AN ABSTRACT OF THE THESIS OF

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Title: THE PARTIAL PURIFICATION-CHARACTERIZATION OF BEAN YELLOW MOSAIC VIRUS

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Factors limiting effective extraction and purification of bean yellow mosaic virus (BYMV) were investigated. Since the use of published techniques for purifying BYMV resulted in low yields of partially purified virus, an improved method for partial purification of this virus was developed. The following treatments of infectious crude extract decreased infectivity: (i) mechanical rupture of the chloroplasts, (ii) homogenization in anionic, nonionic, or cationic surfactants, (iii) addition of the organic solvents acetone, butanol, ethanol, methanol, petroleum ether, diethyl ether, toluene, and chloroform, (iv) centrifugation in high salt concentrations (KBr or CsCl), (v) zone electrophoresis, (vi) homogenization in ethylenedinitrilo-tetra acetic acid (EDTA) or in thioglycollic acid. The following treatments increased infectivity of the crude extract when added to the homogenizing medium: (i) 0.1M KCl, (ii) beta-mercaptoethanol, (iii) urea.

Chenopodium amarantciolor, a local lesion host of BYMV, yielded more virus than the systemically infected hosts evaluated. Virus loss by sedimentation occurred during initial low-speed centrifugation of the crude extract from systemically infected hosts. Relatively little infectivity was lost from C. amaranticolor crude extract during low-speed centrifugation.

Of four severe BYMV isolates tested, isolate 724 yielded the highest amount of partially purified virus. Use of isolate 724 cultured in C. amaranticolor resulted in a high yield of extractable virus.

The best buffer for homogenizing BYMV-infected C. amaranticolor tissue was 0.05M diphosphate, 0.1M KCl within the pH range 8.5-9.0. Re-extraction from pulp increased the total amount of virus extracted from C. amaranticolor by 30 percent.

Preliminary clarification of crude extract was achieved by a low-speed centrifugation followed by a moderate-speed centrifugation. The following treatments decreased yield of partially purified virus: (i) heating or freezing of the crude extract, (ii) acidification (pH 3, 3.5, 4, 4.5, 5, 5.5, 6) of the crude extract, (iii) addition of 0.25 saturated $(NH_4)_2SO_4$ to the clarified supernatant, (iv) clarification by the ether-carbon tetrachloride method, and (v) homogenization of infected tissue in bentonite. Homogenization in 0.45-0.90M sucrose yielded an infectious high-speed pellet, and an infectious density-gradient fraction, but poor clarification. Absorbancy profiles and

infectivity of density-gradient (DG) fractions showed that the polyethylene glycol (PEG) treatment yielded less host contaminants and better banding of partially purified virus in the DG column than did the bentonite, sucrose, or ether-carbon tetrachloride treatments.

Sepharose gel filtration or zone electrophoresis of infectious, concentrated PEG extract did not result in further purification. Zonal rotor centrifugation of clarified supernatant resulted in a concentration of BYMV into a narrow band, but the virus sample was still impure. DG centrifugation of the infectious, concentrated PEG extract was the best purification method. The most highly purified fraction occurred 12-16 mm below the meniscus of the DG column.

Electron microscopy showed that the modal length of the virus particles directly from local lesions was 750 nm, while the modal length of particles in the most purified DG fraction was 680 nm.

The most highly purified BYMV preparation obtainable had an ultraviolet absorption spectrum typical of anisometric viruses and a 260/280 ratio of 1.152. The sedimentation coefficient of the most infectious DG fraction was 2050.5 S. This large value indicated that particle aggregation occurred.

The Partial Purification-Characterization of Bean Yellow Mosaic Virus

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THE PARTIAL PURIFICATION-CHARACTERIZATION OF BEAN YELLOW MOSAIC VIRUS

I. INTRODUCTION

Bean yellow mosaic virus (BYMV) has been known to be economically important since its discovery by Pierce (65) in 1934. It infects bean varieties which are resistant to bean common mosaic virus. In these varieties BYMV causes reduced plant growth, dropping of blossoms, and shortening of pods. Thus, it is not surprising that BYMV can cause a reduction in bean seed yield of 40-45% (42).

Perennial forage legumes such as red clover provide a reservoir of BYMV (38). BYMV can occur in high concentrations in these plants, from which it is transmitted by aphids to neighboring hosts (43).

The wide host range of the many isolates of BYMV make it important in commercial bean production. Pea crops are less affected by this virus, due to the introduction of resistant varieties (38). The geographical distribution of BYMV hosts is virtually worldwide, and the occurrence of the virus generally follows this distribution.

Historically, BYMV has been unusually difficult to purify. The significance of this study is that it extends our knowledge about the

requirements for purifying this virus. Thus, improved purification methods should make it possible to obtain the badly needed information on the in vitro characteristics of this long, flexuous plant virus.

Of the limited number of publications describing successful methods for purifying bean yellow mosaic virus (4, 11, 60, 93, 97, 98), few have appeared to be repeatable in American laboratories. Consequently, the publications reporting the production of antisera against isolates of this virus (9, 10, 12, 13, 39, 97) have also been of limited value. Indeed, the absence of published data on the in vitro characterization of BYMV suggests that there is a need for new and improved methods of obtaining high concentrations of purified, infectious, BYMV nucleoprotein.

Difficulties usually encountered in attempting to purify BYMV by published methods include (i) the marked tendency for the virus to sediment from crude sap during clarification, (ii) failure to remove host constituents without precipitating the virus, and (iii) failure to obtain sufficient quantity of pure virus for analytical or serological studies. Yamaguchi and Matsui (99) were the first workers to speculate on the nature of the difficulty in the purification of long, flexuous plant viruses. They attributed the loss of tulip breaking virus (another flexuous, 18 nm wide and 740 nm long virus), to the pelleting out of the virus particles upon the initial low-speed centrifugation. From this observation they concluded that the virus particles seemed

to be adsorbed to sub-cellular structures.

An alternative explanation for the difficulty in the purification of BYMV, which has not been considered by previous workers, is that the virus occurs naturally in the form of inclusions. The present investigation tested such a possibility in detail. That some long, flexuous viruses, such as BYMV, readily aggregate and are unstable in all but the mildest purification treatments is recognized among virus researchers. Thus, the problem of aggregation and adsorption of BYMV particles must be taken into consideration in any selected purification procedure.

The distinction between the naturally occurring inclusions and aggregations which seem to occur in situ, and the binding of virus particles to sub-cellular organelles and the aggregation between virus particles which occurs after homogenization of the infected tissue, can only be speculated upon. This study recognized the existence of the inclusions and the possibility of adsorption to membranes. From the nature of proteins and from a consideration of the binding forces which might be involved, experiments were designed to free the virus from the bound state.

As Damirdagh and Shepherd (28) pointed out, before in vitro characterization of the viruses belonging to the potato virus Y group (which includes BYMV) can be made, the viruses must be obtained in a purified and unaggregated form. Thus, the aggregation of free virus

particles must be prevented during all stages of the purification.

The main objectives of this study were: (i) to find the virus production host which would yield a large amount of extractable virus, (ii) to develop procedure which would produce a pure, highly infectious, stable, BYMV preparation, (iii) to partially characterize BYMV.

II. LITERATURE REVIEW

Unlike the relative ease with which other viruses of the potato virus Y group have been purified and characterized serologically (for example, watermelon mosaic virus was characterized by van Regenmortel, 93), past attempts to purify bean yellow mosaic virus (BYMV) and to develop antisera to its many isolates have met with limited success. The published techniques for extracting and partially purifying isolates of BYMV are restricted to a few rather standard methods. The application of these methods by the author for the preparation of antigen for BYMV antisera production has been unusually difficult.

BYMV Isolates

Many isolates of viruses causing a yellow mosaic in legumes have been found since 1934, when Pierce (65) recognized the existence of a distinct virus which incited this yellow mosaic disease. Grogan and Walker (40) showed that this virus, called bean yellow mosaic virus, was a distinct entity, though related to bean common mosaic virus, by cross-protection tests.

In 1948 Grogan and Walker (41) described a pod-distorting strain of BYMV which produced symptoms in 7 of 18 legume species tested. Zaumeyer and Fisher (102) found a strain of BYMV which they thought

was similar to the pod-distorting strain but which also produced necrotic local lesions on beans. They stated that unlike all previously reported isolates of BYMV, their strain infected Phaseolus lunatus, Vigna susquipedalis, V. sinensis, Nicotiana tabacum, and N. rustica. In 1953 Thomas and Zaumeyer (89) reported isolating a virus from Phaseolus vulgaris L. varieties which produced chlorotic local lesions on Turkish tobacco. They classified this virus as a severe yellow-mosaic strain of BYMV, in spite of its atypical host range and its lack of cross-protection with typical BYMV isolates.

The choice of the particular BYMV isolate which has been used for purification and antisera production has varied from one worker to another. Wetter (97), chose an isolate of BYMV, which he evidently obtained from Quantz (unpublished). Bercks (11) used four different BYMV isolates for his studies: (i) L778 isolated from white bitter lupine (Lupinus albus L.); (ii) EV 500 from "peas"; (iii) WK from kidney vetch (Anthyllis vulneraria L.), and (iv) an American isolate. Bercks (11) discussed the serological relationships among these four isolates, and between them and antisera against common bean mosaic virus (PVI). He did not actually specify which isolates were the best for purification; apparently he did not find purification a particularly difficult problem.

McCord and Gudauskas (60) described a partial purification of an isolate from Vicia sativa L. which they presumed to be BYMV.

Bancroft and Kaesberg's (4) partial purification of BYMV was done with an isolate from Trifolium pratense L.

Plant Hosts

The various isolates of BYMV are in part differentiated by their host range. Thus there is usually a choice of several plants from which particular BYMV isolate can be cultured.

In his paper on the relationships of legume viruses, Goodchild (39) reported ten species of legume plants which can be used to culture BYMV isolates. These species are Phaseolus vulgaris L., Pisum arvense L., Trifolium subterraneum L., Vicia sativa L., Lupinus angustifolius L., Lathyrus odoratus L., Medicago denticulata Willd., M. laciniata (L.) Mill., M. tribuloides Desr., and Melilotus alba Desr. In their study of common pea mosaic (PV2) Schroeder and Provvinenti (78) indicated that Chenopodium quinoa could be used as a local lesion host for PV2.

Corbett (26) was the first to report using Crotalaria spectabilis Roth. as a local lesion host for quantitative bioassay work on two BYMV strains. Hollings (47) was the first to note that Chenopodium amaranticolor could be used as a local lesion host for many viruses. However, the first report of C. amaranticolor reacting as a local lesion host for BYMV was made by Hollings (48) in 1959.

Partial Purification

For purposes of inoculum production and extraction, BYMV isolates have invariably been cultured in systemically infected hosts such as Vicia faba L., Phaseolus vulgaris L., Pisum sativum L., Solanum tuberosum L., Lycopersicum esculentum Mill. Bancroft and Kaesberg (4) have reported successful extraction of BYMV from systemically infected Phaseolus vulgaris L. var. Bountiful. McCord and Gudauskas (60) reported purifying an isolate of BYMV from Phaseolus vulgaris L. var. Red Kidney. Van Regenmortel (94) obtained better results when purifying BYMV from Phaseolus vulgaris L. than when extracting it from Vicia faba L. Various homogenizing media have been used by these authors to extract the virus from these systemic hosts.

Wetter (97) noted the need for the reducing agents, ascorbic acid and sodium sulphite, described by Steere (84), in the homogenizing medium to prevent melanin formation. In their partial purification of BYMV from "Bountiful", Bancroft and Kaesberg (4) used 0.01M Na_2SO_4 as the homogenizing medium. McCord and Gudauskas (60) also homogenized "Bountiful" by the method described by Bancroft and Kaesberg (4). Van Regenmortel (94) noted that the use of 0.05M citrate buffer, pH 6.7 in the homogenizing media prevented aggregation in his partial purification of tobacco veinal necrosis virus,

PVYn (a strain of potato virus Y), watermelon mosaic virus, and BYMV.

Various methods of achieving satisfactory clarification of crude extract without decreasing the stability of the virus have been published. Pfankuch and Kausche (64) in 1938 and Schneider (77) in 1953 introduced the use of the organic solvent, chloroform, to selectively denature the host plant material. Steere (84) employed the chloroform-butanol clarification method for purifying tobacco ringspot virus from infected Nicotiana alata Link & Otto, Petunia hybrida, Curcurbita pepo L., or Vigna sinensis Endl. The virus was stable in these organic solvents, and thus he was able to obtain highly purified, infectious virus. Porter (66) found freon to be useful in clarification. The disadvantage of this solvent is that it extracts nonviral nucleoprotein from the plant tissues. Milne and Grogan (62) reported the clarification of crude extracts from Cucurbita pepo L. containing isolates of watermelon mosaic virus (WMV) by use of butanol. Van Regenmortel (94) indicated that he obtained clarification of WMV, propagated in Cucrubita pepo L., PVYn (a strain of potato virus Y) propagated in Nicotiana tabacum L., and BYMV propagated in Vicia faba L., or Phaseolus vulgaris L. by chloroform emulsification.

Chibnall (24) introduced the use of ether for purification work. Frampton (35) as well as Frampton and Takahashi (36) employed ether for clarification in the purification of tobacco mosaic virus. Schaffer

and Schwerdt (76) used ether for preliminary steps in the purification of polio virus.

In 1959 Bagnall, Wetter and Larson (3) introduced the ether-carbon tetrachloride method in the purification of potato virus S. In the same year Bercks (2) used the ether-carbon tetrachloride method for the partial purification of Phaseolus virus 1. The following year Bercks (11) reported successful clarification and partial purification of Phaseolus virus 2 by use of ether and carbon tetrachloride. Wetter (97) also used this method to clarify crude sap in the partial purification of BYMV. He noted that the use of the chloroform-butanol method led to a much greater loss of infectivity of BYMV than did the use of the ether-carbon tetrachloride method.

It is interesting to note that in 1964, van Regenmortel (93) reported that low-speed centrifugation, hydrated $CaPO_4$, chloroform, 8 percent butanol, or the ether-carbon tetrachloride method, did not remove the host proteins from crude sap.

It is well known that different isolates of virus may vary in their properties. Thus, the above discrepancy in success by Bercks (12) and Wetter (97) and the lack of success by van Regenmortel using the same ether-carbon tetrachloride method may be partially attributable to the use of different BYMV isolates. Wetter (97) apparently obtained his strains of BYMV from Bercks (11).

Intracellular Inclusions

The possible occurrence of virus particles as intracellular inclusions may be responsible for the difficulty in purifying BYMV. In addition Yamaguchi and Matsui (99) described the loss of tulip breaking virus upon low-speed centrifugation, and attributed this loss to the adsorption of the virus to cellular components.

The presence of intranuclear inclusions in plants infected with BYMV has been used by McWhorter (61), to identify the virus. Edwardson (32) suggested that "pinwheel" or "bundle" types of intracellular inclusions were diagnostic for viruses of the potato Y group.

In their paper on inclusions caused by BYMV, Weintraub and Ragetli (95) assumed that the cytoplasmic inclusions are composed at least partly of virus particles. Rubio-Huertos and Hidalgo (74) also assumed the inclusions caused by severe etch virus to be aggregates of virus particles. Kamei, Honda and Matsui (52) believed the intracytoplasmic bands in BYMV infected Vicia faba cells to be intimately associated with virus particles. Edwardson, Purcifull and Christie (33), however, believed that the cylindrical cytoplasmic inclusions were not composed of virus particles.

Purification

In 1959 Bancroft and Kaesberg (4) published a procedure for the

partial purification of BYMV. They noted the need for the isolation of a high-yielding virus strain and/or hosts, and believed that density-gradient centrifugation techniques would overcome the purification difficulty. These authors found that most of the BYMV infectivity was 26-36 mm below the meniscus of the density-gradient column upon density-gradient rate-zonal centrifugation performed at 24,000 rpm for two hours with an SW 25.1 rotor as described by Brakke (19). Wetter (98) also described a successful partial purification of BYMV by density-gradient rate-zonal centrifugation in an SW 25.1 rotor for two hours, using a 10-50 percent sucrose gradient. McCord and Gudauskas (60) also believed they had partially purified their isolate of BYMV using this method.

In 1964 van Regenmortel (94) reported the purification by density-gradient zone electrophoresis of three viruses belonging to the potato virus Y group. Employing electrophoresis of clarified sap from infected plants, he obtained infectious, opalescent bands of watermelon mosaic virus, potato virus Y, or BYMV.

Serology

Of the several attempts to produce antisera against BYMV, four deserve special mention. The principal workers in these attempts have been Beemster and van de Want (9), Bercks (11), Wetter (97), and Scott (unpublished).

Beemster and van der Want (9) were the first to report the production of antisera to Phaseolus virus 1 and Phaseolus virus 2. For an antigen they used dialyzed sap from systemically-infected Vicia faba L. or Phaseolus vulgaris var. Beba plant. They reported a titer of 1:800 using the agglutination reaction as first described by Chester (23). Their agglutination tests between Phaseolus virus 1 and 2, and their respective heterologous antisera showed that these two viruses were serologically related, but the closeness of this relationship was not defined.

Bercks' (11) work confirmed the serological relationship between BYMV and bean common mosaic virus (BCMV), which Beemster and van der Want (9) had originally observed. Bercks, however, used partially purified BYMV and BCMV antigen stabilized in 10 percent glycine.

Wetter (97) reported obtaining antiserum to BYMV with homologous titer of 1:250,000 by chloroplast agglutination tests. He prepared this antigen by partial purification after clarification by the ether-carbon tetrachloride method. He has obtained better results by intramuscular injection of rabbits with the Freund's (37) adjuvant and BYMV antigen than with BYMV alone. Wetter (97) also noted the anamnastic response upon a secondary injection of BYMV and potato Y virus antigen, following a primary injection with adjuvant plus virus. He found that the booster injection following the primary injection with

adjuvant and virus did not cause an anamnastic response, until the titer had already begun to drop.

Scott (personal communication) apparently achieved a similar success in antiserum production to his isolate of BYMV. However, the evaluation of his BYMV antisera has not been published.

There are many reports of antiserum production to various viruses belonging to the potato virus Y group. Van Regenmortel, Brandes and Bercks (92) produced antiserum to a member of the potato virus Y group with a homologous titer of 1: 16,000 (in microprecipitin tests) by combining intravenous and intramuscular injections. Milne and Grogan (62) also obtained antisera to two strains of watermelon mosaic virus (WMV) which had homologous titers of 1: 512 as determined by microprecipitin tests.

