STUDIES ON LETTUCE NECROTIC YELLOWS VIRUS.

by

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STATEMENT

This thesis contains no material which has been accepted for the award of any other degree of diploma and contains no material previously published or written by another person except where due reference is made in the text.

G.D. McLean

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SUMMARY

A study of some aspects of the purification, serological properties, ultraviolet inactivation of the virus and the interaction of lettuce necrotic yellows virus (LNYV) in Nicotiana glutinosa L was made. This work is described in four separate sections of this thesis, each section of which is followed by a discussion.

1. The purification of the virus presented unusual difficulties due to its complex structure and instability. Leaf homogenates of infected N. glutinosa were clarified by adsorption of host cell material on charcoal and DEAE-cellulose powder and filtration through celite. Virus was then concentrated by centrifugation of clarified extracts. Attempts were made to further purify the virus by density gradient centrifugation, ammonium sulphate precipitation and acidification and it was found that none of these methods were satisfactory. However, purification was achieved by column chromatography on calcium phosphate gel. LNYV purified by this method was used for the production of an antiserum in a rabbit.

2. The serological reactions of the virus, using the agar gel diffusion technique were investigated. It was shown that only fragments of the virus reacted with the antiserum as intact virus particles did not diffuse through the agar in significant amounts.

Evidence is presented that LNYV contains at least two, and possibly three different antigens. These results are discussed in relation to those obtained from more extensive studies on vesicular stomatitis virus, an animal virus structurally similar to LNYV.

3. The virus was found to be approximately eight times more sensitive to ultraviolet irradiation than the U2 strain of tobacco mosaic virus (TMV). The '*in vivo*' and '*in vitro*' dose response curves were similar. When virus-inoculated half-leaves were irradiated at increasing intervals of time after inoculation, the fraction of infection centres surviving increased with time, as found by other workers for TMV and tobacco necrosis virus.

4. Observations were made of the symptoms produced by the SE3 strain of LNYV on *N. glutinosa*. Investigations were also carried out on the distribution, translocation and multiplication of the virus in *N. glutinosa*. LNYV probably resembles many other viruses in its interaction with its host plant; particularly those viruses that produce both local lesions and systemic infection.

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1. INTRODUCTION

In 1954, a destructive virus disease of lettuce was recognized in Victoria which was distinct from lettuce mosaic (Stubbs and Grogan, 1963a). However, until 1959 transmission experiments were unsuccessful and in that year isolates of the virus were incorrectly identified as tomato spotted wilt virus. By 1963, these workers had named the new virus, lettuce necrotic yellows virus (LNYV), and had investigated its host range and other properties. The virus was shown to be mechanically transmissible, unstable and transmitted by the aphid Hyperomyzus lactucae L in a circulative manner (Stubbs and Grogan, 1963b). It was pointed out that it was rare to find a vector (Hyperomyzus) whose only known economic host (lettuce) is not a host of the vector, and the only natural source of the virus (sowthistle) being also the only source of the vector. The control of the virus disease by the destruction of sowthistle has been reported (Stubbs, Guy and Stubbs, 1963).

The partial purification of LNYV was achieved by clarifying leaf extracts with the fluorocarbon Freon-113, followed by differential centrifugation and centrifugation in sucrose density gradients (Crowley et al., 1965). Some of the properties and structure of the virus were then studied (Harrison and Crowley, 1965). The infectivity of virus preparations was found to be

decreased or abolished by treatment with chloroform, diethyl ether or water saturated phenol. The bacilliform or bullet-shaped virus particle was shown to be about 66 mμ wide and about 227 mμ long.

Electron microscopic observations of sections of LNYV-infected N. glutinosa and lettuce leaf mesophyll cells showed the presence of bundles of viral particles in double membrane-bound structures in the cytoplasm (Chambers et al., 1965). The virus was also shown to be present in leaf vein xylem and was extracted from stem xylem of N. glutinosa (Chambers and Francki, 1966).

Recently, many workers have shown a bacilliform or bullet-shape structure for viruses isolated from plants, invertebrates and vertebrates. Simpson and Hauser (1966) summarised the properties of some of these viruses. Since then other viruses have been described which have a bacilliform shape: Flanders virus (Murphy et al., 1966), Kern Canyon virus (Murphy and Fields, 1967), potato yellow dwarf virus (MacLeod et al., 1966), Gomphrena virus (Kitajima and Costa, 1966), northern cereal mosaic virus (Shikata and Lu, 1967), brocolli necrotic yellows virus (Hills and Campbell, 1968), sowthistle yellow vein virus (Richardson and Sylvester, 1968) and rice transitory yellowing virus (Shikata and Chen, 1969). Both aphid and leafhopper transmission has been reported for some of these viruses. Both LNYV and sowthistle yellow vein virus are transmitted by the aphid, Hyperomyzus lactucae L and both viruses infect sowthistle. Gomphrena virus

resembles LNYV to some extent in that both viruses have been mechanically inoculated to a number of host plants. It would seem that extensive serological and biochemical investigations are required to establish if any relationship exists between these viruses.

The results of these studies have been reported in four independent sections of this thesis, each of which contains an introduction to the particular aspect of the study. These sections report studies on:

i) The purification of the virus and the preparation of an antiserum to the virus.

ii) The serological properties of the virus, which concerns the reactions of the virus or treated-virus in gel diffusion tests.

iii) The inactivation of the virus by ultraviolet irradiation.

iv) The symptomatology, distribution, translocation and multiplication of the virus.

2. GENERAL MATERIALS AND METHODS

This section contains those materials and methods which are used throughout this study. Other materials and methods are recorded in each particular section of the thesis.

2A. Virus Isolate

The virus isolate was the SE3 strain of LNYV originally obtained from Sonchus oleraceus L and kindly supplied by Dr. L.L. Stubbs, Victorian Plant Research Institute. Naturally infected lettuce (Lactuca sativa L) were also used as a source of LNYV.

LNYV was maintained in an insect-proof glasshouse on N. glutinosa by mechanical inoculation to healthy plants every 10 to 14 days. Sap was rubbed on leaves dusted with 500 mesh carborundum or celite (Hyflo Super-Cel) was incorporated in the inoculum for the assays in the ultraviolet inactivation experiments.

2B. Infectivity Assay

The infectivity of LNYV was assayed on the most susceptible leaves of N. glutinosa (Crowley, 1967). Because of this variation in susceptibility to infection, in many tests infectivity was compared with that of a standard inoculum applied to the opposite half of each leaf. Because of the instability of the virus, both in sap (Stubbs and Grogan, 1963b) and in partially purified preparations at 20° (Harrison and Crowley, 1965), inocula were kept on ice, prior to inoculation.

3. STUDIES ON THE PURIFICATION OF LNYV AND THE PREPARATION OF AN ANTISERUM TO THE VIRUS

3A. Introduction

Partial purification of lettuce necrotic yellows virus (LNYV) was achieved by Crowley et al. (1965), but virus purified by this method contained much host-cell material as determined by electron microscopy and serological tests. Virus preparations of high purity were required for further studying the properties of the virus.

This section describes the extraction of the virus from infected leaf tissue, the clarification and purification of these extracts. Crowley et al. (1965) found that most methods usually employed in the clarification of extracts from virus-infected plants, either abolished or greatly reduced the infectivity of LNYV preparations. In this study, charcoal (Steere, 1963), Freon-113 (Crowley et al., 1965) and DEAE-cellulose (Whitcomb, 1965) were tested in various combinations and were all found to be potentially useful for the clarification of extracts from LNYV-infected plants.

Three approaches were taken in the further purification of the virus: (a) density gradient centrifugation, (b) preferential precipitation of the virus using ammonium sulphate, methanol or accidification, (c) column chromatography. Column chromatography of concentrated LNYV preparations on calcium phosphate gel proved to

be the most satisfactory method, although variations in the qualities of laboratory-prepared and commercially-obtained gels were observed. Three criteria were used to test the purity of the virus preparations obtained from these columns: serology, electron microscopy and analytical ultracentrifugation.

An antiserum to the virus was prepared and was used in studying the serological properties of the virus (section 4).

Some of the results reported in this section have already been published (see appendix 1, McLean and Francki, 1967).

3B. Materials and Methods

i) Preparation of celite filters. Twenty gm of celite Hyflo Super-Cel) was mixed with approximately 200 ml of distilled water and poured over 2 Whatman No. 1 filter papers, 12.5 cm diameter, in a buchner funnel so as to form a pad $\frac{1}{2}$ cm thick. Such pads were used for the filtration of 100-150 ml of plant extracts. For small volumes of partially purified virus preparations filters were made in 1.5 cm Pyrex filter holders.

ii) Column chromatography. Calcium phosphate gels obtained from commercial sources (see Materials VI) and also prepared in the laboratory according to Siegelman et al. (1965) were used. Columns, 7 cm in height, in glass tubes 1.3 cm in diameter, were prepared as described by Levine (1962). These columns were equilibrated with the required buffers, as described

later, and the flow rate adjusted to between 20-40 ml per hour.

iii) Optical density measurements. The approximate concentration of particulate material in virus preparations was measured in a Shimadzu QR50 spectrophotometer at 260 mμ. Absorption at this wave length was partly due to light scattering.

iv) Density gradient centrifugation. Linear density gradients were prepared in 30 ml Spinco SW 25 tubes using 15% and 45% sucrose solutions in 0.01 M phosphate buffer, pH 7.0. One ml of virus suspension was layered on the surface of the gradient which was centrifuged at 24,000 rpm for 45 minutes in the SW 25 Spinco rotor. Gradients were analysed and fractionated in an ISCO model D density-gradient fractionator and UV analyser (Brakke, 1963).

v) Analytical centrifugation. Analyses were carried out in a Spinco Model E machine. Plain cells (small cell, volume 0.25 ml) were used in the AnD rotor at 9,341 rpm. Photographs were taken at 2 minute intervals.

vi) Electron microscopy. Virus preparations were mixed with an equal volume of 2% phosphotungstic acid (neutralized with KOH to pH 7). Drops of this mixture were applied to copper grids which had been coated with collodion and backed with carbon. Staining with uranyl acetate was carried out by applying a drop of virus suspension to a grid and then floating the grid downwards on

3% unbuffered uranyl acetate solution (pH approximately 4.5). The excess solution was removed with filter paper and the grids air-dried at room temperature. All grids were examined with a Phillips 100B electron microscope.

vii) Serological methods. Precipitin tests were carried out in small tubes, using 0.5 ml of antiserum and 0.5 ml of antigen which was diluted in 0.85% saline and kept for 4 hours with the liquid partially immersed in a water bath at 30°. Gel diffusion tests were carried out by the double-diffusion precipitin technique (Crowle, 1961) using 0.75% agar in 0.01 M phosphate buffer, pH 7.6, containing 0.14 M NaCl and 0.02% sodium azide.

viii) Materials. The following chemicals were obtained from these commercial sources: decolourising charcoal, May and Baker, Dagenham, England; DEAE-cellulose, Bio-Rad Chemicals, Richmond, California or Whatman, England; and calcium phosphate gel (Hypatite C), Clarksom Chemical Company, Williamsport, Pa. or Bio-Rad Chemicals).

3C. Experimental - Purification of the Virus

i) Extraction of the virus from infected leaf tissue. Two methods were used for the extraction of LNYV from infected leaf tissue. These will be referred to as the Freon/glycine method and the phosphate/homogenization method throughout this thesis.

Freon/glycine extraction. This procedure was used by Crowley et al. (1965), and later modified by Crowley (private communication). Ten gm of infected leaves were emulsified with 20 ml of 0.1 M glycine-NaOH buffer (pH 9.3) and 20 ml of Freon-113 in a Servall Omni-mixer, for 10 seconds at 220 volts. The emulsion was broken by centrifugation at 1,000 g for 4 minutes. Aliquots of the top aqueous layer were diluted in 0.1 M glycine-NaOH buffer (pH 8.6), so as to produce 30 to 60 lesions per half-leaf on N. glutinosa.

Phosphate/homogenization extraction. Infected leaves were homogenized in a Waring blender with $1\frac{1}{2}$ volumes of 0.2 M Na_2HPO_4, previously cooled by ice. The extract (pH approximately 7) was strained through cheese-cloth and centrifuged at 2,000 g for 1 minute. The pellets were discarded and the supernatant clarified, sedimented and purified.

ii) Comparison of Freon/glycine and phosphate/homogenization procedures. The infectivity of Freon/glycine prepared extracts was compared to that of the phosphate/homogenization extracts in the following experiment. Two 10 gm samples of infected half-leaves were obtained. One sample was treated as described for the Freon/glycine method. The other sample was homogenized in 60 ml 0.2 M Na_2HPO_4 in the Servall Omni-mixer and centrifuged for 1,000 g for 4 minutes. A sample of the supernatant

was kept for dilution and the remainder was filtered through celite, after the charcoal treatment. Dilutions of each of these three solutions (Freon/glycine extract, phosphate/homogenization extract and the filtrate) were made in 0.1 M glycine-NaOH buffer (pH 8.6), so as to give the same dilution in respect to the initial weight of leaf tissue. The results of infectivity comparisons (Table 1) show that both extraction methods yield approximately the same amount of infectious material.

Table 1

Comparison of the amounts of infectious material obtained after Freon/glycine extraction and phosphate/homogenization

Freon/glycine Phosphate/homogenization	41/33 (a)
Freon/glycine Charcoal-celite filtrate of the phosphate extract	43/35 (a)

(a) Mean local lesions per half-leaf produced by each of the extracts. Each assay was carried out on 12 leaves.

iii) Loss of infectious material on slow speed centrifugation.

The efficiency of release of free virus into the extracting medium

(Na_2HPO_4) during homogenization or the loss of infectious material on slow speed centrifugation was tested in the following way (Flow diagram, Fig. 1). Infected leaves were homogenized in Na_2HPO_4, strained through cheese cloth and centrifuged at 2,000 g for 1 minute. The supernatant was then centrifuged at 3,000 g for 5 minutes. The supernatant was kept and the pellet resuspended in a volume of 0.1 M phosphate buffer, pH 7, equal to that of the original extract. This solution was also centrifuged at 3,000 g for 5 minutes and this procedure was repeated twice. The infectivity of the original supernatant was compared to that of the two subsequent supernatants and the resuspended third pellet. Results of this experiment (Table 2) show that in initial slow-speed centrifugation of a leaf extract infectious material was lost in the pellet.

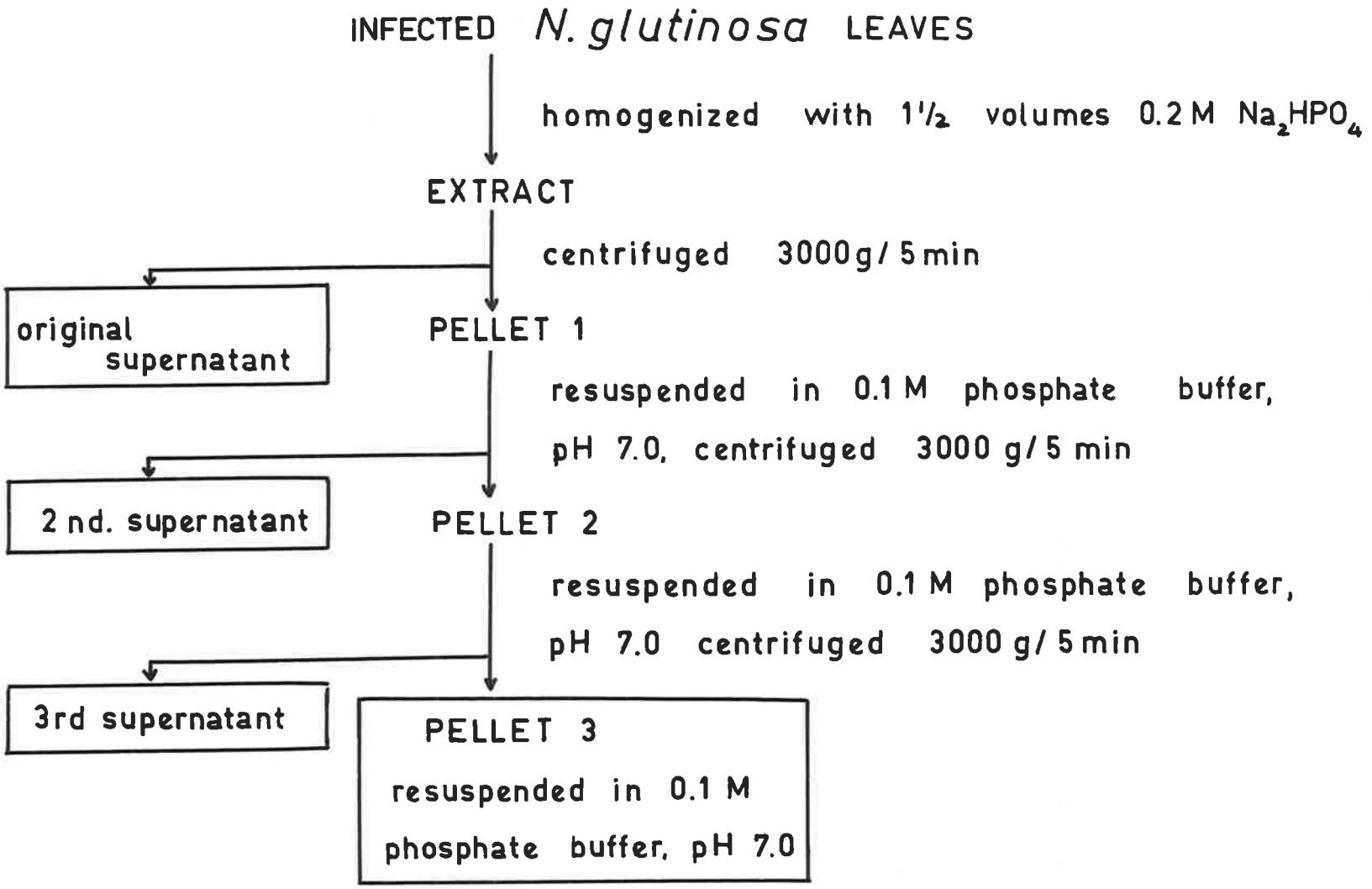
Table 2

Recovery of infectious material from the pellets after successive slow speed centrifugation

Extract assayed	Infectivity relative to original supernatant
2nd supernatant	$^{29}/_{77}$ (a)
3rd supernatant	$^{21}/_{96}$
3rd pellet (resuspended)	$^{18}/_{80}$

(a) Local lesions per half-leaf produced on opposite half-leaves of N. glutinosa by extract (numerator) and original supernatant (denominator). Each assay was carried out on 18 leaves.

Figure 1 Flow diagram, showing the procedure followed to test the loss of infectious material on slow speed centrifugation.


INFECTED N. glutinosa LEAVES
homogenized with 1½ volumes 0.2 M Na_2HPO_4
EXTRACT
centrifuged 3000g/5min
original supernatant
PELLET 1
resuspended in 0.1 M phosphate buffer, pH 7.0, centrifuged 3000 g/5 min
2 nd. supernatant
PELLET 2
resuspended in 0.1 M phosphate buffer, pH 7.0 centrifuged 3000 g/5 min
3rd supernatant
PELLET 3
resuspended in 0.1 M phosphate buffer, pH 7.0

iv) Clarification of extracts. Charcoal (0.05 g/ml) was added to the phosphate/homogenization extract and the mixture shaken for 30 seconds and filtered through celite. DEAE-cellulose (0.01 g/ml) was added to the filtrate and the mixture was shaken for 30 seconds and filtered through celite. Usually 150 ml of extract was filtered through a 12.5 cm diameter celite filter pad. In some experiments the filtrate, after the DEAE-cellulose treatment, was emulsified with Freon-113 and the emulsion broken by centrifugation. The Freon treatment was not included in the final purification schedule.

The extraction and clarification of extracts was completed within 25 minutes and the final filtrate was usually a clear yellow solution if the material used was from plants showing advanced "yellows" symptoms. If the final filtrate still contained green material, much of this could be removed by an additional filtration through celite.

v) Losses of infectious material during the clarification procedures. To check the effectiveness of each clarification treatment, the infectivity of the extract

after each treatment was compared with the phosphate/homogenization extract, after appropriate dilution. Results of these infectivity assays (Table 3) show that there is a loss of 20% to 40% of the infectious material during the charcoal treatment. The loss of infectious material during the DEAE-cellulose treatment, was approximately 50%, while there was a further loss during the Freon-113 treatment (Table 3).

vi) Sedimentation of the virus out of clarified extracts. The filtrate after the DEAE-cellulose treatment was concentrated by centrifugation in a Spinco Model L centrifuge at 50,000 g for 15 minutes. Usually the pellets were light yellow and transparent in appearance. The pellets were suspended in 2 to 4 ml of distilled water and centrifuged at 1000 g for 5 minutes. If this virus suspension contained traces of green material, much of this was removed by filtration of the suspension through a celite micro-filter. This virus suspension will be either referred to as partially purified virus or Pellet 1 virus.

Table 3

Losses of infectious material during the clarification procedure

Infectivity comparison	Dilution (a)		
	$^{1}/_{1}$	$^{1}/_{3}$	$^{1}/_{9}$
Charcoal filtrate phosphate/homogenization extract	$^{59}/_{74}$	$^{30}/_{50}$	$^{27}/_{35}$
DEAE-cellulose filtrate phosphate/homogenization extract	$^{27}/_{48}$	$^{13}/_{32}$	$^{29}/_{49}$
Freon supernatant phosphate/homogenization extract (b)	-	$^{3}/_{28}$	$^{6}/_{40}$

(a) The dilution refers to each extract or filtrate. These results were collated from two experiments. Ratio's represent mean number of local lesions produced per half-leaf on opposite half-leaves of N. glutinosa by the extracts indicated. Each assay was carried out on 18 leaves.

(b) Supernatant after centrifugation of the emulsified DEAE-cellulose filtrate and Freon.

vii) Further purification of the virus.

(a) Electron microscopic observation of partially purified virus suspensions

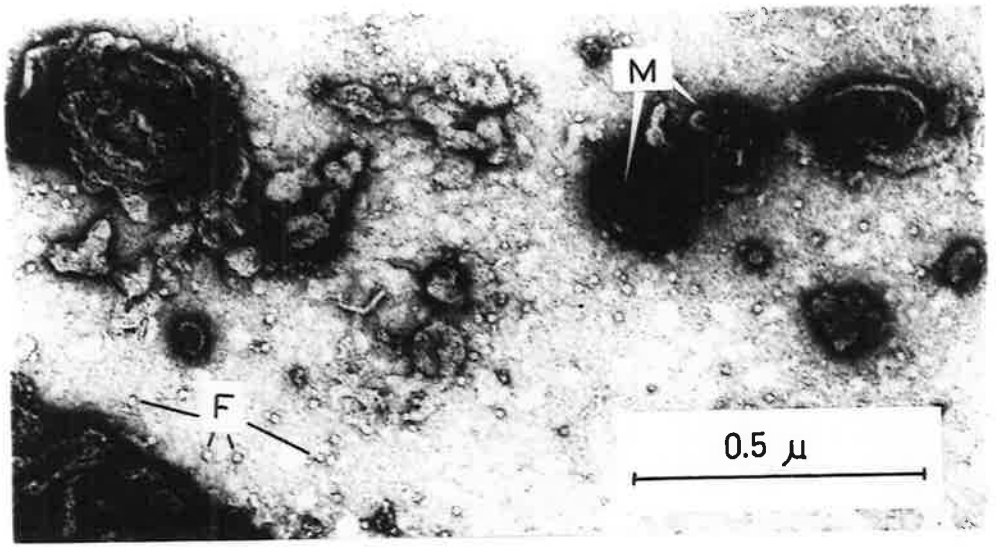
To check the purity of these suspensions, both preparations from healthy and diseased plants which had been extracted in Na_2HPO_4, clarified and sedimented were examined with the electron microscope. In addition to typical LNYV particles (Harrison and Crowley, 1965), electron micrographs of preparations from diseased plants showed much host-cell material similar to that observed in preparations from healthy plants (Fig. 2). Membranous structures, similar to those reported by Harrison and Crowley (1965) were observed as well as numerous small particles, the majority of which were tentatively identified as Fraction 1 protein due to their staining characteristics in uranyl acetate (Miller et al., 1966).

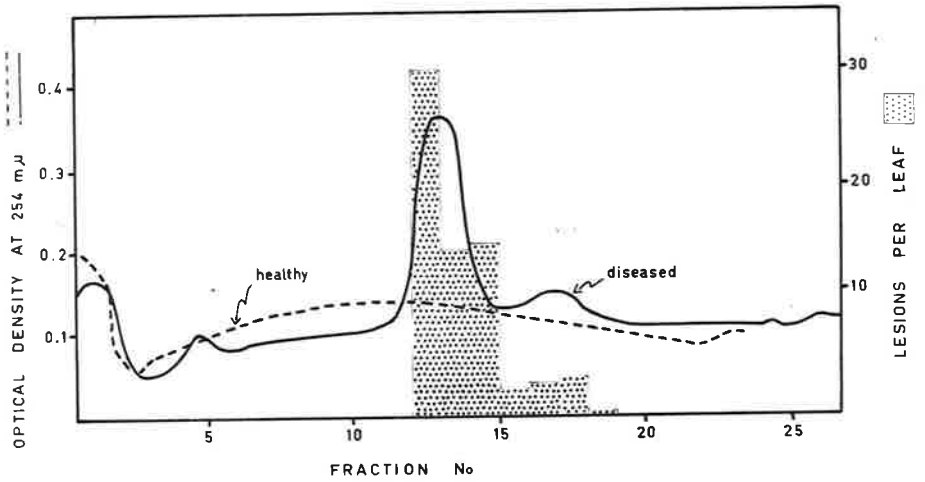
(b) Density gradient centrifugation of partially purified virus suspension

Preparations from healthy and virus-infected leaves, as used for the previous electron microscopic observations, were layered on to 15 to 45% linear sucrose density gradients and centrifuged. Analysis of the gradient, containing the preparation from healthy plants with the ISCO apparatus showed that there was host material throughout the centrifuge tube. Analysis of the gradient, containing the preparations from diseased plants showed

Figure 2 Electron micrograph of material obtained from healthy N. glutinosa plants after clarification of the leaf homogenate and sedimentation at 50,000 g for 15 minutes. Numerous membranous structures (M) and small particles tentatively identified as Fraction 1 protein (F) are shown.

Figure 3 Fractionation of sucrose density gradients on a ISCO density gradient fractionator. Samples (1.0 ml) of healthy and diseased preparations were layered on to 15-45% linear gradients and centrifuged at 24,000 rpm for 45 minutes in the SW 25 rotor of a Spinco centrifuge. Infectivity of each fraction was determined on nine whole leaves of N. glutinosa.


M
F
0.5 μ


OPTICAL DENSITY AT 254 mμ
0.4
0.3
0.2
0.1
healthy
diseased
30
20
10
LESIONS PER LEAF
5
10
15
20
25
FRACTION No

that in addition to the material present in the healthy centrifuge tube, two distinct peaks of optical density were present with which infectivity was associated (Fig. 3).

(c) Ammonium sulphate precipitation of Pellet 1 virus

Saturated $(NH_4)_2SO_4$ was added to 5 ml of a Pellet 1 virus suspension, so as to give a final salt concentration of 60%. This solution was allowed to stand for 30 minutes at 4° and then centrifuged at 1,000 g for 10 minutes. The pellet was resuspended in 5 ml of 0.01 M phosphate buffer, pH 7.2, and the infectivity compared with a sample of the initial Pellet 1 virus suspension. The results of this assay (Table 4) show that less than 50% of the infectivity was recovered from the pellet.

Table 4

Recovery of infectious material after $(NH_4)_2SO_4$ precipitation

Dilution	$^1/_9$ (a)	$^1/_{27}$
$^T/_C$	$^{21}/_{53}$	$^{15}/_{47}$

(a) Local lesions per half-leaf produced on opposite half-leaves of N. glutinosa by resuspended precipitate (T = numerator) and by Pellet 1 virus suspension (C = denominator). Each assay was carried out on 18 leaves.

(d) Methanol precipitation of partially purified virus

Methanol was added to a Pellet 1 virus suspension, so as to give final concentrations of 10, 20, 30, 40 and 50%. The original Pellet 1 virus suspension was diluted with an equal volume of 0.2 M phosphate buffer, pH 7.2. All of these solutions were allowed to stand for 1 hour at 4° and then centrifuged at 1,000 g for 20 minutes. The supernatants were removed from the pellets and centrifuged at 50,000 g for 15 minutes. Both high and low speed pellets were resuspended in 5 ml of 0.02 M phosphate buffer, pH 7.0 and the infectivity of each of these suspensions compared with the initial Pellet 1 virus suspension. The results of these assays (Table 5) show that in all treatments infectious material was recovered from both the pellets and the supernatants. But, there is a gradual decrease in the recovery of infectious material from the resuspended low and high speed pellets for each treatment as the concentration of methanol is increased. The resuspended slow-speed pellet from the buffer/virus suspension treatment was approximately 45% as infectious as the initial Pellet 1 virus suspension (Table 5). The sedimentation of the virus on slow-speed centrifugation agrees with the earlier result (Table 2).

Table 5

Recovery of infectious LNYV from the pellet and supernatant after incubation with various concentrations of methanol

Final methanol concentration	$^{P}/_{C}$ (a)	$^{S}/_{C}$ (b)
10%	$^{21}/_{43}$	$^{81}/_{63}$
20%	$^{35}/_{49}$	$^{69}/_{59}$
30%	$^{50}/_{55}$	$^{64}/_{56}$
40%	$^{30}/_{54}$	$^{41}/_{57}$
50%	$^{10}/_{32}$	$^{22}/_{36}$
0%	$^{16}/_{35}$	$^{46}/_{43}$

(a) Local lesions per half-leaf produced on opposite half-leaves of N. glutinosa by the slow-speed pellets resuspended in phosphate buffer (P = numerator) and by the Pellet 1 virus suspension (C = denominator).

(b) Local lesions per half-leaf produced on opposite half-leaves of N. glutinosa by the supernatants after sedimentation and resuspension in phosphate buffer (S = numerator) and by the Pellet 1 virus suspension (C = denominator).

Each assay was carried out on 10 leaves.

(e) Acidification for the precipitation of the partially purified virus

Aliquots of a Pellet 1 virus suspension were added to an equal volume of 0.05 M acetate buffer solutions at pH's ranging from 3.9 to 5.5. An equal volume of 0.05 M phosphate buffer, pH 7.0, was added to an aliquot of the Pellet 1 virus suspension. These solutions were allowed to stand for 30 minutes at 4° and then centrifuged at 1,000 g for 10 minutes. The supernatants were removed and the pellets resuspended in an equivalent volume of 0.05 M phosphate buffer, pH. 7.0. The infectivity of both the resuspended pellets and the supernatants were compared to that of the initial Pellet 1 virus suspension. The results of these assays (Table 6) show that at pH's of 4.9 or below, only a small proportion of the infectious material was recovered either in the resuspended pellet or in the supernatant. The small amount of infectious material recovered in the pellets at these pH's may not be due to precipitation but may be due to the sedimentation of aggregated virus particles. The failure to recover all of the infectious material at pH's below 4.9 could be due to the fact that virus particles are damaged and rendered non-infectious. At the pH's of 5.1 and 5.5, more infectious material was recovered than at pH 7.0. At pH 5.5, there appeared to be considerably more virus recovered from both the pellet and the supernatant than was present in the initial Pellet 1 virus suspension. This result contracts with the pH stability range of the virus obtained when infected leaves were mashed in buffers of various pH's (Crowley, 1967).

