Purification of Oat Necrotic Mottle Virus with Silver Nitrate as Clarifying Agent*

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SUMMARY

A satisfactory purification procedure for oat necrotic mottle virus involved clarification of the plant juice by silver nitrate, followed by two cycles of differential centrifugation, and then rate-zonal density gradient centrifugation. The concentrate was finally passed through a column of agarose gel. Antiserum to clarified sap from virus-free oats did not react with the final preparation. A medium consisting of 0.1 M-sodium citrate buffer, pH 6.2, containing 0.5 M-urea, was suitable for the concentrated virus. The virus particles were filamentous with a diameter of 11 nm. and a normal length of 720 nm.

INTRODUCTION

The mechanically transmissible oat necrotic mottle virus (ONMV) was first recorded in Manitoba in 1965 (Gill & Westdal, 1966a). Natural local reservoirs of the virus appear to be perennial species of bluegrass, Canada bluegrass, Poa compressa L. and Kentucky bluegrass, Poa pratensis L. (Gill & Westdal, 1966b). Studies were made by Gill (1967) on the symptomatology, host range and stability of the virus. Preliminary electron microscopy (unpublished) indicated that the virus particle was probably filamentous.

This paper reports the successful purification of ONMV by clarification of infectious sap with silver nitrate. The morphology of ONMV is described.

METHODS

Virus. The virus isolate used was from an infected oat plant found in a field in 1965. This was maintained by successive transfers to oats, Avena sativa L. var. Clintland-64, or A. byzantina K. Koch var. Coast Black. The virus was propagated for use in purification studies in Clintland oats grown in wooden flats of soil either in growth cabinets at 18°C ± 2°C, or in a greenhouse. Plants were inoculated at the three-leaf stage by rubbing infectious sap on the leaves. About 14 days later, either whole plants or upper leaves with symptoms were harvested and stored in plastic bags at -12°C.

Virus preparations were assayed for infectivity on Clintland oats at the two- to three-leaf stage. Four or five dilutions in water were prepared for each sample. Assay plants were dusted with 600-mesh corundum powder and each dilution was rubbed on the leaves of 20 plants with a cotton swab. The inoculated plants were kept in a greenhouse for 4 weeks before final counts of infected plants were taken. The ID 50 of the preparation was estimated according to the procedure of Reed & Muench (1938).

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Purification. Virus was purified in the following way. Sap was obtained from frozen tissue using a mechanical juice extractor. The sap was centrifuged for 15 min. at 8,200 g and drops of 0.01 M AgNO₃ were added to the stirred sap until the first sign of flocculation. The mixture was then left at room temperature for 30 min. and then centrifuged for 15 min. at 8,200 g, and the precipitate discarded. Sodium citrate (1 M) was added (1% v/v) to the supernatant fluid, and it was centrifuged for 2½ hr at 70,800 g. The pelleted virus was suspended in a small volume of citrate–urea buffer (0.1 M-sodium citrate, 0.5 M-urea, pH 6.2). The suspension was centrifuged for 15 min. at 8,200 g to remove insoluble matter, and the virus sedimented again by centrifuging at 92,600 g for 2½ hr. The pellet was suspended in citrate–urea buffer and clarified by low-speed centrifugation to give a preparation with a volume about 1/50 that of the sap. This preparation was then layered on top of sucrose density gradients (3 ml. on each gradient of 4, 7, 7 and 7 ml., respectively, of 10, 20, 30 and 40% (w/v) sucrose in citrate–urea buffer solution (Brakke, 1967a)), and centrifuged for 2½ hr at 53,800 g at 2 °. The contents of each centrifuge tube were fractionated using a Model No. 180 ISCO density gradient fractionator and the u.v.-extinction profile recorded. The fractions containing the virus were combined, diluted with buffer, and centrifuged for 2½ hr at 92,600 g. The virus was suspended in a small volume of citrate–urea buffer and passed through a 63 cm. x 1.3 cm. column of agarose gel (Bio-Gel A-15M-100 mesh; Bio-Rad Laboratories, California) equilibrated with the same buffer, and at a flow rate of about 12 ml. per hour. The virus was concentrated from the relevant fractions by centrifuging for 2½ hr at 92,600 g and the pellet was suspended in a volume of citrate–urea buffer equivalent to about 1/400 the volume of initial sap.

