SOME PROPERTIES OF A TOBACCO RINGSPOT VIRUS ISOLATE FROM SOUTH AUSTRALIA

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Summary

A virus has been isolated from *Gladiolus* sp. (cv. Spic and Span) in South Australia. This gladiolus virus has been partially purified and could not be distinguished serologically from a North American strain of tobacco ringspot virus although it is symptomologically unlike the type strain. Gladiolus virus was not transmitted by the aphids *Myzus persicae* (Sulz.), *Aphis craccivora* (Koch), *Aphis gossypii* (Glov.), *Hyperomyzus lactucae* (L.), or *Macrosiphum euphorbiae* (Thos.). The virus failed to be transmitted through soil infested with the nematode *Xiphinema americanum* Cobb, but was transmissible through seeds of *Glycine max* Merr. (cv. Lincoln) and *Nicotiana glutinosa* L.

Partially purified preparations of gladiolus virus contained polyhedral particles about 29 mμ in diameter and in the analytical ultracentrifuge showed four peaks of 21, 57, 99, and 136S. The 21 and 57S components were proteins whereas the 99 and 136S components were nucleoproteins containing about 21 and 35% ribonucleic acid respectively. The molar base ratios guanine : adenine : cytosine : uracil of gladiolus virus are 23·8 : 22·3 : 22·5 : 31·4.

I. Introduction

During 1962 a virus was isolated from a *Gladiolus* plant (cv. Spic and Span) from a commercial plantation at Balhannah, S. Aust. The plant had abnormally short, distorted spikes and small flowers with pointed petals. The virus was difficult to identify and was thought to be new (Francki, Randles, and Chambers 1964). It was referred to as "gladiolus virus" in a paper describing its fine structure (Chambers, Francki, and Randles 1965). In this paper some properties of gladiolus virus are described which indicate that it is a strain of tobacco ringspot virus (TRSV).

II. Materials and Methods

(*a*) *Virus Culture and Purification Procedure*

The virus isolate was cultured in cucumber seedlings (*Cucumis sativus* L. cv. Davis Perfect). The plants were inoculated when at the cotyledonary stage and harvested 10–14 days later when the leaves showed conspicuous symptoms. The purification procedure described by Scott (1963) for cucumber mosaic virus was used and 1 kg of leaf material yielded between 30 and 140 mg of virus.

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(*b*) *Host Range Studies*

Cucumber leaf sap from virus-infected plants was mechanically inoculated, using 500-mesh carborundum as abrasive, to a range of test plants which were grown in an insect-proof glasshouse. The plants were observed for symptoms and after one month they were indexed by mechanical inoculation to cucumber seedlings.

(*c*) *Aphid Transmission Tests*

The aphids used for transmission studies were the following: *Aphis gossypii* (Glov.) raised on cucumber; *Myzus persicae* (Sulz.) and *Macrosiphum euphorbiae* (Thos.) on *Datura stramonium* L.; *Hyperomyzus lactucae* (L.) on *Sonchus oleraceus* L.; and *Aphis craccivora* (Koch) on *Vicia faba* L.

In each transmission test one or two adults or late instar larvae, which had been starved for 1–4 hr and allowed to feed for 15–30 min on a cucumber leaf with conspicuous virus symptoms, were transferred to individual cucumber seedlings. The aphids were sprayed with systemic insecticide 1–2 hr after feeding on the test seedlings. Longer acquisition and test-feeding periods were allowed in tests for persistent transmission.

(*d*) *Nematode Transmission Tests*

Soil infested with *Xiphinema americanum* Cobb was collected from Magill, S. Aust. Gladiolus virus-infected *Chenopodium hybridum* L. and cucumber plants were grown in the nematode-infested soil at 25°C. After 15 days the virus-infected plants were removed and cucumber seeds were sown. The cucumber seedlings were observed for 3 weeks, after which their roots were indexed on cucumber seedlings and the soil was checked for the presence of nematodes.

(*e*) *Seed Transmission Tests*

Young seedlings of *Phaseolus vulgaris* L. (cv. Bountiful and cv. Pinto), *Glycine max* Merr. (cv. Lincoln), and *Nicotiana glutinosa* were mechanically inoculated with gladiolus virus. The plants were indexed on cucumber seedlings 4 weeks later and seed was collected after a further 3 months. Seedlings raised from this seed were observed for symptom development and were indexed on cucumber.