Van Koot et al. (91) used serological evidence to support their conclusion that the virus they isolated from freesias was a BYMV isolate. In 1956 Corbett (27) classified lupine virus number 1 and cowpea mosaic virus as strains of BYMV based on morphological and serological similarities. In the same year, Goodchild (39) reported preparing antisera to BYMV and to pea mosaic virus (PMV) using the methods of Beemster and van der Want (9). Goodchild obtained cross-agglutination reactions between BYMV and PMV which indicated that these isolates were serologically related. In 1957 Tinsley (90) described the production antiserum to a BYMV isolate from

gladiolus. Roland (73) obtained antiserum to a virus, which he thought was an isolate of BYMV, from Vicia faba L.

In 1960 Bercks (13) reported producing three antisera against BYMV with respective homologous titers of 1:128,000, 1:2,048, and 1:256. These antisera reacted against potato virus Y with titers of 1:128, 1:2, and 0 respectively. Bercks (10) found BYMV, potato virus Y, beet mosaic virus, and watermelon mosaic virus all to be serologically related. These viruses are all 720-780 nm long. In 1963 Bartels (8) noted a serological relationship between potato virus A, potato virus Y, tobacco etch virus, and henbane mosaic virus, all members of the potato virus Y group. That same year Bagnall (2) described his SB 29 virus to be serologically related to potato virus Y. Wetter (98) listed eight viruses of the potato virus Y group as being serologically related to BYMV. Brandes (20) concluded that all viruses within this particle-size group were serologically related.

In Vitro Characterization

De Bruyn Ouboter, Beijer and van Slogteren (29), were the first to show an electron micrograph of long, flexuous BYMV particles. Brandes and Quantz (21) examined grids prepared by Johnson's (50) exudate method and determined that BYMV was a particle 15 nm wide and "predominantly" 750 nm long. Bancroft and Kaesberg (4) obtained a value of 20 nm wide and 790 ± 40 nm long for BYMV.

Only four members of the potato virus Y group have been purified sufficiently for in vitro characterization. These four are turnip mosaic virus, maize dwarf mosaic virus, potato virus Y, and tobacco etch virus.

In 1960 Shepherd and Pound (82) reported the purification of turnip mosaic virus. They achieved clarification by utilizing n-butanol and acid precipitation. They obtained a single peak under the schlierien optics, upon analytical ultracentrifugation. However, they did not report a sedimentation coefficient for their virus.

Bancroft et al. (6) reported the purification of maize dwarf mosaic virus, a strain of sugarcane mosaic virus (81), by using chloroform-clarification and ultracentrifugation. They determined the sedimentation coefficient to be 155 ± 3 S.

Delgado-Sanchez and Grogan (30) described the purification of potato virus Y, by chloroform clarification followed by differential and density-gradient centrifugation. They estimated the sedimentation coefficient of potato virus Y to be 154 S by using Brakke's (18) method.

In 1966 Purcifull (68) described the purification of tobacco etch virus (TEV) by utilizing n-butanol clarification and acid precipitation. He obtained a sedimentation coefficient of 154 S for TEV by centrifugation in a Spinco Model E analytical centrifuge employing schlierien optics. He calculated an extinction coefficient of 2.4/cm for a 0.1 percent solution in 261 nm light. TEV exhibited maximal ultraviolet

light absorption between 261 and 265 nm, and minimal absorption at 247 nm and a 260:280 ratio of approximately 1.22.

In 1970 Damirdagh and Shepherd (28) reported that by incorporating 0.1-1.0 percent 2-mercaptoethanol and 0.5M urea during clarification they could prevent lateral aggregation of TEV. and turnip mosaic virus. They suggested that prevention of aggregation was essential before the viruses of the potato virus Y group could be characterized chemically and physically.

III. MATERIALS AND METHODS

Viruses Used

Of the many existing BYMV isolates, four were chosen for use in this investigation. These isolates were labelled Y-9, GG, VFS-2, and 724. The choice of isolates was based on the trueness and invariability of symptoms in Phaseolus vulgaris L. cultivars and Vicia faba L., and on the high infectivity of crude extracts from infected leaves. Y-9 was a severe BYMV isolate from the Willamette Valley used by Dr. W. A. Frazier, Oregon State University for breeding disease resistant beans. Isolate GG was taken from commercial Red Mexican U.I. 34 beans growing in the Columbia Basin, Washington. Isolate 724 was obtained from infected Pisum sativum L. varieties used in pea nurseries near Prosser, Washington. Isolate VFS-2 was obtained from a commercial seed lot of Vicia faba L. in which it was seed-transmitted (43).

Unless otherwise noted, the extraction studies herein reported involved the GG or VFS-2 isolates of BYMV. The homogenization studies involved isolate VFS-2 and the clarification studies involved isolates GG, 724, and VFS-2. Isolate 724 was used in the purification attempts and in the partial characterization studies.

Virus Culture

The four virus isolates studied were cultured in the systemically infected host plants, Vicia faba L. or Pisum sativum L. selection 183F. Isolate GG used for extraction studies, and isolate VFS-2, used in the homogenization and the clarification studies were cultured in V. faba. Isolate 724, used in the purification and partial characterization studies was cultured in Pisum sativum L. selection 183F. These virus cultures then were used to inoculate the local lesion host C. amaranticolor, which was used as the virus production host.

All plants were grown in 46-ounce-size metal cans with holes punched in the bottom for drainage of excess water. Plants used as healthy controls were germinated and grown in a separate greenhouse from inoculated plants. The host plants which reacted to inoculation by a systemic infection (V. faba L. and Pisum sativum L., hereafter referred to as systemic hosts) were grown in a soil mixture consisting of 25 percent peat, 25 percent soil, and 50 percent sand. The local lesion host, C. amaranticolor, used in this study both as a virus-production host and as a bioassay host, required great care to insure that it grew vigorously and that it remained in a succulent, susceptible condition.

The C. amaranticolor seed was free of any seed-borne viruses, and was supplied by Dr. R. O. Hampton, Oregon State University.

C. amaranticolor seeds were allowed to germinate in vermiculite. After reaching the first true leaf stage, seedlings were transplanted to small seven-ounce capacity, foam-plastic cups, containing a fertilized soil mixture. After the seedlings had grown to the third true leaf stage they were transplanted into 46-ounce-size metal cans.

The fertilized soil mixture used in growing C. amaranticolor, contained the following fertilizer in each 46 ounce can:

60 gm. of manure,

30 gm. of dolomite lime,

5 gm. of: 6% (N) - 10% (P_2O_5) - 4% (K_2O),

5 gm. of: 16% (N) - 20% (P_2O_5) - 0% (K_2O).

Vigorously growing C. amaranticolor plants were routinely fertilized with 18% (N) - 18% (P_2O_5) - 18% (K_2O) as required. The plants were watered once or twice daily.

Supplemental lighting was used during the winter, spring, and fall months to maintain a light intensity of at least 600 foot-candles, and a photoperiod of 14 hours. Supplemental lighting consisted of a combination of tungsten incandescent bulbs and fluorescent lamps (two cold white plus two gro-lux tubes per lamp). Greenhouse temperatures were maintained between 16.5-21^{o}C, except during the summer months when the temperature sometimes rose higher.

Plant Inoculation

Leaves of the systemic hosts, V. faba or P. sativum selection 183F, expressing good yellow mosaic symptoms were used as the virus source for inoculating the local lesion host (virus source). Grinding was performed with a mortar and pestle. Inoculum was prepared by homogenizing infected leaf tissue in 4 mls of .02M $diPO_4$ (refer to glossary), pH 7.0 per gm of tissue.

The leaves to be inoculated were lightly dusted with #400 mesh carborundum powder. The leaf was supported with one hand and the inoculum was rubbed gently and uniformly with disposable cotton swabs.

Infectivity Assay

When systemic hosts were employed for bioassay, the cotyledons were inoculated before the first true leaves unfolded. Only healthy, vigorously growing plants were used.

When Chenopodium amaranticolor was used as the bioassay host, either the Latin square method of Youden and Beale (100) or the half-leaf scheme of Rochow (72) were utilized. These inoculation schemes were preferred because they tend to even out differences in susceptibility between different plants and among leaves on the same plant. When the above two schemes were not used, at least two replicate

plants (8-10 leaves) were inoculated per sample to be assayed. Only succulent leaves on vigorously growing plants were used for bioassay.

Partial Purification

Extraction of BYMV from Plant Tissue

Unless otherwise specified, all the compounds evaluated for enhancing BYMV extraction, were of reagent grade.

Hosts. Details of the procedures used in studies leading to the selection of a virus production host are discussed in the Procedures and Results section.

Surfactants evaluated. This study evaluated ten surfactants of technical grade. The two anionic surfactants tested were Igepon T-73 (Sodium-N-methyl-N-oleoyl taurate) obtained from General Aniline and Film Corp., 435 Hudson St., New York 14, N.Y.; and Leonil SA (Sodium dibutyl naphthalenesulfonate) obtained from K&K Lab. Inc., Plainview, New York. The two nonionic detergents tested, Tergitol #7 and Tween 20 (a polyoxyethylene sorbitan monolaurate) and the five cationic surfactants Triton X-45, X-180, X-155, X-100, and B-1456 were obtained from Dr. A. L. Norris, Oregon State University. The bile salt, sodium deoxycholate, was obtained from Doctor's Supply Co., Hospital and Scientific Products, 5714 N. E. Hassald St., Portland, Oregon 97213.

Phenol binding reagents evaluated. Polyclar AT (a cross-linked, insoluble polyvinylpyrrolidone) was used to remove phenols from the crude extract in two experiments involving V. faba L. as the virus production host. This material was obtained from General Analine and Film Corp., and was acid washed as described by Loomis and Battaile (55).

Homogenization of the Chenopodium amaranticolor Leaves

Unless otherwise noted, the tissue to be homogenized was ground with a mortar and pestle. A ratio of one gm of tissue to two mls of homogenizing media (w:2v) was standard. The ground tissue was squeezed through a double layer of cheesecloth to remove plant fiber.

When C. amaranticolor tissue was homogenized, only tissue densely covered by BYMV-induced local lesions was used. The portion of the leaves without local lesions was trimmed away with scissors. Bioassay of tissue exhibiting no local lesions had indicated that it was noninfectious. Therefore, such tissue would have merely increased the host constituents without contributing virus.

Clarification of the Crude Extract

Most clarification treatments involved an initial low-speed and a subsequent moderate-speed centrifugation. These centrifugations

were performed in a Sorvall Model SS-1, centrifuge at 4°C.

Absorbents evaluated. The effect of the two absorbents, Celite and bentonite, was tested in the clarification experiments. Celite was obtained from Fisher Scientific Co., Chemicals Manufacturing Division, Fairlawn, New Jersey. Bentonite was prepared as described by Sequeira and Lister (79), by suspending bentonite powder in 0.05M $diPO_4$, pH 7.6 at 10 gm/200 ml in a Waring blender. This suspension was followed by a centrifugation of 3,000 x g for two minutes. The supernatant was then centrifuged at 6,000 x g for 15 minutes. The resulting pellets were resuspended in one-half the initial volume in buffer and blended again in the Waring blender. After allowing the suspension to stand overnight, the bentonite particles remaining in suspension were again subjected to the above cycles of centrifugation and resuspended. The resulting bentonite suspension was used as the stock preparation from which aliquots were taken for clarification experiments.

Polyethylene glycol used. The coacervate polyethylene glycol 6000 (av. mol. wt. 6000-7500, reagent grade) was obtained from J. T. Baker Chem. Co. A 20 percent w:v stock PEG solution was made in 0.05M $diPO_4$, 0.1M KCl, pH 7.5. This stock solution was used to bring the supernatant to 4 percent PEG as described in the Procedures and Results section.

Ultracentrifugation

Moderate-speed and high-speed centrifugations were performed in a Spinco Model L preparative ultracentrifuge. Moderate-speed centrifugation was performed in the No. 30 rotor at 34,000 x g for 60 minutes. High speed centrifugation was performed in the No. 40 rotor at 100,300 x g for 90 minutes. Oak Ridge screw-cap polypropylene tubes were used. High-speed pellets were resuspended in minimally required amounts of buffer to achieve maximum virus concentration. This predetermined volume, 0.4 ml, was dispensed to the high-speed pellets from disposable (Bacteriological capillary) pipets.

Density-Gradient Centrifugation

Rate-zonal density-gradient centrifugation was used in preparing final samples during the development of extraction, homogenization, clarification, and purification methods. Density-gradient centrifugation was carried out as described by Brakke (16) with slight modifications. Centrifugation was done in a Spinco SW 25.1 rotor using 1- x 3 inch cellulose nitrate tubes. The density-gradient tubes were prepared by layering 3.6 ml. amounts respectively of 100, 150, 200, 250, 300, 350, and 400 mg. of sucrose/ml of 0.02M diphosphate buffer ($diPO_4$, see glossary) containing 0.1M KCl and adjusted to pH 7.5. These seven layers were equilibrated overnight at $4^{o}C$.

Generally, one ml. of virus preparation was layered on top of each density-gradient column. Centrifugation was begun at 8,000 x g (10,000 rpm) for 10 minutes, then increased to 49,300 x g (24,000 rpm) for 90 minutes.

Virus yield resulting after clarification and purification experiments was determined by fractionating the centrifuged columns, producing absorbency profiles, and bioassaying each fraction. Columns were fractionated with an ISCO model D Density Gradient Fractionator. The columns were scanned in a Model UA-2 ultraviolet analyzer, with attached Model 170 servographic recorder. A 10 mm light-path cell was used at a sensitivity range of 0-2.5, with a 254 nm light source. The density gradient fractionator was set at 2 ml/minute, and the fractions were collected in an ISCO model A fraction collector.

The density-gradient fractions were 2 ml. in volume, and generally corresponding fractions from four replicate columns were pooled. The virus with proteinaceous host components in these pooled fractions was concentrated by ultracentrifugation (100,300 x g for 90 minutes). The pellets then were resuspended in 0.4 ml of 0.02M diPO_4, 0.1M KCl, pH 7.5.

Although KBr is usually employed only for equilibrium density-gradient centrifugation, it was used in this study to separate chloroplasts from virus particles in a semi-purified chloroplast suspension,

by centrifugation in a 2.9M KBr solution. The chloroplast rich fraction was layered over a 2.9M KBr solution and centrifuged at 147,600 x g in a Spinco SW 39.1 rotor for three hours.

Gel Filtration

Purification of the highly infectious, concentrated PEG extract was attempted by means of gel filtration. Sepharose 2B, with a fractionating range of 2×10^6 to $20\text{-}30 \times 10^6$ was used. The sepharose beads were poured into a Sephadex K 25/45 column to fill a volume of 135 cm^3. The sepharose column was equilibrated for four hours with the eluant 0.01M diPO_4, 0.1M KCl, pH 7.5. The pressure head was 4.5 cm and the flow rate was 1.12 ml/minute. The sample was brought to 0.5 percent w:v (5 mg. sucrose/ml of buffer) sucrose and applied on the top of the sepharose column with a pasteur pipet. The column was then filled with buffer, and the flow was allowed to start before the sepharose column underwent the characteristic reversable expansion. Five-ml fractions were collected in an ISCO fraction collector, and subsequently concentrated by ultracentrifugation (100,200 x g for 90 minutes). Resultant pellets were resuspended in 0.4 ml of 0.02M diPO_4, 0.1M KCl, pH 7.5, and bioassayed for infectivity.

Zone Electrophoresis

The technique of zone electrophoresis as described by van Regenmortel (93, 94) was employed with slight modifications. The electrophoresis column used was modeled after the apparatus designed by van Regenmortel (94), except that it was only one-third the volume of van Regenmortel's apparatus. The electrophoresis column (Figure 1) consisted of a U-tube with a 16 mm internal diameter. A wide stopcock (S) was fitted on the right arm of the U-tube. The arms of the U-tube were connected to the electrode vessels by glass tubing. The electrode vessels also had stop-cocks at the bottom E_1 and E_2. The gradient (left) arm of the U-tube was fitted with four capillary tubes (C), used for removing fractions after an electrophoresis run. The bottom of the U-tube was fitted with two capillary outlet tubes (B_1 and B_2).

Silver-silver chloride electrodes were used. These electrodes were constructed by doubling, and successively redoubling silver wire (99.9 percent). The positive electrode was placed at the left side of the U-tube (the gradient arm).

A 10-40 percent w:w (mgs. sucrose/mg buffer) sucrose density-gradient was pumped into the left column through tube B_1. Density-gradients were prepared by a twin columned mixing chamber, similar to that designed by Bock and Ling (14). The density-gradient was

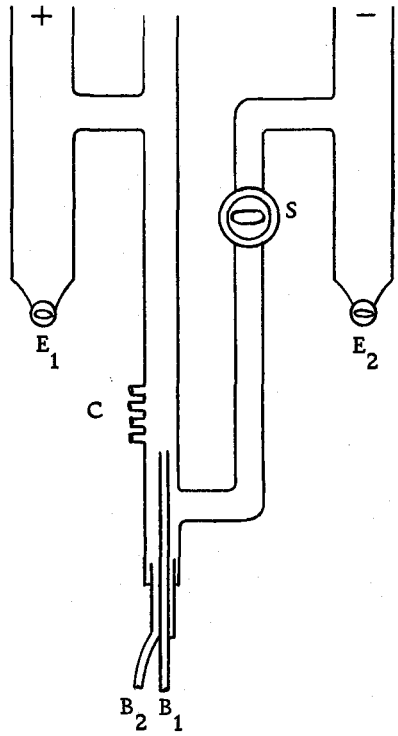


Figure 1. Zone electrophoresis column. The path of the current was 96 cm. Thus the potential gradient was 2.6 volts/cm. The sucrose gradient was pumped into the left arm through the center capillary tube (B_1). A side capillary tube was also installed (B_2). A large stop-cock was in the right arm of the column (S), and smaller stop-cocks were put at the bottom of the electrode vessels (E_1, E_2). A series of four sampling ports were located on the left arm of the column (C).

generally allowed to equilibrate for five hours at 4°C. At a potential difference of 250 volts and a current of 8 ma, formation of light-scattering bands was obtained in 15 hours of electrophoresis. These light-scattering bands were observed by shining a vertical light beam down the gradient column. Five ml factions were collected for assay through the capillary sampling ports on the gradient column. The column was adjusted upward or downward to position bands in relation to sampling ports.