Several clarification methods were tried, and in each sap was expressed in the juicer and cell debris removed by centrifugation for 15 min. at 8,200 g before clarification. Treated preparations were then centrifuged and the supernatant fluids assayed for infectivity. For treatment with organic solvents, chloroform (Schneider, 1953) and butanol–chloroform (Steere, 1956) were used. Both methods were also modified by using equal volumes of solvent and extract. Extracts were also treated by stirring in 1-butanol slowly to final concentrations of 4% or 8% (v/v) or were shaken for 5 min. with an equal volume of trichloroethylene or 1,2-dichloroethane. Treatment at pH values 5.8, 5.3 and 5.0 was made by adding 1 N-HCl slowly to the extract at 4 °. After 5 min., 1 N-NaOH was added to pH 7.0. Extracts were also treated in a water bath at 40 ° for ½ or 1 hr, or with activated charcoal (0.1 g./ml.) removed by filtration after 5 min. The effect of AgNO₃ was assessed by titrating 20 ml. aliquots of extract with various amounts of 0.1% aqueous AgNO₃. Volumes were equalized with water after standing for 30 min. at room temperature.

Ultraviolet extinction spectra of virus preparations were determined in a Beckman Model DK-2A recording spectrophotometer in quartz cells with a 1 cm. optical path length.

Serology. Antiserum to healthy Clintland oats was prepared from sap of frozen whole plants. The sap was centrifuged for 15 min. at 8,200 g and the supernatant liquid was emulsified with an equal volume of Freund's incomplete adjuvant. Two ml. of the emulsion was injected into the muscle of each hind leg of a rabbit on three occasions at weekly intervals, and blood was drawn from the ear 20 days after the final injection.

Healthy oat extract, used as a control antigen for the antiserum, was made from sap that was expressed, clarified and concentrated 20-fold by one cycle of differential centrifugation as for infectious sap. Titres of the antiserum and dilution end points of antigens were determined by the microprecipitin test (Ball, 1961).

Electron microscopy. Infected and healthy oat preparations were examined in an A.E.I. Model 6 B electron microscope. A small drop of the sample was placed on a carbon-coated
Purification of ONMV

grid for 3 min. The liquid was then removed and a drop of 2% potassium phosphotungstate, pH 6.3, applied for 30 sec. Grids with samples from sucrose density-gradient solutions were rinsed with distilled water after removing the excess sample solution.

To determine the size of virus particles, sap was expressed from freshly harvested infected oat leaves in a pestle and mortar. After centrifugation for 15 min. at 8,200 g and clarification with AgNO₃, specimens were prepared for electron microscopy as above. A carbon replica of a diffraction grating (54,864 lines/inch) was used as a standard of measurement. Lengths of virus particles were measured from ×4.5 enlargements with the aid of an opisometer. The common strain of tobacco mosaic virus, used as an auxiliary standard of measurement, was propagated in tobacco. Sap expressed from the leaves was clarified with AgNO₃ and the virus was concentrated and partially purified by three cycles of differential centrifugation.

RESULTS

Propagation of virus

When oats were grown in a growth cabinet and inoculated at the three-leaf stage, tests showed that infectivity of sap from whole plants was highest 14 days after inoculation. Sap from younger leaves with symptoms was more infectious than sap from whole plants. High temperatures in the greenhouse during summer caused early necrosis of infected leaves, and subsequent unsuccessful purification of virus.

Extraction of virus

In these trials, sap extracted by homogenizing tissue (1 g./ml.) in various buffer solutions was filtered through glass wool and centrifuged for 15 min. at 8,200 g before testing for infectivity. Infectivity was usually as high in buffer solutions as in water. This was true for 0.05 M and 0.005 M-sodium ortho-borate at pH 7.5; 0.034 M and 0.05 M-potassium maleate at pH 6.5; and 0.1 M-sodium potassium phosphate at pH 6.5, 7.0, 7.5 and 8.0. There was a progressive decrease in infectivity for 0.5 M-sodium borate and 0.5 M-tris HCl with increasing pH of 7.5, 8.1 and 8.7. One trial with 0.1 M-sodium citrate at pH 5.5, 6.0 and 6.5 gave lower infectivities than with water.