(*f*) *Serological Methods*

Partially purified virus preparations emulsified with Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan) were injected subcutaneously into a rabbit at weekly intervals. The rabbit received three injections each containing approximately 5 mg of virus and was bled a week after the last injection. All serological tests were carried out by the double-diffusion precipitin technique (Crowle 1961) using 0·75% agar in 0·005M borate buffer, pH 9, containing 0·14M NaCl and 0·02% sodium azide.

(*g*) *Ultracentrifugation*

Ultracentrifugal analyses were carried out in a Spinco Model E machine using the AnD rotor at 33,450 r.p.m.

Estimation of sedimentation coefficients was carried out by adding partially purified cucumber mosaic virus (Q strain) to gladiolus virus preparations as a reference macromolecule. Cucumber mosaic virus has an $S_{20,w} = 99S$ after correction to infinite dilution (Wilson, personal communication).

Virus preparations were fractionated by sucrose density-gradient ultracentrifugation in the 39 SW head of a Spinco Model L machine. Gradients were prepared by layering solutions of 50, 40, 30, 20, and 10% sucrose dissolved in 0·005M borate buffer, pH 9, in 2 by $\frac{1}{2}$ in. tubes, and allowing them to equilibrate for 16 hr at 4°C. Aliquots of 0·3–0·5 ml, containing 4–8 mg of partially purified virus were layered on top of the gradients and the tubes were centrifuged for 2 hr at 34,000 r.p.m. Bands were detected by light scattering and removed with hypodermic needles. Fractionated preparations were dialysed overnight against 0·005M borate buffer, pH 9, at 4°C.

(*h*) *Analytical Methods*

Molar base ratios were determined by the technique of Markham (1955). Virus or virus RNA prepared by phenol extraction (Gierer and Schramm 1956) was precipitated by the addition of 3 volumes of ethanol in the presence of Mg^{2+}, dried at 90°C, and hydrolysed in 1N HCl for 1 hr at 100°C. Nucleic acid derivatives were separated by paper chromatography in the isopropanol–HCl solvent (Markham 1955). Optical density measurements were carried out in a cell of 1 cm path length using a Shimadzu QR 50 spectrophotometer.

RNA was estimated quantitatively in nucleoprotein preparations purified by differential centrifugation and fractionated by sucrose density-gradient centrifugation. Preparations were dried at 90°C, hydrolysed with 2N KOH for 24 hr at room temperature and neutralized with 2N $HClO_4$. The resulting mononucleotides were separated by paper chromatography in the isopropanol–NH_3 solvent as described by Matthews (1958). RNA concentration was determined from the optical density at 260 mμ of the eluted mononucleotides.

Protein determinations were calculated from total nitrogen estimations using the microKjeldahl method (Markham 1942) on the assumption that both protein and RNA contain 15% nitrogen.

III. Results

(*a*) *Isolation of the Virus*

Gladiolus virus was isolated from only one plant of the commercial *Gladiolus* cv. Spic and Span. All attempts to isolate a virus from other *Gladiolus* plants showing only the flower and spike distortion were unsuccessful. Gladiolus virus was readily cultured in cucumber seedlings (*Cucumis sativus* cv. Davis Perfect), all transfers being carried out by mechanical inoculations. Unsuccessful attempts were made to transmit gladiolus virus back to *Gladiolus* by patch-grafting, rubbing leaves with carborundum and infectious sap, and by injecting partially purified virus preparations into the corm with a hypodermic syringe.

(*b*) *Host Range and Symptoms*

Gladiolus virus was mechanically inoculated to 35 varieties of 29 species of test plants, and symptoms were observed on 19 of these (Table 1), and some are illustrated in Plate 1, Figures 1–6. On many hosts, gladiolus virus produced variable symptoms according to the light and temperature conditions.

