency for aggregation and shearing in extraction were the major causes of difficulty in our original modal length determinations. Ascertaining particle modal length would require use of only the recommended stains for Closteroviruses, uranyl acetate and uranyl formate. Improvement in preparing these stains would also be of importance in order to overcome problems of precipitation and poor staining encountered during this study.

Antiserum produced to KLS-associated virus has a potential for making further studies of the virus characteristics much easier because it reacts specifically. The low sensitivity of the antiserum to partially purified virus in ELISA tests was basically a problem of antibodies formed to plant antigens introduced into
rabbits with the virus. Pre-adsorption of the globulin with healthy plant antigens overcame the problem of high background but did not increase the titer at which antiserum could detect the virus. Sensitivity of ELISA is ultimately dependent on the specificity, purity and concentration of IgG.

Results with antiserum to fragmented particles of purified virus that cannot recognize intact particles are rather unexpected. However, these results conform with reports by Van Regenmortel (1966) and Lister et al. (1981) that complications are likely to arise in the use of antisera to Closteroviruses due to dissociation of virus particles both during purification, and after introduction into rabbits. Dissociation results in production of antibodies that recognize intact particles, and those which recognize only quartenary associations of protein subunits. The usefulness of such antiserum which recognizes only particle fragments may be very limited for diagnostic purposes.

Although tentatively a serological relationship was detected between CTV and KLS-associated virus, the degree of relationship was not established. This would need to be established with antiserum improved to remove non-specific reactions.

This study has provided preliminary information about the characteristics of KLS-associated virus. More information is still needed on properties that are essential for complete characterization; including definite modal length, pitch of helix, sedimentation coefficient, size and composition of protein and nucleic acid, as well as serological relationships with other known
Closteroviruses. KLS-associated virus probably is a new Closterovirus. The ability to detect this virus in crude sap by ISEM and ELISA provides a useful technique for diagnosis of latent KLS infections. ELISA should help in rapid screening of propagative material, preventing spread of disease through planting material, particularly to areas which are not yet affected.
BIBLIOGRAPHY


APPENDICES
Appendix A: Purification Procedures

Some of the original procedures were modified to suit requirements of the KLS-associated virus. References are provided for detail of the original purification schemes.

1. Procedure of Hiebert and MacDonald (1971) for Purification of Potyviruses (53)

   i) Mix in a Waring blender 100 g infected sisal leaves (chopped), 400 ml 0.5 M Potassium phosphate buffer pH 7.5 and 0.5 g Na$_2$SO$_4$. Add 2 drops Antifoam B, and blend for 1-2 min.
   ii) Strain through cheesecloth; save the filtrate.
   iii) Centrifuge the sap at 9,150 g for 10 min. Save the supernatant.
   iv) To the supernatant add n-butanol up to 8% (v/v) and stir at 4 °C for 4 hrs.
   v) Centrifuge the clarified liquid at 9,150 g for 15 min. Save the supernatant.
   vi) Filter the supernatant through glasswool placed inside a folded Whatman #1 filter.
   vii) To the filtrate add PEG (8,000 MW) to 8% (w/v) of the volume. Stir 1 hr at 4 °C.
   viii) Centrifuge 20 min at 11,700 g. Discard the supernatant.
   ix) Resuspend the pellet in 20 ml of 0.02 M phosphate buffer pH 7.5 containing 0.1% mercaptaethanol. Stir 2 hrs at 4 °C.
   x) Centrifuge 5 min at 11,000 g. Save the supernatant.
   xi) Centrifuge the supernatant 3 hrs at 22,000 rpm in a 50.2 Ti rotor. Discard the supernatant.
   xii) Resuspend the pellet in 5 ml of 0.02 M phosphate buffer, pH 7.5 and stir 2 hrs at 4 °C.
   xiii) Centrifuge 5 min at 4,080 g. Save supernatant. This preparation is partially purified.
   xiv) For rate zonal centrifugation, layer 10 ml of the partially purified preparation over a 10-40% sucrose gradient. The gradient is prepared a day before use in SW 28 rotor tube by layering over one another 8.5 ml of 10, 20, 30 and 40% sucrose in 0.02 M phosphate buffer and refrigerated overnight. Centrifuge 1.5 hrs at 27,000 rpm.
   xv) Remove the light scattering virus band from the gradient, dilute with an equal volume of buffer and centrifuge 4 hrs at 22,000 rpm in a 50.2 Ti rotor. Discard the supernatant. Resuspend the pellet in 1 ml of 0.02 M phosphate buffer pH 7.5. Store frozen at -20 °C.
   xvi) For equilibrium density centrifugation, dilute a partially purified virus preparation with buffer to make the volume 12 ml. To this suspension add 4.628 g of CsCl and stir to dissolve. Fill a SW 40 rotor tube with the suspension and centrifuge 16 hrs at 33,000 rpm. Fractionate the gradient column and dialyse the virus containing fractions against 0.025 M phosphate buffer pH 7.5. Store the virus frozen at -20 °C if not used immediately.
2. **Purification of PSbMV-L (48)**

   i) Mix in a Waring blender and blend for 1 min:
   - 10 g infected sisal leaves (chopped)
   - 40 ml phosphate-DIECA buffer pH 8.0 (0.06 M NaH$_2$PO$_4$ and 0.02 M Dieca)
   - 0.05 g Na$_2$SO$_3$
   - 2 drops Antifoam B.