Zonal Rotor Centrifugation

The technique of density-gradient centrifugation in a zonal rotor as described by Anderson and Cline (1) was evaluated for BYMV purification. The type B-XV Zonal centrifuge rotor was used in an International BD-2 ultracentrifuge. A sucrose gradient (190 ml) was pumped into the rotor spinning at 1500 rpm. Four hundred ml of 64 percent w:w sucrose was then pumped in as an underlay. The 635 ml of supernatant (clarified by the sucrose clarification method) was introduced through the center line, so that it lay over the density-gradient. An overlay of 445 ml of buffer was then pumped over the sample. The seal on the zonal rotor with inlet and outlet tubes, was then replaced with an air tight cap. The chamber door was closed and the rotor was centrifuged at 22,800 rpm for three hours at 20°C. The zonal rotor was then decelerated to 1500 rpm, the chamber door

opened, and the seal replaced. Fifteen ml fractions were collected through the center line by displacing the gradient, through the rotor edge, witn 64 percent w:w sucrose. The percentage of sucrose (w:w) was determined with an Abbe refractometer (Bausch and Lomb).

Spectrophotometry

The ultraviolet absorption spectra and the 260/280 ratios were measured with a Beckman Model DB grating spectrophotometer. The ultraviolet absorption spectra were recorded on a Beckman potentiometric recorder. The measurements were made in 0.02M diPO_4, 0.1M KCl, pH 7.2.

Electron Microscopy

Samples being prepared for electron microscopy were mounted on Formvar coated 400 mesh copper grids, as described in the Procedures and Results section. One percent formalin was used to fix the virus. Two percent phosphotungstate pH 6.9 was used as a negative stain to increase image contrast. Grids were examined in a Phillips 300 electron microscope at 60 Kv.

Analytical Ultracentrifugation

The sedimentation coefficient estimation was performed in a Spinco Model E centrifuge at 7^{o}C using schlieren optics. A 30 mm

double sector cell was used in an AN-J rotor which was spun at 3000 rpm. Photographs were taken at two minute intervals, with a phase plate angle of 70 degrees. The sedimentation coefficient was estimated from the velocity of the refractive index gradient maximum during centrifugation.

IV. PROCEDURES AND RESULTS

Development of Partial Purification Procedure

Extraction of Bean Yellow Mosaic Virus From Plant Tissue

Selection of virus production host. The choice of a virus production host for bean yellow mosaic virus (BYMV) posed the first problem in the partial purification of this virus. Legume hosts which develop systemic sumptoms have been used for this purpose in all previous reports for a number of reasons: (i) the ease of, and short duration required for, growing these plants, (ii) characteristic symptoms of BYMV in these hosts, (iii) the relative freedom from seed-borne latent viruses in these hosts, and (iv) the acceptably high dilution end point (infectivity) of the crude sap from systemically infected hosts.

It has been noted by Bancroft and Kaesberg (4) that one quarter to one third of the virus is lost from the crude extract (CE) upon the initial low-speed centrifugation of 1500 rpm for 10 minutes. Such lost infectivity can be partially recovered by resuspending the chloroplasts from the pellet (Table 1).

Infectivity of the chloroplast fraction. An experiment was designed to elucidate the infectivity of the chloroplast fraction. In order to isolate the chloroplasts, density-gradient centrifugation of the CE

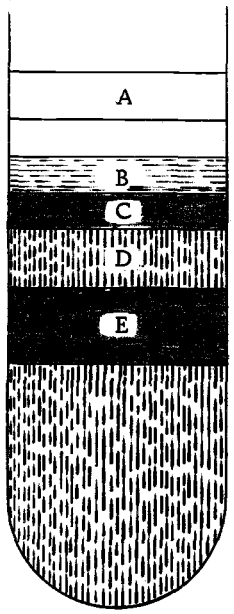
was performed. The CE was prepared by homogenizing BYMV infected Bountiful leaves in 0.01M diPO$_4$, pH 7.5. Five ml of CE were layered on a 40-80 percent sucrose gradient in an SW 25.1 cellulose nitrate tube. Infectivity data from this experiment (Figure 2) demonstrated that the green zones probably consisting of chloroplasts, chloroplast fragments, and BYMV, were very infectious. This chloroplast rich fraction was referred to as the chloroplast fraction, infectious (CFI, see glossary). These results confirmed prior observations on the high infectivity of the resuspended chloroplast portion from a low-speed centrifugation of infectious CE.

Table 1. Loss of infectivity (suspended BYMV)[1] resulting from the first low-speed centrifugation of crude extract.

Treatment	Fraction tested	Infectivity	Dilution
Crude sap (from infected Bountiful leaves homogenized in 0.01M ascorbate, pH 7.0) centrifuged at 2,700 x g for 20 minutes	supernatant	3/9[2]	0
The pellet was resuspended in 0.05M Tris-HCl, pH 9.3, to collect the chloroplast fraction.	chloroplast fraction	2/5	1/2

[1] Isolate GG.

[2] Number of Bountiful plants showing symptoms/number of plants tested.



	Appearance	Infectivity (LL/100 sq. cm) dil. 1/7
4 mm	amber, clear	0.0
3 mm	clear	-
3 mm	white, murky	0.089
3 mm	deep green	9.9
5 mm	light green	1.8
6 mm	deep green	0.78
	diffuse green	-
crude extract		33.0

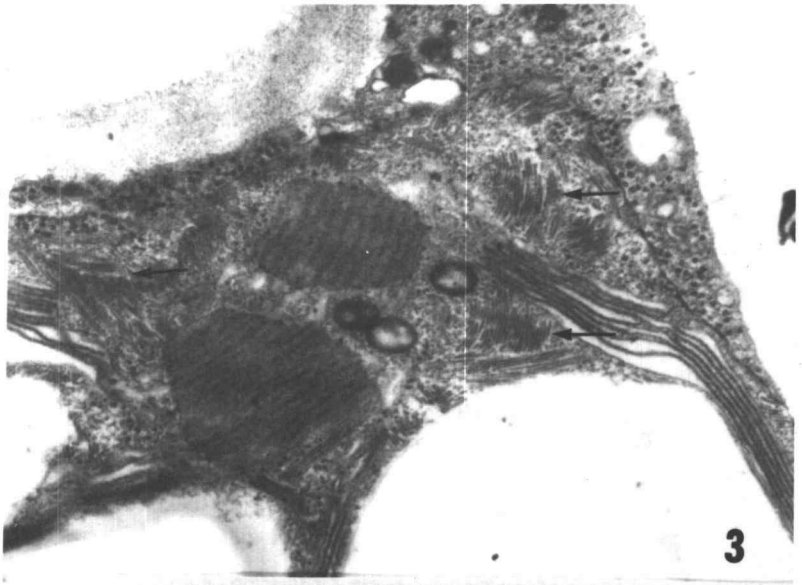
Figure 2. Schematic diagram of density gradient (DG) tube, showing banding and corresponding infectivity after DG centrifugation of crude extract from Phaseolus vulgaris L. var. Bountiful. The zones were: A) amber, clear; B) white, murky; C) deep green; D) light green; E) deep green.

Virus-like particles inside chloroplasts. To better visualize the association of infectivity with the chloroplasts, ultra-thin sections of BYMV-infected leaf tissue (Bountiful var. bean) were examined under the electron microscope. Sections of chloroplasts from a systemically-infected Bountiful plant showed many long, virus-like particles inside the chloroplasts (Figure 3). Although only one plant was studied, it was assumed that this plant was representative of plants infected with BYMV. It was therefore hypothesized that virus particles were bound inside chloroplasts, thus accounting for their loss during low-speed centrifugation.

To test this hypothesis the chloroplast fraction, CFI (see glossary) was mechanically pulverized and assayed for infectivity in comparison with untreated fractions (Table 2). The CFI was obtained by resuspending the topmost portion of the low-speed centrifugation pellet of the CE in 0.01M diPO_4, pH 7.2 buffer. The chloroplasts in this fraction were mechanically ruptured by use of glass beads (53-74 microns) in a Potter Elvejheim-type homogenizer. Prior to their rupture the chloroplasts had been washed in buffer, pelleted down, and resuspended in buffer plus 0.4M sucrose. Infectivity results indicated that the disruption of the chloroplasts decreased the infectivity of the CFI. If the virus particles occurred in the chloroplasts and were actually liberated, then the process of mechanically rupturing these organelles was apparently also harmful to the virus. This

Figure 3. Electron micrograph of an ultrathin section of a chloroplast from a BYMV-infected Phaseolus vulgaris L. var. Bountiful cell. Arrows point to virus-like particles.
Magnification: 34,474 x
Negatively stained in phosphotungstate, pH 6.9.

Figure 4. Electron micrograph of a fibrous mass of particles from which BYMV-like particles extrude. Such fibrous masses occurred in dip preparations of Phaseolus vulgaris var. Bountiful leaf tissue systemically infected with BYMV.
Magnification: 38,400 x
Shadow cast in 80-20%; platinum: palladium.



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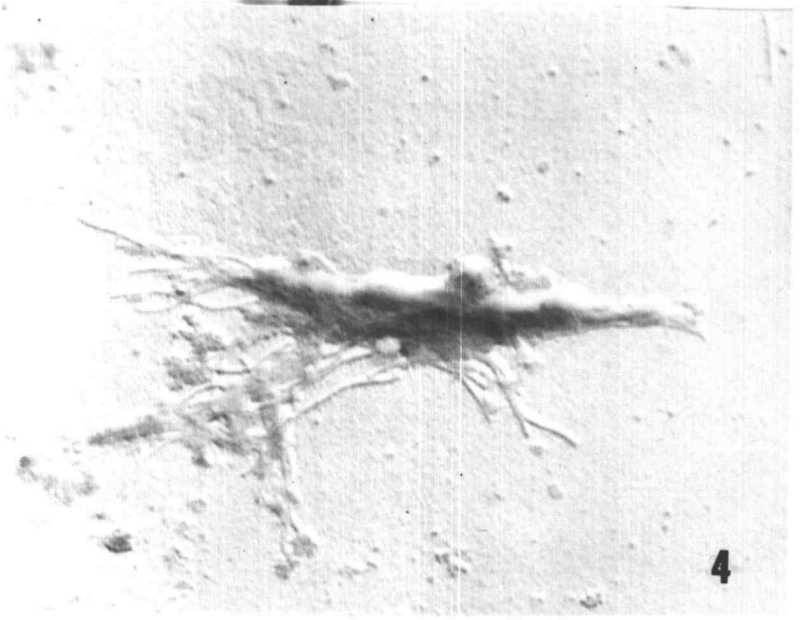

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technique was therefore discarded as a means of increasing the yield of extractable virus.

Table 2. Effect of mechanical rupture of the chloroplasts on the infectivity of the chloroplast fraction.[1]

Treatment	Infectivity
In 0.01M $diPO_4$, pH 7.2:	
1. Whole chloroplasts	14.3 LL/leaf
2. Ruptured chloroplasts	0.0 LL/leaf
In 0.05M Tris-HCl, pH 8.9:	
1. Whole chloroplasts	3/4[2]
2. Ruptured chloroplasts	2/5

[1] Isolate GG.

[2] Number of plants showing symptoms/number of plants tested.

Inclusion and aggregates. Several types of sub-cellular inclusion occur in plants systemically-infected with BYMV (32). BYMV particles sometimes occurred in "packets" of 100 to 500 particles apparently bound together in a membrane (Figure 4). These packets were most easily observed in preparations from cut-dipped leaves. Similar structures have been observed by Purcifull et al. (69) and Weintraub and Ragetli (96). The occurrence of virus particles in such masses could account for drastic losses of BYMV during initial low-speed centrifugation.

An alternative hypothesis was formulated to explain the loss of

infectivity when CE is clarified of chloroplasts, mitochondria, and other membrane-bound organelles. It proposes that the virus particles become adsorbed to the membranes of these sub-cellular organelles.

Effect of surfactants. Surfactants disrupt aggregates of viruses (17). They also dissolve sub-cellular membranes (87). These types of specific action suggested an evaluation of surfactants as instruments for liberating BYMV particles from natural aggregates (membrane-bound packets) or from membranes of organelles.

Nine surfactants were selected for this purpose. In general, CE were prepared by homogenizing Phaseolus vulgaris var. Bountiful leaves infected with BYMV (GG isolate). Chloroplast fractions were prepared as previously described for Table 1.

Two anionic surfactants, Igepon T-73 and Leonil SA, were tested. Five ml of chloroplast fraction, infectious CFI were added to five ml of the Igepon T-73 (100 ppm) and incubated for one hour at 4^{o}C. One ml of CFI was added to one ml of Leonil SA (1000 ppm) and incubated for approximately one hour at 4^{o}C. The treated CFI with the respective, suspended surfactants were directly bioassayed on Bountiful (Table 3).

Five cationic surfactants, all derivatives of the Triton series, were tested. Two ml of CFI were added to 2 ml each of the five cationic surfactants (50 ppm concentration), and incubated for one

Table 3. Effect of surfactants on the infectivity[1] of the low speed supernatant or of the chloroplast fraction from BYMV infected plants.

Surfactant	Infectivity of sample	Percent
Anionic:	Chloroplasts	
Igepon T-73: (100 ppm),		
Control, untreated sample in buffer[2]	11/18	61.2
Treatment, sample in buffer[2] and Igepon T-73	15/17	88.4
Leonil SA: (1000 ppm)		
Control, untreated sample in buffer[3]	2/5	40.6
Treatment, sample in buffer[3] and Leonil SA	0/6	0
Cationic:		
Triton (50 ppm)		
Sample in 0.01M $diPO_4$, pH 7.2		
was added to equal volumes of:		
Control, water	1/4	25.0
Treatment Triton X-45	1/6	16.7
Treatment Triton X-180	0/6	0
Treatment Triton X-155	1/4	25.0
Treatment Triton X-100	0/6	0
Treatment Triton B-1456	1/5	20.0
Nonionic:	Supernatant	
Tergitol #7: (50 ppm)		
Control, untreated sample in buffer[2]	3/11	27.3
Treatment, sample in buffer[2] and Tergitol #7	1/11	9.1
Tween 20: (50 ppm)		
Control, untreated sample in buffer[2]	3/11	27.3
Treatment sample in buffer[2] and Tween 20	2/10	20.0

[1] Number of Bountiful plants showing symptoms/number of plants tested. Both the chloroplasts and the supernatant used for bioassay were prepared from Bountiful systematically infected with the GG isolate of BYMV. The chloroplasts treated with anionic surfactants were diluted v:v in buffer before bioassay. The other treatments were not diluted for bioassay. The above were data collected from experiments conducted over a period of two years.

[2] 0.005M $diPO_4$, pH 7.2.

[3] 0.01M ascorbate, pH 7.2.

hour at 4°C. The treated CFI were then directly bioassayed on Bountiful.

Two nonionic surfactants, Tergitol #7 and Tween 20, were tested. Fourteen ml of infectious crude extract were added to 14 ml each of 50 ppm Tergitol #7 or Tween 20, respectively. The treated CE were then centrifuged at 13,000 x g for 30 minutes. The resulting supernatants, with the suspended surfactants, were then bioassayed directly on Bountiful (Table 3).

Addition of either of the two anionic surfactants tested, Igepon T-73 and Leonil SA, did not increase the infectivity of the chloroplast fraction. Addition of any of the five cationic surfactants resulted in a slight decrease in infectivity. Treatment of the infectious CE with the nonionic surfactants appeared to neither increase nor decrease the infectivity of the low-speed supernatant. None of the surfactants had any visible phytotoxic effect on the Bountiful leaves.

It was concluded that either (i) adsorption or membrane-binding of virus particles was not a limiting factor or (ii) surface active agents were unsatisfactory in resolving this problem. Surfactants were therefore not incorporated into the partial purification procedure.

Effect of agents used in ribosome isolation. This consisted of Strittmater's (87) technique for dissolving microsomal membranes. It was evaluated as a means for separating BYMV particles from

membranes (Table 4). Relatively little infectivity could be detected in the supernatant after low-speed centrifugation, when Tris-HCl buffer was used as the homogenizing medium. No infectivity was detectable upon adding beta-mercaptoethanol or $MgCl_2$ to the medium. Addition of $MgCl_2$ or of beta-mercaptoethanol alone decreased infectivity. Sodium deoxycholate alone decreased infectivity relative to the control. A combination of sodium deoxycholate, $MgCl_2$, beta-mercaptoethanol, and KCl resulted in an increase in infectivity. The addition of 0.6M KCl yielded a two-fold increase in local lesion counts, in spite of the deleterious effect of beta-mercaptoethanol. It is apparent that the use of sodium deoxycholate did not increase the amount of extractable virus.

Effects of moderate KCl concentrations. After having determined that KCl was useful, another experiment was conducted to study the effects of moderate salt concentrations on the infectivity of the supernatant (Table 4). Two grams of infected Bountiful leaves were ground in each of four homogenizing media (Table 4). Supernatants were obtained by centrifuging the CE at 17,000 x g for one hour. The results obtained confirmed the previous indications that the addition of 0.1M KCl increased the amount of virus extractable from systemic hosts. Evidently, 0.1M KCl was as effective as that of 0.6M KCl.

Effect of centrifugation in high KBr concentrations. It was

Table 4. Effects of agents used in ribosome isolation, and of moderate salt concentrations on detectable infectivity of BYMV in the low-speed supernatant.

Homogenizing media	Number of leaves	Infectivity (LL/leaf)
Test I.		
1. 0.025M Tris-HCl, pH 8.6 (control)	6	0.833[2]
2. 0.01M, B-mercaptoethanol, 0.025M Tris-HCl, pH 8.6	6	0
3. 0.005M $MgCl_2$, in 0.025M Tris-HCl, pH 8.6	6	0
4. 0.05% Sodium deoxycholate, 0.025 Tris-HCl, pH 8.6	6	0.166
5. 0.06M KCl, 0.025M Tris-HCl, pH 8.6	6	2.33
6. 0.01M B-mercaptoethanol, 0.005M $MgCl_2$, 0.05% sodium deoxycholate, 0.6M KCl, all in .025M Tris-HCl, pH 8.6	6	2.00
Test II.		
1. 0.01M Na_2SO_3, pH 8.3	18	0.554
2. 0.04M Tris-HCl, pH 8.9	19	1.262
3. 0.1M KCl, 0.04M Tris-HCl, pH 8.9	18	8.66
4. 0.6M KCl, 0.04M Tris-HCl, pH 8.9	17	9.64

[1] Isolate GG

[2] Supernatants for Test I were diluted v:v in buffer before their bioassay.

considered possible that particles could be bound to sub-cellular membranes by ionic bonds which were not influenced by the action of anionic, cationic, or nonionic surfactants. To test this possibility, CFI was exposed to high ionic strength of the salt KBr. The purpose was to neutralize ionic bonding between virus particles and membranes, and to simulatneously separate these structures by centrifugation. KBr was used as the salt because of its higher density than KCl, and of its low cost.

