PURIFICATION AND PROPERTIES OF BROAD BEAN WILT VIRUS

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Summary

Purified preparations of broad bean wilt virus contained many isometric particles about 25 mμ in diameter. The particles were of three sorts with sedimentation coefficients ($S_{20,w}$) of 63, 100, and 126, containing 0, 22, and 33% nucleic acid respectively. The particles with $S_{20,w} = 126$ were infective. The ribonucleic acid of the virus had a molar base content of G 21·8 : A 30·0 : C 17·6 : U 30·6.

Broad bean wilt virus was closely related serologically to PO virus (a virus isolated from peas in North America), but did not react with antisera to broad bean stain, red clover mottle, squash mosaic, or true broad bean mosaic viruses.

I. Introduction

Broad bean wilt virus (BBWV) was first isolated from broad beans (*Vicia faba* L.) growing in Victoria (Stubbs 1947), and has since been isolated from several ornamental and wild plant species growing in New South Wales. The virus can infect a wide range of dicotyledonous plants, and is readily transmitted by sap inoculation, and by aphids in the "non-persistent" manner (Stubbs 1960).

This paper reports studies on the purification and properties of BBWV and its relationship with other viruses.

II. Materials and Methods

For most of the work described here the strain of BBWV used was that originally isolated by Dr. L. L. Stubbs from broad beans growing in Victoria, and stored since then as a desiccated culture. In some tests a strain recently isolated from *Plantago lanceolata* L., and kindly supplied by Professor N. H. White, University of Sydney, was used. Dr. W. T. Schroeder, Cornell University, U.S.A., kindly compared the Victorian isolate of BBWV with two strains of PO virus (Kim and Hagedorn 1959), using antiserum against BBWV.

BBWV was grown in *V. faba* cv. Coles Prolific, plants of which, except when stated, were infected 5–7 days after emergence of seedlings by rubbing with extracts of infected plants buffered with 0·5M phosphate (pH = 7). In some tests, plants were infected using apterous adult *Aphis craccivora* Kock. The aphids were permitted to probe infected plants briefly, and were then transferred to young bean seedlings (five aphids per seedling) and allowed to feed for 24 hr. The aphids were then killed by spraying with an organophosphorus insecticide. The infectivity of virus preparations was compared by rubbing them on the fully expanded, primary leaves of cowpea (*Vignea sinensis* L.) using the half-leaf technique, each treatment being replicated at least 10 times.

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For purification, leaves and shoots showing symptoms were harvested from beans 10–14 days after inoculation. The infected plant tissue was cooled and homogenized in phosphate buffer (0·1M, pH 7·6) containing diethyl dithiocarbamate (0·1M) and thioglycollic acid (0·1%). One millilitre of buffer was used per gram of tissue. The homogenate was kept overnight at 4°C, filtered through muslin, emulsified with chloroform (quarter volume), and centrifuged (10,400 *g* for 20 min). The aqueous phase was collected and the virus purified from it by three cycles of differential centrifugation (100,000 *g* for 90 min, 140,000 *g* for 45 min, and 12,100 *g* for 10 min). The virus pellets were resuspended either in phosphate buffer (0·05M, pH 7·6) or in phosphate buffer containing 0·01M EDTA. The virus preparation was further purified or fractionated by centrifuging in sucrose density gradients. One millilitre of preparation was layered on a gradient of 10–40% sucrose (25 ml total) in 0·01M phosphate buffer, centrifuged at 60,000 *g* for 2·5 hr, and samples collected from the visible zones by puncturing the tube with a hypodermic needle. To minimize mixing of the zones, samples were taken from the trailing edges of the "top" and "middle" zones, and the leading edge of the "bottom" zone. Sucrose was removed from the samples by dialysing them against 0·01M phosphate buffer, pH 7·6.

Ultraviolet absorption spectra were measured using a Shimadzu Q.R. 50 spectrophotometer. Sedimentation coefficients were estimated by a graphical method (Markham 1960) from schlieren diagrams obtained using a Spinco model E analytical centrifuge; various dilutions (in 0·1M KCl) of a virus preparation were analysed, and the sedimentation coefficients at infinite dilution were calculated statistically. Virus preparations were examined and photographed in a Siemens Elmiskop 1A electron microscope using 2% sodium phosphotungstate as negative stain. Sizes of particles were estimated by comparison with particles of tobacco mosaic virus added to the preparation, assuming the width of tobacco mosaic virus particles to be 16 mμ.