   ii) Strain through cheesecloth; save the filtrate.

   iii) Centrifuge 15 min at 4,080 g; save supernatant.

   iv) While stirring, gradually add chloroform to the supernatant up to 7.5% (v/v). Continue to stir 10 min at 4 °C.

   v) Centrifuge the mixture 10 min at 12,100 g. Save supernatant.

   vi) Centrifuge 1 hr at 40,000 rpm in a 50.2 Ti rotor. Discard supernatant, and resuspend the pellet overnight in 10 ml of 0.01 M borate buffer pH 9.0.

   vii) Centrifuge 5 min at 3,020 g. Save supernatant.

   viii) Layer the supernatant over 10 ml of 35% sucrose in borate buffer containing 6% PEG and 0.12 M NaCl. Centrifuge 1.25 hr at 45,000 rpm in a Beckman 65 rotor. Discard supernatant.

   ix) Resuspend the pellet overnight at 4 °C in 1 ml 0.01 M borate buffer pH 9.0. Store frozen at -20 °C.

   x) For CsCl equilibrium density centrifugation, add more buffer to the prep. from step vii to make the volume 12 ml. While stirring, add 4.628 g CsCl to the suspension, then centrifuge 23 hr at 33,000 rpm in SW 40 rotor.

   xi) Collect the virus containing band from the gradient after fractionation and dialyse against 0.025 M phosphate buffer pH 7.5. Store the virus frozen at -20 °C.
3. **Purification of Beet Yellows Virus (10)**

i) Grind 25 g of KLS-infected tissue to powder in liquid nitrogen with a mortar and pestle.

ii) Thaw with 125 ml 0.1 M Tris-HCl buffer pH 7.8 containing 0.01 M Cysteine-HCl.

iii) Strain through cheesecloth.

iv) Centrifuge 5 min at 4,000 g, followed by 10 min at 8,000 g; each time save supernatant.

v) Mix the supernatant with 7 ml of a 50 mg/ml bentonite solution and centrifuge 10 min at 5,900 g. Save supernatant.

vi) To every 100 ml of supernatant add 5g PEG (8,000 MW) and 0.5 ml of 5 M NaCl. Stir to dissolve then centrifuge 15 min at 12,100 g. Discard the supernatant.

vii) Dissolve the pellet in 1/6 original volume of 0.05 M Tris-HCl buffer containing 0.01 M MgCl$_2$ and 0.01 M cysteine-HCl.

viii) Centrifuge 10 min at 5,900 g. Save supernatant.

ix) To the supernatant add PEG (8,000 MW) and NaCl to make respectively 4% and 1% (w/v). Stir to dissolve and centrifuge 10 min at 12,000 g.

x) Resuspend pellet in 4 ml 0.05 M Tris-HCl buffer pH 7.8 containing 0.01 M MgCl$_2$. Stir 2 hr at 4 °C. The preparation is partially purified.

xi) Layer 2 ml of the prep. over a preformed 10-40% sucrose gradient (in 0.05 M Tris-HCl buffer pH 7.8, with 0.01 M MgCl$_2$). Centrifuge 4 hr at 22,000 rpm in a SW 28 rotor.

xii) Fractionate the gradient with an ISCO 640 density gradient fractionator and collect fractions with peak UV-absorbance; pool them.

xiii) Make the collected fractions 4% (w/v) with PEG (8,000 MW). Stir to dissolve and centrifuge 10 min at 12,000 g. Discard supernatant.

xiv) Resuspend the pellet in 1 ml 0.05 M Tris-HCl buffer pH 7.8, with 0.01 M MgCl$_2$.

xv) For further purification, the virus prepared by rate zonal centrifugation is passed through an equilibrium density gradient. Mix 3.1 ml of virus (from step xiv) with 1.6 ml Ca$_2$SO$_4$ solution (conc. of 1.68 mg/ml) in 0.05 M Tris-HCl buffer pH 7.1 containing 0.01 M MgCl$_2$. Centrifuge the mixture 24-30 hrs at 35,000 rpm in a Spinco SW 39 rotor.