The infectivity of homogenate in water was the same as that of sap expressed in a juicer and diluted with water to the same volume. Since the amount of virus in tissues was low, sap was routinely extracted with the juicer to avoid dilution. The macerated tissue was squeezed in a piece of nylon mesh and the extra sap added to that from the juicer.

Clarification of extracts

Organic solvents, adjustment of pH, heating, or the use of charcoal usually decreased the infectivity of the extracts, or did not remove the green pigments (Table 1). Chloroform was the most promising of these agents, though two other trials with this solvent in which only two dilutions were assayed resulted in slight decreases in infectivity. Clear preparations obtained with AgNO₃ were usually slightly more infectious than untreated sap (Table 2).

In routine work, AgNO₃ solution was added to extracts until a granular flocculation was first visible with a lens of ×5 magnification. Then 10% more AgNO₃ solution was usually required to ensure that a clear supernatant fluid resulted after low-speed centrifugation. In later work 0.01 M-AgNO₃ was used to minimize dilution of the preparation during treatment. In 10 such trials the average ID₅₀ of untreated and treated preparations was, respectively, 1/129 and 1/165, indicating an increase of 28% in infectivity. The average increase in infectivity for the solutions clarified in Table 2 was 25%. This method was equally suc-
cessful with an extract prepared by homogenizing infected tissue in 0.5M-potassium maleate at pH 6.5.

In one experiment, sap from healthy oats was divided into two equal parts; one part was clarified with AgNO₃, the other by shaking with an equal volume of chloroform for 4 min. After two cycles of high- and low-speed centrifugation the final pellets were suspended

Table 1. Effect of different clarification methods on the infectivity of extracts* from oats infected with oat necrotic mottle virus

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Untreated</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform (Schneider, 1953)</td>
<td>1/57</td>
<td>1/22</td>
</tr>
<tr>
<td>Chloroform† (1:1)§</td>
<td>1/87</td>
<td>1/74</td>
</tr>
<tr>
<td>Chloroform‡ (1:1)§</td>
<td>1/12</td>
<td>1/15</td>
</tr>
<tr>
<td>1-butanol/chloroform (Steere, 1956)</td>
<td>1/9</td>
<td>Not infective undiluted</td>
</tr>
<tr>
<td>1-butanol/chloroform (1:1)§</td>
<td>1/57</td>
<td>1/67</td>
</tr>
<tr>
<td>1-butanol (4 % v/v)</td>
<td>1/43</td>
<td>Not infective at pH 5.8</td>
</tr>
<tr>
<td>1-butanol (8 % v/v)</td>
<td>1/43</td>
<td>Not infective undiluted</td>
</tr>
<tr>
<td>1-butanol† (4 % v/v)</td>
<td>1/25</td>
<td>1/58</td>
</tr>
<tr>
<td>1-butanol (8 % v/v)</td>
<td>1/25</td>
<td>Not infective undiluted</td>
</tr>
<tr>
<td>Trichloroethylene</td>
<td>1/94</td>
<td>1/128**</td>
</tr>
<tr>
<td>Trichloroethylene</td>
<td>1/51</td>
<td>1/48**</td>
</tr>
<tr>
<td>1,2-dichloroethane</td>
<td>1/66</td>
<td>1/65</td>
</tr>
<tr>
<td>1,2-dichloroethane</td>
<td>1/69</td>
<td>1/42</td>
</tr>
<tr>
<td>5 min.† at pH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.8</td>
<td>1/63</td>
<td>1/58**</td>
</tr>
<tr>
<td>5.3</td>
<td>1/63</td>
<td>1/49**</td>
</tr>
<tr>
<td>5.0</td>
<td>1/63</td>
<td>1/3</td>
</tr>
<tr>
<td>Heated at 40°C for</td>
<td></td>
<td></td>
</tr>
<tr>
<td>½ hr</td>
<td>1/200</td>
<td>1/333**</td>
</tr>
<tr>
<td>1 hr</td>
<td>1/200</td>
<td>1/116**</td>
</tr>
<tr>
<td>½ hr</td>
<td>1/118</td>
<td>1/190**</td>
</tr>
<tr>
<td>1 hr</td>
<td>1/118</td>
<td>1/82</td>
</tr>
<tr>
<td>½ hr</td>
<td>1/36</td>
<td>1/19</td>
</tr>
<tr>
<td>1 hr</td>
<td>1/36</td>
<td>1/13</td>
</tr>
<tr>
<td>1 hr</td>
<td>1/118</td>
<td>1/33</td>
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<tr>
<td>Heated at 40°C† for</td>
<td></td>
<td></td>
</tr>
<tr>
<td>½ hr</td>
<td>1/76</td>
<td>1/35**</td>
</tr>
<tr>
<td>1 hr</td>
<td>1/76</td>
<td>1/44</td>
</tr>
<tr>
<td>½ hr</td>
<td>1/11</td>
<td>1/4</td>
</tr>
<tr>
<td>1 hr</td>
<td>1/11</td>
<td>1/4</td>
</tr>
<tr>
<td>Activated charcoal∥</td>
<td>1/195</td>
<td>1/33</td>
</tr>
<tr>
<td>Activated charcoal‡</td>
<td>1/77</td>
<td>1/44</td>
</tr>
<tr>
<td></td>
<td>1/76</td>
<td>1/55</td>
</tr>
</tbody>
</table>