Table 6

Recovery of infectious INYV from the pellet and supernatant after incubation with acetate buffer at various pH's.

pH	$^{P}/_{C}$ (a)	$^{S}/_{C}$ (b)
3.9	$^{9}/_{48}$	$^{11}/_{52}$
4.3	$^{3}/_{50}$	$^{15}/_{47}$
4.5	$^{7}/_{34}$	$^{11}/_{51}$
4.7	$^{5}/_{37}$	$^{4}/_{54}$
4.9	$^{4}/_{37}$	$^{7}/_{40}$
5.1	$^{21}/_{52}$	$^{44}/_{47}$
5.5	$^{12}/_{41}$	$^{62}/_{46}$

(a) Local lesions per half-leaf produced on opposite half-leaves of N. glutinosa by pellet (P = numerator) and by Pellet 1 virus suspension (C = denominator).

(b) Local lesions per half-leaf produced on opposite half-leaves of N. glutinosa by supernatant (S = numerator) and by Pellet 1 virus suspension (C = denominator).

Each assay was carried out on 18 leaves.

(f) Column chromatography of LNYV on calcium phosphate gel

A preliminary experiment, using columns equilibrated at pH's of 7.0 and 8.0, and eluted with these same buffers indicated that virus was eluted from the columns in the first 15 ml of eluant. But there seemed to be more host material eluted at pH 8.0 than at pH 7.0.

The following experiment was carried out to determine at what pH the virus was eluted from the column. A 2.5 ml sample of a Pellet 1 virus suspension was applied to a column which had been equilibrated at pH 6.6, with 0.01 M phosphate buffer. It had also been found (Table 7), that little virus was eluted from columns which had been equilibrated and eluted with 0.01 M phosphate buffer, pH 6.6. The pH 6.6 column was eluted with 0.01 M phosphate buffer, pH 8.0. Fractions, 5 ml, were collected and pH and optical density determinations were made on each fraction; as well as an infectivity assay after dilution of the fraction. It was found that infectious material was present in the eluate at a pH of approximately 7.5 (Fig. 4). A peak on the optical density trace, corresponded with the position of the infectivity trace (Fig. 4).

To check the pH at which the least amount of host material was eluted from the column and the yield of virus was not impaired, the following experiment was carried out. Columns were equilibrated with 0.01 M phosphate buffers at pH's ranging from 6.6 to 8.1 and the virus was eluted from the columns with the same buffers. The percentage recovery of infectious material in the 15 ml of eluant was determined by comparing the eluant with a sample of the virus suspension which had been added to the column, after appropriate dilution. Results of these assays (Table 7) indicate that at pH 7.3 to 7.8 the maximum amount of host material, as measured by optical density, remains adsorbed to the column and at least 50% of the infectious material is recovered from the column. With some of the columns, at pH's of 7.6 and 7.8, 80 to 100% of the infectious material was recovered from the column, although the recovery as measured by optical density was between 10 and 25% (Table 7).

For the routine purification of LNYV, columns at pH 7.6 were chosen. Such columns, when equilibrated and eluted with 0.01 M phosphate buffer, pH 7.6 yielded a peak of infectious material and a peak of optical density in the initial 15 ml of eluant after discarding the void volume of the column (Fig. 5).

Table 7

Recovery of infectious LNYV from calcium phosphate gel columns

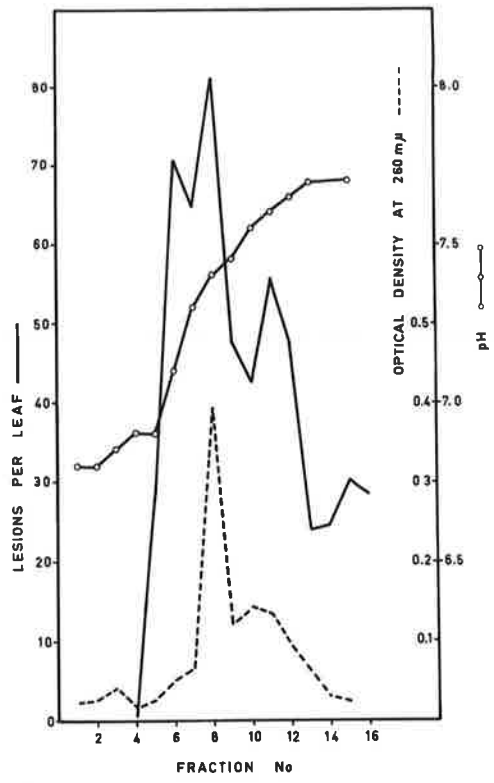
Experiment number	pH of eluting buffer	% Recovery from column (a)	
		Optical density at 260 mμ	Infectivity (b)
1	6.6	2	20
	7.3	4	55
	8.1	26	100
2	7.4	10	73
	7.6	17	106
	7.8	19	100
3	7.4	4	48
	7.6	8	81
	7.8	8	100
4	7.6	14	53
5	7.6	13	52

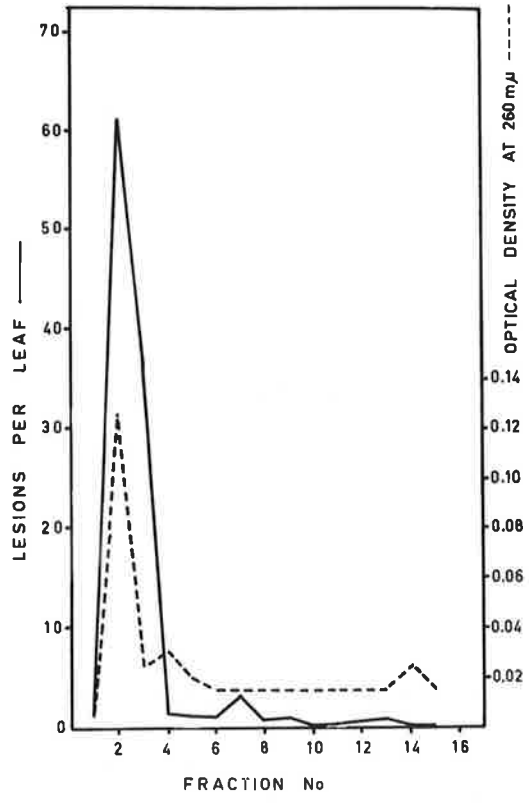
(a) The percentage recovery was determined by comparing the initial 15 ml of eluant from the column and the virus suspension applied to the column after appropriate dilution.

(b) Infectivity assays were carried out on 18 half leaves of N. glutinosa.

Figure 4 Elution of LNYV from a calcium phosphate gel column. Virus preparations were applied to a column equilibrated with 0.01 M phosphate buffer, pH 6.6, and eluted with 0.01 M phosphate buffer, pH 8.0, and 5.0 ml fractions were collected after discarding the void volume of the column. The pH and optical density of each fraction was determined and the fractions assayed for infectivity on nine N. glutinosa leaves, after a 1:3 dilution was made.

Figure 5 Recovery of LNYV from a calcium phosphate gel column equilibrated and eluted at pH 7.6 with 0.01 M phosphate buffer. The optical density was determined for each fraction and the infectivity assayed on N. glutinosa leaves, after a 1:9 dilution was made.


LESIONS PER LEAF
OPTICAL DENSITY AT 260 mμ
pH
FRACTION No


LESIONS PER LEAF
OPTICAL DENSITY AT 260 mμ
FRACTION No

(g) Variations in the properties of commercial and laboratory-prepared calcium phosphate gels

Three types of gels have been used in these studies: several batches of gels were obtained from Clarkson Chemical Company (Hypatite C), one gel was obtained from Bio-Rad Chemicals and laboratory-prepared gels were used (Siegelman et al., 1965). Three properties of the gels were observed to vary: the elution of infectious material from the gel, the retention or elution of host plant material by the gel and in some gels one or both of these two properties varied with the age of the gel. Virus was eluted from one gel, yet it was poorly infectious and the virus peak was located in a different position in a sucrose density gradient.

To compare the properties of different gels, the following experiment was carried out. Equal volumes of a Pellet 1 virus suspension were applied to a commercial calcium phosphate gel and a laboratory-prepared calcium phosphate gel. Both gels had previously been equilibrated with 0.01 M phosphate buffer, pH 7.6, and the virus suspensions were also eluted with this buffer. The 3 fractions containing light scattering material were collected from each gel. With the commercial gel these fractions were 3, 4 and 5, whereas with the laboratory-prepared gel these fractions were 4, 5 and 6. The pooled eluants (9 ml) from each column were each concentrated by sedimentation at 50,000 g for 15 minutes in

the 40 rotor of the Spinco centrifuge. Each pellet was then resuspended in 1.0 ml of distilled water and the suspensions layered on to 15 to 45% linear sucrose density gradients. After centrifugation the density gradient columns were analysed with the ISCO apparatus which showed that there was considerably much more material throughout the tube containing the virus suspension from the commercial gel than from the tube containing the virus suspension from the laboratory-prepared gel (Fig. 6). Visual observations of the gels showed that the laboratory-prepared gel had retained much more host material at the upper portion of the gel column than the commercially obtained gel (Fig. 7). Although these gels retained different amounts of host material, there was no significant difference in the amount of virus eluted from the column, as measured by the virus peaks (Fig. 6) or by infectivity assay.

Although this experiment illustrates the variation of two particular gels, there was also considerable variability between batches of commercial gels and between preparations of laboratory-prepared gels.

viii) Flow diagram of the purification method. The method which has been adopted for the purification of lettuce necrotic yellows virus is depicted in the flow diagram (Fig. 8).

Figure 6 Optical density determinations at different depths in density gradient tubes layered with purified virus from a laboratory-prepared calcium phosphate gel (A) and from a commercial gel (B). The centrifuged gradients were analysed with the ISCO apparatus. Samples (1.0 ml) of each of these preparations were layered on to gradients and centrifuged for 45 minutes at 24,000 rpm in the SW 25 rotor of a Spinco centrifuge.

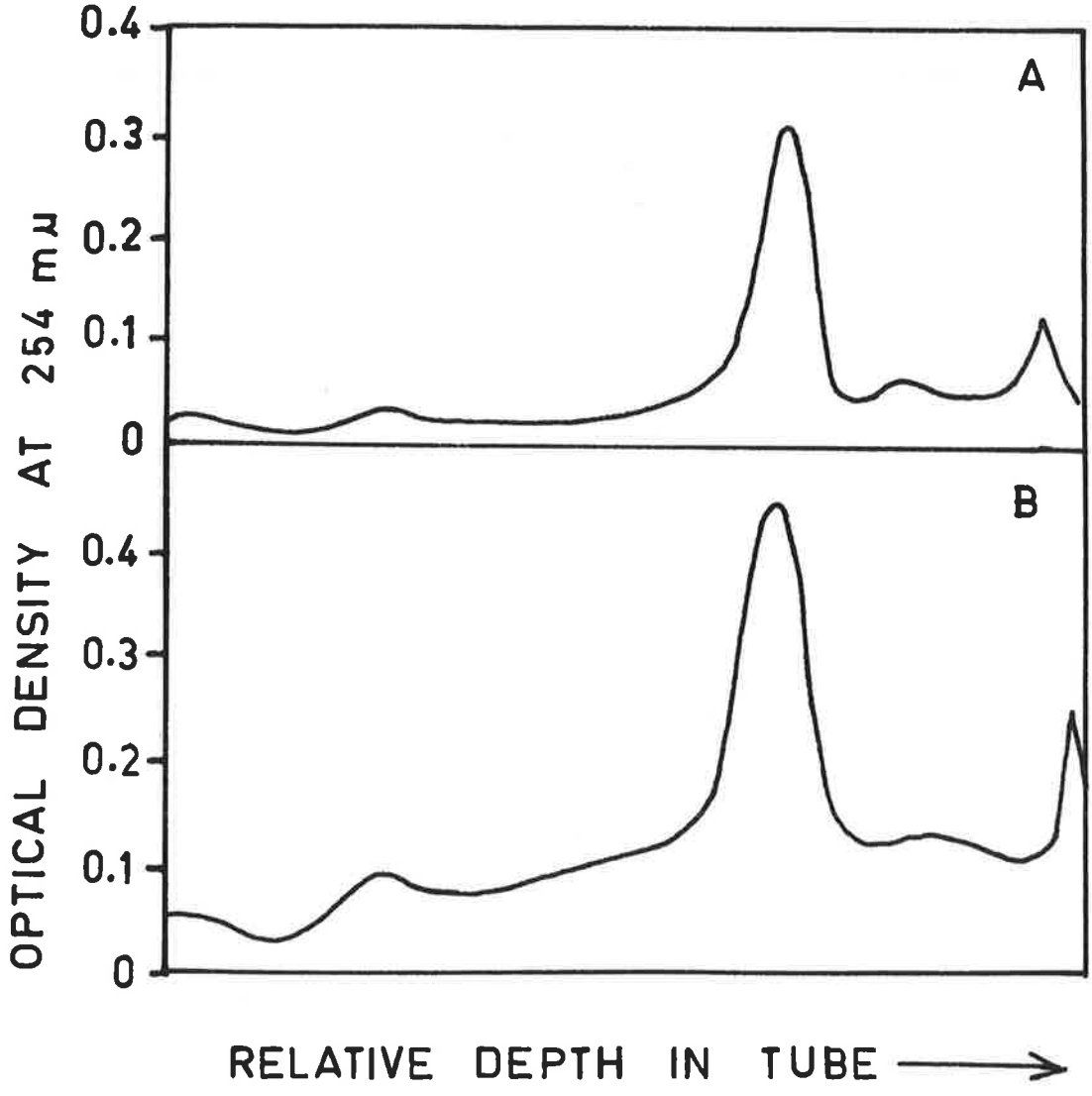
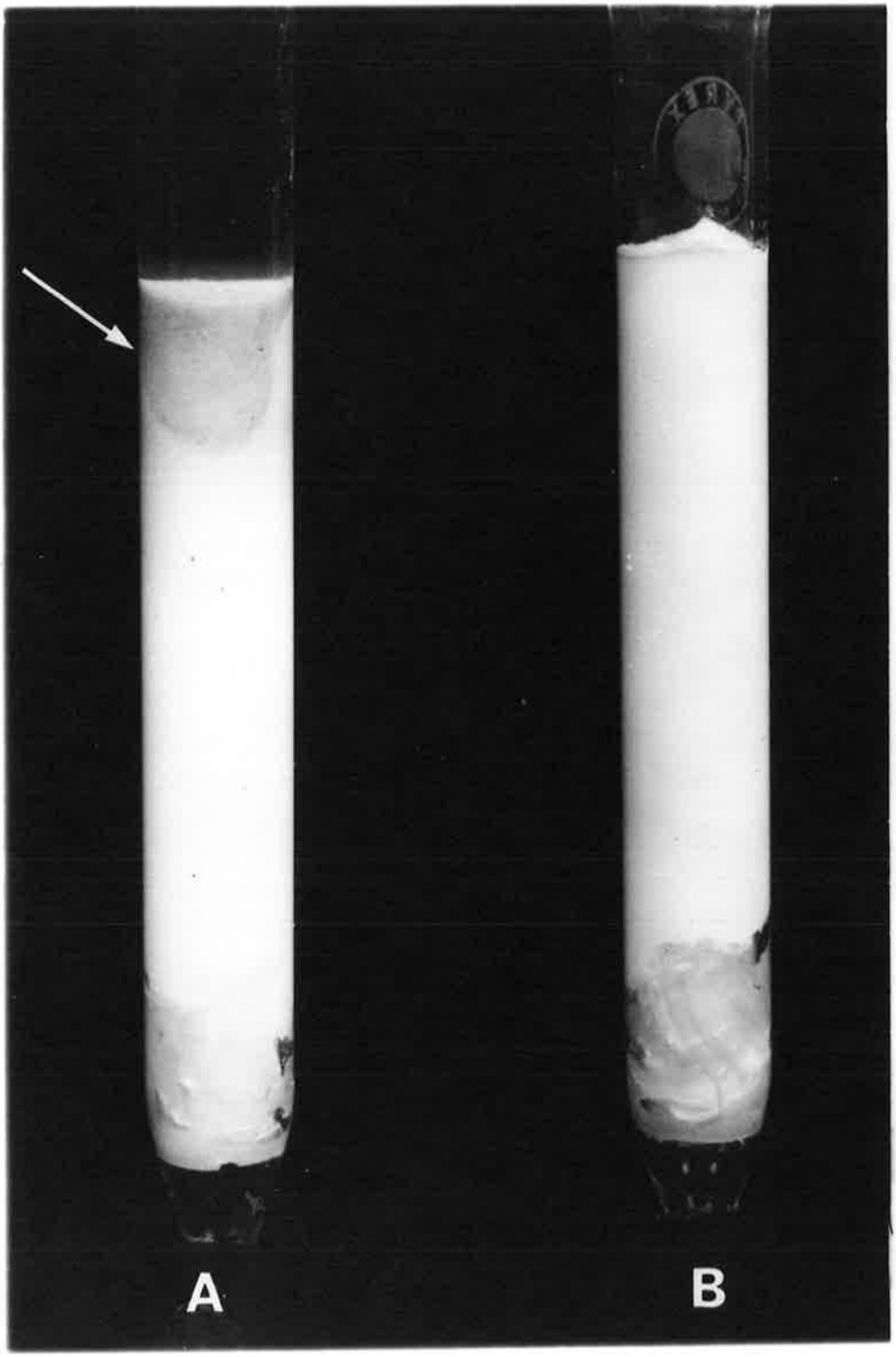

0.4
0.3
0.2
0.1
0
A
B
OPTICAL DENSITY AT 254 mμ
RELATIVE DEPTH IN TUBE

Figure 7 Calcium phosphate gels, after column chromatography of virus suspensions.

(A) Laboratory-prepared gel, made by the method of Siegelman et al. (1965).

(B) Commercially obtained gel, from Clarkson Chemical Company (Batch No. 6338).

Noting, the host material retained at the upper portion of the laboratory-prepared gel (arrowed).


A
B

The Freon-113 emulsification was attempted at point A (Fig. 8) and the preferential precipitation of virus was attempted at point B (Fig. 8).

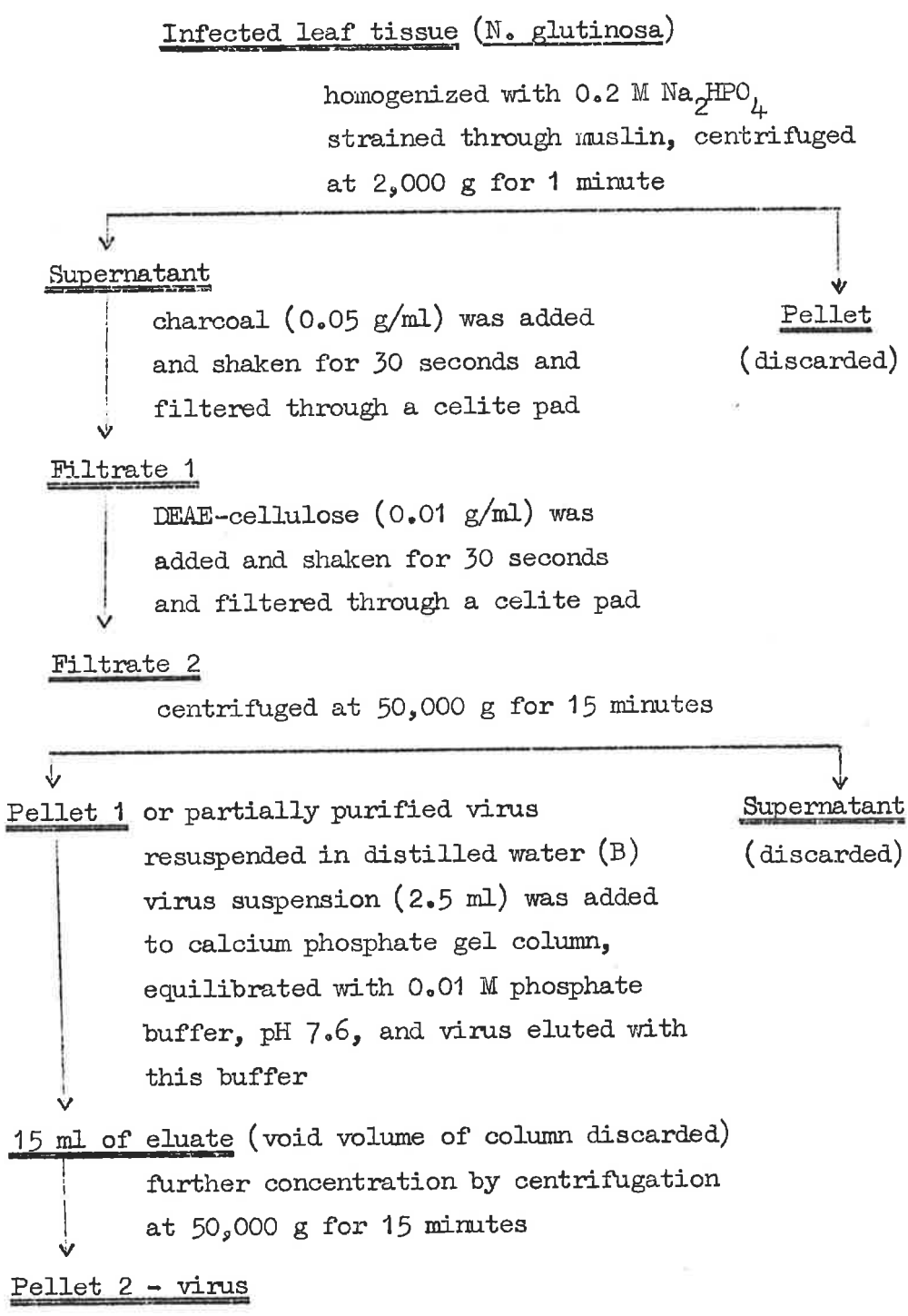
Although this method has been used chiefly for the purification of virus from infected N. glutinosa, it has also been used for the purification of LNYV from infected lettuce plants collected in the field. The clarification procedures (point A, Fig. 8) have also been used successfully in the purification of potato virus X, tobacco mosaic virus and Cymbidium mosaic virus (Francki and McLean, 1968; Appendix 2).

3D. Experimental - Preparation of an antiserum to the virus

i) Preparation of the antiserum. An antiserum was prepared by injecting virus suspensions into a rabbit. The virus suspension was emulsified in Freund's complete adjuvant and injected both intramuscularly and subcutaneously, and further injections were made 1 and 3 weeks later. Virus suspensions were also injected intravenously at 1, 3 and 4 months after the initial inject. Altogether, on these five injection dates, virus recovered from approximately 2 kg of infected N. glutinosa was used.

ii) Testing and titre of the antiserum. The LNYV antiserum reacted with partially purified virus in both gel

Figure 8 Flow Diagram of the purification method



diffusion and precipitin tests. In precipitin tests the titre of the antiserum was $\frac{1}{256}$, when titrated against Pellet 1 virus. The titre of the antiserum when titrated against undiluted sap from infected N. glutinosa was $\frac{1}{32}$. A partially purified virus suspension prepared from INYV-infected lettuce also reacted with the virus antiserum in gel diffusion tests.

3E. Properties and purity of virus preparations

i) Absorption spectrum of purified virus. Purified virus was placed in the normal cell position of the Unicam SP 800 spectrophotometer and the optical density determined in the wave length range, 230 mμ to 300 mμ. The spectrum was also determined when the cell containing the virus suspension was placed in the position, which corrects to some extent for light scattering. There is a substantial decrease in absorption in the 2nd spectrum. due to the light scattering of the large virus particle (Fig. 9).

ii) Examination of virus preparations in the analytical ultracentrifuge. A purified virus suspension was sedimented in the analytical ultracentrifuge and one peak was observed (Fig. 10). This peak indicated that the virus sample was not polydisperse and that the virus sample appeared to be homogeneous.

iii) Electron microscopy of purified virus preparations. Electron microscopy of LNYV preparations showed numerous intact virus particles as well as other particles (Fig. 11) which were tentatively identified as disrupted virus particles by the presence of the characteristic membrane structure (Wolanski et al., 1967).

iv) Serological reactions of purified virus. To check the purity of the virus preparations and also to determine if the virus antiserum contained antibodies to plant material, the following experiment was carried out. An N. glutinosa antigen preparation was obtained by homogenizing leaves with one volume of 0.2 M Na_2HPO_4, straining through cheese cloth and centrifuging at 150,000 g for 1 hour. The pellets were resuspended in 0.2 M phosphate buffer, pH 7.2, and clarified by centrifugation at 12,000 g for 10 minutes. An antiserum to N. glutinosa leaf antigens was supplied by Dr. R.I.B. Francki, for the agar gel tests.

When the N. glutinosa antiserum was tested against the N. glutinosa antigen preparation and purified LNYV (Fig. 12) a precipitin line was observed between the homologous antigen and antiserum. In the test with the LNYV antiserum (Fig. 13), a precipitin line was formed in the test with the purified virus but not with the N. glutinosa preparation. These results provide evidence for both the purity of the virus and the freedom

Figure 9 Absorption spectrum of purified virus preparations, using the Unicam SP 800 spectrophotometer.

(A) Normal cell.

(B) Cell which partially corrects for light scattering.

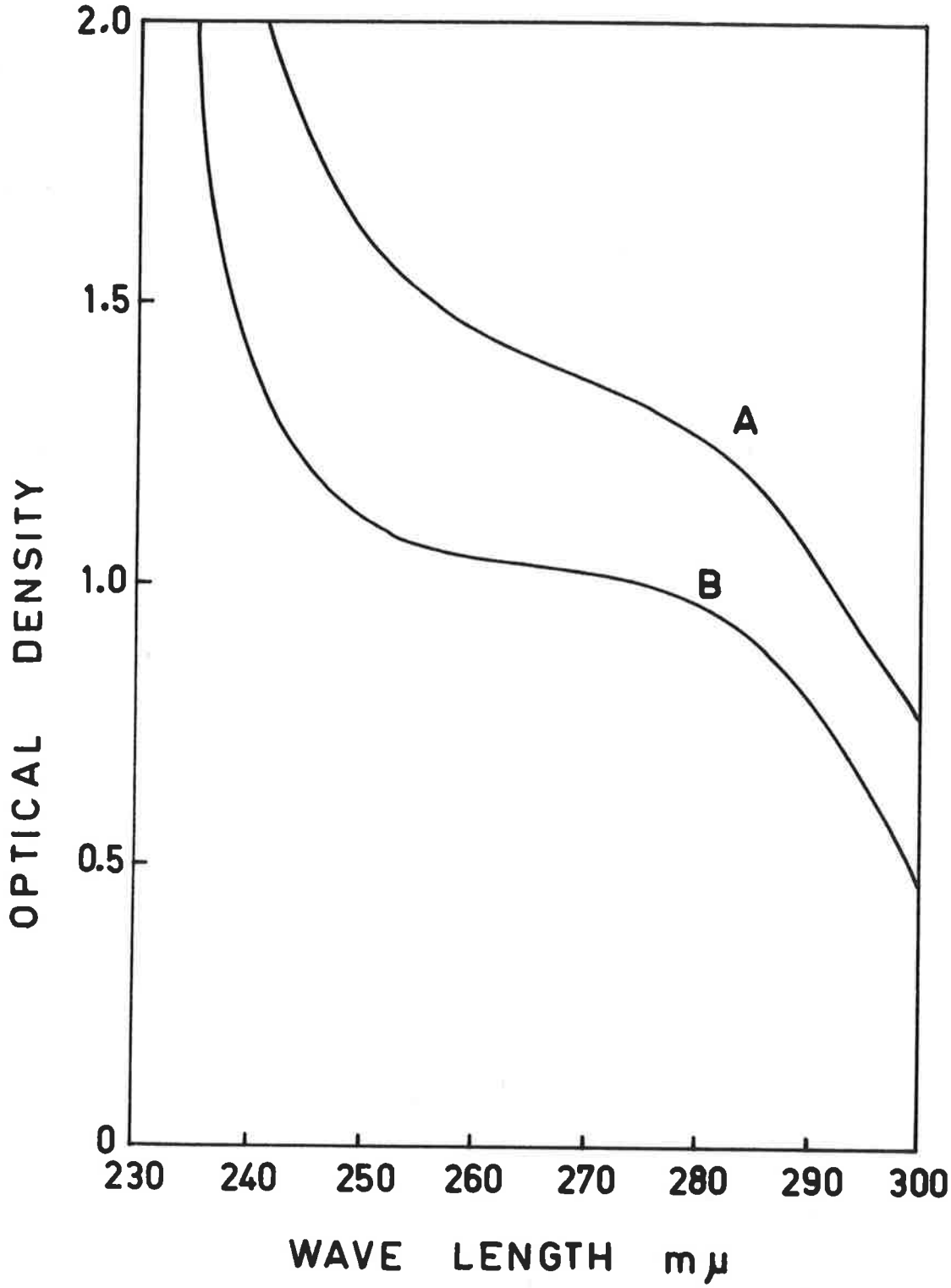
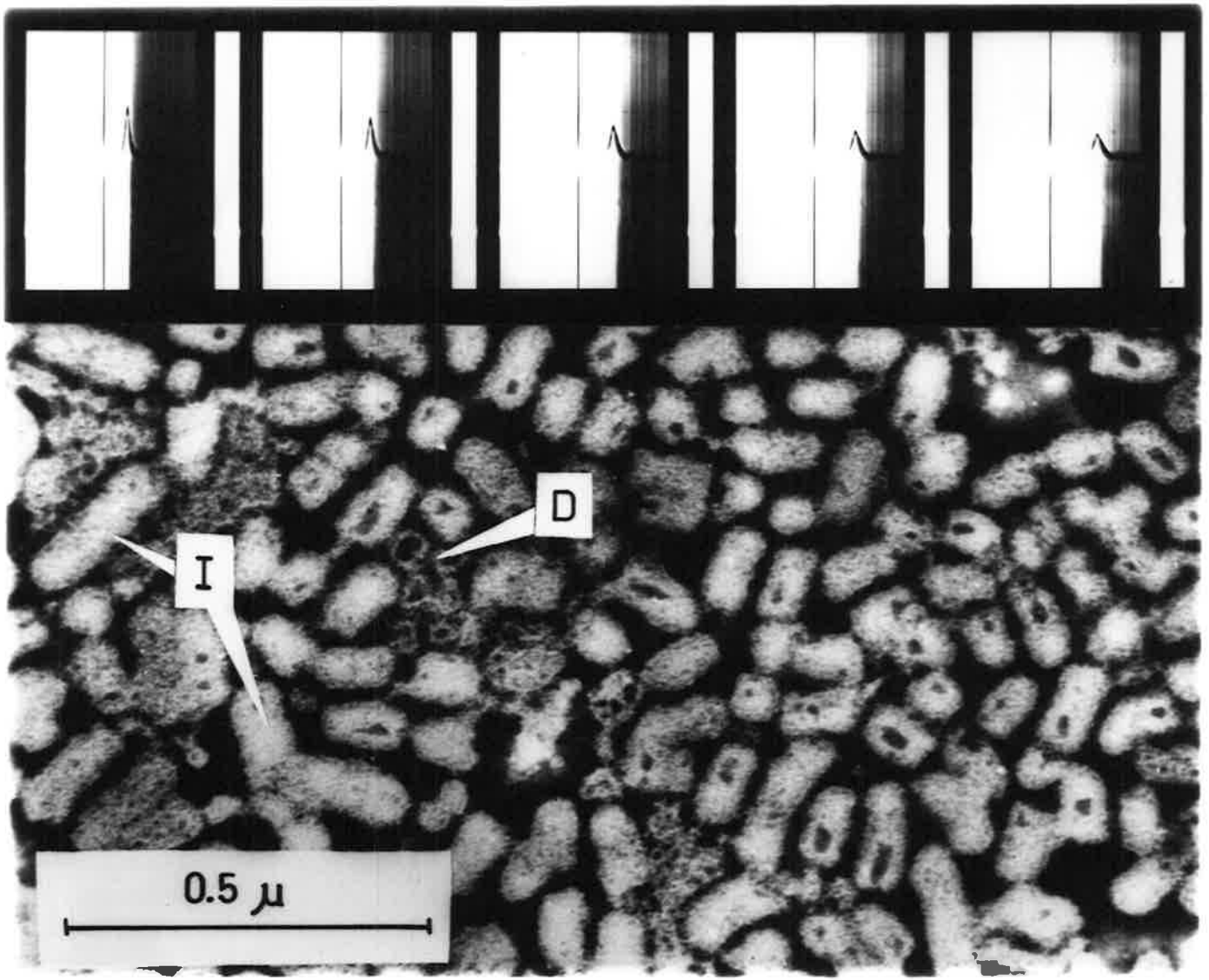

2.0
1.5
1.0
0.5
0
OPTICAL DENSITY
A
B
230
240
250
260
270
280
290
300
WAVE LENGTH mμ

Figure 10 Schlieren pattern of LNYV sedimented in 0.01 M phosphate buffer, pH 7.6.