TABLE 1

SYMPTOMS INDUCED BY GLADIOLUS VIRUS ON THE LEAVES OF SOME SUSCEPTIBLE PLANTS

Host Plant	Symptoms
Chenopodiaceae	
Chenopodium amaranticolor Coste & Reyn.	Necrotic local lesions
Spinacea oleracea L.	Systemic chlorotic spots
Cucurbitaceae	
Cucumis sativus L. cv. Davis Perfect	Mosaic; local ringspots develop on cotyledons under low light intensities
Citrullus vulgaris Schrad.	Necrotic local and systemic lesions
Leguminosae	
Lupinus sp.	
cv. Mixed	Local lesions, and systemic chlorotic spots
cv. Russell	Local lesions
cv. Blue Pearl	Local lesions, systemic chlorotic spots
Pisum sativum L.	
cv. Greenfeast	Slight mottle
cv. White Brunswick	Slight chlorosis
Vigna sinensis Savi cv. Blackeye	Reddish local lesions produced on some plants only; other plants infected systemically, but symptomless
Phaseolus vulgaris L.	
cv. Canadian Wonder	Mosaic, some veinal necrosis
cv. Sutters Pink	Systemic chlorotic spots
cv. Bountiful	Line pattern
cv. Pinto	Veinal necrosis
Scrophulariaceae	
Antirrhinum majus L.	Necrotic local ringspots
Solanaceae	
Datura stramonium L.	Local ringspots develop under low light intensities
Nicotiana tabacum L. cv. Blue Prior	Faint line patterns develop under low light intensities
Petunia hybrida Vilm. cv. Rosy Morn	Faint mottle
Umbelliferae	
Apium graveolens L.	Necrosis and chlorotic ringspots

The virus was recovered from the following nine test plants which showed no recognizable symptoms: *Calendula* sp., *Tagetes erecta* L., *Zinnia elegans* Jacq., *Lepidium sativum* L., *Brassica pekinensis* Rupr. cv. Wong Bok, *Glycine max* cv. Lincoln, *Nicotiana glutinosa*, *Viola* sp., and *Gypsophila elegans* Bieb.

Gladiolus virus failed to infect *Brassica rapa* L. cv. Purple Top Globe, *Zea mays* L., *Secale cereale* L., *Aquilegia* sp., *Lycospersicon esculentum* Mill., *Daucus carota* L., and *Gomphrena globosa* L.

On some hosts such as *Chenopodium amaranticolor* Coste & Reyn., *Citrullus vulgaris* Schrad., *Phaseolus vulgaris* L., and *Antirrhinum majus* L. symptoms were strongly reminiscent of TRSV. However, on other hosts, e.g. *Vigna sinensis* Savi cv. Blackeye and *Cucumis sativus* cv. Davis Perfect, the symptoms differed from those usually associated with TRSV infection.

(*c*) *Transmission Studies*

The virus was not transmitted by any of the five aphid species tested. Three isolates of cucumber mosaic virus were tested simultaneously and each aphid species readily transmitted at least one isolate; *M. persicae*, *A. gossypii*, and *M. euphorbiae* transmitted all three isolates. *M. persicae* was also tested for transmission of gladiolus virus, following acquisition periods of 5 and 6 days, without success.

The virus was not transmitted to any of 20 cucumber test plants in soil infested with *Xiphinema americanum* which had fed on virus-infected *Chenopodium amaranticolor*. Live nematodes were recovered from the soil at the conclusion of the experiment.

The virus was transmitted through the seed of Lincoln soybean, where 13 out of 15 seedlings were shown to be carrying the virus, and in the seed of *Nicotiana glutinosa*, where 9 out of 31 seedlings were infected. Gladiolus virus was not carried in the seed of *Phaseolus vulgaris* cv. Pinto. Infected *P. vulgaris* cv. Bountiful produced seedless pods, and the yield of seed from infected soybean was also markedly reduced.

(*d*) *Properties of Partially Purified Virus Preparations*

Preparations obtained by chloroform clarification and three cycles of ultracentrifugation were opalescent and light green in colour. Absorption spectra between 230 and 300 mμ showed a maximum at 260 mμ and a minimum at 242 mμ and were characteristic of a nucleoprotein. Examination of these preparations in the electron microscope revealed the presence of icosahedral particle of about 29 mμ in diameter as already reported (Chambers, Francki, and Randles 1965). Virus preparations were still infectious after storage at 2–4°C for 10 months. The infectivity of purified virus preparations (0·3 mg/ml in 0·005M borate buffer, pH 9) as well as that of crude sap from infected cucumber plants was destroyed by heating for 10 min at 65°C, but not at 60°C.

On examining virus preparations in the analytical ultracentrifuge, four peaks were observed (Plate 2, Fig. 1) with sedimentation coefficients ($S_{20,w}$) $\approx$ 21, 57, 99, and 136S. In some preparations the 57S component could not be detected (Plate 2, Fig. 2). The four components of the virus preparations could be separated into relatively well-resolved bands in sucrose density-gradients (Plate 2, Fig. 4). The 21 and 57S components exhibited ultraviolet spectra characteristic of proteins whereas the 103S and 143S components resembled nucleoproteins (Plate 2, Fig. 3). The 99 and 136S components were found to have 21 and 35% nucleic acid, respectively. Both nucleoprotein components produced infections when inoculated to cucumber seedlings, but no attempts were made to determine their relative infectivity quantitatively, as a suitable local-lesion assay is not yet available.