* Sap expressed from plants, centrifuged 15 min. at 8,200 g.
† Sap contained 0.02 M-sodium diethyldithiocarbamate.
‡ Sap contained 0.02 M-mercaptoethanol.
§ Equal volumes of extract and solvent or solvent mixture.
∥ 'Norite A' (Matheson, Coleman & Bell, Norwood, Ohio, U.S.A.).
‡† Merck 18351.
** Final preparation greenish and opalescent or cloudy.

in a volume of buffer equivalent to 1/48 that of the original sap. The u.v.-extinctions of the final concentrates were 5.9 and 3.3, respectively, for sap clarified with AgNO₃ and chloroform. Both preparations had u.v.-extinction maxima and minima at 258 and 235 nm.,
respectively. In another trial, healthy oat sap containing 0.01 M-EDTA was clarified with AgNO₃, and the clarified sap passed through one cycle of differential centrifugation. The extinction of the final preparation, representing 1/33 of the original volume of sap, was 1.12 compared to 26.8 for sap not treated with EDTA. This indicated that the concentrates from healthy oat sap, clarified in either way, still contained much particulate material, probably ribosomal in nature (Allfrey, 1963; Brakke, 1967b).

Table 2. Effect of silver nitrate on the ID₅₀ of sap from oat plants infected with oat necrotic mottle virus

<table>
<thead>
<tr>
<th>Trial</th>
<th>0</th>
<th>0.5</th>
<th>1</th>
<th>1.5</th>
<th>2</th>
<th>2.5</th>
<th>3</th>
<th>4</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1/52</td>
<td>1/82</td>
<td>1/73†</td>
<td>---</td>
<td>1/55</td>
<td>---</td>
<td>---</td>
<td>1/40</td>
<td>---</td>
</tr>
<tr>
<td>2</td>
<td>1/12</td>
<td>1/20</td>
<td>1/21†</td>
<td>---</td>
<td>1/20</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>3</td>
<td>1/37</td>
<td>1/50</td>
<td>1/41</td>
<td>1/44†</td>
<td>1/35†</td>
<td>1/30</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>4</td>
<td>1/12</td>
<td>1/22</td>
<td>1/16</td>
<td>---</td>
<td>1/13†</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>5‡</td>
<td>1/90</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>1/71</td>
<td>---</td>
<td>1/85</td>
<td>1/107‡</td>
<td>1/160</td>
</tr>
</tbody>
</table>

* Sap was expressed in a juicer, then centrifuged for 15 min. at 8,200 g and the supernatant fluid treated with AgNO₃.
† Indicates the smallest volume of AgNO₃ with which clarification was achieved.
‡ Sap was obtained from upper leaves only in trial 5; in other trials whole plants were used.