Figure 11 Electron micrograph of a purified LNYV preparation, concentrated by centrifugation and stained in PTA. Numerous intact virus particles (I) and some partially disrupted (D) virus particles are observed.


D
I
0.5 µ

Serological tests in agar gel

Figure 12 (V) Purified LNYV

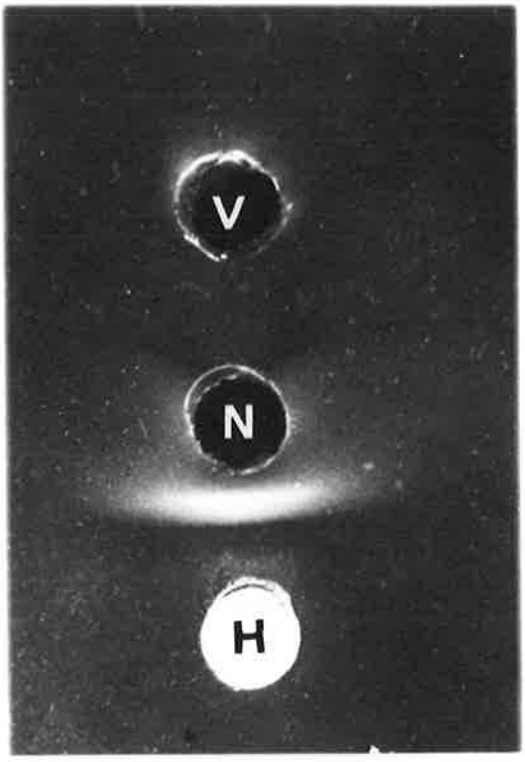
(H) Healthy sap preparation

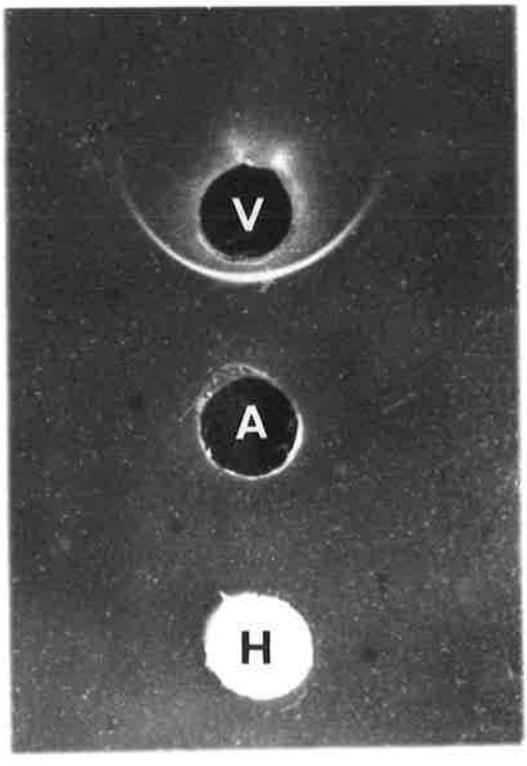
(N) *N. glutinosa* antiserum

Figure 13 (V) Purified LNYV

(H) Healthy sap preparation

(A) LNYV antiserum


V
N
H


V
A
H

of the virus antiserum from antibodies to plant leaf materials.

3F. Discussion

Purification of LNYV is a difficult problem because it is unstable in leaf extracts as determined by infectivity assays (Stubbs and Grogan, 1963b; Crowley, 1967), its large size (Harrison and Crowley, 1966) and its sensitivity to organic solvents commonly used in the clarification of plant extracts (Crowley et al., 1965). The method of purification described (Fig. 8) results in purified virus, as assessed by electron microscopy, serological tests and analytical centrifugation. The low yield of virus may be due to a poor extraction procedure rather than to excessive losses during the clarification and purification procedures. Chambers et al. (1965) showed that LNYV particles in leaf cells are located in the cytoplasm, arranged in bundles enclosed in membrane-bound sacs. For the efficient release of virus from cells, it may be necessary to rupture the membranes enclosing the bundles of virus particles as well as the cell walls. However, if the extraction methods are too severe then the individual particles may be damaged. Crowley (private communication) has shown that mild sonication reduced the infectivity of LNYV in leaf extracts.

The clarification procedure for leaf homogenates is rapid and has the advantage that slow-speed centrifugation,

which can cause high losses of virus (Crowley et al., 1965), is not used. Both charcoal and DEAE-cellulose are effective in removing particulate green material and soluble brown pigments while the celite filtration appears to be efficient in adsorbing green material. It could also be relevant that chloroplast materials are removed without being solubilized to release materials which could adsorb to virus (Ginoza et al., 1954).

This method of clarification has also been used successfully in the purification of potato virus X (PVX), tobacco mosaic virus (TMV) and Cymbidium mosaic virus (Francki and McLean, 1968). As much as 1 g of purified TMV was obtained from 1 kg of infected tobacco leaves.

Attempts to precipitate the virus using ammonium sulphate, methanol and acidification were not successful. In the methanol precipitation and acidification experiments the infectivity of the virus was affected by the treatment. Possibly, the use of ammonium sulphate to preferentially precipitate the virus should have been investigated further. It was also found that density gradient centrifugation of clarified extracts was inefficient in separating virus from host materials as these spread along the entire depth of the columns (Fig. 3), including zones from which virus is recovered.

The method adopted for virus purification was the chromatography of virus suspensions on calcium phosphate gel columns, which were equilibrated and eluted with 0.01 M phosphate buffer, pH 7.6. Such columns were efficient in removing more of the contaminating host material from clarified extracts. For the preparation of purified virus suspensions, it usually took less than 3 hours to purify LNYV from approximately 300 g of infected plant tissue.

Variation was observed in the elution and retention properties of calcium phosphate gels, which had been obtained commercially as well as batches prepared in the laboratory, by the method of Siegelman et al. (1965). Generally little is known about the chromatographic action of calcium phosphate gels. Recently, Bernardi and Kawasaki (1968), using proteins and synthetic polypeptides, found that carboxyl groups react with hydroxyapatite and also that phosphoproteins have a higher affinity for hydroxyapatite than non-phosphoproteins. Further work of this nature may lead to a better understanding of the chromatographic action of these gels.

4. SEROLOGICAL PROPERTIES OF LETTUCE NECROTIC YELLOWS VIRUS

4A. Introduction

A study of the structure, stability, serological reactions and the sedimentation properties of lettuce necrotic yellows virus (LNYV) was made by Harrison and Crowley (1965). The antiserum used by Harrison and Crowley (1965) contained antibodies to host cell material and detailed serological investigations were not carried out.

The structural similarity of LNYV to that of vesicular stomatitis virus (VSV) has been pointed out (Harrison and Crowley, 1965; Chambers et al., 1965). Bradish et al. (1956) studied the biophysical properties of VSV and observed that the major portion of the infectivity of their virus preparations was associated with 35% of the complement-fixing activity of the preparation. They found that the remaining 65% of the complement-fixing activity of their preparations was associated with two discrete components with sedimentation coefficients of about 6S and 20S. In a study of the structure and immunogenicity of the antigens derived from the VS virion treatment after Tween/ether, Brown et al. (1967) observed three types of particles: 'skeletons', 'rosettes' and small particles about the size of the fringes or the spikes of the viral envelope.

This section describes attempts made to determine the nature of the viral antigen which diffuses through agar, the number of antigens of the LNY virion and the relation of these antigens to the structure of the virion. Two main approaches were taken in this study: the effect of Tween/ether on the virus and the observations of lipase-treated virus in gel diffusion tests. The results show that the virion contains at least two antigens.

4B. Experimental

i) Variation in the reaction between purified virus and antiserum. When LNYV preparations purified on different occasions were tested against the virus antiserum in gel diffusion tests, the number of precipitin lines varied from one to three. With some virus preparations, there was a precipitin line close to the antigen well (Figs. 14 and 15). In the virus preparations used in the Tween/ether experiments three precipitin lines were observed (Fig. 16 and also Figs. 23 to 26).

It is difficult to provide a satisfactory explanation for the variation in the number of precipitin lines. There may have been more virus in the preparations used in the Tween/ether experiments and thus the concentrations of the antigens which formed the two precipitin lines closer to the antiserum well was

Variations in gel diffusion reactions

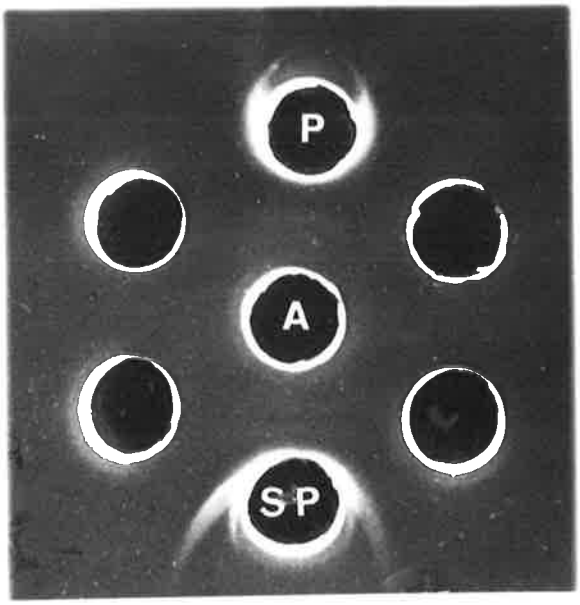
Serological tests in agar gel

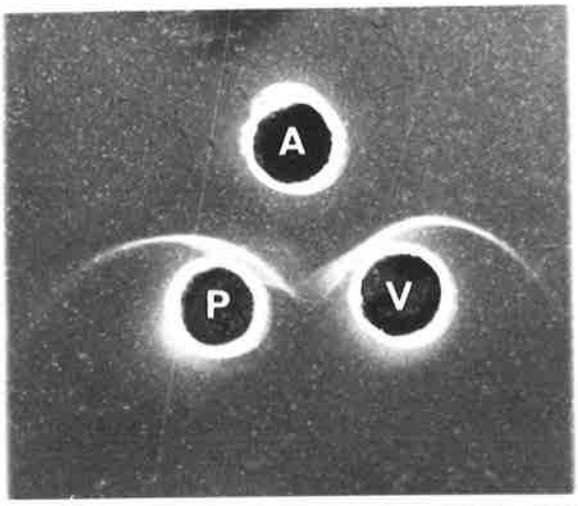
The symbols denote the preparations added to the wells of the agar gel.

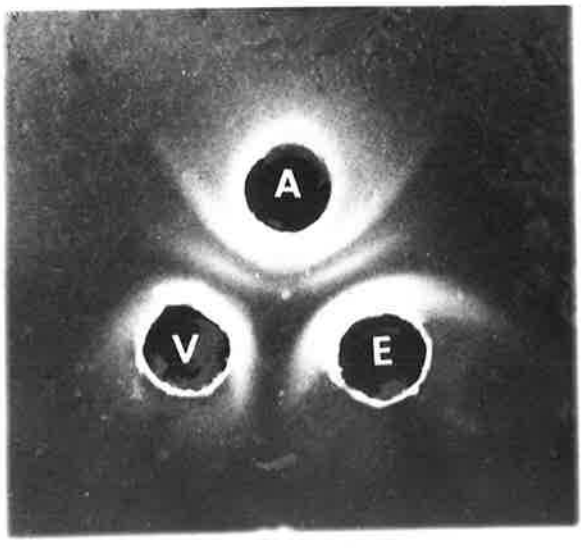
Figure 14
- (P) Pellet 1 virus
- (SP) Sonicated Pellet 1 virus
- (A) LNYV antiserum

Figure 15
- (P) Pellet 1 virus
- (V) Purified LNYV
- (A) LNYV antiserum

Figure 16
- (V) Purified LNYV
- (E) Tween/ether treated LNYV
- (A) LNYV antiserum


P
A
SP


A
P
V


A
V
E

sufficient to form a precipitin line. Another explanation could be that the virus preparations which produced three precipitin lines in gel diffusion reactions, contained many disrupted virus particles.

ii) Effect of Tween/ether on the virus. To investigate the effect of Tween 80/ether on the virus the following experiments was carried out. Brown et al. (1967) used a similar Tween/ether treatment for the disruption of vesicular stomatitis virus. Purified virus, which had been concentrated (Fig. 8), was mixed with an equal volume of Tween 80 (concentration 6 mgm per ml) and the mixture was shaken vigorously for 1 minute. An equal volume of ether was added and the mixture was again shaken for 1 minute. The mixture was centrifuged at 1,000 g for 5 minutes and the ether layer was removed. Any remaining ether was blown off in a stream of nitrogen.

To determine the effect of Tween/ether treatment on the infectivity of the virus, samples of untreated and treated virus were assayed for infectivity. Leaves of assay plants were dusted with bentonite (Singer and Fraenkel-Conrat, 1961) as well as carborundum. Results of the assays for three such experiments show that Tween/ether treatment reduced the infectivity of the virus (Table 8).

Table 8

Effect of Tween/ether on virus infectivity

Experiment number	Virus	Tween/ether-treated LNYV
1	136 (a)	33 (a)
2	97	10
3	> 100	2

(a) The mean number of local lesions per whole leaf. Each assay was carried out on at least 10 leaves.

To determine what effect Tween/ether treatment had on the LNY virion, samples of untreated and treated virus were analysed by sucrose density gradient centrifugation (Figs. 17 and 18). After Tween/ether treatment the majority of the material was at the top of the tube (Figs. 17C and 18B). It was observed that after Tween treatment (Fig. 17B), only a proportion of the material was at the top of the tube. From these density gradient analyses (Figs. 17 and 18), it can be seen that Tween/ether treatment affects the sedimentation property of the virus. The top 1 ml fractions from the top of the gradients (Fig. 17) were collected and these were examined in the electron microscope by Mr. B.S. Wolanski of the University

Figure 17 Optical density determinations at different depths in density gradient tubes layered with untreated virus (A), Tween 80 treated virus (B), and Tween/ether treated virus (C). The centrifuged gradients were analysed with the ISCO apparatus. Samples (0.2 ml) of each of these preparations were layered on to gradients and centrifuged for 30 minutes at 25,000 rpm in the SW 39 rotor of a Spinco centrifuge.

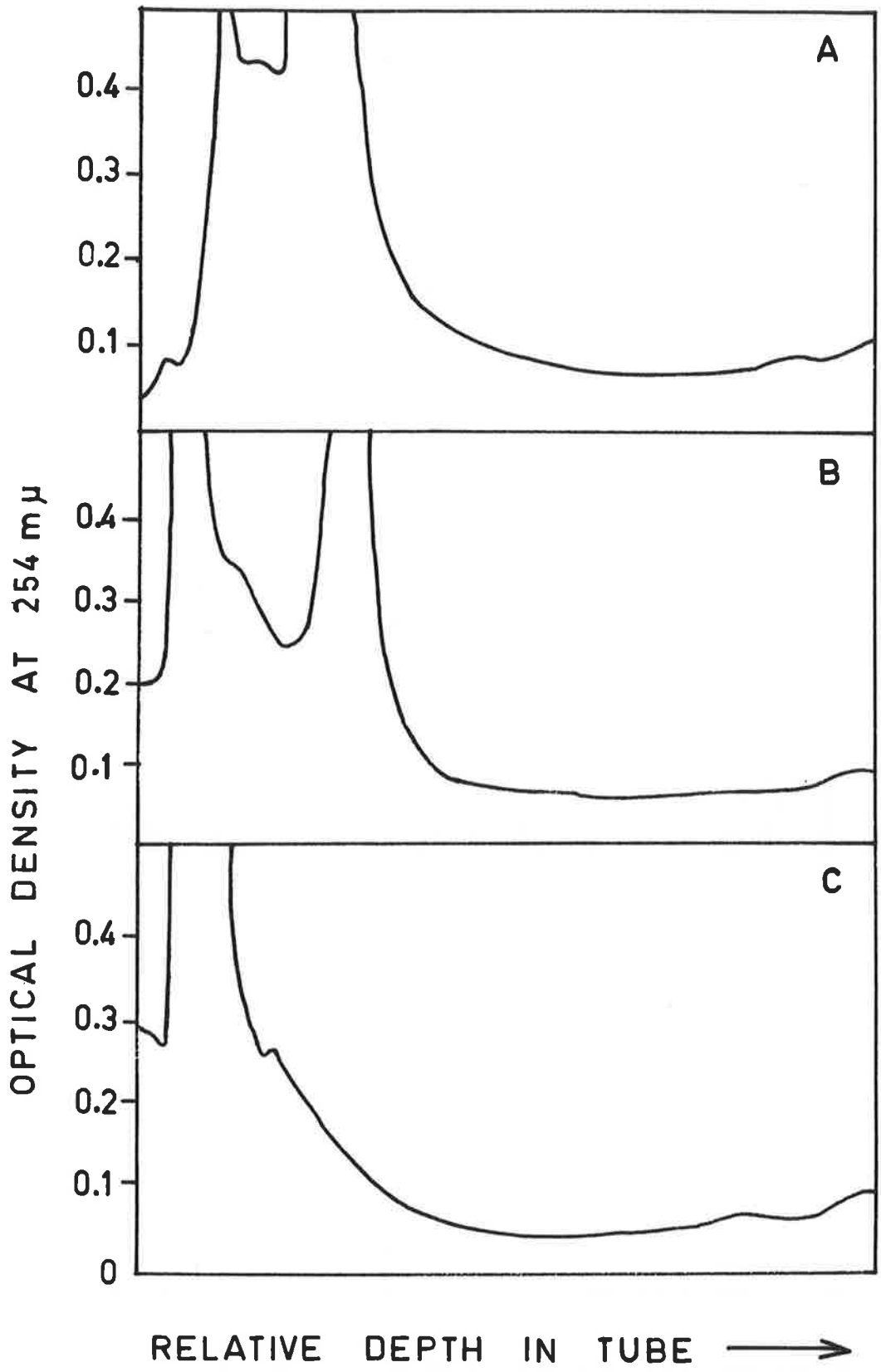
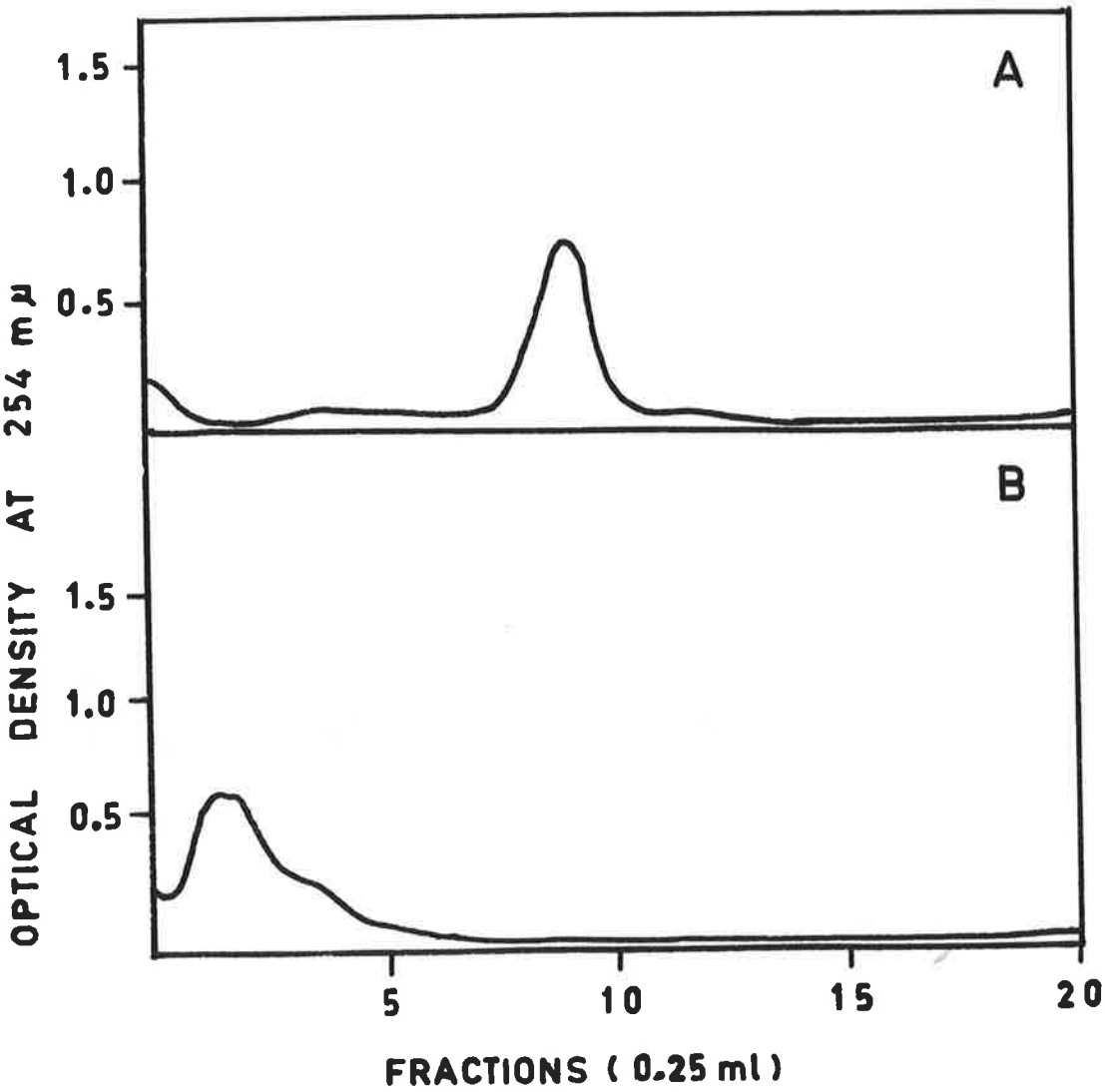

A
B
C
0.4
0.3
0.2
0.1
0
OPTICAL DENSITY AT 254 mμ
RELATIVE DEPTH IN TUBE

Figure 18 Optical density determinations at different depths in density gradient tubes layered with untreated virus (A), and Tween/ether treated virus (B). The centrifuged gradients were fractionated with the ISCO apparatus. Samples (0.2 ml) of each of these preparations were layered on to gradients and centrifuged for 30 minutes at 26,000 rpm in the SW 39 rotor of a Spinco centrifuge.


A
B
1.5
1.0
0.5
1.5
1.0
0.5
OPTICAL DENSITY AT 254 mμ
5
10
15
20
FRACTIONS (0.25 ml)

of Melbourne, after fixation in Osmium vapour and staining in uranyl acetate. Results of these observations (Fig. 19) revealed the presence of rosette-like particles from both untreated and Tween/ether treated virus preparations. Rosettes have been observed in preparations of other viruses (Waterson, 1965; Brown et al., 1966) as well as for preparations of LNYV (Wolanski et al., 1967).

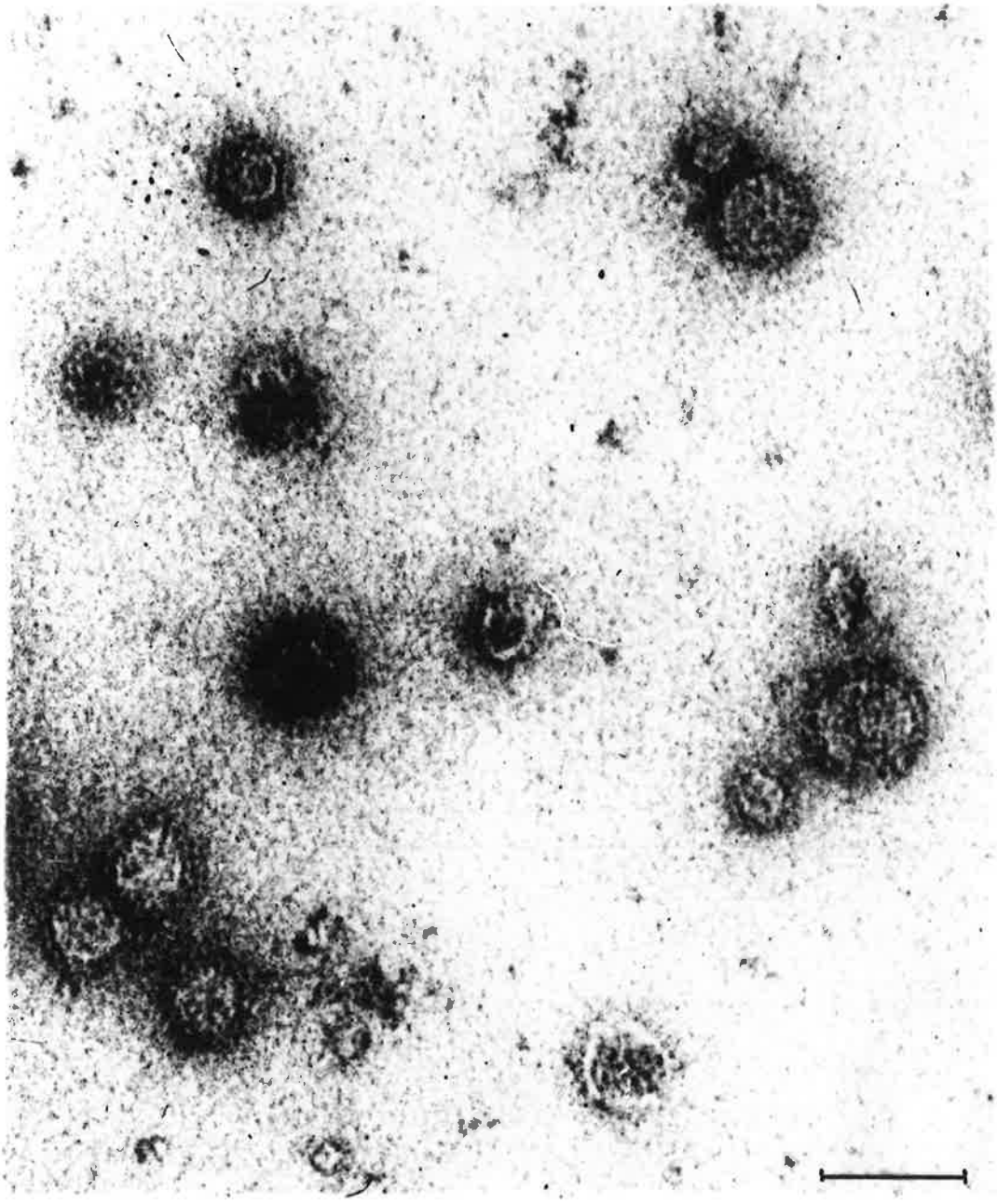
Diameters of the rosettes were measured from the electron micrographs of treated and untreated preparations (Figs. 20 and 21). These results were plotted on a histogram (Fig. 22). Both from the electron micrographs (Figs. 20 and 21) and from the histogram (Fig. 22) it can be seen that the rosettes from the Tween/ether preparation are much smaller than those from the untreated preparation. These results support the suggestion that Tween/ether disrupts the viral envelope.

To determine what effect Tween/ether treatment had on the virus in gel diffusion tests, the treated-LNYV preparations were tested against the LNYV antiserum (Figs. 23 and 24). In these tests three or more precipitin lines were observed when both treated and untreated virus were used (Figs. 23 and 24) but the Tween 80 and the Tween/ether treated preparations diffused further from the antigen wells to form precipitin lines. The slower diffusing antigen formed a much broader precipitin line after

Tween/ether treatment (Figs. 23 and 24). Two other precipitin lines formed closer to the antiserum wells (Figs. 23 and 24). When the gel diffusion plates were observed approximately 3 months later, after storage at 4°, the precipitin lines had intensified considerably (Figs. 25 and 26) and the precipitin lines 'c' and 'd' (Figs. 27 and 28) were observed to cross each other at the point 'e' (Figs. 27 and 28). The crossing or intersection of the precipitin lines suggests that two different antigens were reacting with the virus antiserum.

It is suggested that the precipitin line 'c' (Figs. 27 and 28) may be due to the reaction of rosettes with the LNYV antiserum. The Tween/ether treatment has been shown to cause a breakdown of the virus (Figs. 17 and 18), thus a broader precipitin line further from the antigen well would be expected (Figs. 27 and 28). This suggestion is also supported by the results of the size range of rosettes from untreated and Tween/ether treated virus preparations (Fig. 22). Since rosettes from the Tween/ether preparation were smaller, they would be expected to diffuse at a faster rate from the antigen well.

Figure 19 Electron micrograph taken by Mr. B.S. Wolanski, University of Melbourne. Sample obtained from the top 1 ml of the density gradient containing the untreated virus (Fig. 17A). Magnification X 160,000. The linear scale on the print represents 100 mμ.



Electron micrographs taken by Mr. B.S. Wolanski, University of Melbourne. Magnification X 80,000. The linear scale on the prints represent 100 mμ.

Figure 20 Sample obtained from the top 1 ml of the density gradient containing the Tween/ether treated virus (Fig. 17C).

Figure 21 Sample obtained from the top 1 ml of the density gradient containing the untreated virus (Fig. 17A).