RNA molar base ratios were previously reported erroneously (Francki, Randles, and Chambers 1964). The correct values for the base ratios from unfractionated virus preparations and from isolated 99 and 136S components are given in Table 2. Values from unfractionated preparations and from the two nucleoprotein components are not significantly different.

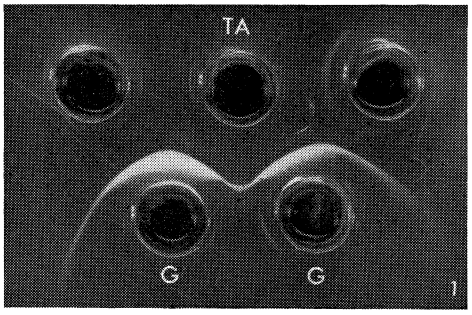
TABLE 2

MOLAR BASE RATIOS OF GLADIOLUS VIRUS PREPARATIONS

Preparation Used	Moles %			
	Guanine	Adenine	Cytosine	Uracil
Virus nucleoprotein	25·1	21·3	22·7	30·9
Virus RNA	24·4	22·6	21·1	31·9
Isolated 99S component	22·9	21·6	23·7	31·8
Isolated 136S component	22·8	23·8	22·5	30·9
Mean	23·8	22·3	22·5	31·4

(*e*) *Serological Tests*

Antiserum obtained from a rabbit immunized with gladiolus virus had a titre of 1/512. When tested against the virus or sap from virus-infected plants a single band of precipitation was observed in gel-diffusion tests.



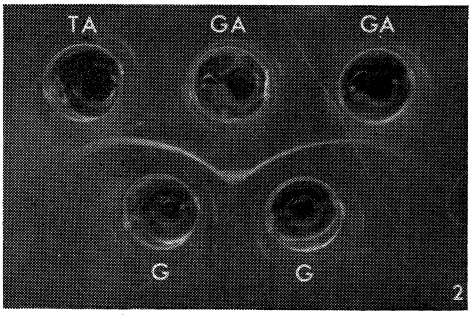


Fig. 1.—Serological reaction in agar gel diffusion between TRSV antiserum (*TA*) and a partially purified gladiolus virus preparation (*G*).

Fig. 2.—Confluence of lines of precipitation between a partially purified gladiolus preparation (*G*) its homologous antiserum (*GA*), and TRSV antiserum (*TA*).

Gladiolus virus reacted strongly with TRSV antiserum (Fig. 1). The line of precipitation in the heterologous reaction with TRSV antiserum was confluent with, and of equal strength to, the homologous reaction between gladiolus virus and its own antiserum (Fig. 2).

IV. Discussion

Data presented in this paper indicate that gladiolus virus is a strain of TRSV and, as far as we are aware, this is the first substantiated record of TRSV in Australia. Gladiolus virus could not be distinguished serologically from a North American TRSV isolate and it has other properties resembling those described for TRSV.

Gladiolus virus has an average particle diameter of *c.* 29 mμ (Chambers, Francki, and Randles 1965) whereas TRSV was found to be 26 mμ in diameter (Steere 1956). However, this discrepancy could be accounted for by the fact that our measurements were made on negatively stained preparations whilst those of Steere (1956) were on shadowed preparations. Gladiolus virus has RNA base ratios similar to those found for TRSV by Kaper and Steere (1959) and the slight discrepancies may be due to the fact that in this work RNA was hydrolysed with 1N HCl whereas Kaper and Steere (1959) used 70% $HClO_4$. Gladiolus virus, like TRSV, does not appear to be transmitted by aphids but is seedborne in soybean. The 136 component of gladiolus virus has a similar RNA content to that reported for TRSV (Steere 1956).

Partially purified preparations of gladiolus virus had two nucleoprotein and usually two protein components. The nucleoprotein appears to be similar to that found in preparations of TRSV (Steere 1956) except that our preparations had a very much higher proportion of the 99S component. However, this may be due to the fact that Steere (1956) used relatively short periods of ultracentrifugation in his purification procedure (30 min at 105, 000 *g*) which would have tended to eliminate the 99S component in preference to the 136S. Components of 21 and 57S have not been found previously in preparations of TRSV and it is still uncertain if they are of virus or host origin. Further work is in progress to investigate the nature and relationships of the various components found in preparations of gladiolus virus.