Antisera were obtained from rabbits injected with concentrated purified virus preparations. Initially each rabbit was injected subcutaneously and intramuscularly with virus emulsified in Freund's incomplete adjuvant, and was then given two further intravenous injections of virus preparations in phosphate buffer at weekly intervals. The rabbits were bled 1 week after the final injection, and at irregular intervals during the following 3 weeks. Serological tests were done using microprecipitin and Ouchterlony gel-diffusion tests (Taylor and Hewitt 1964).

The molar base content of BBWV was determined using purified virus (components with sedimentation coefficients of 100 and 126) from density gradients. The virus was first precipitated by adding two volumes of ethanol and a trace of concentrated magnesium chloride solution. The precipitated virus was washed in 70% ethanol, dried, and hydrolysed in 1N HCl for 1 hr in a boiling water bath. The purine and pyrimidine nucleotides were separated chromatographically (Markham 1955) using as solvent a mixture of isopropanol, concentrated HCl, and distilled water (68·0 : 17·6 : 14·4 v/v respectively), eluted from the chromatograms, and estimated spectrophotometrically. Base contents were calculated using the extinction coefficients given by Markham (1955).

III. Results

(*a*) *Virus Purification*

The best virus preparations were obtained when plants were harvested before they showed much necrosis and when extracts were made immediately; extracts from necrotic or frozen tissue were black and yielded little virus. Plants showing necrosis were not used as inoculum for other plants so that after about 15 transfers a strain of BBWV that gave little necrosis was selected. This strain did not appear to differ from the original isolate in any other of its properties.

Much virus was lost when, at different stages of the purification procedure, the preparation was clarified by centrifugation; the pellets formed were examined in the electron microscope and found to contain often as much virus as the supernatant. The final yield of virus was usually about 1 mg of virus per 100 g infected tissue.

When 0·01M EDTA was added to the phosphate buffer to resuspend the virus after the final sedimentation some host material but little virus was degraded (Fig. 1); however, when 0·01M EDTA was added earlier in the purification schedule the yield of virus was greatly diminished.

(*b*) *Centrifugation of Purified Virus Preparations*

When purified preparations were centrifuged in sucrose density gradients and then examined using "dark background" illumination, three zones were visible about 11, 16, and 20 mm from the meniscus. When the tubes were examined using transmitted light, only the top (11-mm) zone was visible and coloured pale brown. The middle zone was more dense than the others, and the top zone was narrower and more clearly defined when the virus preparation had been treated with EDTA.

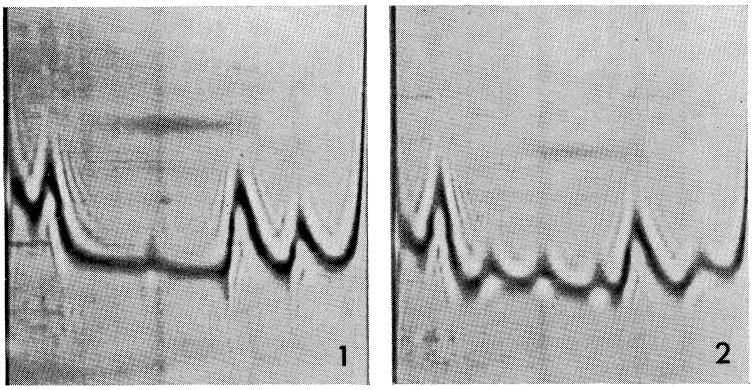
These results were confirmed in the analytical centrifuge using virus preparations from the first cycle of differential centrifugation. Unfractionated preparations, treated with EDTA, contained, in addition to slow sedimenting ($S = 20$) host material, three components with sedimentation coefficients ($S_{20,w}$) of 63, 100, and 126 (Fig. 1). Preparations not treated with EDTA contained two other components (presumably ribosomes), one of which sedimented slightly slower and the other slightly faster than the top component (Fig. 2).