xvi) Fractionate the gradient with an ISCO density gradient fractionator into 0.2 ml fractions. Pool fractions with peak UV absorbance; precipitate the virus with PEG (8,000 MW) and resuspend the pellet in 1 ml 0.05 M Tris-HCl buffer pH 7.8 containing 0.01 M MgCl$_2$. Store the purified virus frozen at -20 °C.
Appendix B: Serological techniques

1. Antiserum collection

i) Wrap the rabbit in a bag or tie the limbs to restrict movement. Clip the peripheral ear vein then touch the ear tip with xylene to stimulate dilation of the vein.

ii) Shave a small area in the middle portion of the ear around the vein to be bled; then surface sterilize the area with alcohol.

iii) With a sterile #22 scalpel make a small, longitudinal slit along the vein. Collect 5-10 ml of blood in a sterile screw top tube.

iv) Leave the blood at room temperature for 1 hr to clot. With the tip of a clean cotton swab or toothpick loosen the clot from the sides of the tube.

v) Incubate the blood at 37 °C for 1 hr. Let cool and refrigerate overnight.

vi) The antiserum will have separated from the blood clot and is floating on top. Decant it off or draw it out with a pasteur pipette. Centrifuge 10 min at 10,000 rpm in a Beckman SS-34 rotor. Quickly pour off the supernatant, which is a clear yellowish liquid.

vii) Store the antiserum in small siliconized vials at 4 °C, containing 0.02% Sodium azide to prevent contamination. Vials are siliconised beforehand by rinsing with Sigmacote (Sigma Chemical Co., St. Louis) and allowed to air dry. It prevents globulins from sticking to the sides of the vials.
2. **CM Affi-Gel Blue Method** of purifying whole antiserum to obtain IgG

i) Prepare a CM Affi-Gel Blue column with a total bed volume of 6 ml for every 1 ml of antiserum to be purified.

ii) Wash the column with 2 volumes of buffer A (1 volume = 10 ml). Buffer A = 0.01 M K$_2$HPO$_4$, pH 7.25 containing 0.15 M NaCl and 0.02% NaN$_3$. When washing the column, add the buffer gently to avoid bubbles forming on the surface of the column, and don't let the surface dry out at any time.

iii) Apply the antiserum to the column. As soon as the antiserum is absorbed in the column start collecting 1 ml portions of the effluent in a set of 15-20, 1 ml test tubes previously arranged in a test tube rack and labelled sequentially. Chase the antiserum through the column with Buffer A, making sure to keep the surface of the column wet all the time when effluent is collected. The column absorbs albumin and plasminogen from the antiserum so that the effluent will contain only immunoglobulin (IgG).

iv) Read the absorbance of each 1 ml fraction of the effluent in the spectrophotometer at 280 nm wavelength.

v) Save the first fraction in the series with highest absorbance at 280 nm for preparation of enzyme-conjugated IgG. Pool the other fractions with peak absorbance readings and read absorbance again. Continue mixing with a little of the dilute fractions and checking absorbance until the absorbance reading of 1.4 is attained. (Absorbance of 1.4 O.D. at 280 nm is equivalent to a concentration of 1 mg/ml IgG in the antiserum). Dilute the first saved fraction with Buffer A in the same way until absorbance of 1.4 O.D. is attained.

vi) Store the IgG in 1 ml fractions in siliconized vials at 4 °C. The IgG may be lyophilized and stored in powder form at 4 °C. Before use it is reconstituted to the original volume with Buffer A.

vii) To regenerate the column, wash it with 2 bed volumes (10 ml each) of 6 M guanidine HCl followed by two volumes of buffer A. Store at 4 °C covered with buffer. The gel's useful life is 8-10 cycles. However, after 5 cycles increase the gel-to-sample ratio by 20% to compensate for loss of absorbing capacity with time.

3. **Conjugation of purified IgG with Alkaline Phosphatase enzyme**

Purified IgG was conjugated with the enzyme by the procedure of Clark and Adams (1977) as outlined:

i) Centrifuge 1000 units (= 176 μl) of Type VII alkaline phosphatase (Sigma No. P4502) at 6,000 rpm for 5 min. in a Beckman SS-34 rotor. Carefully pour off the supernatant (ammonium sulfate carrier).

ii) Dissolve the pellet directly in 1 ml of purified IgG (at a conc. of 1 mg/ml). To avoid losing the pellet, add globulin directly to the centrifuge tube.

iii) Dialyze against 500 ml PBS (phosphate buffered saline) twice at 1 hr interval then overnight at 4 °C to remove the last traces of ammonium sulfate.

iv) Add fresh glutaraldehyde solution to the IgG-enzyme complex (conjugate) to make a final concentration of 0.05% (v/v). Mix well (i.e. dilute 25% glutaraldehyde in distilled water to 3% concentration, then add 20 μl of 3% solution to 1 ml of conjugate).