Concentration by ultracentrifugation

The addition of sodium citrate to the clarified extracts, to a concentration of 0.01 M before ultracentrifugation, sometimes increased the infectivity of the pelleted material when resuspended in buffer solutions. This chemical was therefore added routinely. The addition of EDTA at pH 7.2 to the clarified extracts, to a concentration of 0.01 M, decreased the amount of host material in the resuspended pellets but was omitted because it sometimes decreased virus infectivity.

Several media were tried in attempts to obtain maximum infectivity when resuspending material pelleted once by the ultracentrifuge. A volume of the medium, about 1/8 that of the original clarified extracts containing 0.01 M-sodium citrate, was used. Infectivity was undetectable or very low when water was the medium. Comparative infectivities obtained with several buffers at different pH values are shown in Fig. 1. Maximum infectivity occurred from pH 8.2 to 8.5 with 0.4 M-sodium borate and at pH 9.2 or higher with 0.1 M-tris + HCl. Potassium or sodium-potassium phosphate was the most unreliable of the media tried. On many occasions infectivity was low or undetectable. The optimum pH range for retention of infectivity in 0.1 M buffer solutions was 7.8 to 8.5. Trials with the borate and phosphate buffer solutions in which relative infectivities were greater than 300% (Fig. 1) were those in which virus source material was harvested during spring or early summer. Infectivities with 0.2 M-potassium maleate and 0.1 M-sodium citrate were maximum at pH 6.2 and from about 5.5 to 6.5, respectively. No infectivity was detected with the sodium citrate buffer solution at pH 4.0. In most routine experiments with sodium citrate buffer solutions at pH 6.2 to 6.5, relative infectivities were about 100%. Suspensions of virus in 0.002, 0.01, or 0.02 M-EDTA at pH 6.3 to 8.4 were usually not infective. Maximum infectivity at optimum pH usually occurred with citrate, phosphate, and maleate buffers at 0.1 to 0.4 M concentration. Infectivity with 0.01 M-citrate (pH 6 and 8) or 0.01 M-phosphate (pH 8.3) was low or undetectable.

When extracts were concentrated by two or three cycles of differential centrifugation,
with 0.4 M-sodium borate pH 8.6, 0.1 M-potassium phosphate pH 8.3, or 0.1 M-sodium citrate pH 6.5 as suspending media, the concentration of virus particles increased with each cycle. However, infectivities of these preparations did not show a corresponding increase, and after two cycles of differential centrifugation preparations were sometimes less infective than the clarified sap, probably because of fragmentation or aggregation of the virus particles.

The stability of partly purified virus, prepared by ultracentrifuging clarified sap and suspending the pellets in 0.4 M-sodium borate, pH 8.6, was tested by storage at about 4°C. In three trials infectivity decreased slightly after 20 days, but increased after 56 days in another trial.

![Graph](image-url)
Rate-zonal density gradient centrifugation

Several buffer solutions were tried as solvents for the sucrose in the density-gradient centrifugation of virus preparations. The results (Table 3) indicated that 0.1 M-sodium citrate at pH 6.3 to 6.5 was the most suitable. When collecting 1 ml. fractions of centrifuged gradients prepared with this buffer, the 12th and 13th fractions below the meniscus were the 0.14 and 0.19 respectively.

Fig. 2. U.v.-extinction profiles measured during displacement of the tube contents after density-gradient centrifugation of virus preparations. (a) Sap clarified with AgNO₃; (b) clarified sap given one cycle of differential centrifugation; (c) the concentrate from (b) given a second cycle of differential centrifugation. Four ml. of the preparations were floated on the density-gradient solutions. Preparations in (a), (b) and (c) represent 7.6, 26 and 150 g. of infected tissue, respectively.