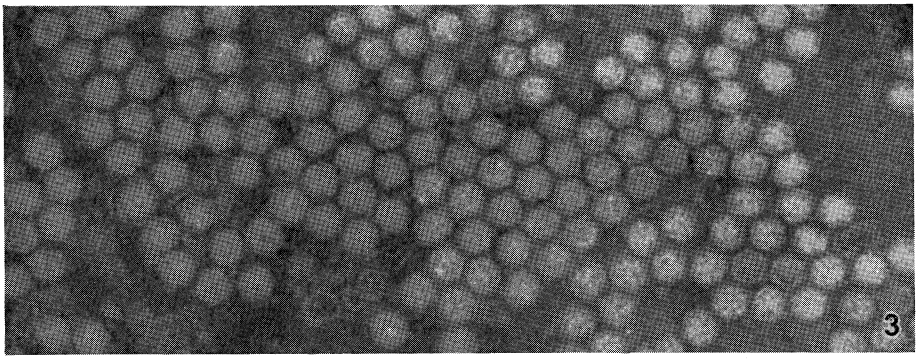
(*c*) *Properties of Fractions Obtained from Sucrose Density Gradients*

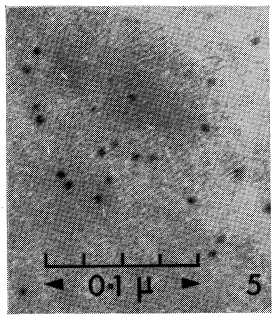
In the electron microscope the samples of the middle and bottom zones were found to contain many isometric particles about 25 mμ in diameter. These particles had an angular outline, usually hexagonal, and showed no obvious subunits. Some particles had been partially penetrated by the negative stain, but the majority seemed intact and had not been penetrated (Fig. 3). The top zone contained particles of two sizes (Fig. 4). The larger particles were the same size as the particles in the middle and bottom zones, but all had been penetrated by stain, to show the "wall" of the particle about 2–3 mμ thick. The smaller particles (Fig. 5) were about 10–12 mμ in diameter, had an electron-absorbing core which could be seen in unstained preparations, and appeared identical to the particles of phytoferritin described by Hyde *et al.* (1963).

The ultraviolet absorption spectrum of the top component (Fig. 6) was typical of a protein, whereas the spectra of the middle and bottom components were typical of nucleoproteins. The ratio of optical density at 280 and 260 mμ was about 0·60 for the middle component and 0·57 for the bottom component. These ratios suggest that the particles in the zones contain about 18–23% and 30–40% nucleic acid respectively (Paul 1959). If it is assumed that the top component only differs from the other components in that it contains no nucleic acid, then the sedimentation coefficients suggest (Reichmann 1965) that the middle and bottom components contain 22 and 33% nucleic acid respectively.

The molar base content of combined middle and bottom zones was determined. The means of seven determinations on two preparations were G $21{\cdot}8\pm0{\cdot}4$: A $30{\cdot}0\pm0{\cdot}4$: C $17{\cdot}6\pm0{\cdot}3$: U $30{\cdot}6\pm0{\cdot}5$.







Figs. 1 and 2.—Analytical ultracentrifuge photographs of purified preparations of BBWV, in phosphate buffer (0·05M, pH 7·6) and KCl (0·1M, pH 7·0), with (Fig. 1) or without (Fig. 2) EDTA (0·01M). Note by comparison of Figure 1 with Figure 2 that the addition of EDTA has eliminated two host components. The three virus components with $S_{20,w}$ values of 63, 100, and 126 are represented by the three peaks (centre and right) in Figure 1 and the corresponding peaks in Figure 2.

Fig. 3.—Electron photomicrograph of bottom zone from density gradient showing particles of BBWV with $S_{20,w} = 126$. Stained in 2% phosphotungstic acid.

Fig. 4.—Electron photomicrograph of top zone from density gradient showing particles of BBWV with $S_{20,w} = 63$ (larger particles) and phytoferritin particles (smaller particles). Note that BBWV particles have been penetrated by the stain and that phytoferritin particles have an electron-dense core.

Fig. 5.—Electron photomicrograph of unstained preparation of phytoferritin illustrating the presence of an electron-dense core.

The infectivity of samples of the three zones was assayed by inoculating them to cowpea leaves; samples from each zone were diluted until the concentration of material in them was similar, and each had an optical density of 0·2 at 260 mμ. The bottom zone was most infective and gave more than 100 lesions on each half leaf, whereas the middle zone gave 0–5 lesions per half leaf, and the top zone was not infective.