Infected Bountiful leaves were homogenized in 0.01M diPO_4 pH 7.2. The CE was centrifuged at 10,000 x g for 40 minutes. The pellet then was resuspended in 0.025M Tris-HCl, pH 8.9 buffer. This resuspension was heavily pigmented and was used as the chloroplast fraction (CFI).

One-half ml of CFI was layered onto 2.9M KBr solution and centrifuged at 147,600 x g in a Spinco SW 39.1 rotor for three hours. The chosen KBr concentration had a density (1.24 gm/ml) greater than that of the chloroplasts, but less than that of the virus particles. Therefore the virus particles were expected to sediment while the chloroplast membranes floated to the top during centrifugation.

After centrifugation the high-speed pellets at the bottom of the tube had a hard, gummy appearance and were light brown in color. These pellets did not resuspend in 0.01M diPO_4, pH 7.2, 0.025M Tris-HCl, pH 8.9, or in distilled water and therefore were not

bioassayed. No fraction of the centrifuged chloroplast suspension was infectious. Thus the use of centrifugation in high KBr concentrations was not useful in separating the virus from the chloroplasts.

Effect of KBr and CsCl on BYMV infectivity. Two experiments were conducted to determine the stability of BYMV (isolate GG) in KBr and in CsCl solutions. In the first experiment one gm of infected Vicia faba tissue was homogenized in 0.05M diPO$_4$, 0.1M KBr, pH 7.5, and incubated for 30 minutes at 4^{o}C. The treated CE was diluted 1/10 and bioassayed on C. amaranticolor (Table 5). No detectable BYMV infectivity remained after this treatment, probably indicating a deleterious effect of bromide.

In the second experiment one gm of Pisum sativum selection 183 infected with BYMV (GG) was homogenized in 0.05M diPO$_4$, 0.1M CsCl, pH 7.6, and incubated for 30 minutes at 4^{o}C. The treated CE was diluted 1/20 and bioassayed on C. amaranticolor (Table 5).

0.1M CsCl decreased BYMV infectivity by approximately 26 percent (Table 5) demonstrating a relatively mild effect of cesium on this virus. The technique of DG centrifugation in KBr or CsCl as a means of purifying BYMV was therefore not useful as a means of purifying BYMV.

Effect of zone electrophoresis. Since the virus particles may have been bound to the chloroplast membranes of hydrogen bonds or salt bridges, attempts were made to separate the virus particles from the membranes by density-gradient-zone electrophoresis. This

technique utilizes the principle that particles migrate in an electric field (column) according to their net ionic charge (88, 93). Assuming the BYMV particles and the bio-membranes differ in net charge, electrophoretic separation would be expected.

Fifteen grams of infected Bountiful leaves were homogenized in 30 ml of 0.01M $diPO_4$, pH 7.2. The CFI was prepared by centrifuging the CE at 9,000 x g for 40 minutes, and resuspending the pellet to yield a suspension consisting mainly of chloroplasts.

The CFI was subjected to density-gradient zone electrophoresis (see Methods, p. 28) as described by van Regenmortel (94). One and a half ml of CFI in 39 percent sucrose were introduced into the sucrose gradient column through the capillary tube at the bottom of the gradient column. The sample stabilized in the region of approximately 39 percent sucrose (10-40 percent sucrose gradient).

Two light scattering zones were visible after 17 hours of electrophoresis. The first zone was greenish in color and was 0-15 mm above the introduced CFI. The second zone was white, and it was 15-19 mm above the point of origin (Figure 5).

The light-scattering zones (Figure 5) were fractionated, and their particulate matter was concentrated by high-speed centrifugation. The pellets were resuspended in 0.01M $diPO_4$, pH 7.2. The lack of infectivity for all light-scattering zones suggested that virus occurred in none of these zones or was inactivated. It is possible that the

Fraction No.	Fraction volume (ml)	Median distance (mm) of fractions from origin	Infectivity of fractions
7	18.0	201	0/5
6	3.1	146.5	0/5
5	17.1	132.5	0/5
4	6.0	94.0	0/6
3	15.0	59.0	0/6
2	5.7	24.5	0/6
1	4.5	7.5	0/6
	0		1/8

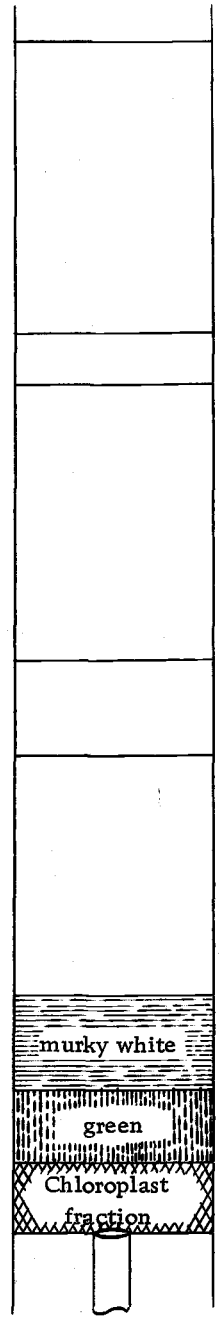

Figure 5. Bands and corresponding infectivity produced by density gradient zone electrophoresis of the chloroplast fraction containing BYMV. Infectivity is indicated as number of Bountiful plants showing symptoms/number of plants tested.

virus associated with the CFI could have migrated heterogeneously due to non-uniform net charges on the particles. This could have also accounted for the apparent dispersion of the virus during electrophoresis. Thus the virus in the CFI apparently was removed from the CFI by electrophoresis, but not concentrated into a zone. Although this method succeeded in separating the host protein from the chloroplast (Figure 5), it was not useful in isolating the virus from the other host components.

Table 5. Effect of KBr and CsCl on the infectivity of BYMV (isolate GG).

Test Material	Infectivity (LL/100 sq. cm)
Test 1.	
Control: Infected V. faba homogenized in buffer.[1]	179.24
Treatment: Infected V. faba homogenized in buffer and 0.1M KBr.	0.0
Test 2.	
Control: Infected P. sativum (183) homogenized in buffer.	125.0
Treatment: Infected P. sativum (183) homogenized in buffer and 0.1M CsCl.	93.12

[1] Buffer was 0.05M diPO_4, pH 7.5.

Effect of organic solvents. The lack of success with previous methods in increasing infectivity, suggested that much harsher

treatments to free the virus from the membranes might be required. Organic solvents were therefore used in an attempt to dissolve membranes. A series of three tests were conducted to determine whether strong solvents enhanced recovery of infectious BYMV.

In the test involving chloroform, infected Vicia faba leaves were homogenized in 0.05M diPO$_4$, 0.1M KCl, pH 7.5. After the CE was emulsified in an equal volume of chloroform, it was centrifuged at 6,000 x g for 15 minutes. The resulting aqueous supernatant was bioassayed on C. amaranticolor (Table 6).

Table 6. Effect of nine organic solvents on the infectivity of BYMV (isolate GG) in the crude extract (CE).

	Material tested	Solvent	Infectivity
1.	First low speed supernatant.	buffer[1] alone	194.0[2] LL/100 sq. cm
		buffer & chloroform	12.46 LL/100 sq. cm
2.	First low speed supernatant.	acetone	0/7[3]
		benzene	1/7
		n-butanol	0/7
3.	Resuspended high-speed pellet.	buffer alone	2/7
		carbon tetrachloride	2/7
		ethanol	0/7
		methanol	0/7
		petroleum ether	0/7
		toluene	0/7

[1] Buffer was 0.05 diPO$_4$, 0.1M KCl, pH 7.5.

[2] Bioassay on C. amaranticolor; no. local lesions per 100 square centimeters of inoculated leaf surface.

[3] Bioassay on Bountiful; no. plants which became infected/no plants inoculated.

The third test evaluated the effects of carbon tetrachloride, ethanol, methanol, petroleum ether, and toluene. The CE was obtained by homogenizing infected Bountiful leaves in 0.01M diPO_4, pH 7.0. An emulsion was formed by adding one part extract to eight parts solvent. It was broken by centrifugation of about 200 x g for 20 minutes. The particulate matter in the partially clarified aqueous supernatant was concentrated by centrifugation (80,700 x g for one hour). The high-speed pellets were resuspended in buffer, and bioassayed on Bountiful (Table 6). In the above three experiments attempts were made to remove the solvent dissolved in the aqueous phase before bioassaying.

Infectivity data indicated that chloroform, acetone, n-butanol, methanol, petroleum ether, and toluene inactivated the virus. Benzene and the carbon tetrachloride treatments produced little loss of infectivity. Clarification by these two solvents, however, was very poor. Thus the nine solvents tested were not useful in the removal of host components.

Yield of virus. The main criterion for selection of a virus production host was that a satisfactory quantity of virus must remain in suspension after the initial low-speed centrifugation. Since all attempts to recover BYMV from the CFI had been unsuccessful, an attempt was begun to determine if a local lesion host, such as Chenopodium amaranticolor might produce virus in a non-bound or

free-particle form. Plants of C. amaranticolor were immediately available and were thus selected for beginning this study. Other local lesion hosts for BYMV were Crotalaria spectabilis Roth (26), and certain selections of Phaseolus vulgaris var. Red Mexican (unpublished). Kado (51) used C. amaranticolor as a systemic host for sowbane mosaic virus. However, there were no reports of C. amaranticolor being used as a virus production host for BYMV.

Three experiments were conducted to test this idea. The infectivity of the low-speed supernatant from extracts of the systemic host, V. faba was compared with that of the supernatant from the local lesion host, C. amaranticolor.

Clarification of the crude extract from V. faba was attempted by use of three organic solvents. It was hoped that these solvents would prepare a partially clarified supernatant for infectivity tests comparable to that useful for purification purposes.

In the first experiment the CE from systemically infected V. faba was obtained by homogenizing leaves in 0.01M diPO_4, pH 7.5, centrifuging at 190 x g for 10 minutes and retaining the supernatant. Four separate aliquots of this supernatant were partially clarified of sub-cellular debris and host proteins by adding one volume of organic solvent per two volumes of supernatant and thoroughly mixing in a separatory funnel. The solvents were pipetted out, and the aqueous supernatants were directly bioassayed on C. amaranticolor. Carbon

tetrachloride decreased infectivity, but neither benzene nor ether were deleterious (Table 7) to the supernatant infectivity.

In the second experiment CE from the local lesion host, C. amaranticolor, was obtained by homogenizing leaves covered with BYMV-induced local lesions in 30 percent w:v sucrose dissolved in 0.05M Tris-HCl, pH 8.6. To obtain the low-speed supernatant, the infectious CE was subjected to 7,500 x g for 30 minutes. This supernatant was then diluted 1/5 and bioassayed on C. amaranticolor. The diluted low-speed supernatant from the local lesion host was more infectious than the nondiluted supernatant from the systemic host (Table 7).

The third experiment was designed to further evaluate the amount of virus which could be extracted from C. amaranticolor, and also to compare the efficiency of extraction in the three most promising buffers. Crude extracts were obtained as above, except that homogenizing media were different (Table 7). In this experiment, however, infectivity of the supernatant was compared after a moderate-speed centrifugation (24,000 x g for 2 hours). This partially clarified supernatant was diluted 1/5 in the buffer being tested and bioassayed on C. amaranticolor (Table 7).

Remarkably, the diluted 24,000 x g supernatants from C. amaranticolor were more infectious than the nondiluted 7,500 x g supernatants from the systemic host V. faba. These results indicated

Table 7. BYMV[1] remaining suspended after low-speed centrifugation from a systemically-infected host, V. faba, and from a local lesion host, C. amaranticolor.

Experiment	Test material	Clarification	Infectivity (LL/100 sq. cm)
			low-speed supernatant
1	V. faba leaves homogenized in 0.01M $diPO_4$, pH 7.5	1. 0.01M $diPO_4$ pH 7.5	111.0
		2. benzene	186.0
		3. CCl_4	32.1
		4. ether	177.0
2	C. amaranticolor leaves covered with BYMV-induced local lesions	1. 30% w:v sucrose in 0.05M Tris-HCl, pH 8.6 (diluted 1/5)	235.0
			moderate-speed supernatant (diluted 1/2)
3	C. amaranticolor leaves covered with BYMV-induced local lesions	1. 0.05M Tris-HCl, pH 8.9	91.0
		2. 30% sucrose in Tris-HCl	73.0
		3. 0.01M $diPO_4$, pH 7.5	188.0
		4. 30% sucrose in $diPO_4$	196.0
		5. 0.05M borate, pH 8.15	166.0
		6. 30% sucrose in borate	128.0

[1] Isolate VFS-2.

[2] Dilution with homologous buffer; without sucrose.

that the amount of virus extractable from this local lesion host was several times greater than from V. faba.

An experiment was designed for a further comparison of the infectivity of a partially purified virus from a local lesion host. The first part of the experiment (Table 8) evaluated the infectivity of the high-speed pellet of BYMV partially purified from V. faba. Infected V. faba leaves were homogenized in 0.02M diPO$_4$, 0.005M DIECA, 0.1M KCl,and 2 gms of Polyclar AT, pH 7.4. This homogenizing media was chosen because preliminary tests indicated that it preserved BYMV infectivity in extracts from this host. Clarification was by moderate-speed centrifugation, 22,000 x g for two hours. The virus plus host material in the supernatants were concentrated by ultracentrifugation (100,3000 x g for 2 hours), and the resulting pellets were resuspended in 0.02M diPO$_4$, 0.1M KCl, 0.005M DIECA, pH 7.4, and bioassayed on C. amaranticolor.

The second part of the experiment was performed to compare the infectivity of the partially purified virus extracted from C. amaranticolor, with the infectivity data already obtained for the systemic host, V. faba (Table 8). In this experiment BYMV-infected C. amaranticolor leaves were homogenized in 30 percent w:v sucrose dissolved in 0.5M diPO$_4$, pH 7.3. Clarification of the crude extract was achieved by one stage of low-speed followed by two stages of moderate-speed centrifugation. The centrifugation was as follows:

Table 8. Infectivity of the partially purified BYMV[1] extracted from V. faba compared with that from C. amaranticolor.

Part	Test material	Homogenizing media	Resuspension media	Infectivity of the high-speed pellet
1	V. faba leaves	A[2]	0.02M $diPO_4$, 0.1M KCl, and 0.005M DIECA, pH 7.4	0.0 LL/ sq. cm
	V. faba leaves	A	same as above	8.0 LL/30 leaves
	V. faba roots	A	0.02M $diPO_4$, pH 7.4	3.2 LL/100 sq. cm
	V. faba roots	A	0.05M Tris-HCl pH 8.1	19.7 LL/100 sq. cm
2	C. amaranticolor leaves covered with BYMV-induced local lesions (Trial 1)	B[3]	0.05M $diPO_4$ pH 7.3	31.8 LL/100 sq. cm
	same as above (Trial 2)	B	same as above	22.8 LL/100 sq. cm

[1] Isolate VFS-2.

[2] Polyclar AT in 0.02M $diPO_4$, 0.1M KCl, and 0.005M DIECA, pH 7.4.

[3] Thirty percent w:v sucrose in 0.05M $diPO_4$, pH 7.3.

17,500 x g for 60 minutes, 29,000 x g for 90 minutes, and 35,000 x g for 70 minutes. The virus in the clarified supernatant was then concentrated by ultracentrifugation (100,300 x g for 90 minutes) and resuspended in 0.05M Tris-HCl, 0.1M KCl, 0.005M $MgCl_2$, pH 8.1.

Infectivity of the high-speed pellets from C. amaranticolor generally exceeded that of pellets from the systemic host, V. faba (Table 8). Thus on the basis of extractable virus, the local lesion host C. amaranticolor was found to be more suitable than the best systemic host.

An experiment was performed (Table 9) to study the combined effect of two aspects of extraction: (i) the recovery of virus from tissue and (ii) the subsequent preservation of this recovered virus. For this experiment 4 grams of infected Bountiful leaves were homogenized in each of 11 media, and BYMV infectivity was tested after storage for three days at $4^{o}C$. The results (Table 9) indicated that BYMV infectivity in crude extracts from V. faba was best preserved by 0.01M $diPO_4$, pH 7.2 and by 0.01M DIECA.

Since C. amaranticolor seemed to be the best host from which to extract the virus, an experiment was devised to monitor the loss of infectivity at each step of the partial purification. Crude extract was prepared by homogenizing BYMV-infected C. amaranticolor leaves in 30 percent w:v sucrose dissolved in 0.05M Tris-HCl, pH 8.9. The lower speed supernatant was obtained by centrifugation of the CE at

24,000 x g for 40 minutes. The moderate-speed supernatant was obtained by centrifugation of the lower-speed supernatant at 34,000 x g for one hour. The virus in this moderate-speed supernatant was concentration by ultracentrifugation (100,300 x g for 90 minutes). The pellets were resuspended in 0.01M $diPO_4$, pH 7.3.

Table 9. Effect of various homogenizing media on BYMV infectivity in crude extract kept for three days at 4°C.

Homogenizing media	Concentration of reagent in homogenizing media (mM)	Infectivity (dilution 1/10)[2]
$diPO_4$, pH 7.2	10	4/6[3]
Tris-HCl, pH 8.4	10	1/5
Na_2SO_3, pH 7.5	10	2/6
Na_2SO_3	5	1/4
DIECA in H_2O, pH 7.9	10	6/6
DIECA in H_2O, pH 8.1	5	3/6
Ascorbic acid, pH 7.6	10	3/6
Ascorbic acid	5	2/6
p-nitrophenol, pH 7.0	50	2/5
Cysteine-HCl, pH 7.6	10	0/6
Cysteine-HCl, pH 7.6	5	2/6

[1] Isolate GG.

[2] Dilution with distilled water.

[3] Number of Bountiful plants expressing symptoms/number of plants inoculated.

The loss of virus upon the low-speed centrifugation was small when C. amaranticolor was used as the production host (Table 10).

Evidently, the virus was not bound up in inclusions or adsorbed to membranes in this host. A comparison of the infectivity of the high-speed pellet with that of the crude sap indicated that at least 43 percent (95.3 LL/100 sq. cm/218 LL/100 sq. cm) of the virus in the CE was recovered in the high-speed pellet (Table 10).

Table 10. Infectivity retention during extraction of BYMV (isolate VFS-2) from infected C. amaranticolor leaves.