Table 3. Comparative efficiency of different buffer solutions for the rate-zonal sucrose density-gradient centrifugation of oat necrotic mottle virus preparations*

<table>
<thead>
<tr>
<th>Buffer solution</th>
<th>pH</th>
<th>No. of trials</th>
<th>Width</th>
<th>Brightness</th>
<th>Infectivity of fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Above zone</td>
<td>In zone</td>
</tr>
<tr>
<td>o.1 M-tris + HCl</td>
<td>8.6</td>
<td>2</td>
<td>Very wide</td>
<td>Low</td>
<td>Medium</td>
</tr>
<tr>
<td>o.1 M-tris + HCl</td>
<td>8.9</td>
<td>1</td>
<td>Very wide</td>
<td>Low</td>
<td>Medium</td>
</tr>
<tr>
<td>o.1 M-tris + HCl</td>
<td>9.2</td>
<td>1</td>
<td>Very wide</td>
<td>Low</td>
<td>—</td>
</tr>
<tr>
<td>o.1 M-tris-citric acid</td>
<td>8.6</td>
<td>1</td>
<td>Very wide</td>
<td>Low</td>
<td>—</td>
</tr>
<tr>
<td>o.1 M-tris, o.1 M-sodium citrate ‡</td>
<td>8.6</td>
<td>1</td>
<td>Narrow</td>
<td>Low</td>
<td>Medium</td>
</tr>
<tr>
<td>o.1 M-potassium phosphate ‡</td>
<td>8.3</td>
<td>4</td>
<td>Narrow or absent</td>
<td>Low or zero</td>
<td>Medium</td>
</tr>
<tr>
<td>o.1 M-potassium phosphate ‡</td>
<td>8.3</td>
<td>1</td>
<td>Wide</td>
<td>Low</td>
<td>Trace</td>
</tr>
<tr>
<td>o.1 M-potassium phosphate ‡</td>
<td>8.3</td>
<td>1</td>
<td>Wide</td>
<td>Low</td>
<td>Trace</td>
</tr>
<tr>
<td>o.1 M-sodium citrate §</td>
<td>6.3 to 11</td>
<td>Narrow</td>
<td>High</td>
<td>Trace or zero</td>
<td>High</td>
</tr>
<tr>
<td>o.1 M-sodium citrate §</td>
<td>6.5</td>
<td>2</td>
<td>Narrow</td>
<td>Low</td>
<td>Trace or zero</td>
</tr>
<tr>
<td>o.1 M-sodium citrate §</td>
<td>8.1</td>
<td>2</td>
<td>Narrow</td>
<td>Low</td>
<td>Trace or zero</td>
</tr>
<tr>
<td>o.1 M-sodium citrate §</td>
<td>8.1</td>
<td>1</td>
<td>Narrow</td>
<td>Low</td>
<td>Trace or zero</td>
</tr>
<tr>
<td>o.1 M-sodium citrate §</td>
<td>8.1</td>
<td>1</td>
<td>Narrow</td>
<td>Low</td>
<td>Trace or zero</td>
</tr>
<tr>
<td>o.1 M-sodium citrate §</td>
<td>8.3</td>
<td>1</td>
<td>Absent</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* Clarified oat extract, concentrated about 8-fold by ultracentrifugation; the pelleted virus was suspended in the buffer solution used as solvent for the sucrose.
† When narrow the zone occurred 24 to 26 mm. below the meniscus for a 3 ml. sample in a 1 in. x 3 in. diameter centrifuge tube.
‡ pH adjusted with citric acid.
§ Containing 0.01 % bovine albumin.
∥ Containing 0.1 % bovine albumin.
¶ Containing normal rabbit serum (1/500, v/v).
most infectious, had the highest concentration of virus particles and corresponded with a light-scattering zone and an u.v.-extinction peak. Light scattering zones, u.v.-extinction peaks and filamentous particles were not associated with these fractions when sap from healthy oats was processed in the same way. Fig. 2 illustrates some of the results obtained in the ISCO density gradient analyser for various virus purification steps.

In one trial, the ID 50 values for clarified sap, and for the virus concentrates suspended in 0.1 M-citrate at pH 6.3 or in buffer solutions with 0.5 M- or 1 M-urea, were 1/64, 1/85, 1/114 and 1/141, respectively. When the three concentrates were analysed by centrifugation on sucrose density-gradient solutions dissolved in the corresponding buffer solutions, the areas of the virus peaks were similar. Thus urea increased infectivity but caused no perceptible increase in the amount of virus.