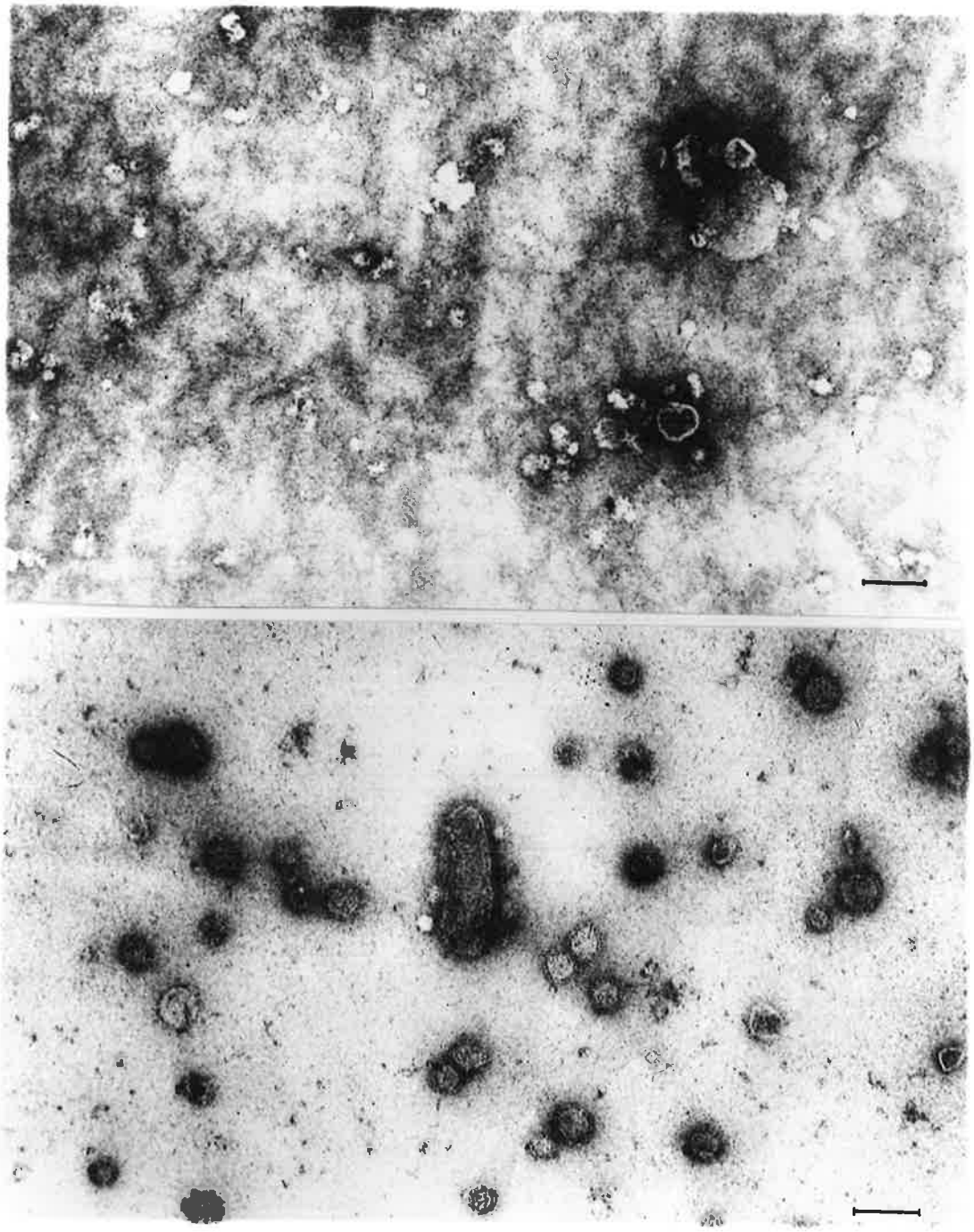
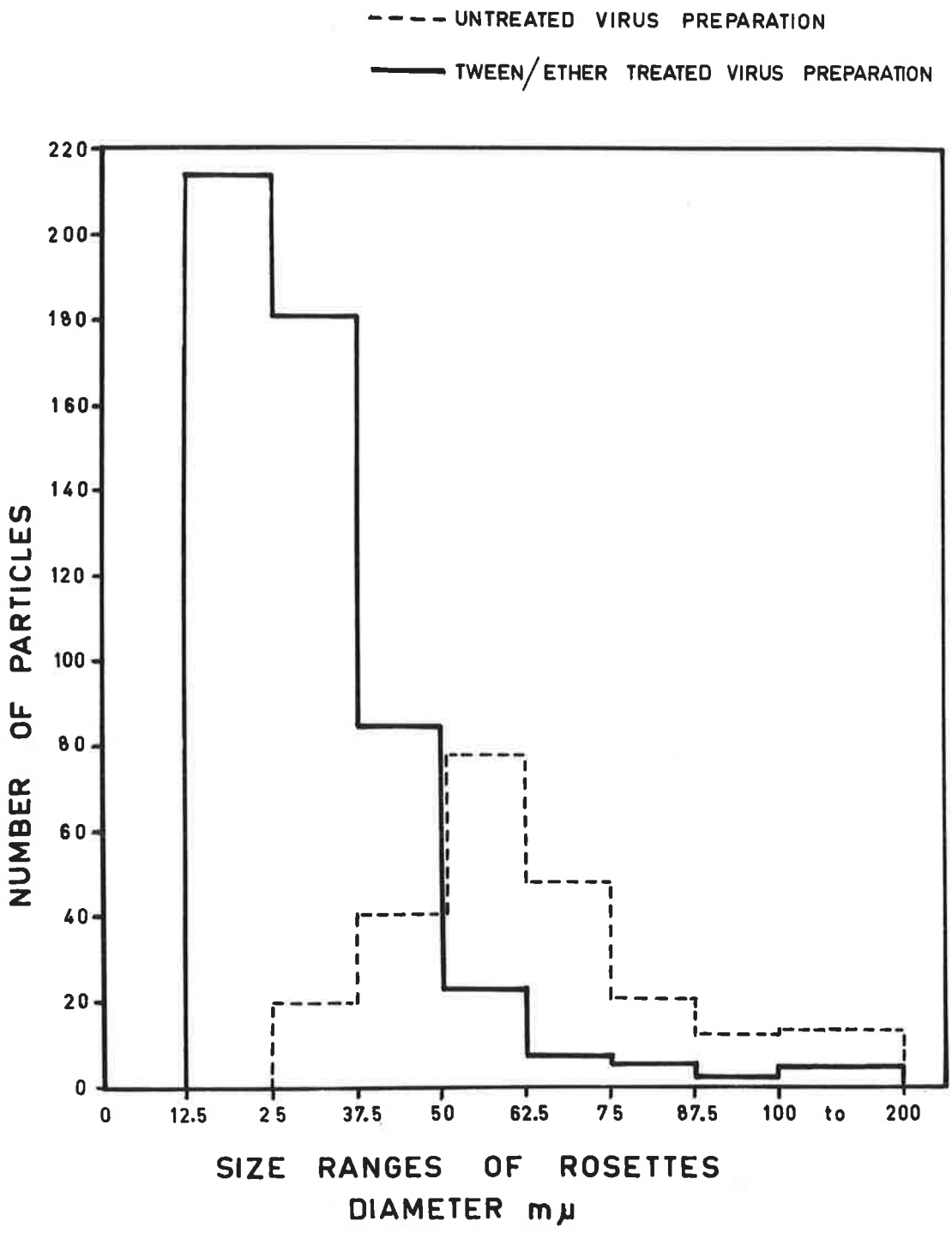


Figure 22 Size distribution of rosettes from untreated and Tween/ether treated virus preparations. The measurements were made from the electron micrograph, segments of which are shown in Figures 20 and 21.


UNTREATED VIRUS PREPARATION
TWEEN/ETHER TREATED VIRUS PREPARATION
NUMBER OF PARTICLES
220
200
180
160
140
120
100
80
60
40
20
0
0
12.5
25
37.5
50
62.5
75
87.5
100 to 200
SIZE RANGES OF ROSETTES
DIAMETER mμ

Serological tests in agar gel

The following symbols denote the preparations added to the wells of the agar gel.

(V) Purified LNYV

(T) Tween 80 treated LNYV

(E) Tween/ether treated LNYV

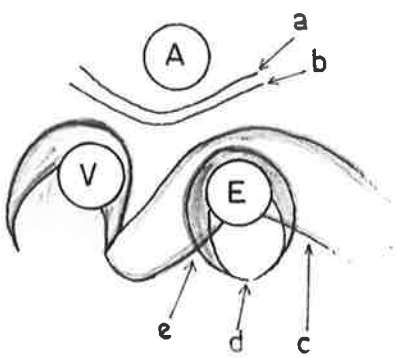
(A) LNYV antiserum

Figures 23 and 24 The antigen preparations were the same as those used for layering on the density gradients (Figure 17 A, B and C).

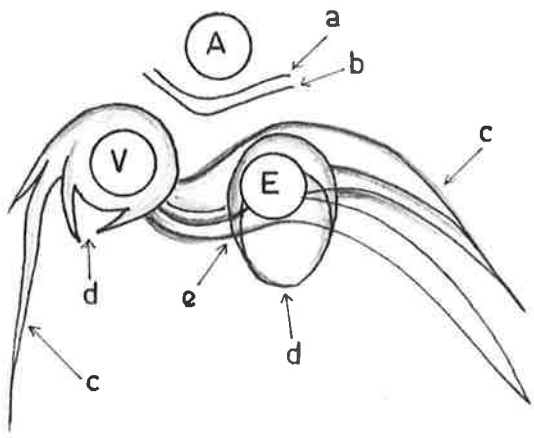
Figures 25 and 26 The antigen preparations were the same as those used for the density gradients (Figures 17 and 18) and were from two separate experiments. The photographs of these gels were taken after 4 months storage at 4^{o}.

Figures 27 and 28 Line drawings of the gel photographs of Figures 25 and 26.

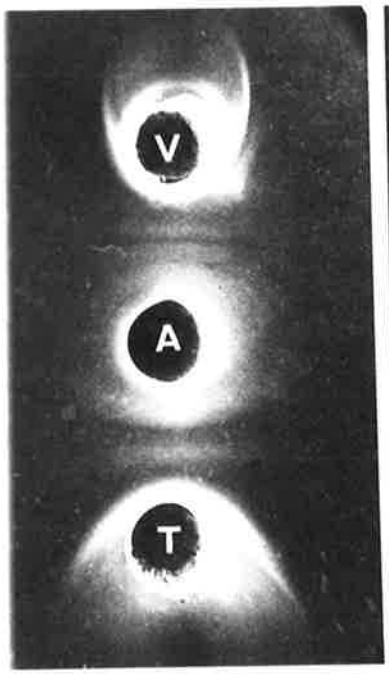
27



28



23



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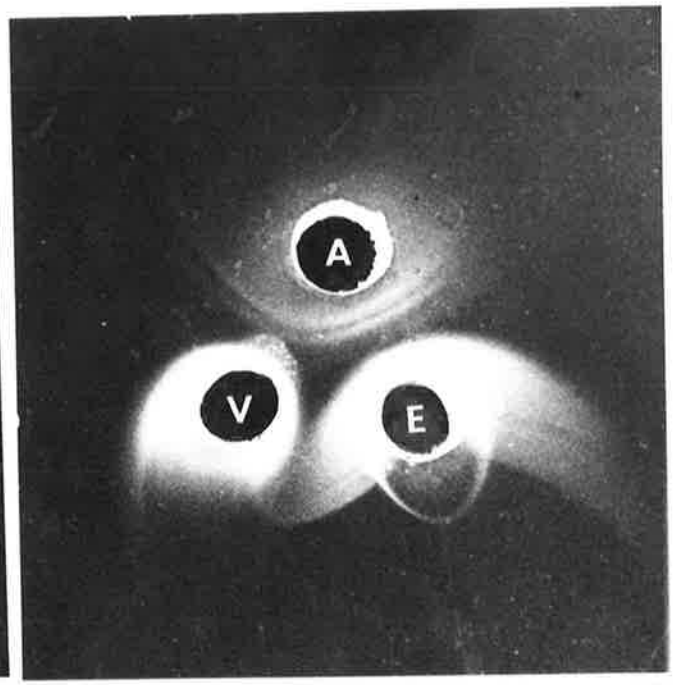
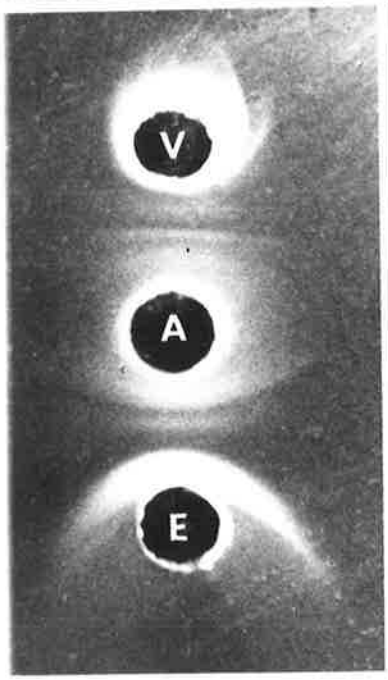
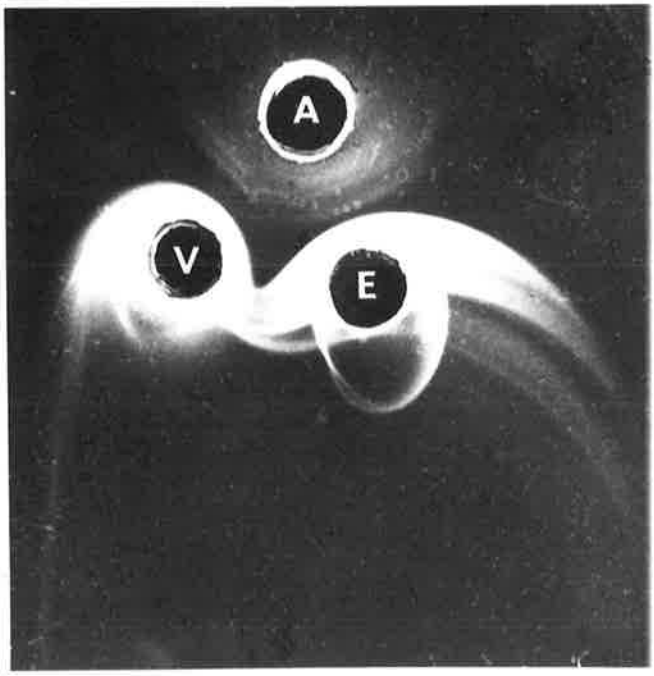






24

26

iii) Diffusion of the virus through agar. To determine the nature of the viral antigen diffusing through agar to produce precipitin lines, the following experiment was carried out. Purified virus was added to the top of a small agar column which had been prepared by melting agar disks in a Pasteur pipette so as to form a column 5 mm in diameter and at least 6 mm long. The agar filter thus prepared was placed at 4° overnight and a sample of purified virus was allowed to pass through it. The filtrate was tested against the LNYV antiserum and subjected to sucrose density gradient centrifugation. After centrifugation, the density gradient columns were analysed with the ISCO apparatus (Fig. 29). For the untreated preparation there was a peak of optical density at the position where infectious material was usually located. However, no equivalent peak of optical density was observed for the filtered virus preparation (Fig. 29). The filtered preparation was approximately one-tenth as infectious as the untreated virus preparation. Possibly, further experiments should have been carried out to investigate the relative amount and the nature of this infectious material.

There was a precipitin line formed when the filtrate was tested against the LNYV antiserum (Fig. 30). This precipitin line was confluent with that line produced by the antiserum and purified virus (Fig. 30). These results suggest that intact

Figure 29 Optical density determinations at different depths in density gradient tubes layered with untreated virus (A), and the filtrate obtained after allowing the virus suspension to diffuse through an agar column (B). The centrifuged gradients were analysed with the ISCO apparatus. Samples (0.1 ml) of each of these preparations were layered on to gradients and centrifuged at 26,000 rpm for 30 minutes in the SW 39 rotor of a Spinco centrifuge.

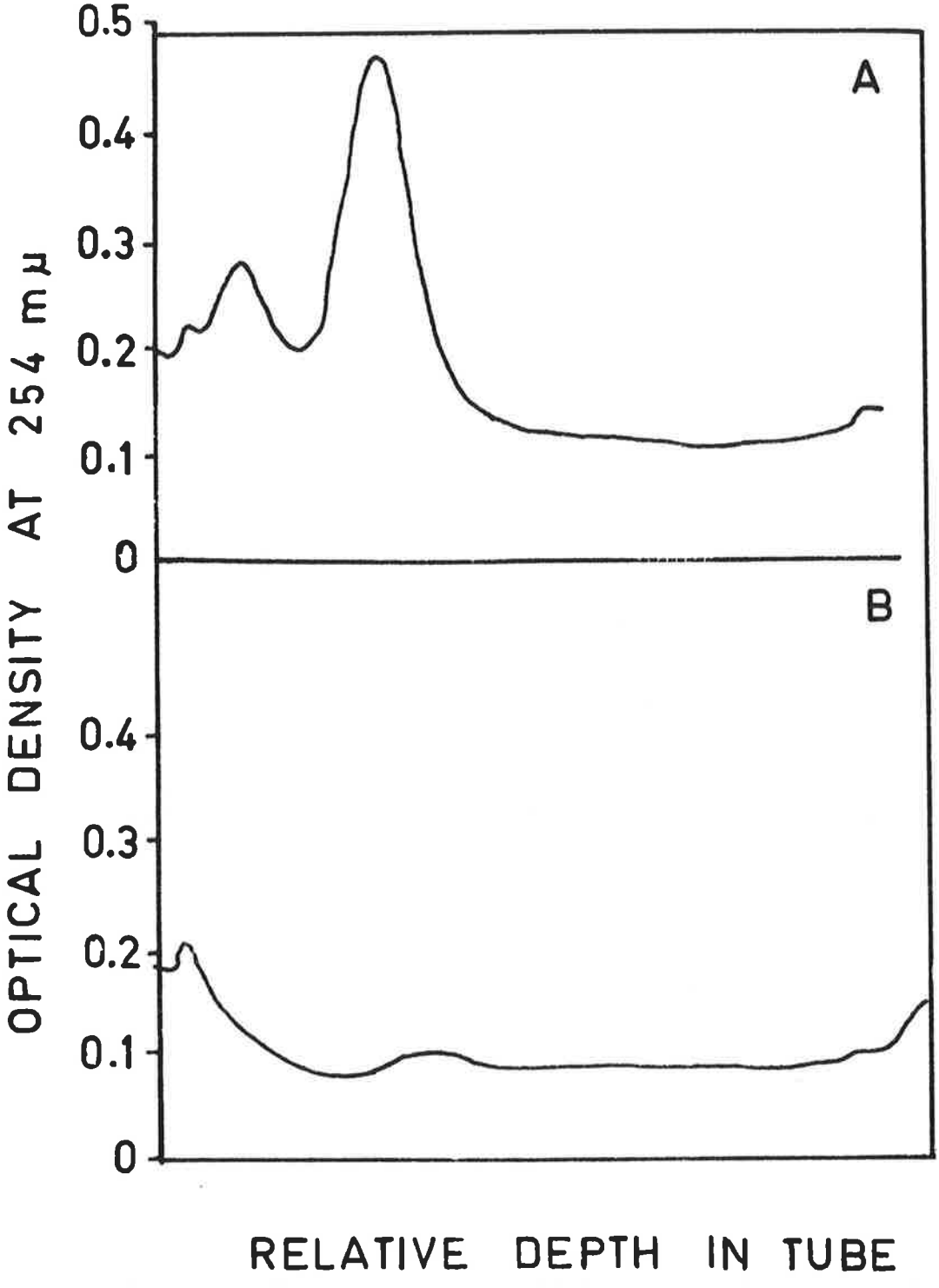

0.5
0.4
0.3
0.2
0.1
0
A
B
0.4
0.3
0.2
0.1
0
OPTICAL DENSITY AT 254 mμ
RELATIVE DEPTH IN TUBE

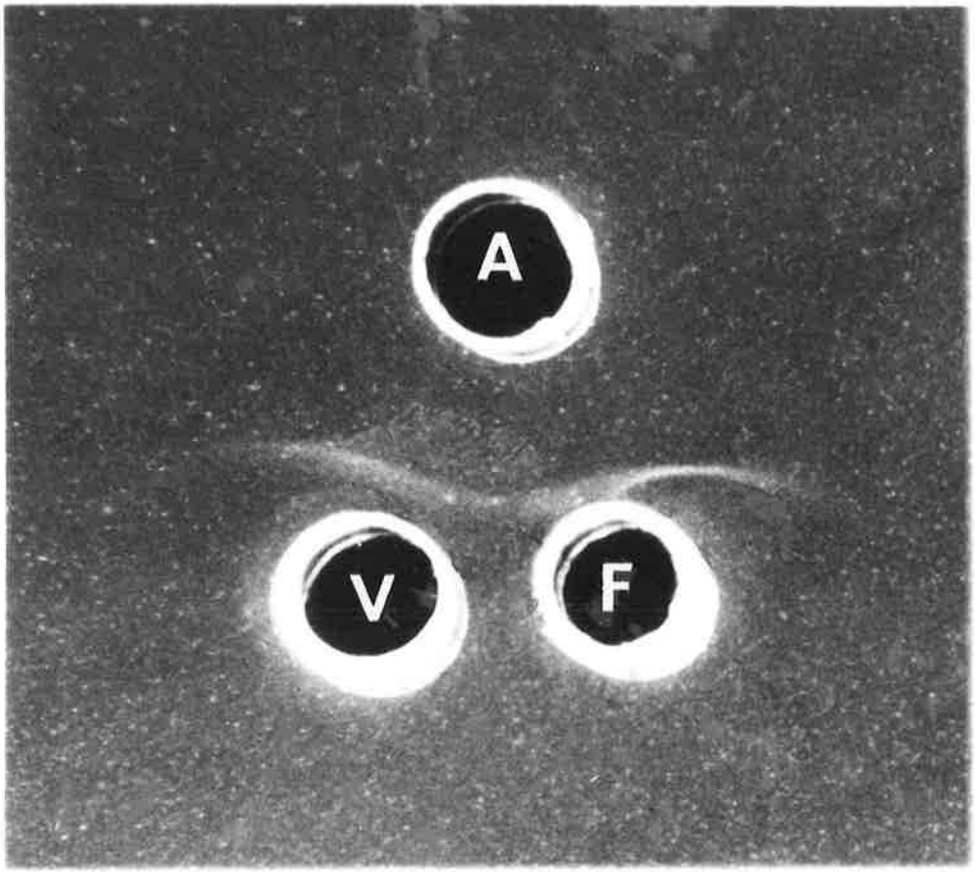
Figure 30 Serological tests in agar gel

These preparations were the same as those layered on to the density gradients (Fig. 29A, B).

(V) Purified LNYV

(F) LNYV preparation filtered through an agar column

(A) LNYV antiserum


A
V
F

virus does not diffuse through agar and that the viral antigen reacting in the gel diffusion tests was a sub-viral particle.

iv) The number of viral antigen. In this experiment an attempt was made to obtain evidence for the existence of several antigens in the LNY virion. Lipase solution was used to disrupt the virus (Sigma Chemical Co., St. Louis, Missuori; type 2, crude, from hog pancreas, containing diastase and trypsin) at a concentration of 1 mgm per ml and was suspended in 0.05 M phosphate buffer, pH 5.8 with 0.001 M $CaCl_2$ (Einset and Clark, 1958). An equal volume of the lipase solution was added to an aliquot of purified virus suspension and this was allowed to stand at 25° for 24 hours before being used in gel diffusion tests.

Sap from infected N. glutinosa was prepared by mincing infected leaves for 10 seconds in a Servall Omni-mixer, at 220 volts. The sap was expressed from the brei through cheese cloth and used for the agar gel diffusion tests.

In some experiments the antigen preparations were added to the wells of the agar gel several hours prior to the addition of antiserum. This procedure resulted in the precipitin lines being formed slightly further out from the antigen wells, as well as a better resolution of the precipitin lines (Figs. 31, 33 and 34).

Purified virus, lipase-treated virus and sap from infected N. glutinosa plants were tested against the LNYV antiserum (Figs. 31, 32, 33 and 34). Purified virus produced only one precipitin line whereas infectious sap produced two precipitin lines and the lipase-treated virus produced three precipitin lines (Figs. 31, 32, 33 and 34).

Line drawings of Figures 31 to 34 are shown in Figures 35 to 38 respectively. From Figure 35, the precipitin line 'a' produced by the purified virus is confluent with the precipitin line 'b' produced by the sap preparation. It has already been suggested that the precipitin lines 'a' and 'b' (Fig. 35) may be due to the reaction between rosettes and antiserum. The sap preparation produced an additional precipitin line 'c' (Fig. 35) which is approximately midway between the antigen and antiserum wells. From Figures 36, 37 and 38, the precipitin line 'c' is confluent with the line 'd'. Also the precipitin line 'a' is confluent with the lines 'b' and 'e'. From Figures 37 and 38, the precipitin lines 'c' and 'd' cross line 'e' and the lines 'f' and 'c' cross each other. These observations provide evidence for the existence of at least two antigens in the LNY virion. Since line 'f' also crosses line 'c' (Fig. 37), this observation suggests the possibility of a third antigen in the LNY virion. However, a relationship between the antigens 'c' and 'f' has not been excluded.

Serological tests in agar gel

The following symbols denote the preparations added to the wells of the agar gel.

(V) Purified LNYV

(D) Undiluted sap from infected N. glutinosa

(L) Lipase treated LNYV preparation

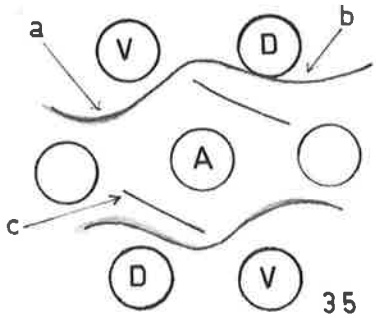
(A) LNYV antiserum

Figures 31 and 34 Antigen preparations added to the wells of the gel 16 hours prior to the addition of antiserum.

Figure 32 Antigens and antiserum added to the gel at the same time.

Figure 33 Antigen preparations added to the wells of the gel, 7 hours prior to the addition of antiserum.

Figures 35, 36, 37 and 38 are line drawings of Figures 31, 32, 33 and 34 respectively.



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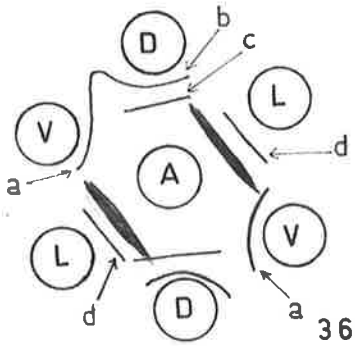

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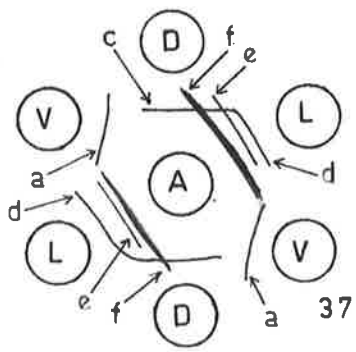

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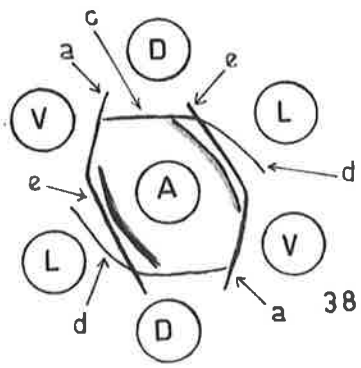
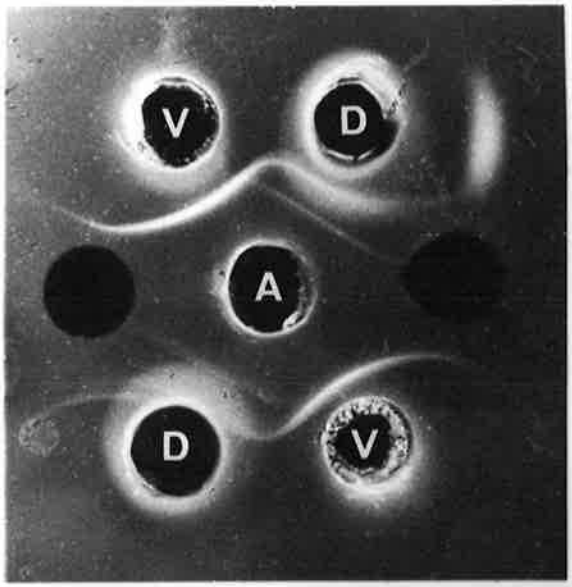


38

31



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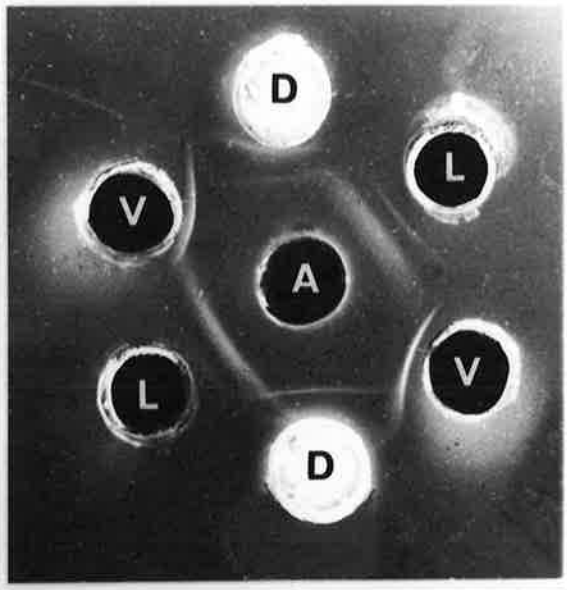
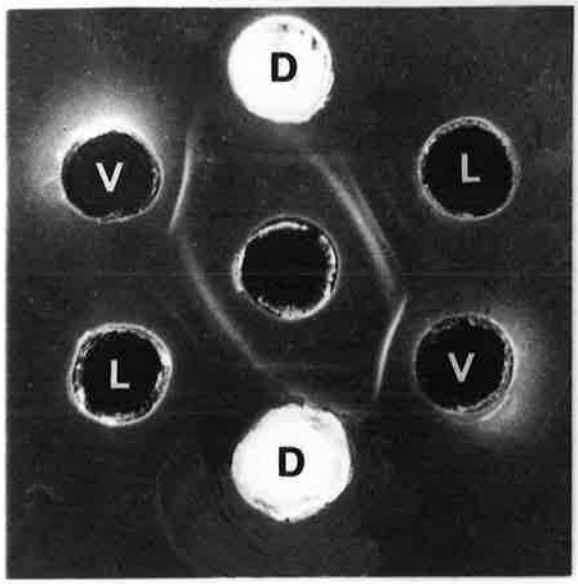
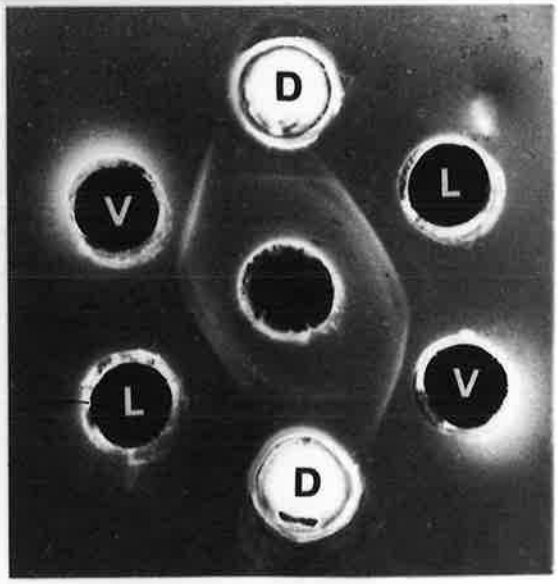




33



34

In some gels the slow diffusing antigen from infected sap (Fig. 35, line 'b') was not always visible (Figs. 33 and 34). This may be due to the fact that the antigen forming the precipitin line 'a' diffused much faster than the antigen forming the precipitin line 'b' (Fig. 31), even when the antigen preparations were added to the wells 16 hours before the addition of antiserum to the wells of the gel.

Further results, which may support the conclusion that the LNY virion contains at least two antigens, were obtained from the titration of the LNYV antiserum with undiluted sap from infected N. glutinosa using the agar gel technique (Figs. 39 and 40). Two precipitin lines were observed which had titres of $\frac{1}{2}$ and $\frac{1}{32}$. There may be two possible explanations for this result. It could be that the sap has two different antigens reacting with their homologous antibodies or it could be that two different antibodies (7S and 19S) had been produced in the rabbit in response to the one antigen.