Test	Treatment	Infectivity (LL/100 sq. cm)
1	C. amaranticolor	
	crude sap[1]	4,550.0
	low-speed spin supernatant	5,340.0
2	C. amaranticolor	
	crude sap	218.0
	supernatant after moderate-speed spin	42.9
	supernatant after high-speed spin	10.2
	high-speed pellet	95.3

[1]Samples were diluted 1/10 with 0.025M Tris-HCl, pH. 8.6 before bioassay on C. amaranticolor, except sample 2-a, above which was diluted 1/5.

Selection of the virus isolate. Because strains of viruses differ quantitatively in producing recoverable nucleoprotein (85) four isolates of BYMV were evaluated for virus yield from inoculated C. amaranticolor (Table 11). In this experiment (Table 11) the amount of extractable virus, rather than the total virus present in the crude sap, was

determined.

Table 11. Comparison of virus concentration of four BYMV isolates in C. amaranticolor.[1]

BYMV isolate	Infectivity of the resuspended high-speed pellet (LL/100 sq. cm)
GG	66.4
724	294.0
Y-9	4.8
VFS-2	30.7

[1] Virus was partially purified by ultracentrifugation of the PEG extracts.

BYMV-infected C. amaranticolor leaves were homogenized in 0.05M diPO_4, 0.1M KCl, pH 8.7. The CE was partially clarified by centrifugation at 15,000 x g for 50 minutes, and the resulting supernatant was centrifuged at 35,000 x g for one hour. This partially clarified supernatant was further clarified and concentrated by 4 percent polyethylene glycol (PEG) in 0.1M KCl, 0.05M diPO_4, pH 7.2. The PEG was sedimented by centrifugation at 11,000 x g for 30 minutes. The virus was resuspended in buffer from the PEG sediment. The virus and host proteins in the PEG extracts were concentrated by ultracentrifugation (100,300 x g for 90 minutes). The high-speed pellets for each of the four isolates were resuspended in 0.02M diPO_4, 0.1M KCl, pH 7.5 and bioassayed on C. amaranticolor as described in the Materials and Methods section.

From this experiment it was concluded either that isolate 724 yielded the highest amount of extractable virus, or that the particles were more infectious than those of other isolates.

Homogenization of the Chenopodium amaranticolor Leaves

Re-extraction. As Stanley (83), Price (67), and others have shown, the extraction of virus from plant tissue can be increased by a second extraction from the pulp. An experiment was performed to determine whether re-extraction from C. amaranticolor pulp increased virus yield.

One gram of BYMV-infected C. amaranticolor leaves was homogenized in 2 ml of 0.05M diPO$_4$, 0.1M KCl, pH 7.3. The homogenate was pressed through cheese cloth to yield the first CE. Three-tenths of a gram of the pulp retained in the cheese cloth was re-extracted in 6 ml of buffer, and this homogenate was pressed through cheese cloth to yield the second sample of CE.

The second extraction from C. amaranticolor leaf pulp yielded 30 percent of the infectivity of the first extract (Table 12). Re-extraction therefore was standard in obtaining BYMV from infected C. amaranticolor tissue.

Effect of homogenizing medium. The best homogenizing medium for extracting a given virus from a particular host must first be determined before the highest virus yield can be obtained. An experiment

was performed to compare the relative efficiency of five buffers in extracting the virus from C. amaranticolor leaves. These five were selected as standard buffers from plants virus literature, and from preliminary experiments on buffers.

Table 12. Infectivity of homogenate (crude sap)[1] obtained by first and second extraction from BYMV[1] infected C. amaranticolor leaf tissue.

Test material	Treatment	Infectivity (LL/100 sq. cm)
C. amaranticolor leaves covered with BYMV-induced local lesions.	1. First crude extract[3]	5.8
	2. Re-extraction from the pulp[4] left after first extraction.	1.75

[1] Final extracts diluted 1/10 before bioassay.

[2] Isolate VFS-2.

[3] Homogenized in 0.05M diPO$_4$, 0.1M KCl, pH 7.3.

[4] The pulp was drier than the fresh tissue, but dilution was not affected significantly.

Five grams of BYMV-infected C. amaranticolor leaves were homogenized in each buffer to be tested. The low-speed supernatant was obtained by centrifugation, 13,000 x g for 50 minutes. Further clarification was accomplished by centrifuging this supernatant at 26,000 x g for 50 minutes. The virus with accompanying host materials was concentrated by ultracentrifugation (100,300 x g for 90 minutes). The resulting pellet was resuspended in 0.01M of the

respective buffer being tested (Table 13).

Table 13. The comparative efficiency of five homogenizing buffers in extracting the VFS-2 isolate of BYMV from C. amaranticolor.

Buffer at pH 8.0 with 0.1M KCl	Infectivity of supernatant after low-speed centrifugation (LL/100 sq. cm)	Infectivity of high-speed pellet (LL/100 sq. cm)
1. 0.05M $diPO_4$	0.45	137.2
2. 0.05M HPO_4^--citrate	0.99	95.4
3. 0.05M borate	0.48	88.4
4. 0.05M Tris-HCl	0.0	76.2
5. 5% $NaHCO_3$	0.0	0.0

The quantity of virus remaining in the low-speed supernatant for all buffers was evidently near the sensitivity limit of the local lesion host. Infectivity of resuspended high-speed pellets was high for samples extracted in all buffers except $NaHCO_3$ in which all detectable BYMV infectivity was lost. The amount of extractable virus was greatest in a medium of 0.05M $diPO_4$, 0.1M KCl. Results of this experiment led to the selection of 0.05M $diPO_4$ as the buffer which would best stabilize the virus during homogenization and subsequent clarification.

Effect of pH of $diPO_4$ buffer. The effect of pH range on BYMV

extraction in $diPO_4$ buffer was studied for two reasons. First, a pH divergent from the isoelectric point of the virus was needed to prevent the loss of virus during homogenization. Secondly, a pH which was least harmful to the virus was essential to ensure maximum infectivity and stability of BYMV in the CE.

Optimum pH for the homogenizing buffer was determined by testing BYMV infectivity at 11 pH values (Table 14). CE was prepared by homogenizing BYMV infected C. amaranticolor leaves in 0.05M $diPO_4$, 0.1M KCl at these pH values. The supernatant was obtained by centrifuging CE from infected C. amaranticolor at 13,000 x g for 30 minutes. The pH was not checked after homogenization.[1]

In the first of two tests (Table 14) not only did the virus in the CE appear to withstand the pH range of 4-11 of the homogenating media, but it did not precipitate within that range. In the second test, (Table 14), a further evaluation of the infectivity in the pH range of 7.5-10.0 was made. The supernatant appeared to possess the greatest infectivity in the pH range 8.5-9.0, but infectivity differences in this test were not considered significant.

Effects of reagents used to prevent aggregation. Viruses of the

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[1] The pK_2 of phosphoric acid is 7.12; thus the buffering effect of diphosphate buffer at pH values below 6 and above 8 decreased considerably. The organic acids in the leaves probably decreased the pH of the homogenizing buffer in the cases where pH below 6 or above 8 were tested.

Table 14. Effect of pH range of 0.05M $diPO_4$, 0.1M KCl on BYMV (isolate VFS-2) infectivity.

Test	pH[1]	Infectivity of supernatant (LL/100 sq. cm) after 1/2 dilution with homogenizing buffer
1	4.0	9.88
	5.0	6.3
	6.0	11.1
	7.0	8.6
	8.0	18.7
	9.0	28.7
	10.0	22.0
	11.0	12.6
2	7.5	24.7
	8.0	34.2
	8.5	52.2
	9.0	53.0
	9.5	40.1
	10.0	44.0

[1] pH values between six and eight were obtained by the appropriate combination of KH_2PO_4 and K_2HPO_4. pH values below six or above eight were obtained by the addition to $diPO_4$ buffer of 0.1N HCl or 0.1N KOH respectively.

potato Y group tend to aggregate to each other both laterally and end-to-end (28, 82). The forces causing this aggregation are not clearly understood. If the aggregation occurred as a result of intermolecular disulfide bonding, the reducing agents, thioglycollic acid and beta-mercaptoethanol, might prevent it. If the aggregation resulted from hydrogen bonding, then low concentrations of urea might prevent coupling by competing for the hydrogen bonds. However, if the aggregation were due to hydrophobic forces (28), then high concentrations of urea might be required to break or prevent the hydrophobic bonds (54). The chelating agent EDTA was employed to tie-up the Mg^{++} and thus cause the ribosomes to dissociate (15). This dissociation of ribosomes into their subunits might reduce coupling with virus particles and prevent aggregation of ribosomes to form structures approximating the density of virus particles. Both of these effects would tend to facilitate BYMV purification.

A series of three tests were conducted to evaluate the effect of these reagents on the CE infectivity (Table 15). The CE was prepared by homogenizing BYMV-infected C. amaranticolor leaves in 0.05M diPO_4, 0.1M KCl, pH 7.5 and the reagent to be evaluated.

Test-1 indicated that thioglycollic acid slightly decreased the CE infectivity, while beta-mercaptoethanol substantially increased infectivity and EDTA slightly increased infectivity. Test-2 however, indicated that EDTA actually caused a slight decrease in CE

Table 15. Effect on BYMV[1] infectivity by selected homogenizing media.

Test	Treatment	Infectivity (LL/100 sq. cm) (Dilution, 1/50)
1	1. buffer, control[2]	0.86
	2. buffer with 0.01 M β-mercaptoethanol	4.1
	3. buffer with 0.2% Thioglycollic acid	0.5
	4. buffer with 0.005M EDTA	1.9
		(Dilution, 1/25)
2	1. buffer, control	12.0
	2. buffer with 0.0005M EDTA	7.1
	3. buffer with 0.005M EDTA	7.4
	4. buffer with 0.01M EDTA	9.8
	5. buffer with 0.05M EDTA	7.9
	6. buffer with 0.1M EDTA	5.0
		(Dilution, 1/25)
3	1. buffer, control	28.7
	2. buffer with 0.1M urea	39.6
	3. buffer with 0.2M urea	39.0
	4. buffer with 0.5M urea	39.0
	5. buffer with 1.0M urea	38.7
	6. buffer with 2.0M urea	29.3

[1] Isolate VFS-2 in crude sap.

[2] Crude extract from BYMV-infected C. amaranticolor homogenized in 0.05M diPO$_4$, 0.1M KCl, pH 7.5.

infectivity. The third test, evaluating the effect of urea, indicated that several concentrations of urea added to the homogenizing medium slightly but consistently increased infectivity.

Because these reagents failed to drastically increase BYMV infectivity and because their specific effects on virus particle structure and stability were not known their use was not adopted.

Clarification of the Crude Extract

Preliminary clarification. As previously indicated (Table 10), low-speed centrifugation did not appreciably reduce the infectivity of the C. amaranticolor derived supernatant. Thus, a low-speed centrifugation was the initial step in clarification. Subsequent clarification treatments of the partially clarified extract involved the general approaches of precipitating non-infectious materials and removing them by differential centrifugation.

Effect of heating and freezing. In an attempt to denature and coagulate the noninfectious components, the infectious CE was subjected to a heating and a freezing treatment. BYMV-infected C. amaranticolor leaves were homogenized in 15 percent w:v sucrose, dissolved in 0.05M diPO_4, 0.1M KCl, pH 7.2. The CE was clarified by two centrifugation steps. It was centrifuged at 29,000 x g for one hour, the pellet was discarded, and the resulting supernatant was again centrifuged at 34,000 x g for 50 minutes. The pellet was

discarded and the supernatant was then treated by freezing or heating (Table 16).

Table 16. Effect of heating or freezing of infectious supernatant on clarification and infectivity of the low and high speed pellets.

Part	Treatment	Appearance	Infectivity (LL/100 sq. cm)
1	Resuspended low-speed pellet		
	1. Control[2]	light green	2.36
	2. 50°C for 10 minutes	light green	0.856
	3. 0°C for one hour	light green	4.12
2	Resuspended high-speed pellet		
	1. Control[3]	tan pellet	92.7
	2. 50°C for 10 minutes	blackish pellet	73.4
	3. 0°C for one hour	tan pellet	50.0

[1] Isolate VFS-2.

[2] Untreated low-speed pellet resuspension at 4°C for one hour.

[3] Untreated high-speed pellet resuspension held at 4°C for one hour.

Virus which had precipitated after each treatment was collected by low-speed centrifugation of the treated supernatant (11,000 x g for 15 minutes). The virus remaining in suspension was concentrated by ultracentrifugation (100,300 x g for 90 minutes). The high-speed pellets were resuspended in 0.05M Tris-HCl, 0.1M KCl, pH 8.1,

and bioassayed on C. amaranticolor.

The heat treatment caused no visible clarification of the supernatant, but it did result in a black, high-speed pellet which was less infectious than the control. Since the low-speed pellet was less infectious than the control, the loss of infectivity was not due to a precipitation of the virus upon heating. The freezing treatment also caused no visible clarification, and it did cause a reduction in infectivity of the high-speed pellet. The presence of 15 percent sucrose may have had an effect on the virus upon freezing. The high infectivity of the low-speed pellet indicated that much of the virus precipitated after the freezing treatment. Since neither treatment clarified the supernatant and both decreased infectivity, the heating and freezing treatments were decidedly not useful.

Effect of salt precipitation. The use of $(NH_4)_2SO_4$, (0.4 saturation) was found by Markham (57) to precipitate many plant proteins. Since these precipitated proteins do not readily resuspend, the method of salt precipitation has been used by Kassanis (53), and others, to selectively precipitate the noninfectious protein.

An experiment was performed to evaluate the usefulness of this clarification technique (Table 17). Five grams of BYMV-infected C. amaranticolor leaves were homogenized in 0.05M diPO_4, 0.1M KCl, pH 7.5. The CE was partially clarified by centrifugation at 15,000 x g for 50 minutes. Half of the supernatant was treated with one part

saturated $(NH_4)_2SO_4$ to three parts supernatant; the other half was used as the control. After stirring for one hour at 4°C, both the treated sample and the control were centrifuged at 11,000 x g for 30 minutes to sediment precipitated host proteins. The resulting supernatants were then bioassayed.

Table 17. Effect of saturated $(NH_4)_2SO_4$ on infectious[1] supernatant.[2]

Treatment	Appearance	Infectivity (LL/100 sq. cm)
1. Control: Infectious supernatant in buffer[3]	Clear, bright-greenish supernatant; tiny green low speed pellet.	385.6
2. Treatment: Infectious supernatant in buffer[3] and (0.25 sat.) $(NH_4)_2SO_4$.	Opaque, black-green supernatant; large, dirty-green pellet.	24.4

[1] Isolate 724 of BYMV.

[2] Saturated $(NH_4)_2SO_4$ was added (1v salt: 3v crude extract), after which the resultant precipitate was centrifuged at 11,000 x g for 30 minutes. The pellet was resuspended in 0.05M diPO$_4$, 0.1M KCl, pH 7.5, and bioassayed on C. amaranticolor as described in the Material and Methods section.

[3] Buffer: was 0.05M diPO$_4$, 0.1M KCl, pH 7.5.

The results (Table 17) show that the addition of saturated $(NH_4)_2SO_4$ to the partially clarified sap had a deleterious effect on the virus. Instead of selectively precipitating the noninfectious protein, the high salt concentration either precipitated both virus and host protein or simply inactivated much of the virus. These results agree

with van Regenmortel's (93) evaluation of salting out with $(NH_4)_2SO_4$.

Effect of organic solvents. In previous experiments (Table 6) the effect of several solvents on the infectivity of the CE from BYMV-infected plants was determined. The next objective was to explore the use of some of these and of other organic solvents to denature or precipitate host materials.

The use of n-butanol and chloroform (77, 84) was found to denature host proteins but to stabilize plant viruses. Results of the author's experiments (Table 6) however, indicated that n-butanol inactivated BYMV in the CE from Bountiful bean plants, and that chloroform decreased the infectivity of the low-speed supernatant.

Another solvent which has occasionally been useful in the selective precipitation of proteinaceous host materials is 20-50 percent ethanol as described by Steere (86). However, this technique is not feasible with BYMV, because this virus is inactivated by ethanol (Table 6).

Porter (66) used fluorocarbon for preliminary clarification of CE. Steere (86) noted, however, that fluorocarbons extracted non-viral nucleoproteins from plant tissue. Thus, the use of this solvent would probably increase the amount of impurity of the partially purified virus. For this reason, fluorocarbons were not considered further.

Bercks (11) and Wetter (97) described a successful use of ether and carbon tetrachloride in the partial purification of BYMV. These solvents were therefore evaluated (Table 18). The chelating agent, DIECA (0.000005% or 2.23×10^{-9}M) was added to the ether to act as a preservative. The peroxides present in the ther were not removed by use of Fe_2SO_4 as described by Wetter (97).

Table 18. Effect of the ether-carbon tetrachloride clarification method on BYMV (isolate GG) infectivity.

Treatment	Infectivity of crude sap (LL/100 sq. cm)
V:V (buffer: crude extract)	16.1
V:V (ether-CCl_4: crude extract)	2.9

The ether-carbon tetrachloride clarification was performed as described by Wetter (97) and Bercks (11). Infected P. vulgaris L. var. Bountiful leaves were homogenized in 0.2 percent ascorbate and Na_2SO_3, 0.01M diPO_4, pH 7.3. The treated sample was stirred in v:v diethyl ether: crude sap at 8°C for 15 minutes. After decanting the ether, an equal volume of CCl_4 was added. After the mixture was shaken to dissolve the residual ether in the CCl_4, the CCl_4 was removed. The aqueous portion was then bioassayed on C. amaranticolor as described in the Materials and Methods section. Infectivity results indicated that the addition of ether and carbon tetrachloride

decreased infectivity of the CE.

Effect of pH adjustment. van Regenmortel (93) has pointed out that 80 to 90 percent of the non-viral protein stays in solution at pH 4 to 5. However, Sequeira and Lister (78) used acidification to precipitate a flexuous virus. Thus, acidification of the CE could be used to clarify the extract, provided the virus could be resuspended and remain stable.

An experiment was designed to evaluate the usefulness of this clarification technique. One gm of BYMV-infected C. amaranticolor leaves was homogenized in 0.02M diPO_4, 0.1M KCl at the eight respective pHs (Table 19). The CE was centrifuged at 15,000 x g for 50 minutes to pellet down the virus which had precipitated at the pH tested. The pellet was resuspended in 0.05M diPO_4, 0.1M KCl, pH 7.5 to neutralize the pH and solubilize the precipitated virus. Both the low-speed supernatant and the resuspended low-speed pellet were bioassayed on C. amaranticolor.