Agarose-gel chromatography

When virus preparations clarified with AgNO₃ and concentrated by two cycles of differential centrifugation were passed through agarose-gel columns, the virus emerged immediately after the void volume. With 5 ml. fractions the infectivity, number of virus particles, and u.v.-extinction were highest in fractions 7 or 8. Extinction was also high for the non-infective fraction 13. Results were similar for columns equilibrated with citrate buffer solutions or with those containing 0.5 M-urea.

Table 4. The dilution end points of oat necrotic mottle virus preparations when tested with healthy-oat antiserum

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Volume of preparation</th>
<th>Dilution end point of preparation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Sap clarified with AgNO₃</td>
<td>1/1</td>
<td>1/2048</td>
</tr>
<tr>
<td>2. Clarified sap after 1st cycle of differential centrifugation</td>
<td>1/6.4</td>
<td>1/32</td>
</tr>
<tr>
<td>3. Clarified sap after 2nd cycle of differential centrifugation</td>
<td>1/40</td>
<td>1/32</td>
</tr>
<tr>
<td>4. Concentrate from 2nd cycle centrifuged in sucrose-density gradients, and the virus reconcentrated by ultracentrifugation</td>
<td>1/88</td>
<td>1/8</td>
</tr>
<tr>
<td>5. Sucrose-density concentrate passed through agarose-gel column, and the virus reconcentrated by ultracentrifugation</td>
<td>1/280</td>
<td>No reaction</td>
</tr>
</tbody>
</table>

* Determined by microprecipitin test (Ball, 1961).

Testing for host contaminants in virus preparations

The optimum dilution of healthy-oat antiserum was 1/2 in titrations against twofold dilutions of the healthy-oat extract. This dilution was used when testing virus preparations. The titre of the antiserum was 1/256 for extract diluted 1/2. Dilution end points for preparations in a typical virus-purification procedure are shown in Table 4. The u.v.-extinction spectrum of the preparation obtained after concentrating material from the relevant sucrose density-gradient fractions (Table 4, Step 4) resembled that of a virus. There was a broad maximum at 259 nm and a minimum at 246 nm. Extinction ratios E₂₅₉/E₂₈₀ and E₂₅₉/ E₂₄₆ were 1.40 and 1.11, respectively. The pellet obtained after reconcentrating the virus from the agarose-gel column (Table 4, Step 5) was 3 mm. in diameter, vitreous and colourless,
Purification of ONMV

and dissolved readily and completely in buffer solution. The solution contained a high concentration of virus particles and no impurities were visible. There was a wide range of particle lengths.

Particle morphology

The concentration of virus particles in preparations made by the leaf-dip method (Brandes, 1964) or from epidermal strips (Hitchborn & Hills, 1965) was so low that sap expressed from leaves, and then clarified, was used. An average of about 2.5 particles/plate was photographed at an electron-optical magnification of 40,000 ×. The lengths of 178 particles were measured.

Fig. 3. Distribution of particle lengths of oat necrotic mottle virus. The class interval is 10 nm.

Fig. 4. Electron micrograph of oat necrotic mottle virus particles negatively stained with potassium phosphotungstate.

The normal length was determined by arranging lengths into a series of classes each 10 nm. wide. Most particle lengths fell into the 710 to 719 nm. class (Fig. 3). Eight particles were shorter than 500 nm. and five longer than 900 nm. An average value was calculated
for all particles with lengths from 690 to 749 nm. The mean of 723 nm. was accepted as the
normal length. The average length for all 178 particles was 722 nm. The lengths of the two
longest particles were 1438 and 1442 nm. – almost exactly twice the normal length.

The average width of 26 particles measured from different plates was 11.4 nm. in tests
with either the diffraction grating or the average width of 26 particles of tobacco mosaic
virus (TMV), photographed on the same plates, as the dimensional standard. The width
of TMV was accepted as 18 nm. (Williams, 1959).

Particles were flexuous (Fig. 4) and the phosphotungstate stain usually penetrated for a
short distance into the central portion of the ends of each filament.