4C. Discussion

Although this work has not established the nature of the viral antigen reacting with the LNYV antiserum in gel diffusion tests, the results obtained from the filtration of a virus suspension through an agar column and the Tween/ether experiments

Figure 39 Serological tests in agar gel

Titration of LNYV antiserum with sap obtained from infected N. glutinosa.

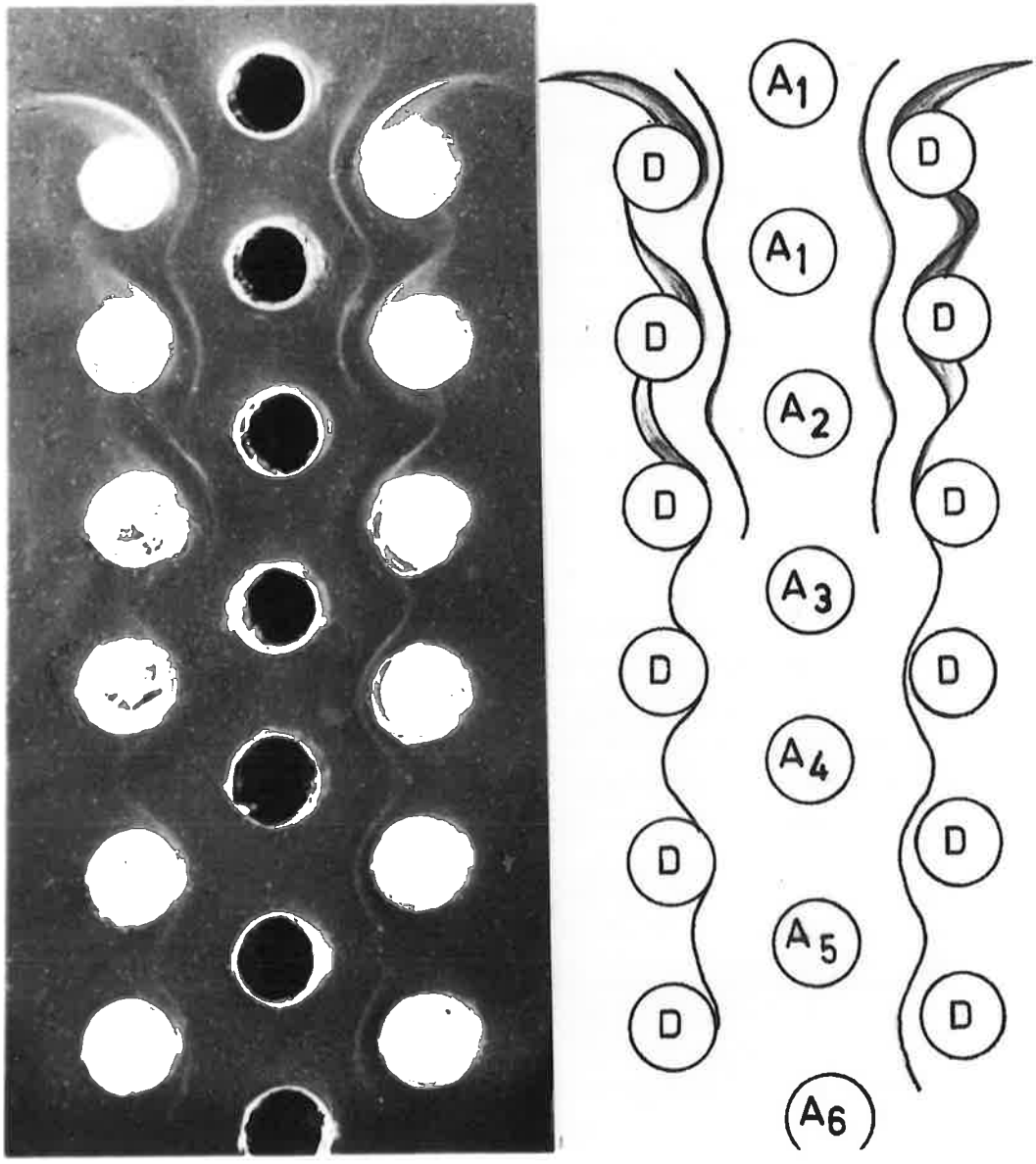
(D) Undiluted sap from infected N. glutinosa

(A) LNYV antiserum

A_1	undiluted antiserum
A_2	$^1/_2$
A_3	$^1/_4$
A_4	$^1/_8$
A_5	$^1/_{16}$
A_6	$^1/_{32}$

Figure 40 Line drawing of Figure 39.

Figure 41 Apparatus for irradiating plants with ultraviolet light.


A1
D
A1
D
A2
D
A3
D
A4
D
A5
D
A6
D
D
D
D
D
D

show that intact virus does not diffuse through agar to form precipitin lines in gel diffusion tests. However, intact virus may diffuse through agar in small amounts which were not detected by the optical density determinations.

Assuming that virus particles do not diffuse through agar, then antigenic sub-viral particles must be reacting with the LNYV antiserum in gel diffusion tests. From the electron microscopic observations, the density gradient analysis and the gel diffusion tests with Tween/ether treated virus (Figs. 20, 21; 17, 18; and 25, 26) it would seem that rosettes are the viral antigen which reacts with the LNYV antiserum. Rosettes and pleomorphic virus particles were observed by Wolanski et al. (1967) in electron micrographs of some negatively stained virus preparations. Wolanski and Francki (1969) suggested that procedures used in the purification of LNYV, as well as subsequent preparations for electron microscopy, may contribute to the different structures observed in electron micrographs. They showed that the pH of the phosphotungstic acid stain affected the shape of the virus particle, the staining characteristics of the virus and the length of the virus particle.

The origin and significance of rosettes is not known. They have not been observed in sections of infected leaf or in sections of infected vector cells (Chambers et al., 1965;

O'Loughlin and Chambers, 1967). The results of other workers support the suggestion that rosettes are degradation or disruption products of the virus. LNYV particles became disrupted on storage at 4^{o} for several weeks (Wolanski et al., 1967). These workers examined stored preparations in the electron microscope and observed that the virus particles were devoid of the envelope. Atchison et al. (1967) reported that salicyaldoxime and diethylthiocarbamate, specific chelators of copper and iron, inactivated LNYV. They suggested that virus inactivation was caused by the removal of Cu^{++} and Fe^{++} from the virus preparations, which allowed any lipases present to degrade the viral membrane. It is possible that lipolytic enzymes may be present in small amounts in virus preparations and degradation of the virus is taking place in such preparations.

Possibly an analogy may be drawn with the disruption of another bacilliform virus. Black et al. (1965) observed many disrupted virus particles in a partially purified virus preparation of potato yellow dwarf virus. When MacLeod et al. (1966) studied the intracellular localization of potato yellow dwarf virus bacilliform particles were observed, but no typically disrupted particles were seen. Waterson (1965) has proposed a model of rosette formation either from untreated or Tween/ether treated Measles virus, which could apply to the formation of rosettes in LNYV preparations.

Results of this work show that the LNY virion has at least two antigens and possibly three. In Figures 27 and 28 the intersection of the precipitin lines was observed (point 'e'). From the results of the gel diffusion tests with the lipase-treated virus preparations (Figs. 36, 37 and 38) there is evidence for the presence of at least two antigens since the precipitin lines 'd' and 'b' cross.

Brown et al. (1967) concluded that rosettes accounted for a considerable proportion of the immunogenic activity of Tween/ether treated VSV preparations. They also observed that a mass of small particles, which were thought to be the fringes or spikes of the viral envelope, produced two precipitin lines in agar gel diffusion tests. With LNYV, it is possible that precipitin lines 'a' and 'b' (Figs. 27 and 28) could also be due to the fringes or spikes of the LNYV envelope.

Although there is no conclusive evidence concerning which particular antigens are due to any particular component of the LNY virion, the following suggestions are offered on the basis of the structural similarity of LNYV and VSV. Recent work on the proteins of VSV (Kang and Prevec, 1969; Wagner et al., 1969) indicates that the VS virion has at least three proteins. Kang and Prevec characterized three major and one minor protein by polyacrylamide gel electrophoresis, and they concluded that

two of these proteins were from the envelope and a third from the nucleoprotein core of VSV. Wagner et al. (1969) identified three major and three minor proteins of VSV; one of the major proteins was thought to be from the viral envelope. Thus, for LNYV the following hypothesis is proposed: The LNY virion may have three antigens, two of which are present in the viral envelope and a third which is present in the internal nucleoprotein component of the virus. It is suggested that the precipitin line 'd' (Figs. 27 and 28) may be due to the nucleoprotein component of the virus while the precipitin lines 'a' and 'b' (Figs. 27 and 28) may be due to the envelope proteins of the virus. Further work on LNYV is required to resolve the number of protein components of the virus.

5. INACTIVATION OF LETTUCE NECROTIC YELLOWS VIRUS BY ULTRAVIOLET IRRADIATION

5A. Introduction

Ultraviolet irradiation has been used to study replication of bacteriophage (Luria and Latarjet, 1947) and to study the early events in the replication of tobacco mosaic virus (TMV) (Siegel and Wildman, 1956; Dijkstra, 1964), tobacco necrosis virus (TNV) (Bawden and Harrison, 1955) and red clover mottle virus (Bawden and Sinha, 1961).

The sequence of the early events in plant virus replication has been divided into four separate phases, based on the changes in the sensitivity of the infection centres to inactivation by ultraviolet light (Siegel and Wildman, 1956). During the first, second and third phases the survival curve was exponential, whereas in the fourth phase the secondary rise in resistance was expressed by a change in the survival curve from exponential to multitarget. They showed that in the first phase the sensitivity of the infection centres to irradiation was constant, but Dijkstra (1964) observed a decrease in sensitivity during this period.

Irradiation of the leaf before inoculation was shown by Bawden and Kleczkowski (1960) to markedly affect its capacity to

support virus multiplication. Hence they inferred that conclusions drawn from previous experiments (Bawden and Harrison, 1955; Siegel and Wildman, 1956), in which leaves were irradiated at different time intervals after inoculation, were possibly invalid. Goodchild (1961) reported that 4 minutes of irradiation prior to inoculation did not affect the capacity of the leaves to support virus multiplication. In his experiments TMV-inoculated leaves of N. glutinosa were irradiated for periods not exceeding 90 seconds.

Photoreactivation has been described as the reversal with near ultraviolet or visible light of ultraviolet damage to a biological system (Jagger, 1958). Bawden and Kleczkowski (1955) reported the photoreactivation of five plant viruses, but failed to observe this phenomenon for TMV. However, Goodchild (1961) observed photoreactivation of TMV at $3\frac{3}{4}$ hours after inoculation but not in the intervening period after inoculation. The photoreactivation of TMV nucleic acid has also been reported (Bawden and Kleczkowski, 1959).

Lettuce necrotic yellows virus (LNYV) was found to be very sensitive to ultraviolet irradiation (Owusu and Crowley, unpublished results). This fact, together with the other properties of the virus (Harrison and Crowley, 1965), prompted this investigation into the effects of ultraviolet light on the replication of the virus.

Some of the results reported in this section have already been published (see Appendix 3, McLean and Crowley, 1969).

5B. Materials and Methods

LNYV (SE3 isolate) and the U2 strain of TMV supplied by Dr. S.G. Wildman (University of California, Los Angeles) were used. LNYV inoculum for the 'in vivo' experiments was extracted from infected leaves using the Freon/glycine method (see Section 3C(i)), while inoculum for the 'in vitro' experiments was obtained from infected leaves using the charcoal/DEAE-cellulose clarification method (see Section 3C(iv)). Dilutions of the virus extract were made in 0.1 M glycine/NaOH buffer (pH 8.6), so as to produce between 30 to 60 lesions per half-leaf. Purified TMV (which was supplied by Dr. N.C. Crowley) was prepared by precipitation with ammonium sulphate, followed by two cycles of high and low speed centrifugation. Celite was added to all inocula at the rate of 0.5 gm per 10 ml.

The infectivity tests for the 'in vitro' studies were carried out by half-leaf comparisons on young leaves of N. glutinosa plants. For the 'in vivo' studies, plants were pruned to the two most susceptible leaves (Crowley, 1967) prior to inoculation. At least ten plants were used for each treatment.

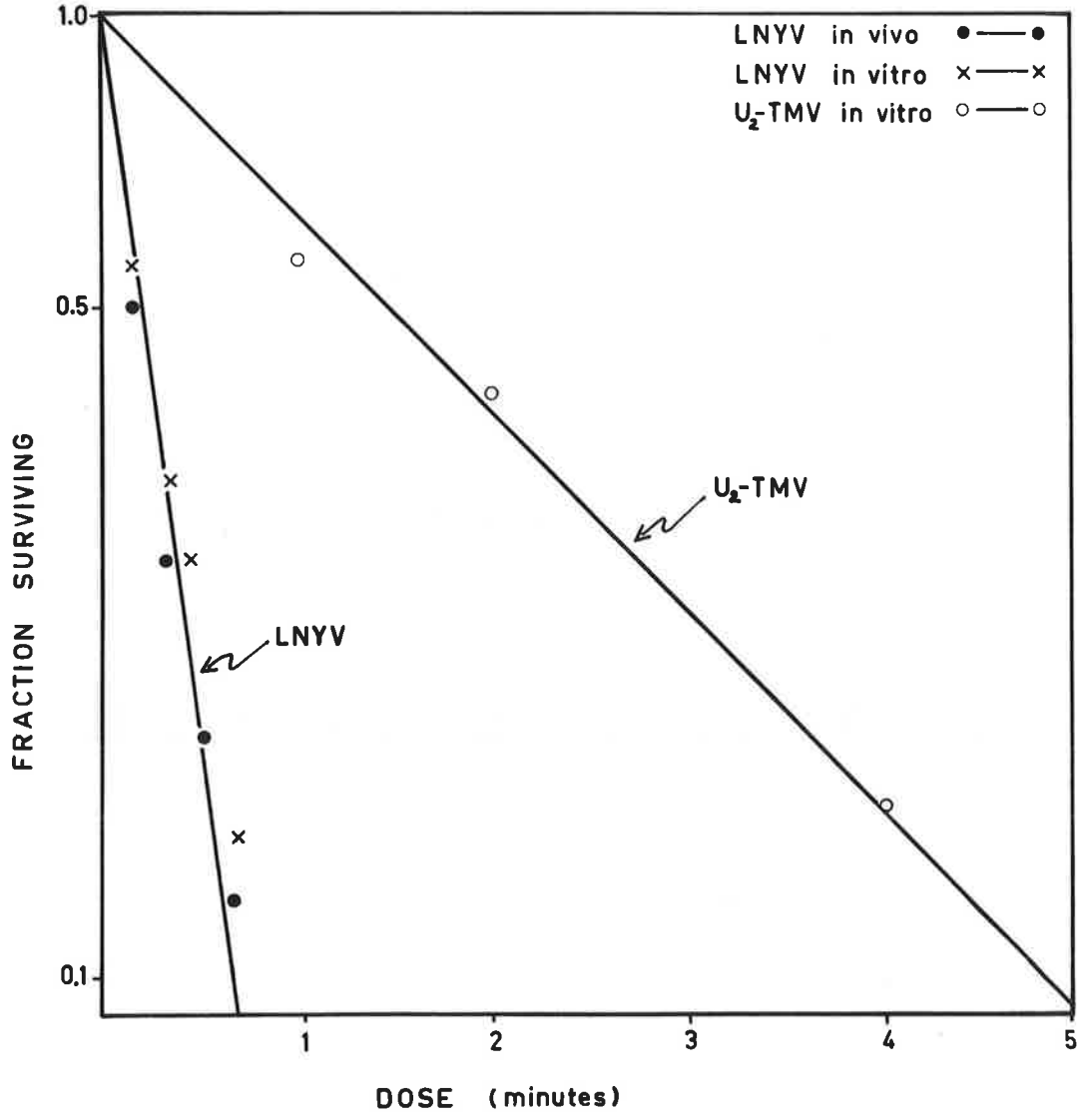
One half of every leaf was covered with aluminium foil and the pot containing the plant was placed in a larger pot, so as to support each leaf horizontally and to maintain each leaf at the same distance from the ultraviolet light source; a 240 volt, 50 watt, Oliphant "Sterilamp" (Fig. 41). According to the manufacturer's specifications this lamp emits 700 ergs per square cm at 1 metre with 90% of the irradiation concentrated at 2537 Å. A voltage stabilizer was used in all experiments to avoid variation in output due to fluctuations in the mains supply. In the 'in vivo' and 'in vitro' experiments the inoculated plants or the petri dish of clarified plant extract were placed 60 cm from the light source. In the 'in vitro' experiments the clarified extract was stirred with a magnetic stirrer while being irradiated.

5C. Experimental

i) Ultraviolet dose response curve for LNYV and U2-TMV.

The infectivity survival curves for purified U2-TMV and clarified LNYV irradiated 'in vitro', and for LNYV irradiated within 30 minutes of inoculation to N. glutinosa are shown in Figure 42. The 'in vitro' and 'in vivo' curves for LNYV coincide, showing that the observed effect is on the virus and not on the host. LNYV appears to be approximately eight times more sensitive to ultraviolet irradiation than U2-TMV.

Figure 42 Survival curves (dose response) for LNYV and TMV when irradiated with UV '*in vitro*' and for LNYV-inoculated plants ('*in vivo*'), irradiated within 30 minutes of inoculation.


1.0
0.5
0.1
FRACTION SURVIVING
1
2
3
4
5
DOSE (minutes)
LNYV in vivo
LNYV in vitro
U2-TMV in vitro
U2-TMV
LNYV

ii) Effect of irradiation on N. glutinosa prior to inoculation with LNYV. Half-leaves of N. glutinosa were irradiated for varying times, ranging from 1 to 10 minutes, and were then immediately inoculated with virus. In two experiments, 5 minutes of irradiation caused no significant reduction in lesion numbers on the irradiated half-leaves when compared with the non-irradiated. However, 10 minutes irradiation affected the plant's capacity to support virus multiplication and the lesion numbers on irradiated half-leaves were 66% and 40% of those produced on the non-irradiated.

iii) Effect of a period of darkness following irradiation. Leaves of N. glutinosa were inoculated with LNYV and half-leaves were irradiated for 30 seconds after inoculation. Ten irradiated plants were placed under lights in the growth room (22°), while another 10 irradiated plants were immediately transferred to a dark chamber for 24 hours. In one typical experiment, the percentage of the lesion numbers on the irradiated half-leaves to those on non-irradiated half-leaves was 19% for the group of plants placed in the dark chamber and 17% for the group of plants kept under normal light conditions.

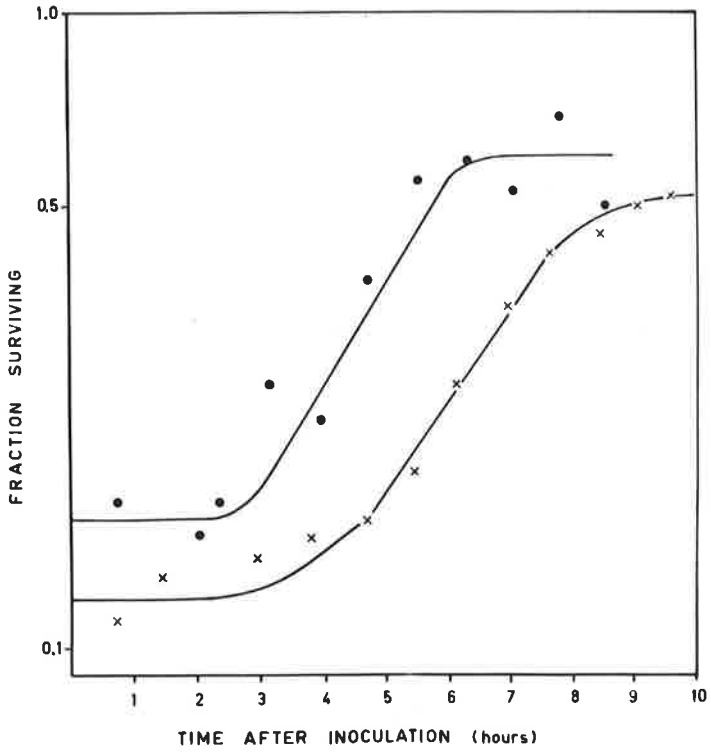
iv) Constant-dose irradiation of inoculated leaves at varying times after inoculation. Leaves of N. glutinosa plants were inoculated with LNYV in a constant

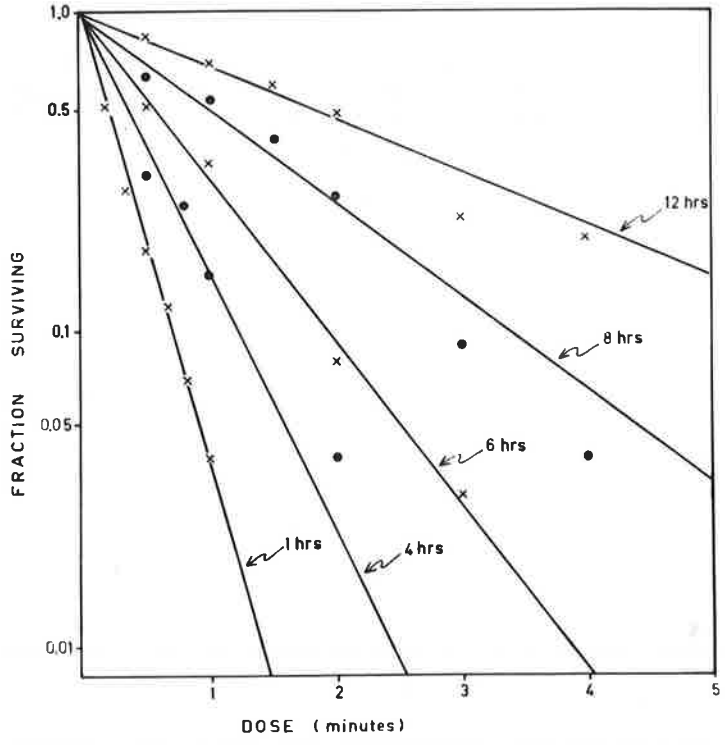
temperature room at 22°. Half-leaves were irradiated with a constant dose (30 seconds) of ultraviolet light at different times after inoculation. This dose is one-tenth to one-twentieth of that required to affect the capacity of the leaf to support virus multiplication. Three phases were evident in the survival curves for two experiments (Fig. 43), selected from several experiments with similar curves. In the first phase the percentage of inactivated infection centres was constant for approximately 3 hours, while in the second phase, lasting about 2 hours, there was a sharp decrease in the percentage of inactivated infection centres. In the third phase the percentage of inactivated infection centres was constant. The survival curves obtained for LNYV (Fig. 43) resembled those obtained by Bawden and Harrison (1955) for TNV and by Siegel and Wildman (1956) for U2-TMV.

v) Changes in the survival curve, for leaves irradiated at different time intervals after inoculation. In this series of experiments leaves of N. glutinosa were inoculated in a constant temperature room at 24 to 26°. Dose response curves were obtained by irradiating half-leaves at increasing intervals of time after inoculation (Fig. 44). The slope of the survival curve decreased as the time interval increased after inoculation. This experiment was repeated several times so that

Figure 43 Survival curves obtained when LNYV-inoculated leaves were irradiated with a constant dose (30 seconds) of UV at varying times after inoculation. Each curve represents a separate experiment.

Figure 44 Survival curves obtained when LNYV-inoculated leaves were irradiated with an increasing dose of UV at different time intervals after inoculation.


1.0
0.5
0.1
FRACTION SURVIVING
1
2
3
4
5
6
7
8
9
10
TIME AFTER INOCULATION (hours)


1.0
0.5
0.1
0.05
0.01
FRACTION SURVIVING
12 hrs
8 hrs
6 hrs
4 hrs
1 hrs
1
2
3
4
5
DOSE (minutes)

survival curves for irradiation at 1, 4, 6, 8 and 12 hours after inoculation, were determined at least three times each. There was no evidence for the existence of multitarget survival curves even at 12 hours after inoculation, which is 4 to 6 hours after the commencement of the third phase (Fig. 43).

5D. Discussion

It was found that less than one-tenth of the irradiation dose, that was required to affect the capacity of the leaves to support virus multiplication, was used to inactivate LNYV infection centres. Thus the criticism levelled by Bawden and Kleczkowski (1960) at similar work with TNV (Bawden and Harrison, 1955) and TMV (Siegel and Wildman, 1956) cannot apply to LNYV. However, the survival curves obtained when LNYV-inoculated leaves were irradiated with a constant dose at varying times after inoculation (Fig. 43) are quite similar to those obtained for TMV and TNV. Both TMV and TNV consist of RNA and protein, while LNYV is more complex in that it contains a membrane of lipid material in addition to protein and nucelic acid (Wolanski et al., 1967).

It was suggested by Siegel et al. (1957) that phase 1 (the period of time in which the fraction of infection centres surviving after irradiation was constant) was the time required for RNA and protein to separate. Bawden (1964) questioned the validity of this interpretation. Recently, Streeter and Gordon

(1967) compared the relative sensitivities to inactivation by irradiation of the U1-TMV and U2-TMV strains to those of the hybrid viruses obtained by reconstituting the protein of either strain with the RNA of the other. They observed that the sensitivity of the hybrid virus was similar to that of the strain which supplied the protein coat. This indicates that the relative sensitivities of the two natives strains are, in part, a function of their respective protein coats. Streeter and Gordon (1968) extended their studies on the role of the protein coat in the ultraviolet inactivation of these two TMV strains. They obtained evidence which suggests that irradiation affects the protein-RNA cross-linking of these two TMV strains to various degrees. Further, they reported that the inactivation rate for U1-RNA and U2-RNA was three times that obtained for the intact U2 virus. Thus there is a discrepancy between the results of Streeter and Gordon (1968) and Siegel et al. (1956) who found that U2-TMV and U1-RNA and U2-RNA showed similar rates of inactivation. Another observation which is in conflict with the results of Siegel and Wildman (1956) and Siegel et al. (1957) is the fact that no lag period (phase 1) was observed by Dijkstra (1964) when TMV-inoculated N. glutinosa were irradiated at different times after inoculation.

One possible explanation for the interpretation of results obtained from ultraviolet inactivation of infection centres may be an effect due to the screening produced by the layers of plant cells as the virus passes through these layers. Benda (1955) provided evidence that the epidermis transmits less than 50% of incident ultraviolet light.

Results obtained from irradiation of virus-inoculated leaves reflect changes produced on infection but do not show their nature (Bawden, 1964) or explain them (Siegel, 1966). One of the conflicts arising out of such work is whether one or more virus particles enters an infectible site. Rappaport and Wu (1962) and Wu (1964), working with two strains of TMV, suggested that more than one particle enters an infectible site and, therefore, multitarget survival curves are to be expected. The results of Siegel and Wildman (1956) were interpreted as showing that a transition from exponential to multitarget survival curves occurred, but Siegel (1966) has subsequently stated that such a transition has not been demonstrated. With LNYV multitarget survival curves were not observed.

Elucidation of the infection process of a plant virus has recently been studied more profitably using the technique of epidermal stripping. Ehara and Misawa (1967a, b), studying the infection of cucumber mosaic virus in cowpea and tobacco,

suggested that the separation of viral protein and nucleic acid takes 2 hours, and this event can only occur in the epidermis. These results require confirmation.

LNYV, like TMV and tobacco rattle virus (Kleczkowski, 1960), does not appear to exhibit the phenomenon of photoreactivation. Possibly, with these viruses, ultraviolet irradiation causes both functional and genetic damage, while with the plant viruses which are photoreactivable the damage is only functional. This suggestion arises out of the work of Symonds and McCloy (1958). They concluded that when phage-infected bacteria were irradiated photoreactivable damage is wholly functional and not associated with genetic damage, while the damage by ionizing radiations and the non-photoreactivable damage inflicted by ultraviolet irradiation is both functional and genetic. More recently Kleczkowski (1967) has demonstrated two types of repair mechanisms when virus-inoculated leaves were irradiated. Damage caused to TNV by UV irradiation can be repaired in darkness in Chenopodium amaranticolor, but not in French bean or tobacco. However, photoreactivation was observed in French bean and in tobacco, but not in C. amaranticolor. Thus, the damage repaired in the dark in C. amaranticolor appears to be repaired in the light in French bean and tobacco.

Kleczkowski and McLaren (1967) have shown that ultraviolet irradiation caused at least two kinds of damage to free TMV-RNA, one of which was photoreversible, but the photoreactivable damage did not occur when intact virus was irradiated.

6. THE SYMPTOMATOLOGY, DISTRIBUTION, TRANSLOCATION AND MULTIPLICATION OF LNYV IN NICOTIANA GLUTINOSA L.

6A. Introduction

Stubbs and Grogan (1963b) obtained many isolates of LNYV from lettuce (Lactuca sativa L.) and sowthistle (Sonchus oleraceus L.) which infected N. glutinosa, Petunia hybrida Hort., Gomphrena globosa L. and Datura stramonium L. The majority of the isolates from lettuce and sowthistle produced necrotic local lesions on N. glutinosa. Some isolates produced mild symptoms on N. glutinosa. One of the isolates producing local lesions which was isolated from S. oleraceus has been studied in this laboratory for over 6 years and has been referred to as the SE3 strain. Symptom development of this strain on N. glutinosa is as follows: local lesions, which may be either chlorotic or necrotic, appear 6 to 8 days after inoculation, and about 7 to 10 days after inoculation systemic symptoms develop on the young terminal leaves which show vein clearing, curling of the leaves and also yellowing at 12 to 15 days after inoculation.

This section describes observations of the symptoms produced on LNYV-infected N. glutinosa. Although the variation in symptom expression has not been correlated with environmental differences, it appears that the physiological age of the plant

also affects symptom production. A study of the distribution of LNYV in the inoculated and non-inoculated leaves, the egress of virus from the inoculated leaves, and the rate of increases of virus in inoculated and systemically infected leaves was made.

6B. Experimental

i) Observations of symptoms produced on LNYV-infected plants

(a) The model LNYV-infected N. glutinosa

As an aid in simplifying the description of the symptoms and the experiments in this section, the leaves of the N. glutinosa plant will be referred to in a uniform manner (Fig. 45). If there is one or more symptomless leaves above the inoculated leaves, such leaves will be designated leaf '0'. The inoculated leaves will be referred to as leaves L1, L2, L3 and L4 down the plant, while the systemically infected leaves will be referred to as leaves S1, S2 and S3 up the plant (Fig. 45).

(b) Virus isolates

In this study the SE3 isolate (Stubbs and Grogan, 1963b) was used. For the majority of experiments infected plants resembled the plant shown in Figure 46, but

Figure 45 The model LNYV-infected N. glutinosa

1. The number of inoculated leaves may vary from 2 to as many as 4 or even 5 leaves.

2. There may be no leaves with symptoms or there have been as many as 5 symptomless leaves above 3 inoculated leaves.

3. In some infected plants there were no systemically infected leaves, while in other infected plants there were 3 or 4 systemically infected leaves.