The high infectivity of the resuspended low-speed pellet indicated that considerable BYMV was precipitated at all pH values tested. Thus, it was considered a potentially feasible clarification method provided most of the host protein remained in solution. However, under the conditions of these tests the majority of BYMV remained suspended after acidification. Thus, there were no real differences in infectivity over the pH range tested, within fractions. For this

reason pH adjustment was not incorporated into the partial purification procedure.

Table 19. Effect of acidification (clarification) on BYMV[1] infectivity in crude extract.

Fraction tested	Infectivity (LL/100 sq. cm) after homogenization at pH's indicated							
	3.0	3.5	4.0	4.5	5.0	5.5	6.0	7.5
1. Low-speed supernatant diluted 1/3	16.3	9.7	13.9	15.7	19.3	20.3	11.0	3.7
2. Low-speed pellet resuspended in 0.26 ml buffer[2], then diluted 1/20	7.8	8.7	17.6	9.9	7.5	9.1	6.9	4.3

[1] Isolate VFS-2.

[2] The resuspension of the pellet and all dilutions were with 0.05M diPO$_4$, 0.1M KCl, pH 7.5.

Effect of sucrose clarification. Dorner, Kahn and Wildman (31) showed that Fraction-1 protein (carboxydismutase enzyme) constitutes the major portion of the soluble proteins extractable from leaves. Thus the main host protein impurity in partially purified virus samples extracted from C. amaranticolor probably consists of F_1 protein and ribosomal subunits.

The work of Lyttleton and Ts'o (56) has shown that carboxydimutase is a chloroplast enzyme. To prevent the chloroplasts from rupturing, Commoner et al. (25) used 0.5M sucrose in the homogenizing media. Therefore, in the present study 0.90M (30% w:v)

sucrose in the homogenizing media was evaluated as a preliminary clarification method.

Two experiments (Tables 20, 21 and Figures 6 and 7) were designed to evaluate the efficacy of this sucrose clarification method in reducing the amount of host protein in the partially purified BYMV sample. In these experiments CE was obtained by homogenizing BYMV-infected C. amaranticolor leaves in 30 percent w:v sucrose dissolved in 0.05M diPO$_4$, 0.1M KCl, pH 7.0. Clarification of the CE was achieved by three cycles of successively higher speed centrifugation: 17,500 x g for one hour, 29,000 x g for 90 minutes, and 34,000 x g for one hour. The first low-speed supernatant was diluted v:v with buffer thus reducing sucrose concentration to 15 percent. Concentration of the virus and host proteins in the clarified supernatant was achieved by ultracentrifugation (110,300 x g for 90 minutes). The pellet was resuspended in 0.05M Tris-HCl, 0.1M KCl, and 0.005M MgCl$_2$, pH 8.1.

This second high-speed pellet (P-HS$_2$ obtained by a low-speed centrifugation of the first high-speed pellet, see glossary) was highly infectious (Table 20) but it was also impure. Thus an attempt was made to remove host constituents from pellet P-HS$_2$ and from the third high-speed pellet (P-HS$_3$) by density gradient (DG) centrifugation (Table 20, Figure 6). The first DG centrifugation was performed by layering 4 ml of resuspended P-HS$_2$ onto a 10-40 percent sucrose

Table 20. Effect of sucrose clarification followed by density-gradient (DG) centrifugation on yield of partially purified BYMV.[1]

Treatment	Infectivity (LL/100 sq. cm)
1. Crude sap[2]	4.1
2. Second high-speed pellet ($P\text{-}HS_2$)	62.9
3. Third high speed pellet ($P\text{-}HS_3$)	155.45
4. First DG centrifugation of $P\text{-}HS_2$; Two ml DG fractions; ml from meniscus:	
6.5 - 8.5 ml	27.4
8.5 - 10.5 ml	303.5
10.5 - 12.5 ml	162.0
12.5 - 14.5 ml	87.9
14.5 - 16.5 ml	18.0
5. First DG centrifugation of the $P\text{-}HS_3$ from pooled and concentrated $P\text{-}HS_2$; ml from meniscus:	
3.5 - 5.5 ml	40.4
5.5 - 7.5 ml	153.0
7.5 - 9.5 ml	224.0
9.5 - 11.5 ml	209.0
11.5 - 13.5 ml	142.0
13.5 - 15.5 ml	6.2
15.5 - 17.5 ml	3.4

[1] Isolate VFS-2.

[2] The crude sap was bioassayed in 30 percent sucrose. The sucrose reduced its infectivity when inoculated on C. amaranticolor.

Table 21. Effect of first and second cycles[1] of density-gradient (DG) centrifugation on BYMV[2] zonation and yield.

Treatment	Infectivity (LL/100 sq. cm) Trial A	Trial B
1. Crude sap: diluted[3] 1/3	7.1	1.44
2. Infectious fraction from first cycle of DG centrifugation	102.0	53.4
3. Second cycle of DG centrifugation of the infectious fraction from the first DG centrifugation:		
Density gradient tube fractions from the meniscus:		
6.5 - 8.5 ml	0.0	
8.5 - 10.5 ml	2.44	
10.5 - 12.5 ml	105.0	
12.5 - 14.5 ml	27.5	
14.5 - 16.5 ml	13.7	
16.5 - 18.5 ml	4.8	
4.0 - 6.0 ml		0.26
6.0 - 7.5 ml		13.5
7.5 - 9.0 ml		10.2
9.0 - 11.0 ml		6.0
11.0 - 12.5 ml		3.7
12.5 - 14.0 ml		0.4

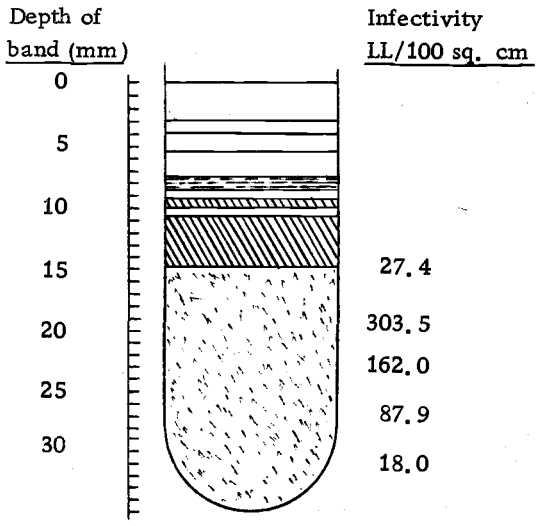
[1] Second high-speed pellet resuspended in 0.4 ml of 0.05M Tris-HCl, 0.1M KCl, and $MgCl_2$, pH 8.1; DG centrifuged, infectious fraction collected, subjected to differential centrifugation, and again to DG centrifugation.

[2] Isolate VFS-2 of BYMV.

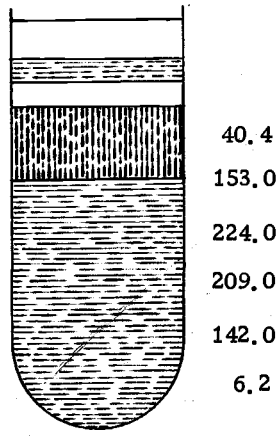
[3] Dilution with 0.05M diPO_4, 0.1M KCl, pH 7.0.

Figure 6. Schematic diagrams of density gradient (DG) columns from the first DG centrifugation. A) Zonation obtained upon the first DG centrifugation of the second high speed pellet ($P\text{-}HS_2$). B) Zonation obtained upon the first DG centrifugation of the third high speed pellet ($P\text{-}HS_3$). The 10-40% (w:v) sucrose gradient was centrifuged at 8,000 x g for 10 minutes, followed by 49,530 x g for 90 minutes.

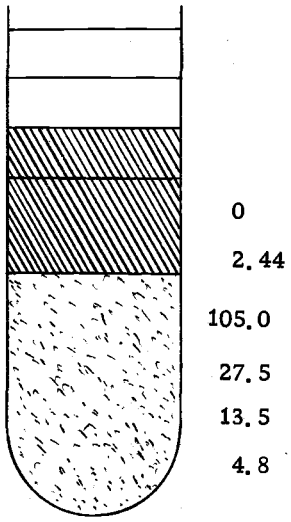
Figure 7. Schematic diagram of density gradient (DG) columns from the second DG centrifugation. Two trials of the same treatment (A & B) showing type of zonation obtained during second cycle DG centrifugation of infectious fraction from first DG centrifugation of $PH\text{-}S_2$. The 10-40% (w:v) sucrose gradient was centrifuged at 8,000 x g for 10 minutes, followed by 49,530 x g for 90 minutes.


Depth of band (mm)
Infectivity LL/100 sq. cm
0
5
10
15
20
25
30
27.4
303.5
162.0
87.9
18.0

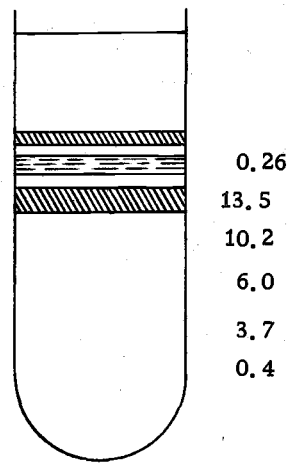
6A


40.4
153.0
224.0
209.0
142.0
6.2

6B


0
2.44
105.0
27.5
13.5
4.8

7A


0.26
13.5
10.2
6.0
3.7
0.4

7B

gradient. The DG was centrifuged in an SW 25.1 rotor at 8,000 x g for 10 minutes, followed by 49,530 x g for 90 minutes. The resultant light-scattering bands (Figure 6A) were collected and respectively pooled. The virus plus host protein in the bands were then concentrated by ultracentrifugation (100,300 x g for 90 minutes), and the pellets were resuspended in 0.05M Tris-HCl, 0.1M KCl, and 0.005M $MgCl_2$, pH 8.1. The resuspensions then were bioassayed on C. amaranticolor.

Pellet, $P\text{-}HS_2$, was 15 times more infectious than the crude sap. Some of the fractions from centrifuged DG columns also were more infectious than this high-speed pellet. The resuspension volume for all pellets was the same. Concentration of the second high-speed pellet by a second cycle of differential centrifugation slightly increased total yield of BYMV from DG columns, but resulted in a more diffuse zone of virus (Table 20, Figure 6B).

Most of the infectivity after a DG centrifugation of pellet $P\text{-}HS_1$ was in the fraction 8.5-10.5 ml from the meniscus (Table 20). This infectious fraction was collected, and its particulate matter was concentrated by ultracentrifugation (100,300 x g for 90 minutes). The pellets were resuspended in 0.05M Tris-HCl, 0.1M KCl, and 0.005M $MgCl_2$, pH 8.1 for further study.

A second cycle of DG centrifugation was repeated in its entirety in an experiment designed to remove the host material from the

infectious fraction obtained from the first cycle of DG centrifugation of pellet P-HS_2. This infectious fraction, was also more infectious than the CE (Table 21). The second DG centrifugation resulted in an equally discrete zonation of the virus but in a lower infectivity of the fractions (Table 21, Figure 7) than did the first cycle of DG centrifugation (Table 20, Figure 6A). Two trials of the second cycle of DG centrifugation were performed. In the first trial (Figure 7A) the rpm was gradually increased from 8,000 x g to 49,530 x g. In the second trial (Figure 7B) the rpm was increased rapidly, thus causing a great degree of shearing in the sedimenting particles. These results indicated that the best zonation and infectivity was obtained from the first cycle of DG centrifugation of the resuspended pellet, P-HS_2.

Effect of adsorbents. Two experiments were conducted to evaluate the two adsorbents, bentonite and Celite, for clarifying CE (Table 22). Infected V. faba leaves were homogenized in 0.05M diPO_4, pH 7.6 and the respective trial adsorbent. CE was centrifuged at 9,000 x g for 15 minutes. The resulting supernatant was clarified by stirring in successive aliquots (1 ml of 0.025-0.4 percent adsorbent per 10 ml of supernatant) of adsorbent followed by a centrifugation of 10,000 x g for 15 minutes. This procedure was repeated until no further clarification was evident.

Infectivity assay results suggested that clarification by bentonite decreased detectable BYMV, probably by adsorption of the virus onto

Table 22. Effect of bentonite and Celite on crude extract (CE) clarification and supernatant[1] infectivity.

Test[2] No.	Treatment	Appearance of supernatant bioassayed	Infectivity[3] (LL/100 sq. cm)
1	a. CE in buffer[4] only, control	Bright, translucent, green, supernatant	376.0
	b. CE in buffer and 0.2% bentonite	Bright, translucent green, supernatant	70.2
	a. CE in buffer only, control	Bright, translucent green, supernatant	112.0
	b. CE in buffer and 0.025% Celite	Bright, translucent green, supernatant	95.1
2	a. CE in buffer only, control	Dark green, translucent supernatant	37.7
	b. CE in buffer and 0.4% bentonite	Opaque, black-green supernatant	17.1
	a. CE in buffer only, control	Dark green, translucent supernatant	49.5
	b. CE in buffer and 0.4% Celite	Dark green, translucent supernatant	56.8

[1]
[2]Supernatants were obtained by low-speed centrifugation of the infectious CE from Vicia faba.
[3]Test 1 and 2 differed only in the concentrations of adsorbents.
[4]Isolate VFS-2 of BYMV.
0.05 $diPO_4$, pH 7.6.

bentonite particles. Clarification with Celite did not appear to reduce infectivity. By comparison with untreated supernatant, however, Celite treatment provided no reduction in pigment density or opacity of the infectious supernatant.

Effect of polyethylene glycol. In their paper on filamentous viruses, Sequeira and Lister (79) obtained satisfactory clarification of plant extracts by polyethylene glycol (PEG) treatment. The use of PEG and KCl for precipitation of plant viruses was introduced by Hebert (46). On the basis of their results a series of four tests was herein designed to evaluate the efficiency of clarification by use of PEG (Table 23).

In these tests BYMV-infected C. amaranticolor leaves were homogenized in 0.05M diPO_4, 0.1M KCl, pH 7.5 or in 0.05M diPO_4, 0.3M KCl, pH 7.5. The CE was centrifuged at 11,000 x g for one hour. The resulting supernatant was centrifuged at 34,000 x g for 50 minutes to yield a partially clarified infectious supernatant.

In the first test aliquots of this supernatant were further clarified with selected PEG and salt concentrations (Table 23). PEG was stirred into the supernatant for one hour at 8°C. The PEG was then sedimented at 11,000 x g for 30 minutes, and the virus was extracted from the PEG sediment for a period of 14 to 18 hours in 0.02M diPO_4, 0.1M KCl, pH 7.5. Both the supernatant after PEG sedimentation and the PEG extract were subjected to ultracentrifugation (100,300 x g for

Table 23. Evaluation of BYMV[1] sedimentation by polyethylene glycol (PEG).

Test 1 — Optimum PEG and KCl concentration:

Treatment		Infectivity[2] (LL/100 sq. cm)	
PEG percent	KCl(M)	supernatant	pellet
0	0.1	16.4	--
2	0.1	16.2	8.1
4	0.1	0.0	29.7
2	0.3	71.0	20.6
4	0.3	0.0	37.8
6	0.3	0.0	0.41

Test 2 — Four percent PEG, 0.1M KCl:

Treatment	Infectivity (LL/100 sq. cm)
1. Supernatant after PEG clarification concentrated by high speed centrifugation.	0.0
2. Extract from PEG sediment, concentrated by high speed centrifugation.	40.6
3. Resuspended PEG sediment after extraction.	0.25

Test 3 — PEG clarification in $diPO_4$ buffer at two different pH's:

pH maintained throughout test	Infectivity of high speed pellet (LL/100 sq. cm)
1. 7.5	56.2
2. 8.7	46.2

Test 4 — Resuspension of high speed pellets in two buffers; PEG extract concentrated by high speed centrifugation and resuspended in:

Treatment	Infectivity (LL/100 sq. cm)
1. 0.02M $diPO_4$, 0.1M KCl, pH 7.3	49.7
2. 0.025M Tris-HCl, 0.1M KCl, pH 8.1	46.0

[1] Isolate VFS-2.

[2] The supernatant and the resuspended pellets were diluted 1/10 in the 0.02M $diPO_4$ buffer plus homologous KCl concentration before bioassay.

90 minutes), and the resultant pellets were resuspended in 0.02M Tris-HCl, 0.1M KCl, pH 8.1 for bioassay on C. amaranticolor.

The results of this test (Table 23) indicated that all detectable BYMV was precipitated with the PEG sediment when 4 percent PEG, 0.1M KCl was used. A second test (Table 23) confirmed this and indicated that the virus was readily extractable from this PEG sediment.

As previously shown (Table 14), the optimum pH for extraction of BYMV from C. amaranticolor was between 8.5 and 9.0. In a third test PEG clarification at pH 7.5 was compared with that at pH 8.7. The results (Table 23) indicated that PEG clarification in 0.05M $diPO_4$ at pH 7.5 yielded slightly higher infectivity of the partially purified pellet than at pH of 8.7.

Previous experiments (Table 13) indicated that 0.05M $diPO_4$, 0.1M KCl, pH 8.0 was the most efficient buffer for the extraction of BYMV from C. amaranticolor. Although Tris-HCl buffer was found to readily resuspend high-speed pellets, $diPO_4$ buffer was selected for the partial purification procedure because chlorophyll present in the high-speed pellets was less soluable in this buffer.

Comparison of four clarification methods. Although bentonite (p. 80), ether-carbon tetrachloride (p. 72), and sucrose (p. 74) clarification had not proven useful in previous experiments, the PEG treatment yielded good results. Thus, an experiment was designed

to compare the relative effectiveness of the foregoing four methods in achieving a partial purification of BYMV.

For each method an equal amount of BYMV-infected C. amaranticolor leaves was used as the virus source. Three ml of the partially clarified and concentrated virus were layered on top of the density-gradient. DG centrifugation was performed in a 10-40 percent w:v sucrose gradient, which had been equilibrated for 36 hours. The DG columns were centrifuged in an SW 25.1 rotor at 8,000 x g for 10 minutes and then at 49,300 x g for 90 minutes. The sucrose treatment resulted in DG bands (Figure 6), but the PEG, bentonite, and ether-carbon tetrachloride treatments did not. The infectivity was highest, respectively, at 20 mm, 32 mm, 49 mm and 32 mm below the meniscus for the PEG, ether-carbon tetrachloride, bentonite, and sucrose treatments. The virus in the DG fractions was concentrated by ultracentrifugation (100,300 x g for 90 minutes), and the pellets were resuspended in 0.02M diPO_4, 0.1M KCl, pH 7.3. These resuspended pellets then were bioassayed on C. amaranticolor.