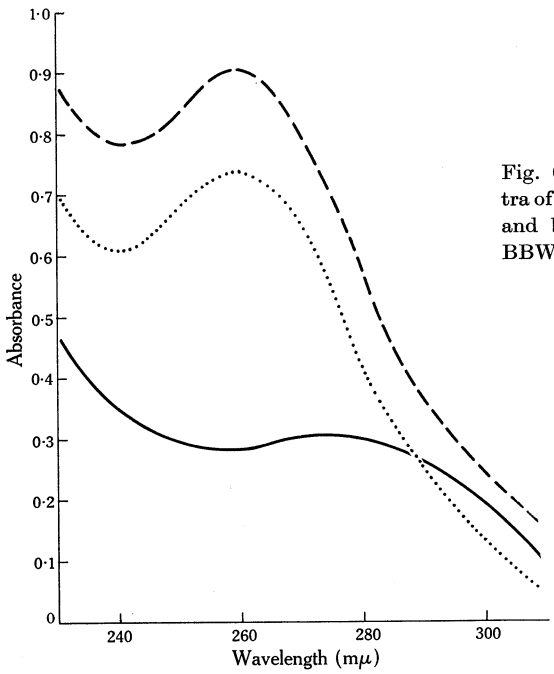


Fig. 6.—Ultraviolet absorption spectra of the top (———), middle (· · · · ·), and bottom (– – – –) components of BBWV.

Wood and Bancroft (1965) showed in experiments with bean pod mottle and cowpea mosaic viruses, which, like BBWV, have three types of particles, that the middle component enhanced the infectivity of the bottom component. Similar experiments were done with BBWV. In two experiments equal amounts (estimated spectrophotometrically at 260 mμ) of middle and bottom components were more than twice as infective as bottom component alone. However, in three other experiments the infectivity of the bottom component was not enhanced by adding middle component.

(*d*) *Serology*

Two antisera with titres of 1/256 and 1/1024 reacted clearly in gel-diffusion tests with purified virus preparations, giving a single band of precipitate. Poorly defined bands of precipitate were obtained with sap from infected broad bean plants, but were much clearer when the sap was extracted by grinding the plant tissue directly into a buffer containing a reducing agent (1 ml/g tissue of 0·5M phosphate buffer, pH 7·6, containing 0·1% thioglycollate and 0·1M diethyldithiocarbamate).

In gel-diffusion tests the Victorian and New South Wales isolates of BBWV seemed identical, and were both closely related serologically to PO virus (Schroeder, personal communication). Neither of the BBWV isolates reacted with antisera prepared against broad bean stain, red clover mottle, squash mosaic, and true broad bean mosaic viruses in either gel-diffusion or microprecipitin tests.

IV. Discussion

The method found best for purifying BBWV in the present work is similar to methods used successfully with other such viruses. The purified preparations seemed free of most host components except some phytoferritin, which is apparently a common contaminant of purified virus preparations (Lister, Bancroft, and Nadakavukaren 1965; Corbett and Grant 1967), often overlooked in the past.

There was no obvious reason for the inconclusive results in the experiments to show whether the component of BBWV with $S = 100$ enhanced the infectivity of the component with $S = 126$. However, there have been other conflicting reports on this phenomenon. Wood and Bancroft (1965) showed enhancement with the components of cowpea mosaic virus, but van Kammen (1967) could not confirm their results, and Fulton (1967), working with tobacco streak virus, found enhancement using one species of host plant but not with another.

BBWV is closely related to the PO virus isolated from peas in North America, but also shares many of its properties with other viruses. Its particles resemble closely those of viruses of the cowpea mosaic virus group (CMV group) — bean pod mottle, broad bean stain, cowpea mosaic, F1, MF, radish mosaic, red clover mottle, squash mosaic, and true broad bean mosaic viruses (Gibbs *et al.* 1966; Gibbs, Guissani-Belli, and Smith 1968) — in their size, appearance, sedimentation behaviour, nucleic acid content, and composition. Yet BBWV is readily transmitted by aphids, whereas some viruses of the CMV group are transmitted by beetles and others have no known insect vectors. Also, BBWV has hosts in a wider range of plant families than most viruses of the CMV group.

As several CMV-group viruses are seed-borne, some in broad beans, we suspected that during glasshouse experiments BBWV might have been contaminated with a virus of that group. However, tests showed that both the Victorian and New South Wales isolates of BBWV used in this study were readily transmitted by aphids, and purified virus preparations made from plants infected by aphids were indistinguishable in serological tests, in the electron microscope, and in the analytical centrifuge from preparations made from sap-inoculated plants.

The close similarity of BBWV and CMV-group viruses suggests that further attempts should be made to see whether aphids are the vectors of broad bean stain, F1, MF, red clover mottle, and true broad bean mosaic viruses.

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