The failure to demonstrate transmission of gladiolus virus in soil containing *Xiphinema americanum* could suggest that it differs from TRSV, but such failure could also be explained by the inability of our strain of the nematode to transmit typical TRSV. Because typical TRSV is not available in Australia, the ability of our nematode isolate to transmit this virus has not been tested.

The host range and symptoms produced by gladiolus virus are not typical of TRSV and this illustrates how inadequate can be the identification of a virus by symptomology alone. Gladiolus virus failed to give symptoms typical of TRSV on plants usually considered as reliable differential hosts, such as cowpea and *Nicotiana glutinosa*. On the results of host range studies, gladiolus virus could be regarded as a very mild strain of TRSV by virtue of the very mild symptoms on many hosts usually severely affected by TRSV and its failure to produce any symptoms on other known hosts of TRSV.

V. Acknowledgments

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VI. References

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TOBACCO RINGSPOT VIRUS

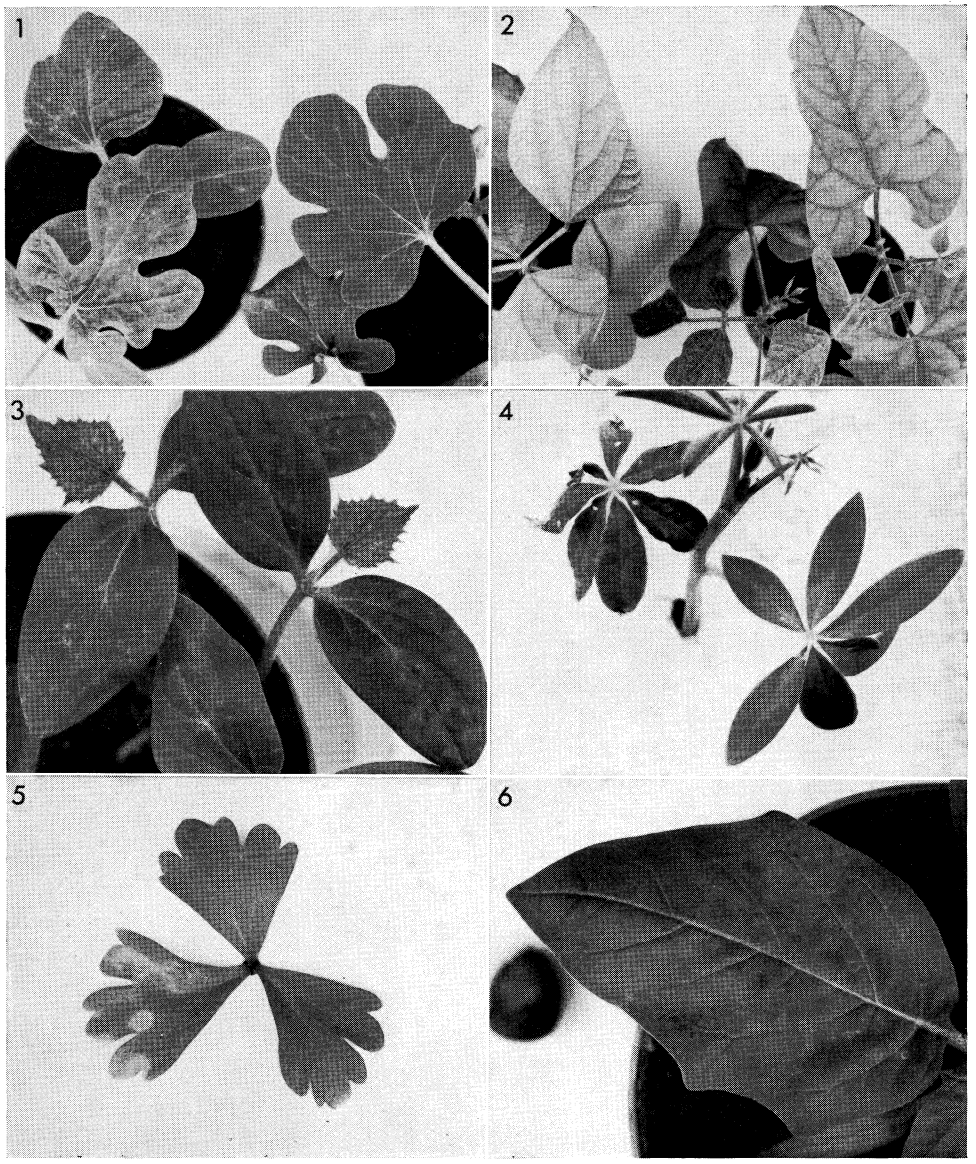
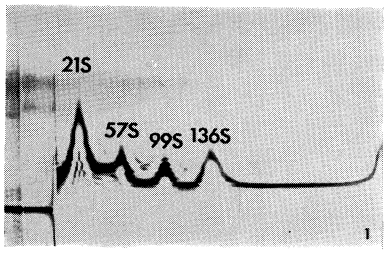
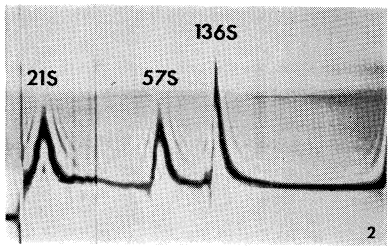
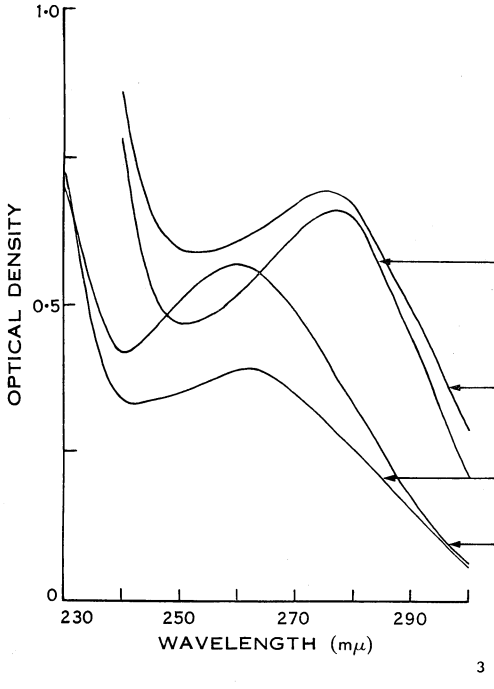