For the PEG treatment, BYMV-infected C. amaranticolor leaves were homogenized in 0.05M diPO_4, 0.1M KCl, pH 7.5. The CE was centrifuged at 15,000 x g for 50 minutes, and the resulting supernatant was centrifuged at 34,000 x g for one hour. This partially clarified supernatant was brought to 4 percent PEG, 0.1M KCl. After stirring for one hour at 8°C, the PEG in the supernatant was

sedimented by a centrifugation of 11,000 x g for 30 minutes. The PEG sediment was extracted in 0.02M diPO$_4$, 0.1M KCl, pH 7.3 for 14 hours. The virus plus host proteins in this PEG extract were concentrated by ultracentrifugation (100,300 x g for 90 minutes). The pellet was resuspended in 0.02M diPO$_4$, 0.1M KCl, pH 7.5, subjected to DG centrifugation, and the DG columns were scanned photometrically. Fractions were taken and bioassayed on C. amaranticolor (Figure 8A). A sharp infectivity peak occurred in an area of low optical density (254 nm). The 8 LL/100 sq. cm at the peak indicated that a moderate amount of virus was concentrated in this area. Host-derived materials, presumably ribosomes and soluble proteins accounted for most of the optical density.

For the ether-carbon tetrachloride method (Figure 8B), the crude sap and supernatant were prepared as for the PEG method. Diethyl ether and carbon tetrachloride were added to an equal volume of supernatant, and after homogenization the aqueous phase was recovered. The virus plus host constituents in this partially clarified aqueous phase were concentrated by ultracentrifugation (100,300 x g for 90 minutes). The pellets were resuspended in 0.02M diPO$_4$, 0.1M KCl, pH 7.5. These resuspended pellets were subjected to DG centrifugation. The DG columns were scanned photometrically, fractionated, and the fractions were bioassayed on C. amaranticolor. The fractions contained only trace quantities of infectious BYMV (Figure 8B).

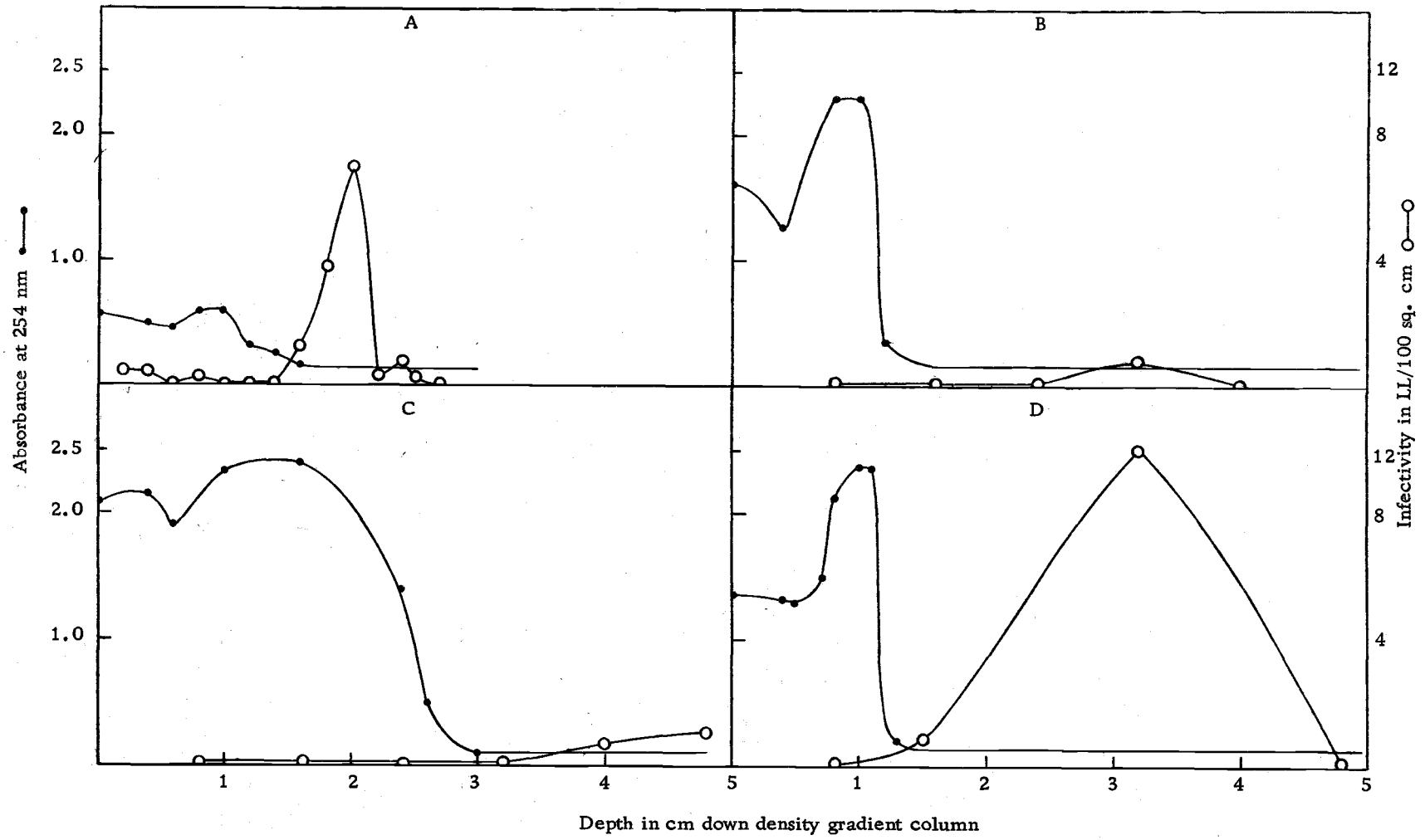


Figure 8. Absorbance and infectivity profiles (254 nm) of refined plant extracts containing BYMV. A) Polyethylene glycol treatment of moderately centrifuged (clarified) extract. B) Ether-carbon tetrachloride clarification. C) Bentonite clarification. D) Sucrose clarification.

Infectivity occurred in a region of low optical density. On the basis of bioassay results and ultraviolet absorbancy profiles, it was concluded that light-scattering DG bands contained principally host materials.

In the bentonite clarification method (Figure 8C), the homogenizing medium was 0.05M diPO$_4$, 0.1M KCl and 10 ml of bentonite/100 ml of buffer, pH 7.5. Addition of 2 ml of one percent bentonite/50 ml was repeated three times, followed by centrifugation at 6,000 x g for 15 minutes. For further clarification this supernatant was centrifuged at 34,000 x g for one hour. The virus plus host constituents in the supernatant were concentrated by ultracentrifugation (100,300 x g for 90 minutes). The pellets were resuspended in 0.02M diPO$_4$, 0.1M KCl, pH 7.5. This resuspension was subjected to DG centrifugation, and the DG columns were scanned photometrically. The DG fractions were collected and concentrated as in the above two methods. Again, the fractions contained only trace amounts of infectious BYMV (Figure 8C). Infectivity occurred in a region of low optical density, while the host materials accounted for most of the optical density.

In the sucrose clarification experiment, the homogenizing media was 15 percent w:v sucrose dissolved in 0.05M diPO$_4$, 0.1M KCl, pH 7.5. The partially clarified supernatant was prepared by subjecting the CE to three successive centrifugations: 15,000 x g for 50 minutes, 27,000 x g for one hour, and 34,000 x g for one hour. The

virus plus host proteins in the partially clarified supernatant were concentrated by ultracentrifugation (100,300 x g for 90 minutes), and the resulting pellets were resuspended in 0.02M diPO_4, 0.1M KCl, pH 7.5. The resuspended pellets were subjected to DG centrifugation and scanned photometrically. The DG columns were fractionated, BYMV plus host constituents were concentrated by ultracentrifugation, and the resulting pellets were resuspended as above. The resuspended pellets were bioassayed on C. amaranticolor. Infectivity occurred in a wide region of the DG column (Figure 8D), principally in a low optical density region, while host materials accounted for virtually all of the optical density. The infectivity peak of 10 LL/100 sq. cm indicated moderate amounts of BYMV were concentrated in that area.

A comparison of these four methods indicated that the ether-carbon tetrachloride and bentonite methods yielded little infectious BYMV. From this evaluation of partial purification methods, PEG was concluded to be superior because infectious BYMV banded into a more compact DG band and was accompanied by less material than resulted by the sucrose method.

Comparison of clarification methods followed by PEG treatment. A comparison of precursory clarification methods was needed to provide maximal BYMV content before PEG precipitation. Four tests were designed to compare the infectivity of partially purified BYMV obtained by ether-carbon tetrachloride clarification, bentonite

clarification, and sucrose clarification each followed by the PEG treatment, and by the PEG treatment alone. These four tests were also designed to show whether or not absorbancy profiles reflected the increased purity of BYMV resulting from PEG precipitation.

Clarification by the ether-carbon tetrachloride method (p. 72), the bentonite method (p. 80), and the sucrose method (p. 74) were performed as previously described. Following clarification, the respective extracts were precipitated by adding 4 percent PEG, 0.1M KCl.

PEG treatment without previous clarification yielded the most infectious preparation (Table 24).

Table 24. Effect of PEG treatment and PEG combined with three clarification procedures on the infectivity of the partially purified virus.[1]

Test	Treatment	Infectivity (LL/100 sq. cm) of partially purified virus
A	PEG treatment of low-, and moderate-speed centrifuged crude extract.	16.3
B	Ether-CCl_4 followed by PEG treatment	0.26
C	Bentonite followed by PEG treatment	4.2
D	Sucrose followed by PEG treatment	9.7

[1] Isolate 724 of BYMV.

These results confirmed previous indications that these precursory clarification treatments were unsatisfactory for extracting infectious BYMV.

Absorbancy profiles of DG columns (Figure 8) obtained from the three clarification treatments prior to PEG treatment indicated the presence of host constituents in the partially purified virus sample. Absorbancy profiles of DG columns of the three clarification treatments subsequent to PEG treatment showed the same general pattern (Figure 9). However, the amount of host material in the infectious DG fraction was much lower when the clarified extract had been treated with 4 percent PEG, 0.1M KCl (Figure 9). Thus, PEG seemed to selectively precipitate BYMV excluding much of the host proteins. For this reason it probably represented the single most beneficial step in producing partially purified BYMV. On the basis of these various results a flow diagram was constructed (Figure 10) to summarize the method found most useful for partially purifying BYMV.

Evaluation of Purification Procedures

Attempts were made to further purify BYMV, since a purified virus is necessary for *in vitro* studies. A technique was needed which would separate BYMV particles from host proteins and nucleoproteins in partially purified virus preparations, and which would concentrate the virus particles without decreasing their stability.

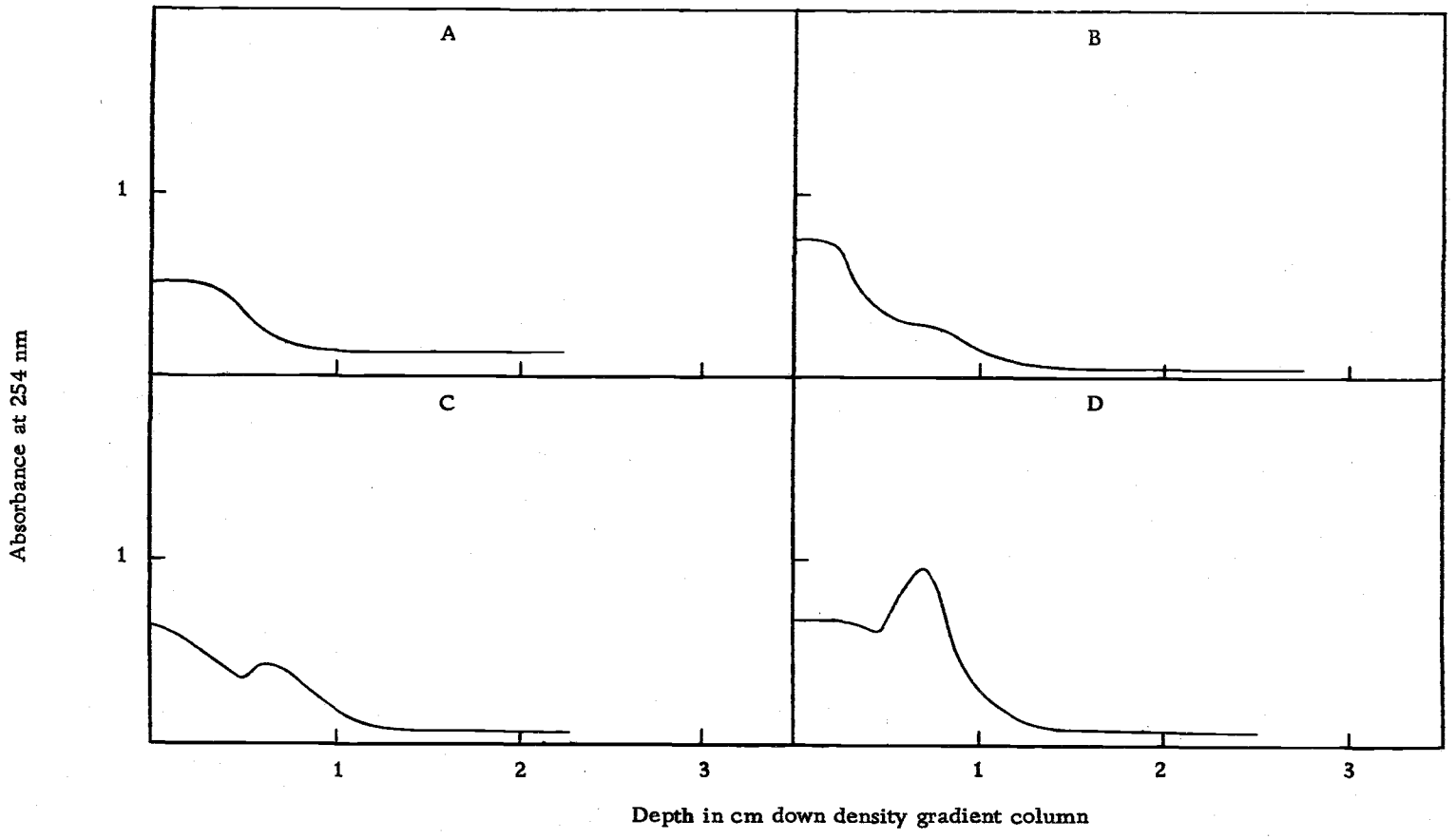


Figure 9. Absorbance profiles (254 nm) of refined plant extracts containing BYMV. A) Polyethylene glycol (PEG) treatment of moderately centrifuged (clarified) extract. B) Ether-carbon tetrachloride clarification followed by PEG precipitation. C) Bentonite clarification followed by PEG precipitation. D) Sucrose clarification followed by PEG precipitation.

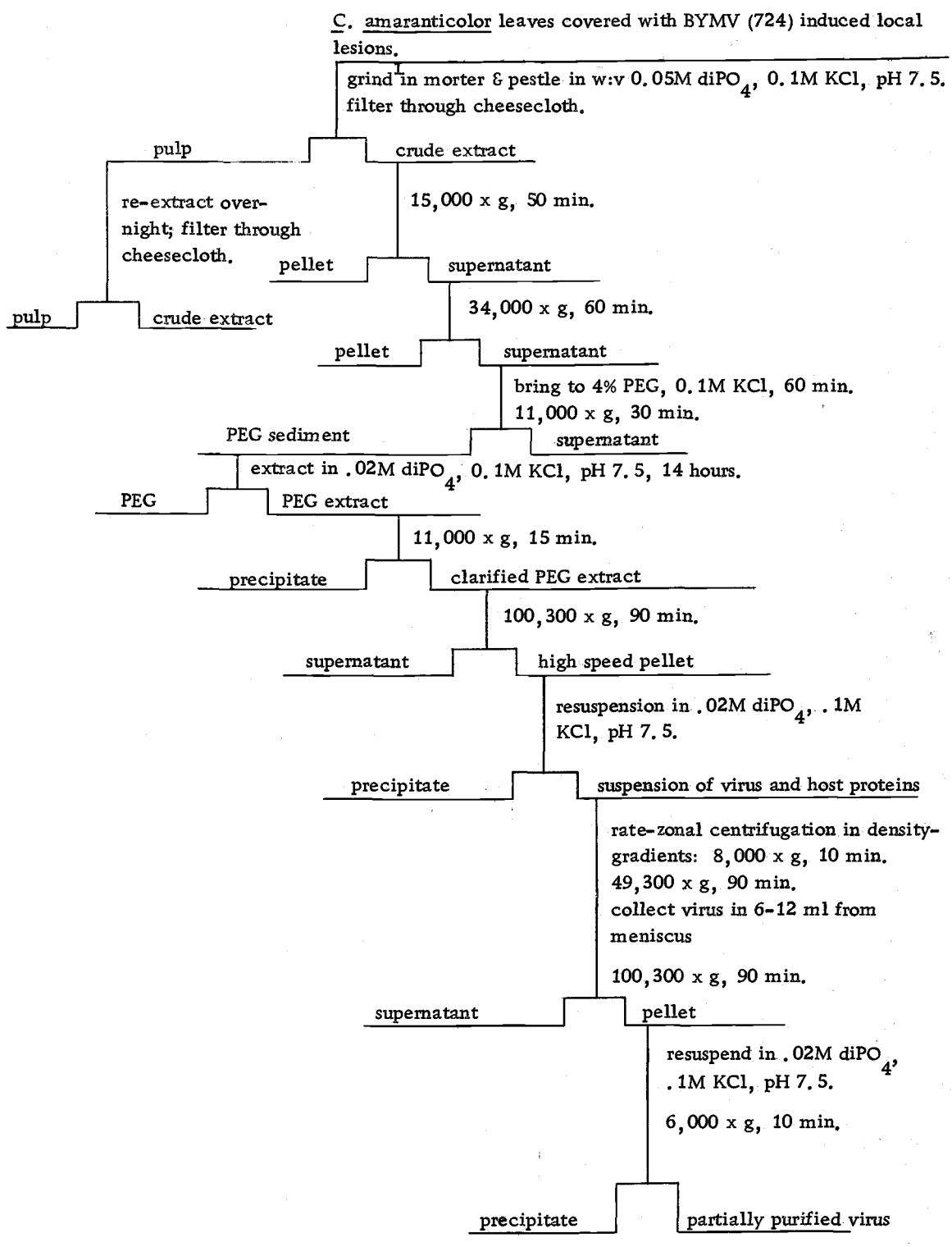


Figure 10. Partial purification procedure for bean yellow mosaic virus.