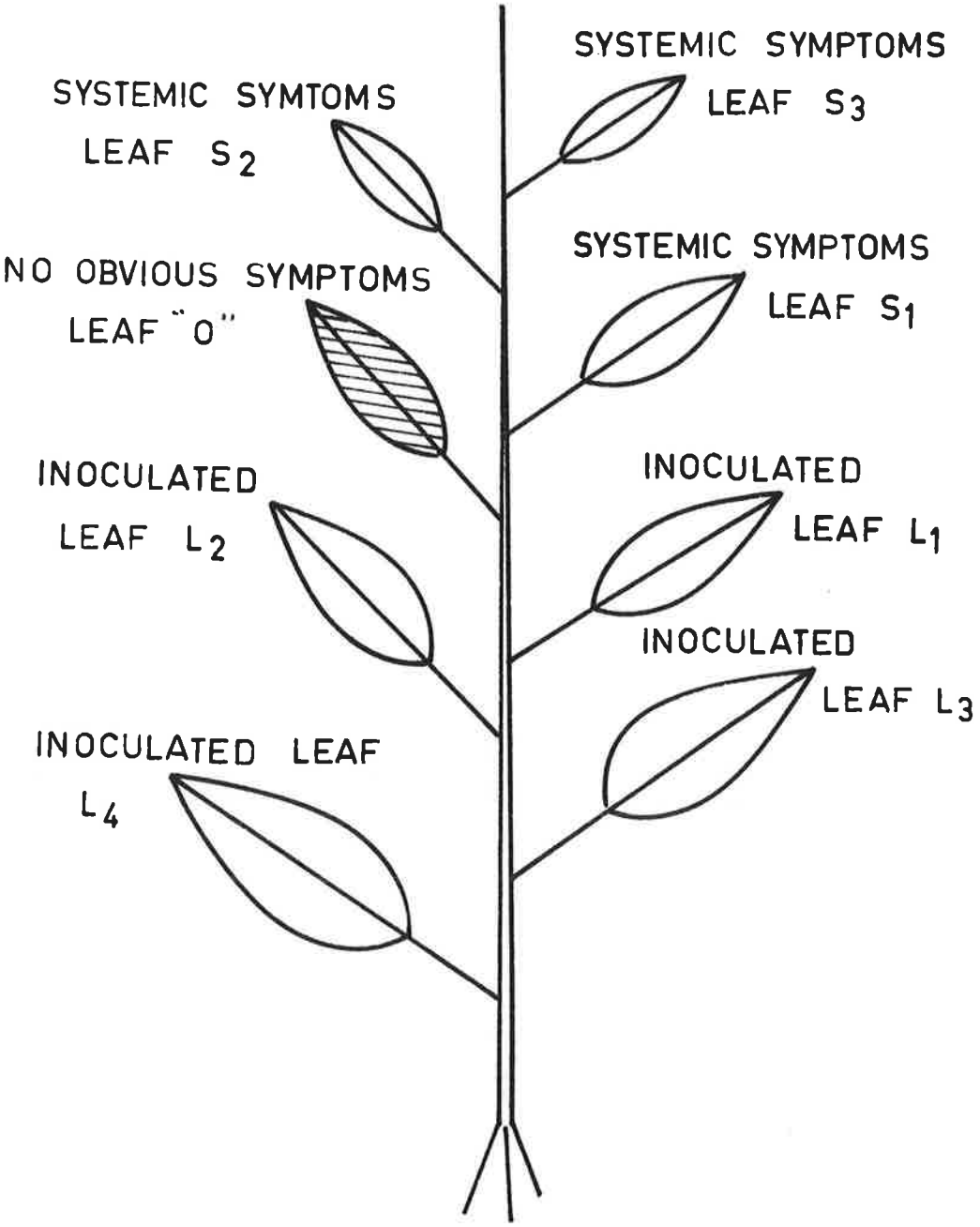
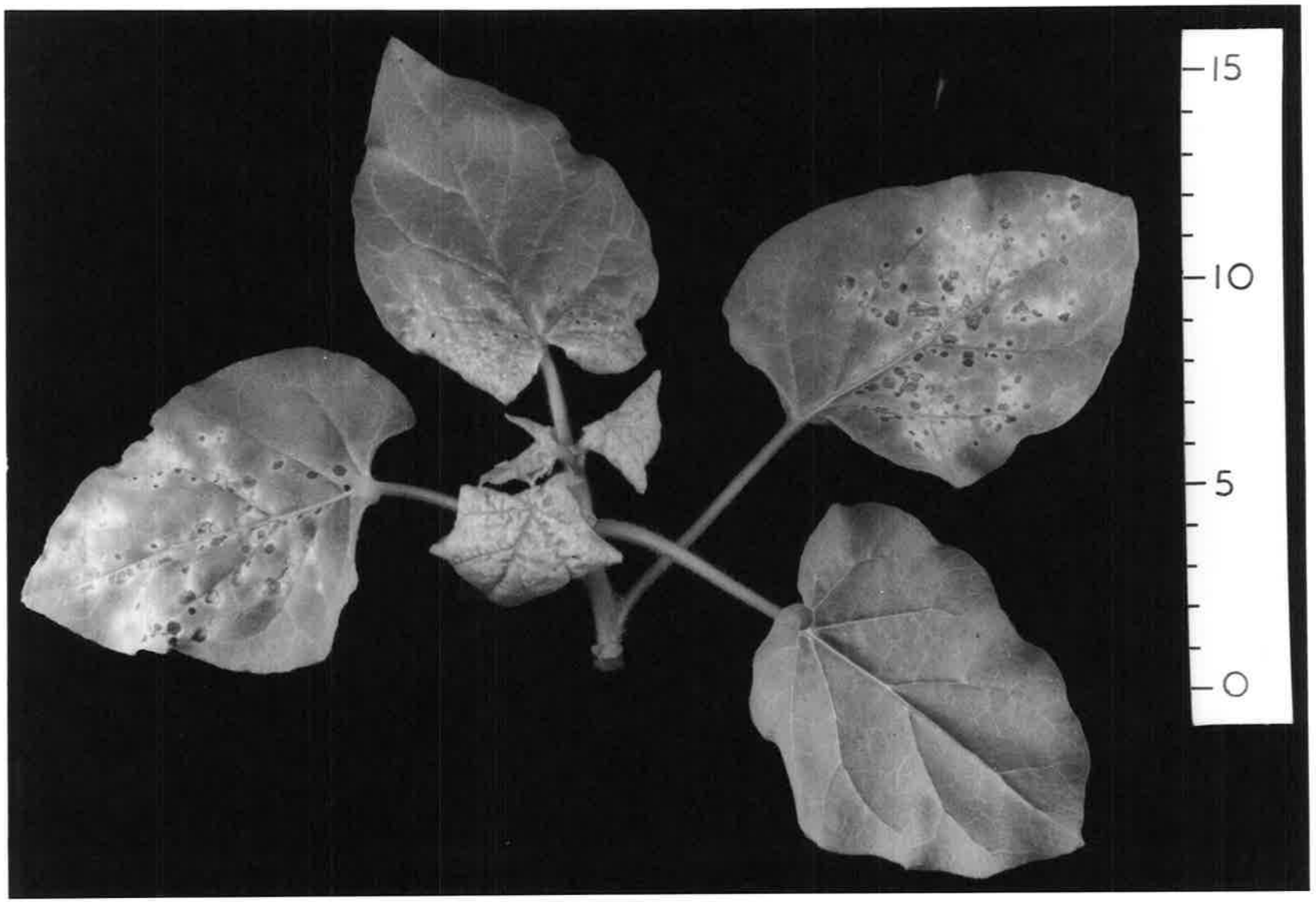

SYSTEMIC SYMPTOMS
LEAF S_3
SYSTEMIC SYMTOMS
LEAF S_2
SYSTEMIC SYMPTOMS
LEAF S_1
NO OBVIOUS SYMPTOMS
LEAF "O"
INOCULATED
LEAF L_1
INOCULATED
LEAF L_2
INOCULATED
LEAF L_3
INOCULATED LEAF
L_4

Figure 46 Symptoms produced by the SE3 isolate of LNYV on *N. glutinosa*, at 16 days after inoculation. Scale in cm.


15
10
5
0

some infected plants did not have a symptomless leaf above the top inoculated leaf. Isolates of LNYV were also obtained from lettuce and sowthistle, but these isolates produced a mild type of symptom when inoculated on to N. glutinosa (Fig. 47).

(c) Symptom variation in LNYV-infected N. glutinosa due to plant variation

Some plants from a group which had been inoculated at the same time differed from others in the absence or presence of a leaf '0' on the plant (Figs. 48 and 49).

(d) Environmental conditions and symptom development

In some inoculated plants, where a small number of lesions developed (Fig. 50), there were three leaf '0's and then leaves S1, S2 and S3. In other inoculated plants, local lesions were observed but systemic symptoms were absent (Fig. 51). This plant was chosen from a group of such plants, which had been grown under abnormally high light conditions.

ii) Distribution of LNYV in N. glutinosa.

(a) Concentration of LNYV in leaves

To compare the amount of virus in different leaves of the same plant an LNYV-infected N. glutinosa plant,

Figure 47 (A) Healthy *N. glutinosa*, same age as (B).

(B) Symptoms produced by a mild isolate of LNYV from lettuce on *N. glutinosa*. Note stunting of the plant and abnormally shaped upper leaves.

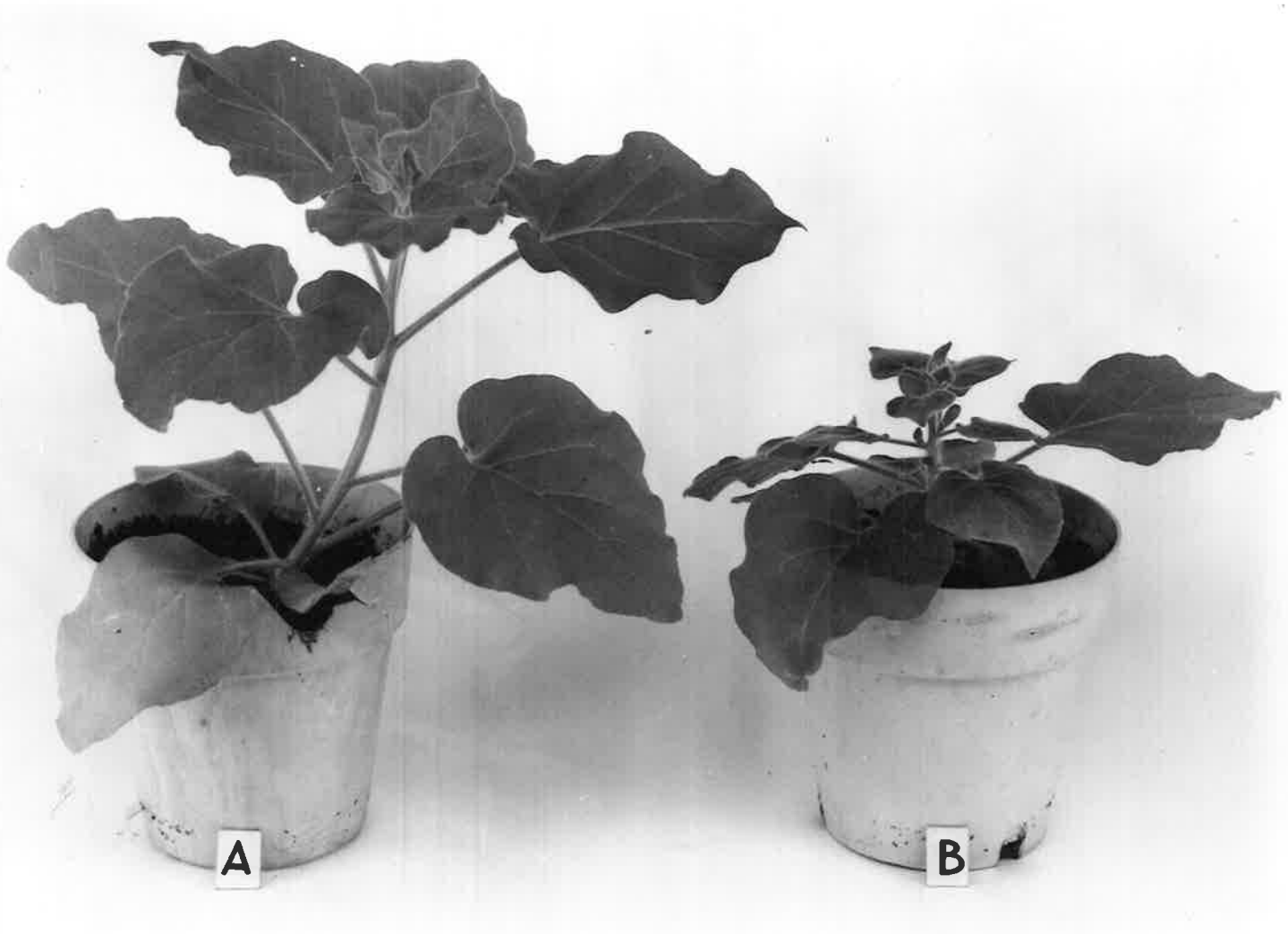

A
B

Figure 48 LNYV-infected N. glutinosa at 12 days after inoculation. Note the first leaf above the inoculated leaves (arrowed) shows no symptoms.

Figure 49 LNYV-infected N. glutinosa at 12 days after inoculation. Note the first leaf above the inoculated leaves (arrowed) has vein clearing towards the base of the leaf.

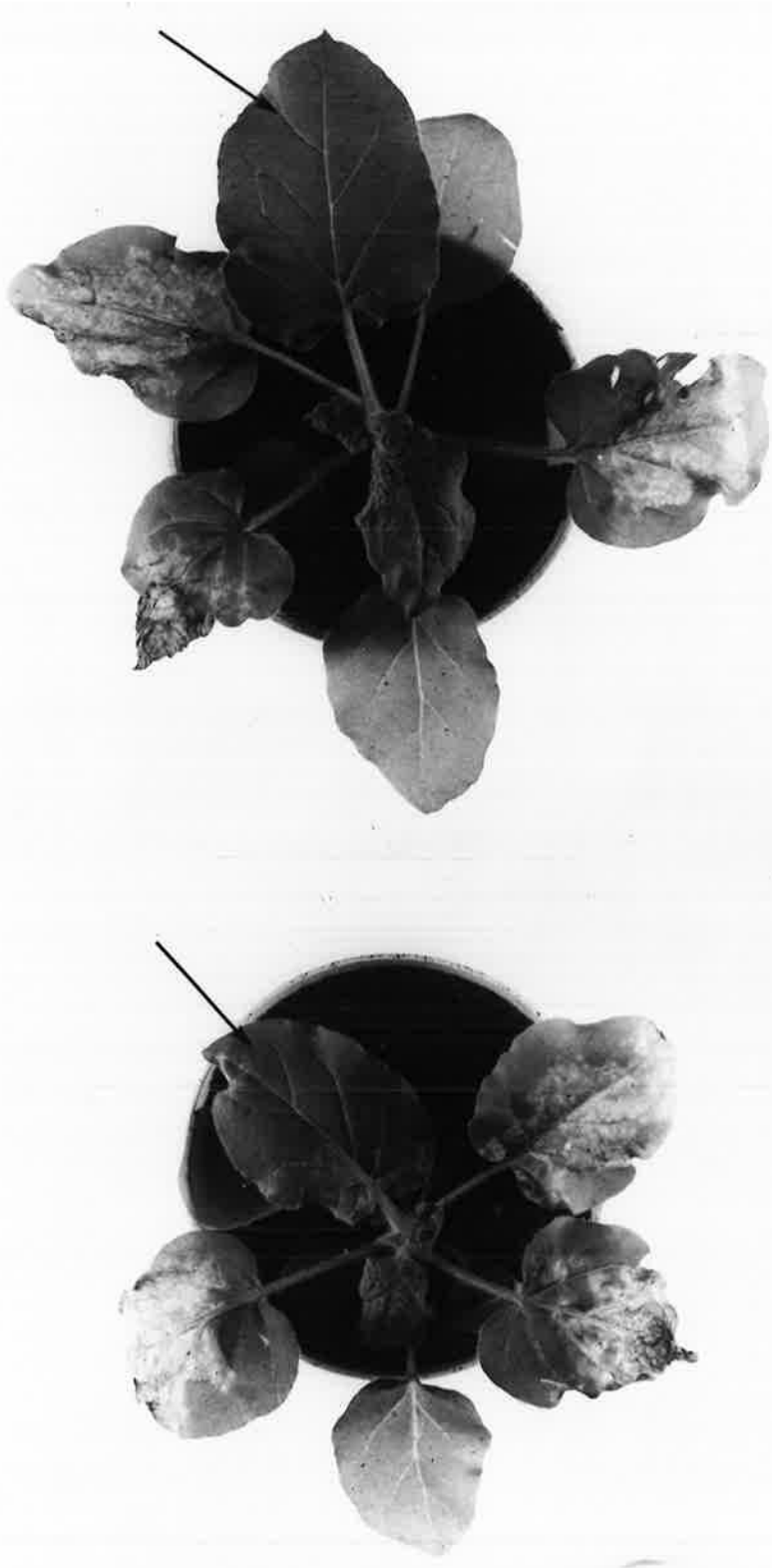
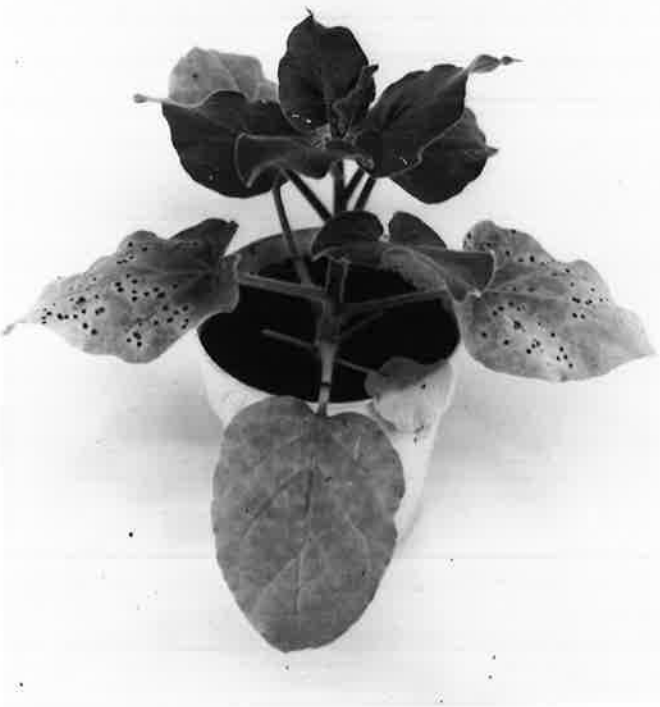


Figure 50 LNYV-infected N. glutinosa at 20 days after inoculation. Note the 3 symptomless leaves (leaf '0's) between the inoculated leaves and the systemically infected leaves.

Figure 51 LNYV-infected N. glutinosa, at 19 days after inoculation. Note the absence of symptoms on the non-inoculated leaves.



which had been inoculated 12 days previously, was chosen. Leaves L1, L2 and L3 had been inoculated and local lesions had developed. Leaf '0' was devoid of symptoms while leaves S1, S2 and S3 had developed systemic symptoms. Each leaf was weighed, then mashed in a volume of distilled water which would give the same dilution for each leaf on a weight to weight basis. These extracts were then further diluted in distilled water, and the infectivity of extracts from each leaf was compared by half-leaf assay.

Results of these comparisons (Table 9) show that leaves L2 and L3 contained approximately the same amount of virus. No virus was detected in leaf '0', while leaves S1, S2 and S3 contained between five and eight times as much virus as was present in leaf L1. This experiment was repeated and similar results were obtained.

Although no virus was detected in leaf '0' in the experiment, further observations were made on the appearance of symptoms on the first leaf above leaf L1. In one experiment 50% of the plants contained a leaf '0', while in two other experiments 88% and 92% of the plants contained a leaf '0' (Table 10).

Table 9

Comparison of extractable virus from leaves of LNYV-infected N. glutinosa

$L2/_{L1}$ (a)	$L3/_{L1}$	leaf '0' $/_{L1}$	$S1/_{L1}$	$S2/_{L1}$	$S3/_{L1}$
$17/_{8}$ (b)	$18/_{7}$	$0/_{5}$	$29/_{5}$	$69/_{8}$	$32/_{6}$

(a) The positions of the inoculated and systemic leaves have been referred to in Figure 40.

(b) Local lesions produced on opposite half-leaves of N. glutinosa by the extracts from the leaves of an infected plant. Each assay was carried out on 18 leaves.

Table 10

Observation of the number of plants with a leaf '0'

Experiment number	Number of plants with a leaf '0'
1	$57/_{113}$ (a)
2	$84/_{91}$
3	$83/_{95}$

(a) The number of plants with a leaf '0' (numerator) and the total number of plants inoculated (denominator).

Even though leaf '0' exhibits no symptoms, it is possible that virus may be present in this leaf in quite small amounts. Forty-two of the plants from experiment 1 (Table 10) with a leaf '0' were chosen and each leaf '0' was mashed in 1 ml of distilled water and the extract assayed for infectivity. Sixteen of the forty-two extracts were found to be infectious.

To check if the maturity of the last inoculated leaf at the time of inoculation correlated with the presence or absence of leaf '0' the leaf lengths were measured on inoculated leaves prior to inoculation. Results (Table 11) show that if the last inoculated leaf was $1\frac{1}{4}$" to $1\frac{1}{2}$" long then a leaf '0' was present on the plant. If the last inoculated leaf was $\frac{3}{4}$" long then leaf '0' was absent from the plant. It was observed that some of the inoculated leaves (leaf L1) on some plants exhibited both local lesions and vein clearing at the base of the leaf (Fig. 52C).

(b) Distribution of virus in an inoculated leaf

To determine virus distribution, a leaf disk containing a lesion and a leaf disk adjacent to this lesion were taken from a 10 day old LNYV-infected N. glutinosa plant. Each of these disks was mashed in 0.25 ml of distilled water and the infectivity of the extracts was compared by half-leaf assay.

Table 11

Correlation of the length of the last inoculated leaf (L1), at the time of inoculation, with the presence or absence of a leaf '0', on LNYV-infected N. glutinosa

Plant number	Length of leaf L1 at inoculation	Length of leaves at 13 days after inoculation		
		'0'	S1	S2
1	$\frac{3}{4}$"	+	$1\frac{5}{8}$"	$\frac{3}{4}$"
2	$\frac{3}{4}$"	+	$1\frac{3}{4}$"	$\frac{7}{8}$"
3	$\frac{3}{4}$"	+	$1\frac{7}{8}$"	1"
4	$\frac{3}{4}$"	+	$1\frac{3}{8}$"	$\frac{1}{2}$"
5	$1\frac{1}{4}$"	$2\frac{5}{8}$"	2"	1"
6	$1\frac{1}{2}$"	$2\frac{1}{2}$"	$1\frac{7}{8}$"	$\frac{7}{8}$"
7	$1\frac{3}{8}$"	$2\frac{1}{2}$"	$2\frac{3}{8}$"	$1\frac{1}{4}$"
8	$1\frac{1}{2}$"	$2\frac{1}{2}$"	$1\frac{7}{8}$"	1"

(+) indicates the absence of leaf '0' on the plant.

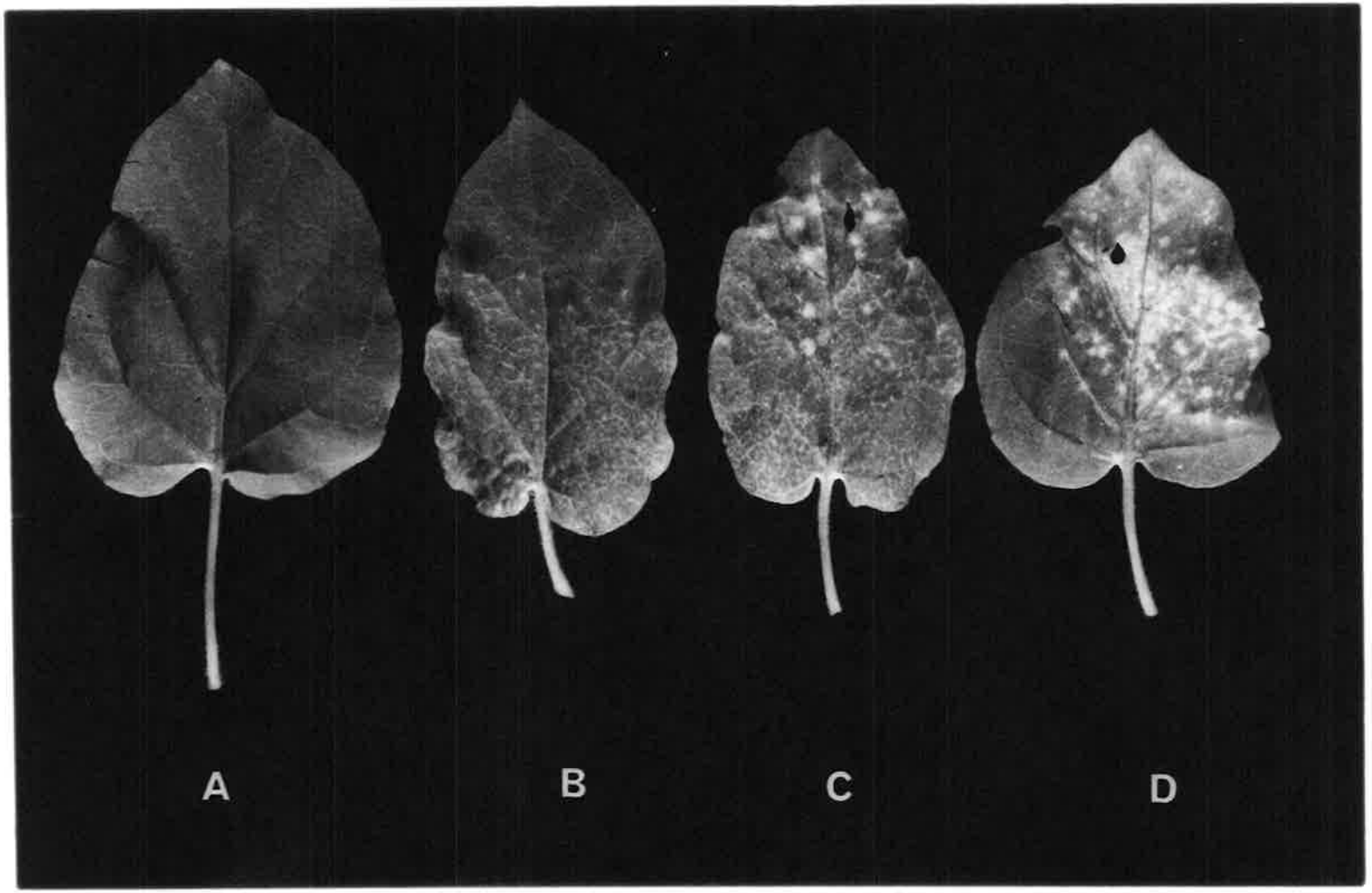
Figure 52 Leaves obtained from the LNYV-infected N. glutinosa plants at 14 days after inoculation.

(A) Leaf '0' $2\frac{1}{2}$" long

(B) Leaf S1 $2\frac{3}{8}$" long

(C) Leaves L1 and S1 $2\frac{1}{4}$" long

(D) Leaf L1 $2\frac{1}{8}$" long


A
B
C
D

Results of several of these comparisons (Table 12) show that there is considerably less virus in the leaf disk adjacent to the lesion than from the leaf disk containing the lesion.

Table 12

Levels of virus extracted from leaf disks containing a lesion and one from an area adjacent to it

Experiment	Tissue adjacent to a lesion / Tissue containing a lesion (a)
1	$^{0}/_{9}$
2	$^{0}/_{5}$
3	$^{1}/_{24}$
4	$^{12}/_{50}$

(a) Local lesions per half-leaf produced on opposite half-leaves of N. glutinosa by the extract of a leaf disk from the area adjacent to a lesion (numerator) and by the extract of a leaf disk containing a lesion (denominator). Each assay was carried out on 9 leaves.

(c) Amounts of virus in leaf tissue containing a lesion and from systemically infected leaf tissue

A leaf disk containing a lesion from an inoculated leaf and a leaf disk from the base of a systemically infected leaf (leaf S2) were taken from a 10 day old LNYV-infected N. glutinosa plant. Each of these disks was mashed in 0.5 ml of distilled water and the infectivities of the extracts were compared. Results of two of these comparisons showed that approximately the same amount of virus was extracted from the leaf tissue containing a lesion, and from leaf tissue taken from a systemically infected leaf (Table 13).

(d) Concentration of LNYV in various regions of systemically infected leaves

The rate of development of vein clearing in systemically infected leaves was observed to procede at different rates. To determine if the amount of infectious material correlated with the development of symptoms, leaf disks (5 mm diameter) were taken from the base and tip of each of two systemically infected leaves (leaves S2 and S3), from an LNYV-infected plant at 7 days after inoculation. Each leaf disk was mashed in 0.5 ml of distilled water and the extracts obtained from the tip and base of each leaf were assayed for infectivity. This procedure was repeated at 10 days after inoculation for leaves S1, S2 and S3.

Table 13

Ratio of infectious material extracted from a systemically infected leaf and an inoculated leaf

Days after inoculation	Tissue from base of a systemically infected leaf: Tissue containing a lesion (a)	
	Experiment 1	Experiment 2
8	$^{37}/_{45}$ (a)	$^{46}/_{43}$ (a)
9	$^{91}/_{69}$	$^{65}/_{66}$
10	$^{40}/_{33}$	$^{54}/_{49}$

(a) Local lesions per half-leaf produced on opposite half-leaves of N. glutinosa by the extract of a leaf disk from the base of a systemically infected leaf (numerator) and by the extract of a leaf disk containing a lesion (denominator). Each assay was carried out on 12 leaves.

Results of these comparisons (Table 14) show that in leaf S3, the rate of virus increase, based on infectivity assay, in the tip relative to the base, was as high as 10 fold. In six experiments this rate of increase varied from 3 to 18 fold. However, in leaf S2 the ratio of virus extracted from the tip as compared to the base did not change significantly over the 2 assay days. Leaf S1 has a negligible amount of virus at the tip compared to that at the base.

To investigate the rate of increase of infectious material at the base and tip of two adjacent systemically infected leaves, with respect to the base of the lower leaf (S2), the following experiment was carried out. The extracts obtained after mashing a leaf disk from the tip and base of leaf S3 and the tip of leaf S2 in 1 ml of distilled water were each compared with the extract obtained from the base of leaf S2. Results of one such assay (Table 15 and Fig. 53) show that the extract from the leaf disk from the tip of leaf S3 contained much more infectious material than the extract from the leaf disk from the tip of leaf S2, at 11 days after inoculation. There was not a large difference in the amount of infectious material extracted from the bases of leaves S2 and S3 (Table 15 and Fig. 53), although there was slightly more infectious material recovered from the base of leaf S3 at 9 days after inoculation. This experiment was repeated and a similar result obtained.

Table 14

Comparison of infectivity of extracts of leaf disk samples from the tip and base of systemically infected N. glutinosa

Leaf number (a)	Tip/Base (b)					
	Experiment 1		Experiment 2		Experiment 3	
	Day 7 (c)	Day 9	Day 7	Day 9	Day 8	Day 10
S3	1/3	26/25	1/5	24/10	0.3/2	21/6
S2	2/7	4/11	1/6	0/21	.25/14	3/39
S1	-	1/63	-	0/16	-	1/21

(a) The system of numbering the leaves of the plant was depicted in Figure 45.

(b) Local lesions per half-leaf produced on opposite half-leaves of N. glutinosa by extracts of leaf disk from tip (numerator) and from base (denominator). Each assay was carried out on 12 leaves.

(c) Days of assay after inoculation with virus.