v) Leave 4 hrs at room temperature. A yellow-brown color should develop, indicating that the enzyme has been fixed to the IgG molecule.

vi) Dialyze against 500 ml PBS, twice at 1 hr interval then overnight at 4 °C, to remove the glutaraldehyde.

vii) Add bovine serum albumin at a conc. of 5 mg/ml and store at 4 °C. (Dissolve 0.1 g of bovine serum albumin in 100 μl distilled water in a microcentrifuge tube; then add 50 μl of this solution to 1 ml of the dialyzed conjugate.) Bovine serum albumin stabilizes the conjugated IgG.
Purification of whole antiserum by precipitation with Na$_2$SO$_4$.

i) To 2.5 ml antiserum, add an equal amount of distilled water.

ii) Prepare 36% Na$_2$SO$_4$ in distilled water, and add 5 ml of this solution to the diluted antiserum (1:1 ratio). Mix and let stand 10 min at room temperature to precipitate IgG.

iii) Centrifuge 15 min at 7,000 rpm in a Sorvall SS-34 rotor at room temperature. Keep the white pellet.

iv) Wash the pellet with 10 ml of 18% Na$_2$SO$_4$ and centrifuge as before. Resuspend pellet in 1.0 ml PBS and dialyze at 4 °C against 500 ml PBS; twice at 1 hr interval then overnight.

v) If there is a precipitate after dialysis, remove by centrifugation in a Sorvall SS-34 rotor at 5,000 rpm for 10 min at room temperature. Save the supernatant. That is the stock IgG solution.

vi) Read the absorbance of stock IgG in the spectrophotometer at 280 nm. Estimate the concentration of IgG in the preparation using the extinction coefficient (A$_{280}$) = 1.5. The relationship is:

\[ \text{Conc.} = \frac{\text{Absorbance reading}}{\text{Extinction coefficient}} \times \text{dilution} \]

(Dilution is considered only if the reading was above the range of the spectrophotometer and the preparation was diluted to make absorbance reading.) Add NaN$_3$ to 0.02% and store frozen, or lyophilize and store at 4 °C for long term storage.

vii) To dilute the stock IgG to the concentration of 1 mg/ml for coating ELISA plates, use the concentration of stock IgG calculated from absorbance reading before; i.e.,

\[ \text{Vol. of stock IgG required} = \frac{\text{Volume of dilute IgG}}{\text{Concentration of stock IgG}} \]

Dilute to the needed volume with PBS containing 0.02% NaN$_3$. Store at 4 °C.

(b) For conjugation with alkaline phosphatase enzyme:

i) Dilute stock IgG with PBS to get 0.56 ml IgG with a concentration of 2 mg/ml.

ii) Combine with 0.5 ml of alkaline phosphatase enzyme preparation (Sigma Type VII) at a concentration of 5 mg/ml.

iii) Dialyze against PBS at 5 °C with several changes and overnight.

iv) Place measured volumes in small test tubes at room temperature. Add enough 10% glutaraldehyde in distilled water to make 0.2% solution. Incubate 2 hr at room temperature.

v) Dialyze at 5 °C against 500 ml of PBS; at least 2 changes at 1 hr interval then overnight.

*A modified procedure of Clark and Adams, 1977 (Hampton, personal communication).*
5. **Buffers used in ELISA and ISEM**

All buffers listed were prepared to a final volume of 1 l with distilled water, and pH adjusted with either NaOH or HCl. Except for PBS, all were stored at 4 °C.

**PBS (Phosphate Buffered Saline)**

8.0 g NaCl  
0.2 g KH$_2$PO$_4$  
0.2 g KCl  
2.9 g Na$_2$HPO$_4$.12H$_2$O  
0.2 g NaN$_3$

**ELISA 'coating buffer' pH 9.6**

1.59 g Na$_2$CO$_3$  
2.93 g NaHCO$_3$  
0.20 g NaN$_3$

**ELISA 'sample buffer' pH 7.4**

1 l PBS-Tween  
20.0 g Polyvinylpyrrolidone (PVP) - MW 10 or 40  
2.0 g Ovalalbumin  
0.2 g NaN$_3$

**ELISA 'substrate buffer' pH 9.8**

97 ml Diethanolamine  
0.2 g NaN$_3$  
(Start with 800 ml water, add HCl till desired pH is obtained then increase volume to 1 l.)

**ELISA 'washing buffer' (PBS-Tween) pH 7.4**

1 l PBS  
0.5 ml Tween 20

Sorensen's phosphate buffer, pH 7.0

9.078 g/l KH$_2$PO$_4$ - Solution A  
9.470 g/l Na$_2$HPO$_4$ - Solution B  
Mix 392 ml A + 608 ml B to obtain buffer pH 7.0.