DISCUSSION

The silver nitrate clarification method was simple and reliable and offered more precise
control than other methods because the amount of AgNO₃ required was easily determined
by the incipient flocculation point. This end point apparently varied according to the amount
of host proteins in the preparation. Silver nitrate was approximately as efficient as chloro-
form in removing host material and there was no need to use solvent-resistant centrifuge tubes
or to separate different liquid phases after treatment. Chloroform treatment occasionally
lowered infectivity but there was either no loss or an increase of infectivity with AgNO₃.
This increase may have been due to the destruction of a virus inhibitor. An increase in the
infectivity of TMV in tobacco juice treated with AgNO₃ was reported by Gill & Yarwood
(1964).

Silver nitrate probably reacts with the host proteins to form insoluble silver salts (White,
Handler, Smith & Stetten, 1959). There appeared to be considerable latitude in the end
point before the AgNO₃ caused a decrease in the infectivity of the ONMV preparation. Juice
from tobacco plants infected with TMV was also satisfactorily clarified without apparent
decrease in the amount of virus. Furthermore, the treatment with the TMV preparation
was mild since most of the virus particles in the clarified preparation were near normal
length. The AgNO₃ method may be useful with other sensitive viruses and may be advan-
tageous in retaining maximum infectivity when virus concentration in the tissues is low.

Many filamentous viruses have been difficult to purify because of their tendency to aggre-
gate or degrade in certain media. The relatively high infectivity obtained when borate
buffer was used to resuspend ONMV after ultracentrifugation parallels that obtained when
this buffer solution was used in the purification of some other filamentous viruses (Schade,
ever, the high optimum pH of about 8.4 for ONMV was more like that required for turnip
mosaic virus (Schade, 1960/61) than that for the other viruses which were purified at
a pH near neutrality.

Sodium citrate with potato virus X (Reichmann, 1959), and citrate buffer solution at
pH 8.0 with wheat streak mosaic virus (Brakke & Ball, 1968), prevented aggregation when
used as media in purification. The citrate ion was also useful with ONMV, but the optimum
pH was acidic. Also, with partially purified preparations of potato virus X, aggregation
and presumably loss in infectivity occurred at a concentration of 0.05 M but not of 0.005 M
(Reichmann, 1959). By contrast, high ONMV infectivity occurred with citrate buffer
solutions at 0.4 M but not at 0.01 M. The observation that EDTA reduced the infectivity of
ONMV may indicate that some divalent cation is necessary to stabilize the virus structure
(Wells & Sisler, 1969).

Because borate buffer solutions cannot be used in agarose-gel chromatography, and since
Purification of OMNV

citrates buffer solutions gave satisfactory results, borate was not tried as a solvent for the sucrose solutions. The incorporation of urea in the citrate buffer solutions during the later purification steps may have increased the infectivity of the virus by prevention of some aggregation and fragmentation of particles (Damirdagh & Shepherd, 1970). Nevertheless, considerable fragmentation of the virus did occur, presumably due to shearing forces during the several concentration steps involved.

The average diameter of 11.4 nm. found for the ONMV particles falls within the general range of diameters reported for a number of filamentous viruses (Varma et al. 1968). The normal length of 720 nm. for ONMV would place this virus in the potato virus Y group according to the classification system of Brandes (1964), though attempts at transmission of the virus with several aphid species have failed (Gill, 1967). There are several filamentous viruses of grasses with normal or mean particle lengths in this range. Thus soil-borne oat mosaic virus has a length of 660 nm. (Gold, McKinney & Scott, 1959); *Hordeum* mosaic virus of 683 to 698 nm. (Slykhuis & Bell, 1966); wheat streak mosaic virus of 700 nm. (Brandes & Wetter, 1959); ryegrass mosaic virus of 700 nm. (Brandes, 1964); *Agropyron* mosaic virus of 717 nm. (Slykhuis & Bell, 1966); maize dwarf mosaic virus of 700 to 750 nm. (Sehgal, 1968); sugarcane mosaic virus of 750 nm. (Brandes, 1964); and cocksfoot streak virus, of 750 nm. (Brandes, 1964). Although biological and other properties of ONMV appear to distinguish it from these other viruses, serological studies should establish whether or not ONMV is related to viruses of similar morphology.

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REFERENCES


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