Table 15

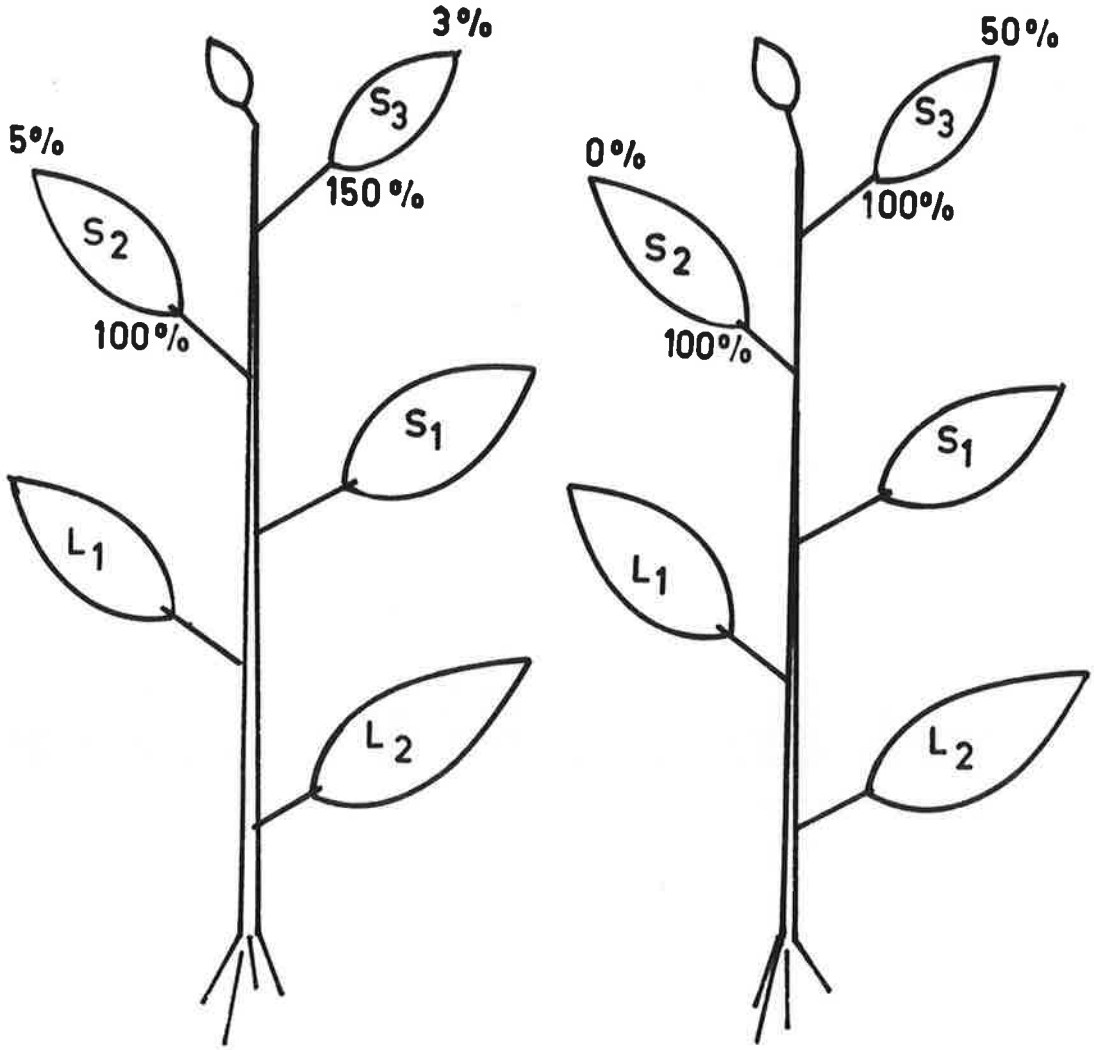
Comparison of infectivity of extracts of leaf disks taken from the base and tip of two adjacent systemically infected N. glutinosa leaves

Number of days after inoculation	Base S3 / Base S2(a)	Tip S3 / Base S2(a)	Tip S2 / Base S2(a)
9	45/30	1/35	2/39
11	66/64	19/37	0/69

(a) These three ratios represent the number of local lesions per half-leaf produced on opposite half-leaves of N. glutinosa by the extracts from the various leaf disks. Each assay was carried out on 12 leaves.

Figure 53 Comparison of infectivity of extracts of leaf disks taken from the tip and base of 2 systemically infected N. glutinosa leaves. These comparisons are expressed as a percentage of the infectivity of the leaf disk obtained from the base of leaf S2.

These data are presented in Table 15.



AFTER INOCULATION

(e) Presence of LNYV in root tips

To determine if the virus had migrated into the root tip, the following experiment was carried out. Root tips were removed from N. glutinosa plants which had been grown in aerated nutrient water culture solution (Hewitt, 1952) and which had been inoculated 14 days previously. Root tip sections, 0.5 mm in length, were cut using a coleoptile microtome (Nitsch and Nitsch, 1956). The 1st, 2nd, 3rd, 4th, 6th, 8th and 10th sections were obtained from a batch of root tips and each batch was mashed in one drop of 0.1 M glycine-NaOH buffer, pH 8.5, and the extracts were assayed for infectivity. Results of these assays (Table 16) show that infectious material was recovered from the distal 0.5 mm of the root tips. In two of the three experiments (Table 16), there was as much infectious material recovered from the 3rd section as from the 10th section.

iii) Egress of LNYV, PVX and CMV from inoculated leaves of N. glutinosa.

(a) Time of egress from inoculated leaves

To investigate the rate of egress of the SE3 isolate of LNYV (Stubbs and Grogan, 1963b), an isolate of potato virus X (PVX) described by Francki and McLean (1968) and the Q strain of cucumber mosaic virus (QCMV) (Francki et al., 1966), the following type of experiment was carried out.

Table 16

Infectivity of extracts of root tip sections from LNYV-infected N. glutinosa

Days after inoculation	Number of root tips	Infectivity of extracts from root tip sections (a)						
		1st	2nd	3rd	4th	6th	8th	10th
14	10	+(b)	15	16	14	11	11	15
16	20	+(b)	7	22	30	50	48	73
16	20	13	23	43	40	26	48	48

(a) The extracts were assayed for infectivity on 12 whole leaves of N. glutinosa, the results being expressed as local lesions per leaf. Root tip sections were 0.5 mm long.

(b) + indicates that the assay plants, although showing no local lesions, developed systemic symptoms.

N. glutinosa plants were inoculated with virus and at various intervals after inoculation the inoculated leaves were removed from a batch of these plants. Plants were later observed for the development of systemic symptoms. Results of these experiments (Table 17) show that there was much variation in the time taken for LNYV and PVX to egress from the inoculated leaves. In some experiments, LNYV had egressed out of the inoculated leaves of all the plants in the batch by the 4th day (experiment 5), while in other experiments it took longer for LNYV to egress out of the inoculated leaves (Table 17). In some experiments PVX and CMV had egressed out of the inoculated leaves by the 2nd and the 3rd day, which is a slightly faster rate of egress than observed for LNYV (Table 17). These experiments were not carried out under controlled environmental conditions, however, this does not explain all the variability in the results. Possibly another explanation for these results could be due to the different distances which the virus has to move from the site of the local lesion to the vascular system.

iv) Rate of increase of LNYV in inoculated and systemically infected leaves of N. glutinosa.

(a) Rate of increase of virus in inoculated leaves

To determine the rate of increase of virus in inoculated leaves in LNYV-inoculated N. glutinosa, a leaf disk

Table 17

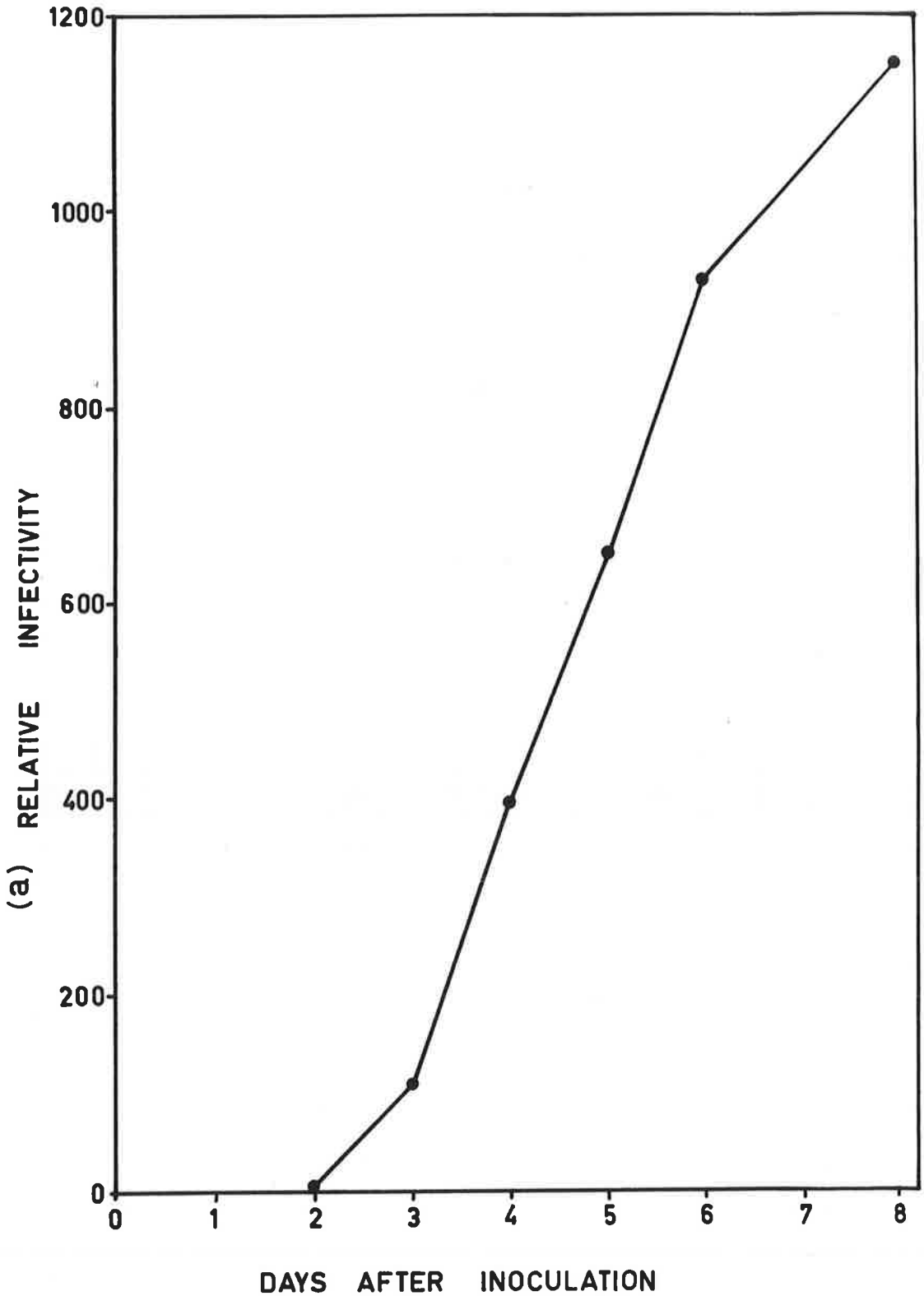
Egress of LNYV, PVX and CMV from the inoculated leaves of N. glutinosa

Expt	Virus	Removal of inoculated leaves at these times after inoculation (a)								
		$1^2/_3$	2	$2^2/_3$	3	$3^2/_3$	4	$4^2/_3$	5	6
1	PVX	$^{14}/_{20}$	$^{12}/_{20}$	X	$^{8}/_{17}$	X	$^{5}/_{15}$	X	$^{21}/_{21}$	X
1	LNYV	X	X	X	$^{1}/_{13}$	X	$^{3}/_{18}$	X	$^{13}/_{18}$	$^{22}/_{22}$
2	PVX	$^{6}/_{11}$	$^{7}/_{11}$	$^{5}/_{11}$	$^{9}/_{12}$	$^{6}/_{10}$	$^{10}/_{11}$	X	X	X
2	LNYV	$^{1}/_{10}$	$^{0}/_{9}$	$^{1}/_{12}$	$^{1}/_{11}$	$^{0}/_{11}$	$^{0}/_{9}$	$^{2}/_{9}$	$^{7}/_{8}$	X
3	CMV	X	X	X	$^{24}/_{24}$	X	$^{24}/_{24}$	X	$^{24}/_{24}$	X
3	LNYV	X	X	X	$^{8}/_{24}$	X	$^{5}/_{24}$	X	$^{16}/_{24}$	X
3	PVX	X	X	X	$^{24}/_{24}$	X	$^{24}/_{24}$	X	$^{24}/_{24}$	X
4	PVX	X	$^{20}/_{24}$	X	$^{23}/_{24}$	X	X	X	X	X
5	LNYV	X	X	X	$^{8}/_{23}$	X	$^{20}/_{20}$	X	$^{20}/_{20}$	X
6	LNYV	X	X	X	$^{1}/_{23}$	X	$^{0}/_{23}$	X	$^{20}/_{24}$	X
7	LNYV	X	X	X	X	X	$^{11}/_{24}$	X	$^{21}/_{24}$	X

(a) Numerator, number of plants showing systemic symptoms; Denominator, number of plants inoculated; X not tested.

Figure 54 Rate of increase of LNYV in inoculated leaves of N. glutinosa.

(a) Relative Infectivity: The assay test which produced 20 to 50 lesions per whole leaf, multiplied by the dilution of the extract.


1200
1000
800
600
400
200
0
(a) RELATIVE INFECTIVITY
0
1
2
3
4
5
6
7
8
DAYS AFTER INOCULATION

was taken from one of the inoculated leaves of each of 24 plants. These disks were mashed in 3.0 ml of 0.01 M phosphate buffer, pH 7.0, and dilutions of the extract made in buffer and assayed for infectivity. This sampling and assay procedure was commenced on the 2nd day after inoculation and repeated at daily intervals until the 8th day. The dilution of the extract which produced a mean number of local lesions per leaf in the range 20 to 50 was chosen, and a relative infectivity for each assay was thus obtained. Results of one such experiment (Fig. 54) show that virus was detected on the 2nd day after inoculation and increased until the 8th day after inoculation.

(b) Effect of light intensity on the rate of increase of virus in systemically infected leaves

To investigate the rate of increase of virus at different light intensities two experiments were carried out. N. glutinosa plants were inoculated with virus and transferred to a growth cabinet (23°); four of the plants were placed at a light intensity of 900 f.c. and another four at 30 f.c. At 9 and 16 days after inoculation, leaf disk samples from the base of the systemically infected leaves (usually leaf S2) of each of the four plants at both light intensities were taken. Each leaf disk was mashed in 1.0 ml of distilled water and the infectivity of the extracts from the leaf disks at the two light intensities compared. Results of these assays (Table 18) show that there

were differences in the amount of infectious material recovered from the leaf disks at the two light intensities.

Table 18

Comparison of infectivity of extracts of leaf disks from systemically infected leaves of N. glutinosa at 30 and 900 f.c.

Days after inoculation	Ratios of infectious material from leaf disks of plants at 30 and 900 f.c. (a)			
	Experiment 1	Experiment 2	Experiment 3	Experiment 4
9 days	$^{0}/_{34}$	$^{0}/_{37}$	$^{0}/_{32}$	$^{0}/_{48}$
16 days	$^{49}/_{47}$	$^{15}/_{16}$	$^{4}/_{10}$	$^{18}/_{29}$

(a) Leaf lesions per half-leaf produced on opposite half-leaves of N. glutinosa by the extract of a leaf disk from infected plant at 300 f.c. (numerator) and by the extract of a leaf disk from infected plant at 900 f.c. (denominator). Each assay was carried out on 12 leaves.

In the second of these experiments, inoculated N. glutinosa plants were transferred to a growth cabinet (19 to 22°) and placed at light intensities of 170 f.c. and 1,600 f.c. The sampling and

assay procedure was as described for the previous experiment except that leaf disks were taken at 10, 11, 12 and 13 days after inoculation. Results of the infectivity assays (Table 19) show that there was a considerable difference in the amounts of infectious material extracted from the leaf disks. In one of the three experiments (Table 19), at 10 days after inoculation the ratio of the mean number of local lesions per leaf produced by the extracts from the two light intensities was $\frac{15}{1}$. At 13 days after inoculation this ratio was $\frac{8}{31}$ (Table 19). These differences in the rates of virus increase at the two light intensities could be due to either differences in the rate of multiplication of virus in the leaves or a difference in the rate of transport of virus to the systemically infected leaf.

6C. Discussion

The results of these studies on the virus-host interactions were shown to be very variable. The variation in symptom expression has been shown in Figures 47, 51 and 52. Variation was also observed in the amount of infectious material recovered from the base and tip of systemically infected leaves (Tables 14 and 15; Fig. 54), and in the rate of egress of virus from the inoculated leaves (Table 17). The light intensity was observed to affect the amount of infectious material recovered from the systemically infected leaves of N. glutinosa (Tables 18 and 19).

Table 19

Comparison of infectivity of extracts from leaf disks from systemically infected leaves of N. glutinosa at 170 and 1,600 f.c.

Days after inoculation	Ratios of infectious material from leaf disks of plants at 170 and 1,600 f.c. (a)		
	Experiment 1	Experiment 2	Experiment 3
10 days	$0.6/0.14$	$25/1$	$15/1$
11 days	$17/15$	$79/9$	$13/18$
12 days	$11/43$	$97/38$	$2/6$
13 days	$3/42$	$31/33$	$8/31$

(a) Local lesions per half-leaf produced on opposite half-leaves of N. glutinosa by the extract of a leaf disk from infected plant at 1,600 f.c. (numerator) and by the extract of a leaf disk from infected plant at 170 f.c. (denominator). Each assay was carried out on 12 leaves.

Some inoculated plants exhibited no symptomless leaves above the inoculated leaves (Fig. 49), while other plants had as many as three symptomless leaves (leaf '0's) above the inoculated leaves (Fig. 51). There were other inoculated plants which exhibited no systemic symptoms, yet there were approximately 70 lesions on each of two inoculated leaves (Fig. 52). In some inoculated plants, where the inoculum concentration was low, local lesions were not observed but systemic symptoms developed. Thus with LNYV, both local lesions and systemic symptoms, local lesions only, or systemic symptoms only may be observed on an inoculated plant.

LNYV was found to be present in varying amounts in the inoculated and systemically infected leaves of a LNYV-infected plant (Table 9). On a whole leaf basis, the systemically infected leaves contained much more virus than the inoculated leaves (Table 9), while no infectious material was extracted from leaf '0'. However, when the leaf '0's from 42 plants were each mashed in 1 ml of distilled water, 16 of the extracts were shown to be infectious after an infectivity assay. It was also shown that in the inoculated leaf, most of the infectious material was recovered from leaf tissue containing a lesion, and very little, if any, was recovered from leaf tissue adjacent to the lesion (Table 12). There was no significant difference in the

amount of infectious material recovered from tissue containing a lesion and tissue from the base of a systemically infected leaf (Table 13). These results suggest that the cells in inoculated and systemically infected leaves may have a similar potential for virus multiplication.

There was considerable variation in the number of infected plants which contained a leaf '0' (Table 10). But when measurements were made of leaf L1 at the time of inoculation it was found that there was a correlation between the presence of a leaf '0' and the length of the top inoculated leaf (L1) (Table 11).

Two suggestions can be made for the lack of symptoms on the first leaf above the inoculated leaf (leaf '0') on some infected plants. Either, at the time of egress of virus from the inoculated leaves (Table 17) the cells of leaf '0' may be resistant to virus multiplication or, at the time of egress of virus from inoculated leaves most transport may be out of the leaf '0', rather than to this leaf and thus the entry of virus into the leaf may be restricted. The latter of these suggestions refers to a restriction of entry of virus into leaf '0', while the former concerns a reduced multiplication of virus in the leaf.

There was a considerable difference in the amount of virus extracted from the tip and base of adjacent systemically infected leaves (Tables 14 and 15). At 7 days after inoculation, there was more virus at the base than at the tip of leaves S2 and S3 (Table 14). Two days later there was no significant increase in the ratio of the amount of infectious material extracted from the base and tip of leaf S2. But, in leaf S3, there was a significant change in this ratio (Tables 14 and 15; Fig. 54). Several suggestions may be made for the differences in the rate of increase of virus in leaves S2 and S3. A suggestion which could explain the concentration of virus in leaves S1 and S2 is that the cells at the tip of the leaf, being physiologically older than the cells at the base (Avery, 1933), are more resistant to virus multiplication. Virus is presumably transported throughout the entire leaf in the vascular system and multiplication of the virus may then occur at varying rates, depending on the position of the cells in the leaf, relative to the tip or base. A suggestion which could explain the distribution of virus in leaves '0', S1, S2 and S3 relates to the relative transport of virus to these leaves. Beemster (1958) studied the translocation of PVX in the potato (Solanum tuberosum L) and observed that virus translocation was dependent on the flow of assimilates in the stem. He observed that translocation in young plants was both upwards and downwards, while in older plants translocation was often found to

be only downwards. Bennett (1960) obtained results with sugar beet yellows, sugar beet mosaic and sugar beet curly top virus-infected plants which indicated a correlation between virus movement and carbohydrate translocation. Movement of all three viruses into the non-inoculated shoots of the beet plant was accelerated by defoliation of these shoots and this acceleration was associated with the transport of carbohydrates. With LNYV, leaf S1 is older than leaf S3, and thus different translocation patterns of both carbohydrates and virus might be expected. Another suggestion which may help to explain the amount of infectious material extracted from the tip and base of leaf S3 on the second assay day (Tables 14 and 15; Fig. 54) involves the possible inhibition of virus multiplication in meristematic cells. Wu et al. (1960), when studying TMV-inoculated cell cultures, found that the meristematic cell types were more resistant to TMV infection and/or subsequent virus multiplication than more mature cell types. They inferred that the synthetic activities involved in TMV multiplication were competitive with those involved in cell division. Thus in LNYV-infected N. glutinosa, leaf S3 being the youngest systemically infected leaf, this suggestion may be relevant. Solberg and Bald (1963) have suggested that the hypothesis of Wu et al. (1960) may help to explain their results where the stem apex of

Nicotiana glauca Grah was invaded to different degrees by different strains of TMV.

It is difficult to provide a satisfactory explanation for the differences in the amount of LNYV extracted from the base and tip of leaves S2 and S3. Other workers have shown that with TMV and turnip yellow mosaic virus the dark green areas of the leaf contained much less virus than light green areas (Fulton, 1951; Solberg and Bald, 1962; Atkinson and Matthews, 1967; Chalcroft and Matthews, 1967). With LNYV, as well as for other viruses, it is difficult to distinguish between translocation of virus to a systemically infected leaf and multiplication of virus in that leaf.

Variation was observed in the rate of egress of LNYV and PVX from the inoculated leaves of N. glutinosa (Table 17). Possibly, such factors as the inoculum concentration, the rate of virus multiplication at the primary infection site, and the distance of these sites from the vascular system may influence the rate of egress. Beemster (1959) investigated the rate of movement of potato leaf roll virus, potato virus X, potato virus Y and turnip mosaic virus out of the inoculated leaves of Physalis floridana Rydb. He repeated the experiment monthly for 16 months and observed that there were differences between experiments for the rate of movement of each virus into the stem

of the plant. Beemster pointed out that these differences were not apparently caused by seasonal influences. Results of this study on the egress of LNYV and PVX are difficult to explain at present.

Light intensity affected the amount of infectious material extracted from the base of systemically infected leaves (Tables 18 and 19). These differences could be due either to a difference in the rate of translocation of virus to these leaves or a difference in the rate of multiplication of virus in these leaves. Wardlaw (1968) has pointed out that both the movement of assimilates and the rate of photosynthesis are reduced under low light intensities. Possibly, with LNYV, both of these factors (reduced photosynthesis and reduced translocation) may affect the rate of increase of virus (Tables 18 and 19).

This study on the interaction of LNYV with one of its host plants, N. glutinosa, has shown that in many respects LNYV resembles other viruses that produce local lesions and systemic infection (Schneider, 1965). Further work is required to resolve the factors affecting the invasion and multiplication of the virus in the non-inoculated leaves of the plant. Such work is required to be carried out under controlled environmental conditions.

7. IMPLICATIONS OF THIS WORK

LNYV is the first bacilliform plant virus to be studied extensively, although several other such viruses have been described. Studies on the structure of potato yellow dwarf virus (PYDV) were first reported by Black *et al.* (1948), but it was not until 1966 that it was shown to be bacilliform and structurally similar to LNYV (MacLeod *et al.*, 1966). LNYV also resembles PYDV in that both viruses are unstable (Stubbs and Grogan, 1963b; Black, 1938) and both multiply in their insect vectors (O'Loughlin and Chambers, 1967; Sinha, 1965). *Gomphrena* virus resembles LNYV in its host range and structure, but no vector transmission has been reported for it (Stubbs and Grogan, 1963b; Harrison and Crowley, 1965; Kitajima and Costa, 1966). *Gomphrena* virus differs from LNYV in that Kitajima and Costa (1966) observed it in the nucleus of infected cells, while LNYV has been observed in the cytoplasm (Chambers *et al.*, 1965). Sowthistle yellow vein virus (SYVV) is structurally similar to LNYV (Richardson and Sylvester, 1968), but is not mechanically transmissible and seems to be associated with the cell nucleus. However, as both viruses are transmitted by *H. lactucae* and both infect sowthistle, it seems possible that they may be related.

Some of the results reported in this thesis may be profitably extended to other bacilliform plant viruses. The

method of purification for LNYV may be applicable to other viruses. An investigation to determine if these viruses are serologically related should be carried out. These viruses may share a similar sensitivity to ultraviolet irradiation.

In respect to LNYV, several suggestions for further work may be made. It may be possible to use other extraction methods to obtain virus from leaf tissue, which are superior to those available at present. Further work is required to determine the number of antigens in the LNY virion and their relationship to the structure of the virus. Polyacrylamide gel electrophoresis may be profitably used in this study. Since the various interactions of most viruses with their host plants are not understood, further studies on the LNYV-host interaction are desirable. Since LNYV has been shown to produce a wide variety of symptoms on N. glutinosa, studies on the physiology and biochemistry of virus-infected plants may be valuable. Most important of all, studies on the nature of the LNYV nucleic acid and its replication should be carried out.

ADDENDUM 1

Purification of Lettuce Necrotic Yellows Virus by Column Chromatography on Calcium Phosphate Gel

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Lettuce necrotic yellows virus (LNYV) was purified from infected *Nicotiana glutinosa* plants. Leaf homogenates were clarified by adsorption of host cell material on charcoal and DEAE-cellulose powder and filtration through Celite. Virus was concentrated by centrifugation and purified by column chromatography on calcium phosphate gel at pH 7.6. Electron microscopic examination of virus preparations purified in this way showed typical LNYV particles and negligible contamination by host cell materials.

INTRODUCTION

Partial purification of lettuce necrotic yellows virus (LNYV) was achieved by Crowley *et al.* (1965) and this enabled Harrison and Crowley (1965) to study some of its properties. Virus purified by this method contains much host-cell material and hence is unsatisfactory for critical physical and chemical studies. This paper describes a method of obtaining highly purified preparations of this virus.

MATERIALS AND METHODS

Virus isolate and infectivity assays. The S.E.3 isolate of LNYV (Stubbs and Grogan, 1963) was used throughout this work. The virus was propagated in *Nicotiana glutinosa* L. and infectivity was assayed by the local lesion method on the same host (Crowley, 1967).

Preparation of Celite filters. Sufficient Celite (Hyflo Super-cel) was mixed with distilled water and poured over two layers of Whatman No. 1 filter paper in a Büchner funnel so as to form a pad 0.5 cm thick. Usually, for the filtration of 100–150 ml of crude plant extracts, filters of 12.5 cm diameter were prepared from 20 g of Celite. For small volumes of partially purified virus preparations filters were made in 1.5 cm Pyrex filter-holders.

Column chromatography. Calcium phosphate gel (Hypatite C, obtained from Clarkson Chemical Co. Inc., Williamsport, Pennsylvania) was used to prepare columns 7 cm in height in glass tubes 1.3 cm in diameter. The columns were equilibrated with the required buffers as described later, and the flow rate adjusted to between 20 and 40 ml per hour.

Optical density measurements. The approximate relative concentration of particulate material in virus preparations was measured in a Shimadzu QR50 spectrophotometer at 260 mμ. Absorption at this wavelength was largely due to light scattering (McLean and Francki, unpublished results).

Density gradient centrifugation. Linear density gradients were prepared in 30 ml Spinco SW 25 tubes using 15% and 45% sucrose solutions in 0.01 *M* phosphate buffer, pH 7.0. One milliliter of virus preparation was layered on the surface of the gradient and centrifuged at 24,000 rpm for 45 minutes in the SW 25 Spinco rotor. Gradients were analyzed and fractionated in an ISCO model D density-gradient fractionator and flow densitometer (Brakke, 1963).

Electron microscopy. Specimens were stained with either phosphotungstic acid (PTA) or uranyl acetate and examined in a Philips 100B electron microscope as described by Francki (1966).

EXPERIMENTAL

Extraction and Clarification

Crowley *et al.* (1965) found that most methods usually employed in the clarification of extracts from virus-infected plants, either abolished or greatly decreased the infectivity of LNYV preparations. We have now tested charcoal (Steere, 1963), calcium phosphate gel (Fulton, 1959), freon 113 (Crowley *et al.*, 1965), bentonite (Dunn and Hitchborn, 1965), and DEAE cellulose (Whitcomb, 1965) alone and in various combinations and have found them all potentially useful for the initial clarification of extracts from LNYV-infected plants. The most successful procedure which we have adopted is described below.

Infected *N. glutinosa* leaves were homogenized in a Waring blender with 1.5 volumes of cooled 0.2 *M* Na_2HPO_4. The extract (pH approx. 7) was strained through cheesecloth and centrifuged at 2000 *g* for 1 minute. Charcoal (0.05 g/ml decolorizing charcoal, May and Baker, Dagenham, England) was then added to the supernatant and the mixture was shaken for 30 seconds and filtered through Celite. DEAE-cellulose (0.01 g/ml Cellex-D, Bio-Rad Chemicals, Richmond, California) was added to the filtrate and the mixture was shaken for 30 seconds and filtered through Celite.

The above procedures were completed within 20 minutes, and the final product was usually a clear yellow solution if the material used was from plants showing advanced "yellows" symptoms. However, if the preparation still contained some green material, much of this could be removed by an additional filtration through Celite.

Concentration and Purification

The virus was concentrated by centrifugation in a Spinco model L centrifuge at 50,000 *g* for 15 minutes; usually the pellets were light yellow and transparent in appearance. After resuspension in distilled water, the solution was centrifuged at 1000 *g* for 5 minutes. Occasionally traces of green material were present which were then removed by filtration of the resuspended virus through Celite. Attempts at further purification were made by precipitation with either $(NH_4)_2SO_4$, methanol (Cox *et al.*, 1947), or acidification to pH 3.5. All these procedures precipitated the virus, but only small amounts of infectious material were resuspended, and hence these methods were not investigated further.