Fig. 1.—Local and systemic lesions on water melon (left) compared with a healthy leaf (right).
Fig. 2.—Mottle with vein-yellowing on young leaves of Bountiful bean.
Fig. 3.—Local ringspots on the cotyledons of cucumbers, and mosaic on the first leaves. (Plants grown under shaded conditions.)
Fig. 4.—Chlorotic and necrotic ringspotting on Blue Pearl lupin.
Fig. 5.—Chlorotic ringspotting on celery, preceding a necrotic ringspotting.
Fig. 6.—Local ringspots on the inoculated leaf of *Datura stramonium*. (Plant grown under shaded conditions.)

TOBACCO RINGSPOT VIRUS







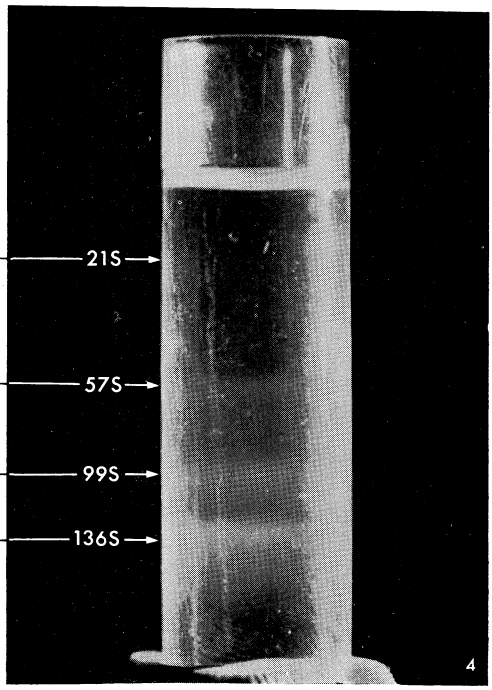


Fig. 1.—Schlieren diagram of a gladiolus virus preparation in 0·005M borate buffer, pH 9, with 21, 57, 99, and 136S components. Sedimentation from left to right. Photograph taken 11 min after reaching a speed of 33,450 r.p.m. at bar angle of 60°.

Fig. 2.—Schlieren diagram of a gladiolus virus preparation in 0·005M borate buffer, pH 9, with 21, 57, and 136S components. Sedimentation from left to right. Photograph taken 12 min after reaching a speed of 33,450 r.p.m. at bar angle of 60°.

Figs. 3 and 4.—Ultraviolet absorption spectra (Fig. 3) of the 21, 57, 99, and 136S components of gladiolus virus separated by density-gradient centrifugation in sucrose (Fig. 4).