Eventually it was found that the most efficient method of further purifying the virus was by column chromatography on Hypatite C. When virus was applied to a column equilibrated with 0.01 *M* phosphate buffer, pH 6.6, it was adsorbed. On elution

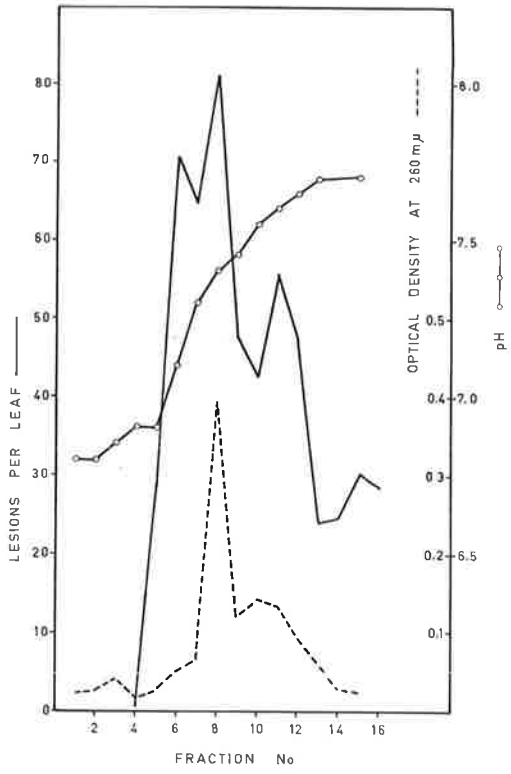

FIG. 1. Elution of LNYV from a Hypatite C column. Virus preparations were applied to a column equilibrated with 0.01 *M* phosphate buffer pH 6.6 and eluted with 0.01 *M* phosphate buffer pH 8.0 and 5 ml fractions were collected. Infectivity of each fraction diluted 1:3, was assayed on 9 *N. glutinosa* leaves.

of the column with 0.01 *M* phosphate buffer, pH 8.0, the virus was released when the pH of the eluate was between 7.0 and 7.6 (Fig. 1). Using buffers of low molarity ensured that most host plant materials remained adsorbed to the column.

For the routine purification of LNYV we have found that it is convenient to equilibrate the column to a pH at which the virus is not adsorbed and hence is recovered in the initial volume of the eluate. Results of experiments carried out with columns equilibrated at selected pH's are summarized in Table 1. These data indicate that at pH 7.3–7.8 the maximum amount of material remained adsorbed to the column and a large proportion of the infectivity was recovered. We have found that columns equilibrated and eluted with 0.01 *M* phosphate buffer pH 7.6 are suitable for the preparation of LNYV

TABLE 1

RECOVERY OF INFECTIOUS LNYV FROM HYPATITE C COLUMNS

Expt. No.	pH of each equilibrated column	Per cent recovery from column[a]	
		OD_{260}[c]	Infectivity[b]
1	6.6	2	20
	7.3	4	55
	8.1	26	100
2	7.4	10	73
	7.6	17	106
	7.8	19	100
3	7.4	4	48
	7.6	8	81
	7.8	8	100
4	7.6	14	53
5	7.6	13	52

[a] Recovery was determined by analysis of the initial 15 ml of eluate and the material applied to the column after appropriate dilution.

[b] Infectivity assays were carried out on 18–54 half-leaves of *N. glutinosa*. Number of lesions produced by the eluate are expressed as a percentage of the number of lesions produced by an aliquot of the virus material which was applied to the column.

[c] Optical density at 260 mμ.

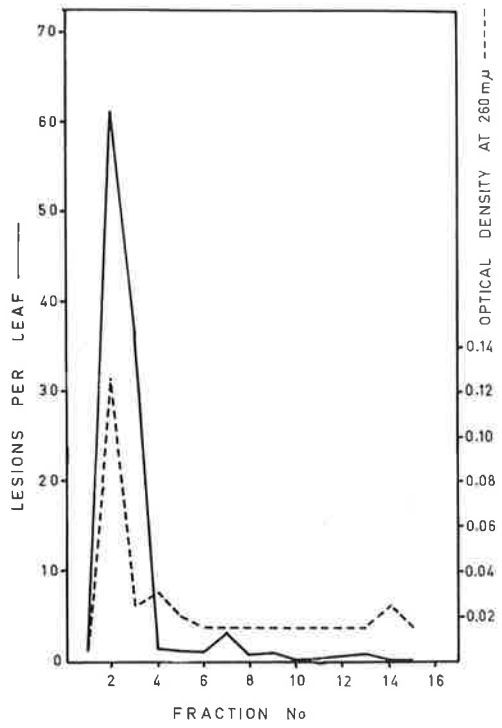

FIG. 2. Recovery of LNYV from a Hypatite C column equilibrated and eluted at pH 7.6 with 0.01 *M* phosphate buffer. Each 5-ml fraction was diluted 1:9 and assayed on 9 *N. glutinosa* leaves.

(Fig. 2). In routinely purifying LNYV we use such columns to which we apply 2.5 ml of virus suspension and recover the initial 15 ml of eluant after discarding the void volume of the column. If required the virus can be concentrated by centrifugation at 50,000 *g* for 15 minutes.

The method described above has also been used successfully to purify LNYV from infected lettuce plants collected in the field.

Efficiency of the Purification Procedure

Extraction of virus from leaf material. The efficiency of release of free virus into the extracting medium during homogenization was tested in the following way. A leaf extract, from infected plants, was fractionated as depicted in Fig. 3. Results of this experiment (Table 2) indicate that in the initial slow-speed centrifugation of a leaf extract a considerable amount of extractable virus was lost in the pellet.

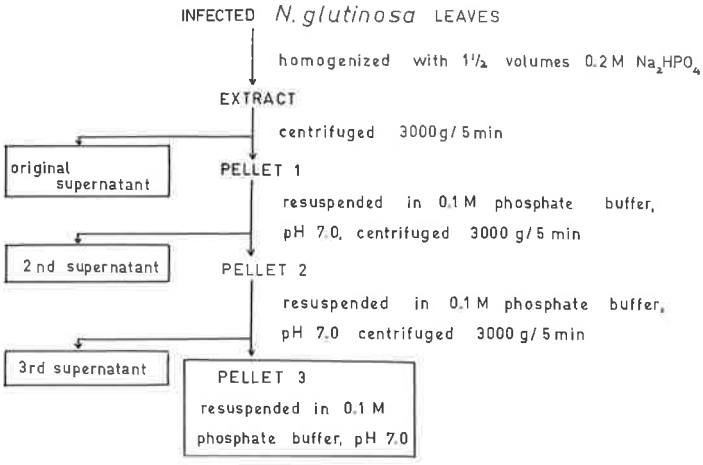

FIG. 3. Fractionation procedure of extract from LNYV-infected *N. glutinosa* leaves by slow speed centrifugation (see Table 2 for results).

TABLE 2

RECOVERY OF LNYV INFECTIOUS MATERIAL FROM FRACTIONS AFTER SLOW SPEED CENTRIFUGATION

Extract assayed	Infectivity relative to original supernatant
2nd supernatant	29/77[a]
3rd supernatant	21/96
3rd pellet (resuspended)	18/80

[a] Local lesions produced on opposite half-leaves of *N. glutinosa* by extract (numerator) and original supernatant (denominator). Each assay was carried out on 18 leaves.

Clarification and concentration. During the clarification procedure the loss of infectious material did not exceed 25%, as shown by comparing the infectivity of the leaf extract with the filtrates obtained after the charcoal and DEAE-cellulose treatments. Preparations, clarified by charcoal and DEAE-cellulose absorption followed by Celite filtration and concentrated by centrifugation, were examined by electron microscopy and zonal density-gradient centrifugation. In addition to typical LNYV particles (Harrison and Crowley, 1965), electron micrographs of preparations from diseased plants showed much host cell material similar to that observed in preparations from healthy plants (Fig. 4). Membranous structures, similar to those reported by Harrison and Crowley (1965) were observed as well as numerous smaller particles, the majority of which were thought to be fraction I protein (Miller *et al.*, 1966). Analysis with the ISCO apparatus of density-gradient columns containing preparations from healthy plants showed that they contained material throughout the tube. Analysis of preparations from diseased plants showed that in addition to the material found in healthy preparations, two distinct peaks of optical density were present with which infectivity was associated (Fig. 5).

Column chromatography. Data summarized in Table 1 show that when eluted at pH 7.6, about 10% of material added to the column, as measured by optical density at 260 mμ, was recovered. However, not less than 50% of the infectious material added to the column was recovered and in many experiments recovery was better than 80%. This indicates an efficient purification step. Electron micrographs of LNYV preparations subjected to the entire purification procedure show numerous virus particles (Fig. 6). Particles lacking the characteristic bacilliform shape of LNYV were also observed. These were almost always found to be virus fragments or disrupted virus particles by the presence of the characteristic virus membrane structure (Francki, Wolanski, and Chambers, in preparation).

The infectivity of purified LNYV prepara-

tions declined relatively slowly when these were stored at 0°. Virus recovered from Hypatite C columns and producing about 50 lesions per leaf when inoculated immediately after purification, produced only a very few lesions after storage for a week at 0°. In one experiment virus purified from 100 g of leaf material and concentrated to 0.5 ml after column chromatography was stored at 4°. After 8 days the preparation diluted 1:100 produced 48 lesions per leaf on *N. glutinosa*.

DISCUSSION

Purification of LNYV presents a difficult problem on account of its instability in leaf extracts (Stubbs and Grogan, 1963; Crowley, 1967), its large size (Harrison and Crowley, 1965) and its extreme sensitivity to organic

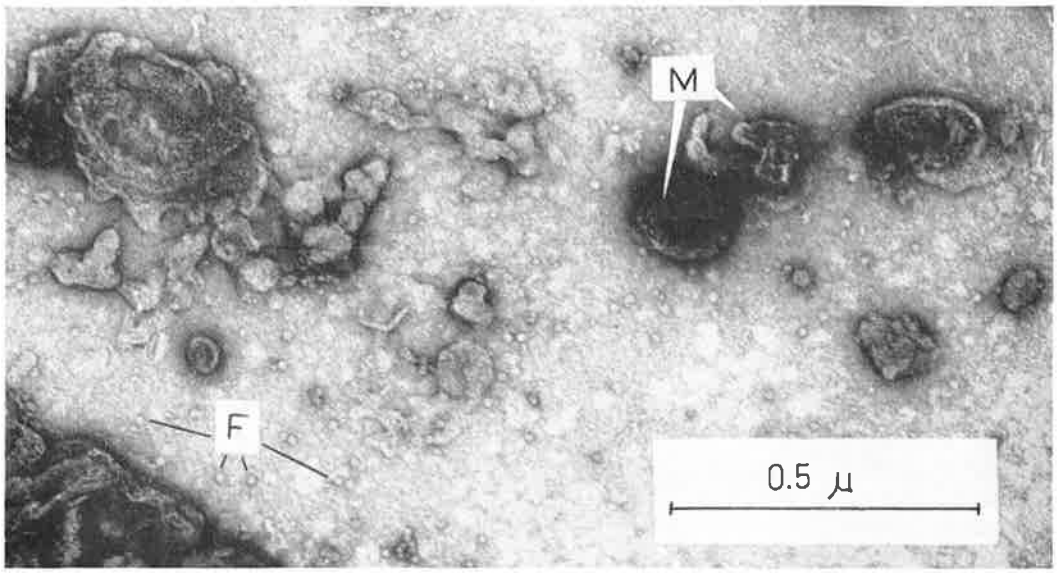

FIG. 4. Electron micrograph of material (stained with uranyl acetate) obtained from healthy *N. glutinosa* plants after clarification of the leaf homogenate and sedimentation at 50,000 *g* for 15 minutes. Numerous membranous structures (*M*) and small particles thought to be fraction I protein (*F*) are observed.

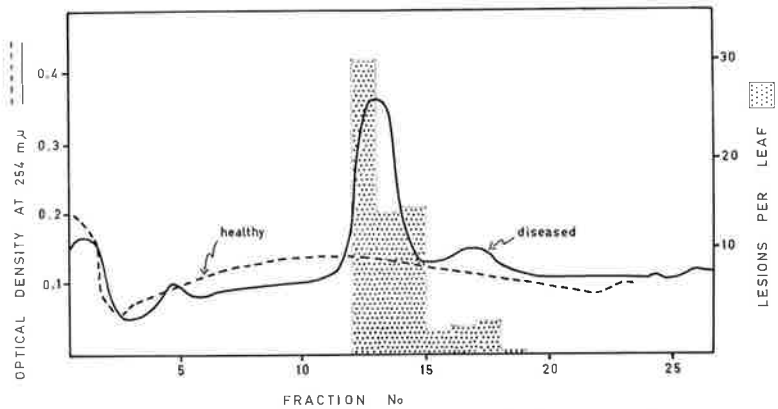

FIG. 5. Fractionation of sucrose density gradients on a ISCO density gradient fractionator. Samples (1.0 ml) of healthy and diseased preparations were layered on to 15–45% linear gradients and centrifuged for 45 minutes at 24,000 rpm in the SW 25 rotor of a Spinco centrifuge. Infectivity of each 1 ml fraction was determined on 9 whole *N. glutinosa* leaves.

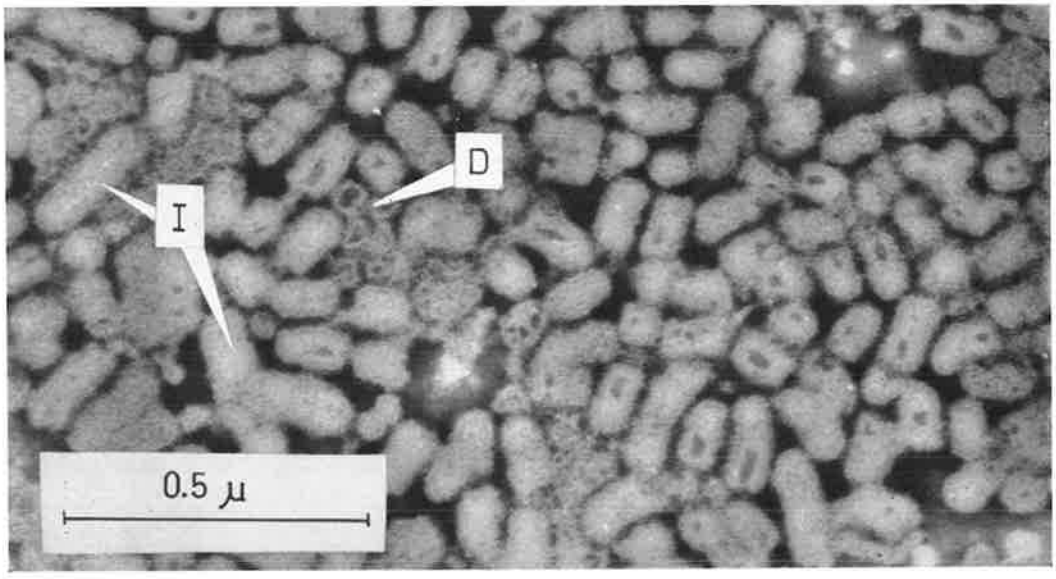

FIG. 6. Electron micrograph of a preparation of LNYV purified by the method described and concentrated by centrifugation and stained in PTA. Numerous intact (*I*) and some partially disrupted (*D*) LNYV particles are observed.

solvents commonly used in the clarification of plant extracts (Crowley *et al.*, 1965). The method of preparation described here results in highly purified virus, but only a small amount of virus is obtained from a relatively large amount of infected plant material. The low yield of virus appears to be due to the relatively poor extraction procedure rather than to excessive losses during the purification steps. Chambers *et al.* (1965) showed that LNYV particles in leaf cells are located in the cytoplasm, arranged in bundles enclosed in membrane-bound sacs. For the efficient release of virus from cells, it is thus necessary to rupture the membranes enclosing the bundles of virus particles, as well as the cell walls. However, if the extraction methods are too severe then the individual virus particles may be damaged. Crowley (private communication) has actually shown that mild sonication reduces the infectivity of LNYV in leaf extracts.

Our clarification of leaf homogenates is rapid and has the advantage that slow-speed centrifugation, which can cause high losses of virus (Crowley *et al.*, 1965), is not used. Both charcoal and DEAE-cellulose are effective in adsorbing particulate green material and soluble brown pigments while the Celite filtration appears to be efficient in removing green material.

It has been demonstrated that density gradient centrifugation of concentrated LNYV preparations is relatively inefficient in separating virus from host materials as these spread along the entire depth of the columns (Fig. 5), including zones from which virus is recovered. However, passage of virus through a column of Hypatite C equilibrated and eluted at pH 7.6 resulted in a satisfactory method of removing most of the contaminating material.

In addition to the preparation of highly purified virus suspensions, the method described in this paper has the advantage that it is relatively rapid to perform. It usually takes us less than 3 hours to purify LNYV from 300–400 g of plant material.

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ADDENDUM 2

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ADDENDUM 3

Inactivation of Lettuce Necrotic Yellows Virus by Ultraviolet Irradiation

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Inactivation of Lettuce Necrotic Yellows Virus by Ultraviolet Irradiation

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Lettuce necrotic yellows virus (LNYV) is approximately eight times more sensitive to ultraviolet irradiation than the U_2-strain of tobacco mosaic virus (TMV). The *in vivo* and *in vitro* dose-response curves of LNYV to ultraviolet irradiation are similar. When virus-inoculated half-leaves are irradiated at increasing intervals of time after inoculation with virus, the fraction of infection centers surviving increases with time, as found by other workers for TMV and tobacco necrosis virus.

INTRODUCTION

Ultraviolet irradiation has been used to study the process of growth of intracellular bacteriophage (Luria and Latarjet, 1947) and to study the infection by tobacco mosaic virus (TMV) (Siegel and Wildman, 1956; Dijkstra, 1964), tobacco necrosis virus (TNV) (Bawden and Harrison, 1955), and red clover mottle virus (Bawden and Sinha, 1961).

The sequence of events in virus infection has been divided into four separate phases, based on changes in the sensitivity of the infection centers to inactivation by ultraviolet light (Siegel and Wildman, 1956). During the first three phases the survival curve was exponential, whereas in phase four the secondary rise in resistance was expressed by the change in the survival curve from exponential to multitarget. They showed that, in phase one, the sensitivity of the infection centers to irradiation was constant; however, Dijkstra (1964) observed a decrease in sensitivity during this period.

Irradiation of the leaf before inoculation was shown by Bawden and Kleczkowski (1960) to markedly affect its capacity to support virus multiplication. Hence they inferred that conclusions drawn from previous experiments (Bawden and Harrison, 1955; Siegel and Wildman, 1956), in which leaves were irradiated at different intervals after inoculation, were possbily invalid. However, Goodchild (1961) showed that 4 min irradiation of plants prior to inoculation did not reduce their capacity to support virus multiplication. In his experiments TMV-inoculated *Nicotiana glutinosa* L. leaves were irradiated for periods not exceeding 90 sec.

Lettuce necrotic yellows virus (LNYV) was found to be very sensitive to ultraviolet irradiation and later it was found to be eight times more sensitive to irradiation than the U_2-strain of TMV (McLean, Owusu, and Crowley, unpublished results). This and the unusual properties of LNYV (Harrison and Crowley, 1965) prompted us to investigate further the effects of ultraviolet light on the infection process of the virus. Results of these studies are the subject of this paper.

MATERIALS AND METHODS

LNYV (S.E.3 isolate; Stubbs and Grogan, 1963) and the U_2 strain of TMV supplied by Dr. S. G. Wildman (University of California, Los Angeles) were used in these studies. Inoculum for the *in vitro* virus inactivation studies was clarified by the method of McLean and Francki (1967). Inoculum for the *in vivo* studies was prepared by homogenizing 10 g of infected *N. glutinosa* leaves

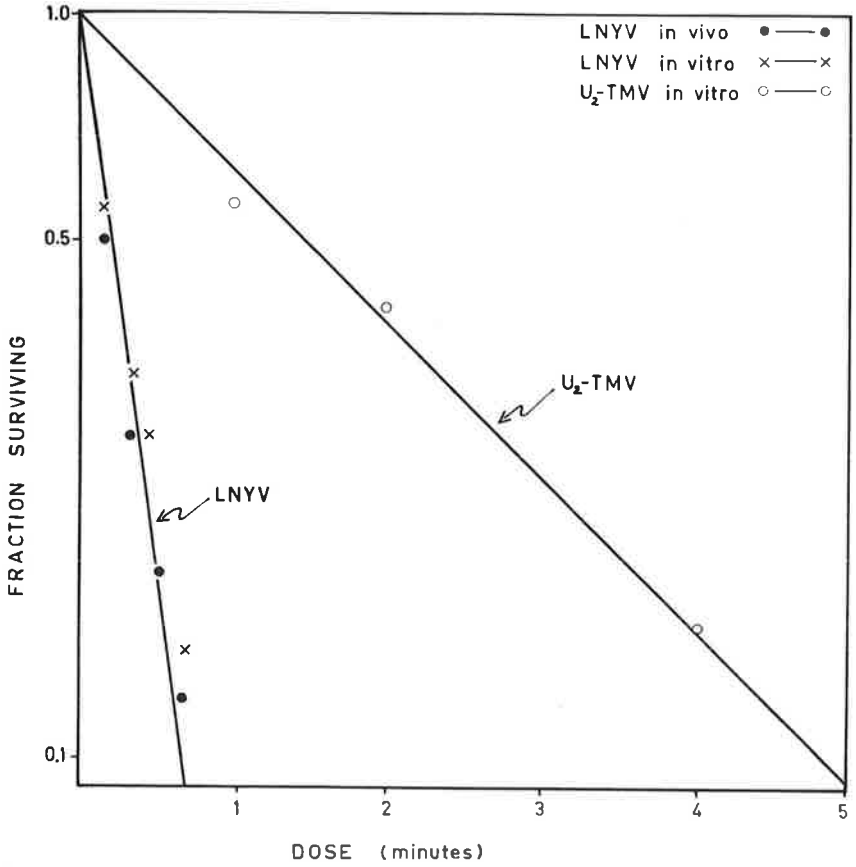

FIG. 1. Comparison of the survival curves obtained when LNYV and TMV were irradiated *in vitro*, and when LNYV-inoculated plants were irradiated within 30 min of inoculation.

with 20 ml of glycine buffer (pH 9.3) and 20 ml of Freon 113 (Crowley and Harrison, 1965), and breaking the emulsion by centrifugation for 4 min at 1000 *g*. Aliquots of the top aqueous layer were diluted in glycine buffer (pH 8.6), to produce 30–60 lesions per half-leaf. TMV was purified by precipitation with ammonium sulphate, followed by two cycles of high and low speed centrifugation. Celite was added to all inocula at the rate of 0.5 g per 10 ml.

The infectivity tests for the *in vitro* studies were carried out by half-leaf comparisons on young leaves of *N. glutinosa* plants. For the *in vivo* studies, plants were pruned to the two most susceptible leaves (Crowley, 1967) prior to inoculation. One half of every leaf was covered with aluminum foil and the pot containing the plant was placed in a larger pot, so as to support each leaf horizontally and to maintain each leaf at the same distance from the ultraviolet light source, a 240 V, 50 W, Oliphant "Sterilamp." According to the manufacturer's specifications this lamp emits 700 ergs per square centimeter at 1 metre with 90% of the irradiation concentrated at 2537 Å. A voltage stabilizer was used in all experiments to avoid variation in output due to fluctuations in the mains supply. In the *in vivo* and *in vitro* experiments, the inoculated plants or the petri dish of clarified plant extract were placed 60 cm from the light source. In the *in vitro* experiments the clarified extract was stirred with a magnetic stirrer while being irradiated.

EXPERIMENTAL

Ultraviolet Dose-Response Curve for LNYV and U_2-TMV

The survival curves for purified U_2-TMV, clarified LNYV, irradiated *in vitro*, and for LNYV irradiated within 30 min of inocula-

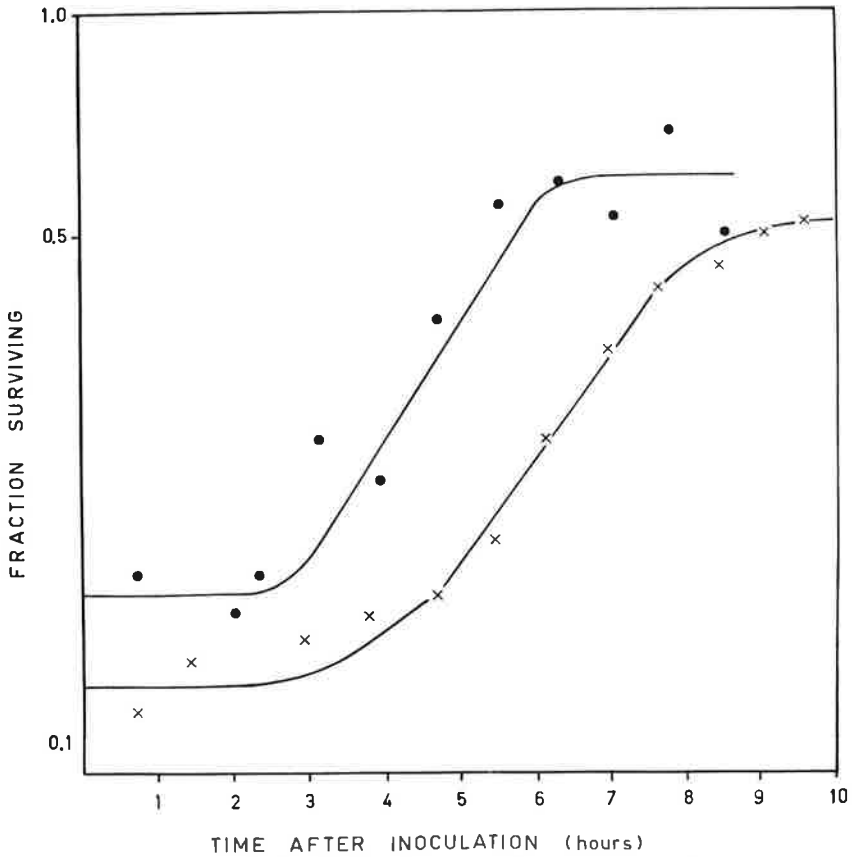

FIG. 2. Survival curves obtained when LNYV-inoculated leaves were irradiated with a constant UV dose (30 sec) at varying times after inoculation. Each curve represents a separate experiment.

tion to *N. glutinosa* are shown in Fig. 1. The *in vitro* and *in vivo* curves for LNYV coincide, showing that the observed effect is on the virus and not on the host. LNYV appears to be approximately eight times more sensitive to irradiation than U_2-TMV.

Effect of Irradiation on N. glutinosa prior to Inoculation with LNYV

Half-leaves of *N. glutinosa* were irradiated for varying times ranging from one to ten minutes and were then immediately inoculated with virus. In two experiments, 5 min of irradiation caused no significant reduction in lesion numbers on the irradiated half-leaves when compared with the nonirradiated. However, 10 min irradiation affected the plant's capacity to support virus multiplication and the lesion numbers on irradiated half-leaves were 66% and 40% of those produced on the nonirradiated.

Constant-Dose Irradiation of Inoculated Leaves at Varying Time Intervals after Inoculation

Leaves of *N. glutinosa* leaves were inoculated with LNYV in a constant temperature room at 22°. Half-leaves were irradiated with a constant dose (30 sec) of ultraviolet light at different times after inoculation. This is one-tenth to one-twentieth of the dose required to reduce the capacity of the leaf to support virus multiplication. Three phases are evident in the survival curves for two experiments (Fig. 2) selected from several experiments. Resistance of the infection centers to inactivation does not change in the first phase which lasts about 3 hours; it increases sharply in the second phase which lasts 2 hours, and again shows no change in the third phase. The survival curve obtained for LNYV (Fig. 2) resembles the curves obtained by Siegel and Wildman (1956) for

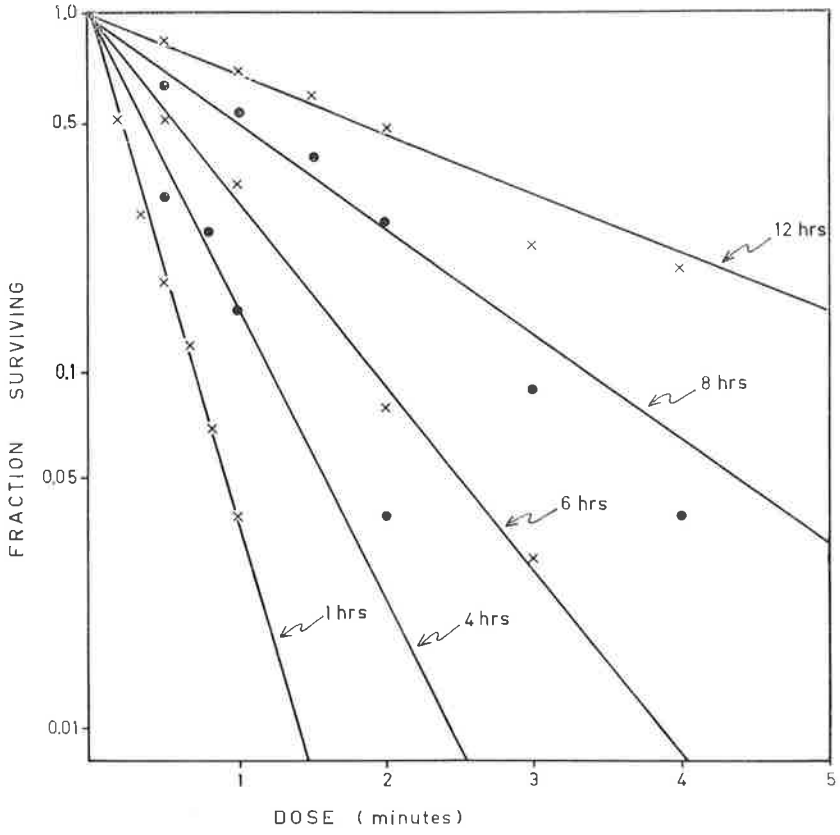

FIG. 3. Survival curves obtained when LNYV-inoculated plants were irradiated with increasing UV dose at different time intervals after inoculation.

for U_2-TMV and by Bawden and Harrison (1955) for TNV.

Changes in the Survival Curve, When Plants Are Irradiated at Different Time Intervals after Inoculation

In this series of experiments *N. glutinosa* leaves were inoculated with LNYV in a constant temperature room at 24–26°. Figure 3 shows the survival curves of infection centers after irradiation at increasing times after inoculation. The slope of the survival curve decreases as the time interval increases after inoculation. This experiment was repeated several times so that curves for irradiation at 1, 4, 6, 8, and 12 hours after inoculation, were determined at least 3 times each. Inspection of the data gave no evidence for the existence of multitarget survival curves even 12 hours after inoculation, which is 4–6 hours after the commencement of phase three (the plateau in Fig. 2).

DISCUSSION

In studying the changes in resistance to ultraviolet light-inactivation of infection centers following inoculation with LNYV, less than one-tenth of the dose found necessary to reduce the capacity of the leaves to support virus multiplication was applied. Thus the criticism leveled by Bawden and Kleczkowski (1960) at similar work with TNV (Bawden and Harrison, 1955) and TMV (Siegel and Wildman, 1956) cannot apply to LNYV. However, the changes in the fraction of LNYV infection centers surviving, increased with time (Fig. 2) in a manner similar to that found for TNV and TMV.

It is interesting to note that the survival curves for LNYV (Fig. 2) are quite similar to those obtained for TMV and TNV which are simpler viruses, consisting of RNA and protein. LNYV, however, appears to be more complex in that it contains a membrane of

lipid material, in addition to RNA and protein (Wolanski, *et al.*, 1967).

Results obtained from irradiation of virus-inoculated leaves reflect changes produced by infection but do not show their nature (Bawden, 1964) or explain them (Siegel, 1966). One of the conflicts arising out of such work is whether one or more virus particles enter an infectible site. Rappaport and Wu (1962) and Wu (1964), working with two strains of TMV, indicated that more than one particle enters an infectible site, and therefore multitarget survival curves are to be expected. The results of Siegel and Wildman (1956) were interpreted as showing that a transition from exponential to multitarget survival curves occurred, but Siegel (1966) has subsequently stated that such a transition has not been demonstrated. With LNYV we did not observe multitarget survival curves. Thus the results obtained from ultraviolet inactivation of infectious centers lack a reasonable interpretation at the present time.

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