[1]Samples maintained at 4°C throughout procedure.

The only methods which had proven useful for partially purifying BYMV were the physical treatments of differential centrifugation and selective precipitation. Thus four additional physical methods were evaluated in attempts to obtain a suspension of purified BYMV including gel filtration, density-gradient zone electrophoresis, rate-zonal centrifugation by zonal rotor, and a further evaluation of density-gradient centrifugation.

Gel filtration. Sepharose gel filtration, a technique which takes advantage of particle size differences, was used in an attempt to achieve separation of virus particles from host proteins. The assumption was that the long flexuous BYMV particles would be excluded from the Sepharose beads thus migrating rapidly between beads while the smaller ribosomal subunits and fraction 1 protein particles would diffuse through the gels at a slower rate. The virus was therefore expected to elute more rapidly than host proteins.

The partially purified virus sample was obtained by ultracentrifugation (100,300 x g for 90 minutes) of the PEG extract (Figure 10). Three ml of this infectious, partially purified virus sample were eluted through a Sepharose 2B column at a flow rate of 1.12 ml/minutes. The eluant was 0.01M diPO_4, 0.1M KCl, pH 7.5. Fractions were collected and their particulate matter was concentrated by ultracentrifugation. The pellets were resuspended in 0.02M diPO_4, 0.1M KCl, pH 7.5. The virus eluted in two regions (Figure 11), but most of

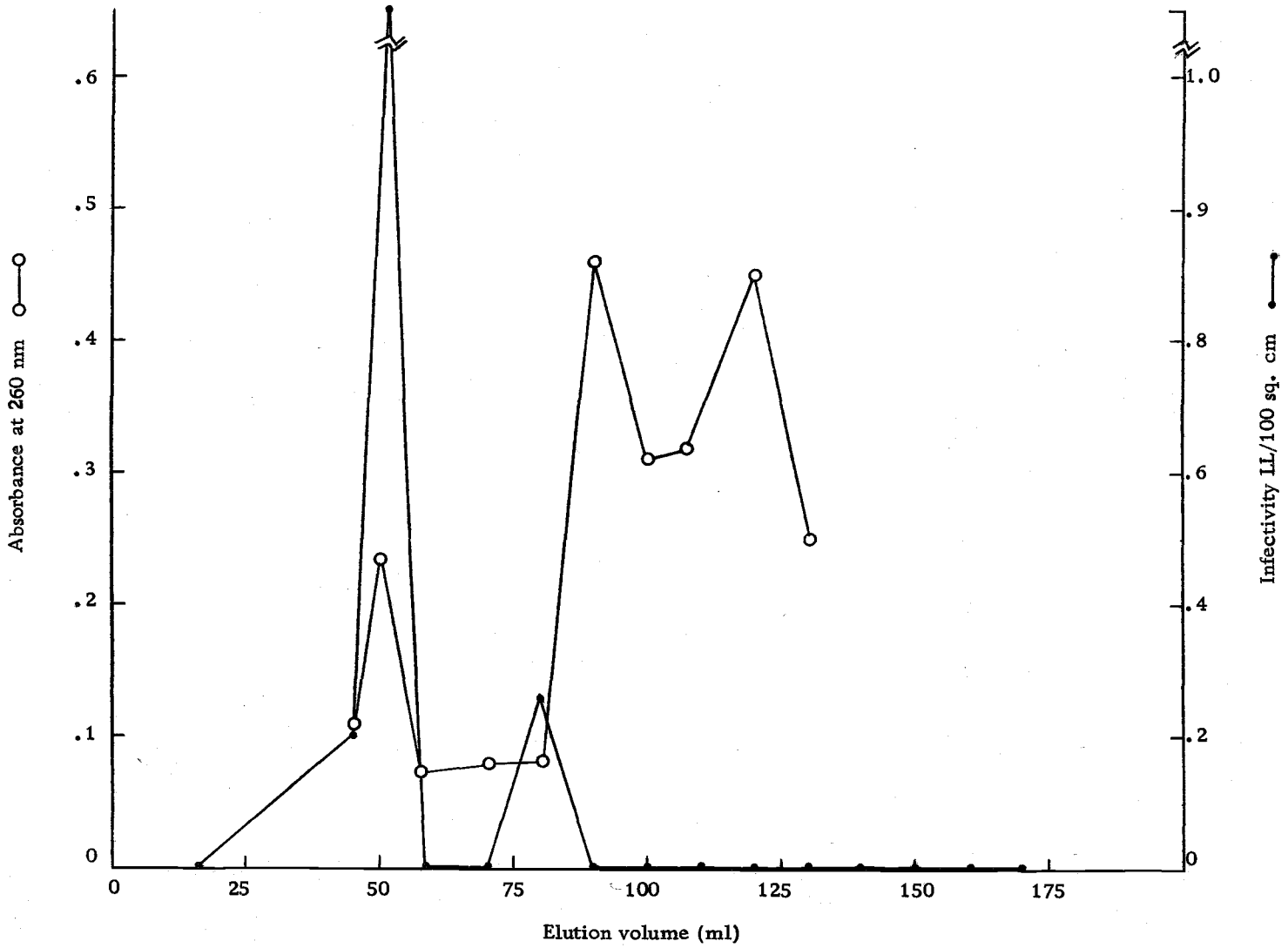


Figure 11. Infectivity and absorbance profiles produced by gel (Sepharose 2B) filtration of a partially purified BYMV preparation.

it came through as an isolated fraction between 44 ml and 55 ml elution volume. The high optical density at 254 nm shown by the two peaks at 90 ml (260/280 ratio = 1.3) and at 120 ml (260/280 = 1.09) were probably due to polyribosomes and fraction 1 protein respectively.

Although a sharp peak of infectivity was obtained, the infectivity of fractions between 44 and 55 ml was very low relative to the infectivity of the partially purified sample introduced at the top of the gel (Table 24A). The dilution followed by reconcentration of the sample by ultracentrifugation resulted in an unacceptable loss of infectivity. For this reason gel filtration was unsuitable for BYMV purification.

Zone electrophoresis. This technique has been used to achieve a separation of viruses from the noninfectious plant proteins present in partially purified virus preparations (92, 93, 94). Previously, this technique was employed in an attempt to separate virus particles from chloroplasts. Here it was used to separate virus particles from host proteins on the basis of their different rates of migration in an electric field.

The technique of zone electrophoresis used in this experiment was that described by van Regenmortel (93). Partially purified virus was obtained by ultracentrifugation (100,300 x g for 90 minutes) of PEG extract (Figure 10). One and a half ml of this infectious, partially purified virus sample in 39 percent sucrose were introduced

into the zone-electrophoresis column through the lower center capillary tube (Figure 1). Upon completion of electrophoresis, two light-scattering bands had formed in addition to the region where the sample was introduced. These two zones occurred at 7-10 cm and at 13-17 cm above the capillary tube (point of sample introduction). Five-ml fractions were collected, and the suspended materials were concentrated by ultracentrifugation. The pellets were resuspended in 0.02M diPO_4, 0.1M KCl, pH 7.5, and bioassayed.

Infectivity results indicated that BYMV became distributed throughout the electrohphoresis column and was thus markedly diluted. The infectivity detectable in fractions was low relative to that of the partially purified sample introduced into the column. It is not felt that virus inactivation per se was a factor. Although absorbance (260 nm) was highest at the light-scattering zones (Figure 12), only background levels of infectivity were measured in these zones. Thus, these zones were probably concentrations of host materials. The 260/280 ratios for these zones were less than 1.3, thus the host materials probably consisted mainly of fraction 1 protein rather than ribosomal nucleoprotein. Results of this experiment indicated that zone electrophoresis failed to isolate or concentrate BYMV particles.

As previously described (Figures 8A, 9A) PEG precipitation yielded partially purified virus with less accompanying host protein than any other method tried. Sucrose clarification resulted in

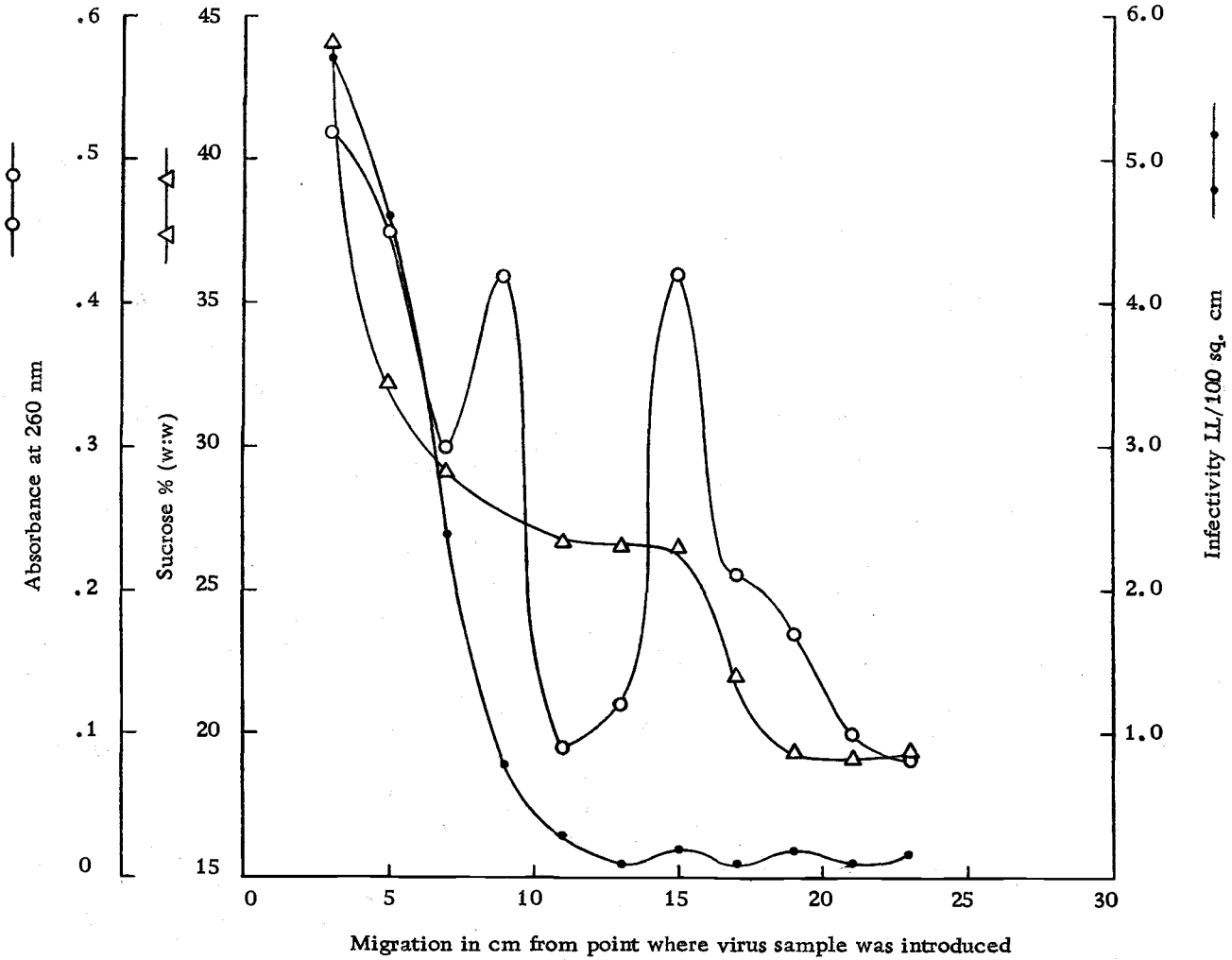


Figure 12. Infectivity and absorbance profiles obtained by density-gradient zone electrophoresis of a partially purified BYMV preparation. Linearity of sucrose concentration gradient is shown.

preparations which were highly infectious, but contained large amounts of host derivatives (Figures 8D, 9D). An experiment was therefore, performed to compare results from sucrose clarification followed by DG centrifugation in a zonal rotor with those obtained previously by use of PEG (Figure 10). It was anticipated that by using the zonal rotor, with large volumes of partially clarified supernatant, concentration of BYMV and simultaneous separation from the host proteins could be achieved.

Zonal rotor centrifugation. In this experiment the CE was obtained by homogenization of BYMV-infected C. amaranticolor leaves. The homogenizing medium was 30 percent w:v sucrose dissolved in 0.05M diPO_4, 0.1M KCl, 0.005M $MgCl_2$, 1 gm phenyl thiourea/liter of buffer, pH 7.3. Preliminary clarification of the CE was achieved by three centrifugation steps: 17,400 x g for one hour, (the supernatant was then diluted v:v in buffer) 29,000 x g for 90 minutes, and 34,000 x g for 50 minutes. Six hundred and thirty-five ml of this partially clarified supernatant was introduced into the zonal rotor. The zonal rotor was run for three hours at 22,800 rpm. The value of w^2t was derived as follows: $w^2t = \frac{(2 \times 3.14 \times rpm)^2}{60} = \frac{(2 \times 3.14 \times 22,800)^2}{60} \times 10,800 = 6.15 \times 10^{10}$ sec.$^{-1}$. The density gradient formed was 8-64 percent w:w (see glossary).

Upon completion of the centrifugation, 15 ml fractions were collected, and the virus plus host constituents were concentrated by

ultracentrifugation (100,300 x g for 90 minutes). The pellets were resuspended in 0.025M Tris-HCl, 0.1M KCl, 0.005M $MgCl_2$, pH 8.1, and bioassayed on V. faba. All detectable infectivity occurred in fractions 22 through 25 (Figure 13). Except for possible undetectable virus loss, the BYMV contained in 635 ml of partially clarified supernatant had been concentrated to 60 ml. Ultracentrifugation of these fractions gave large, dark green pellets. The resuspension of these pellets was a translucent, deep green color.

The results of this experiment showed that a combination of the sucrose clarification and zonal rotor methods would concentrate the virus. The deep green appearance and the high 260/280 ratio of the infectious fraction (Table 25), indicated that as in the sucrose clarification alone (Figures 8A, D and 9A, D), more host material occurred than when PEG precipitation was employed. For these reasons zonal rotor centrifugation was finally rejected as a means of purifying BYMV.

Density-gradient centrifugation. Application of the technique of DG centrifugation provided better concentration and purification of BYMV (Figure 8A and 10) than the above methods. Thus, further attempts at purification by employing DG centrifugation were made.

Of the four main methods evaluated for preparing a partially purified virus (PEG treatment, ether-CCl_4 with PEG, bentonite with PEG, and sucrose clarification with PEG, Table 24), the PEG

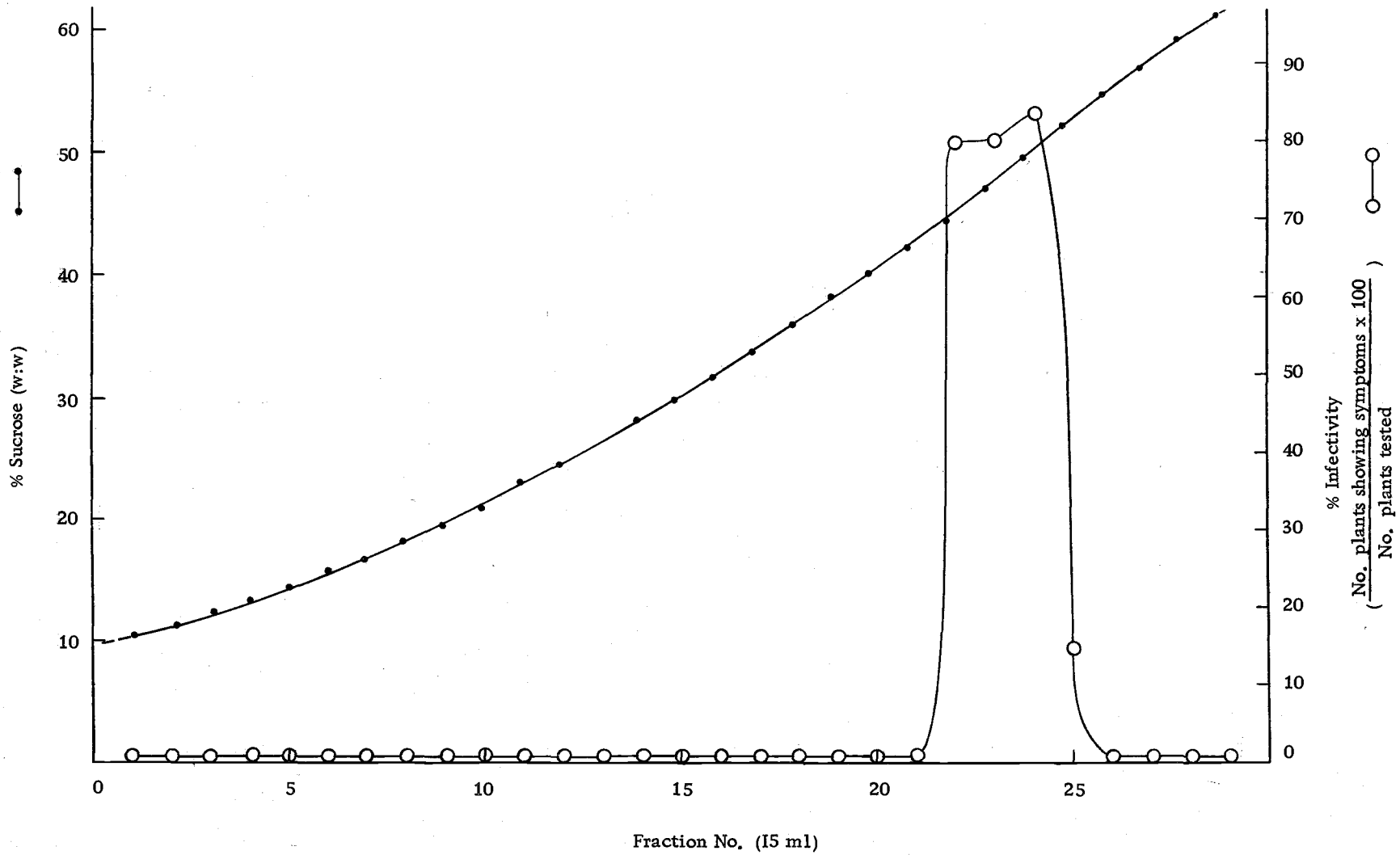


Figure 13. Zonal rotor density gradient centrifugation to purify a partially clarified BYMV sample. A B-XV zonal rotor was used ($w^2t = 6.150 \times 10^{10}$ sec^{-1}). The distribution of sucrose concentration in the zonal rotor with corresponding infectivity profile are shown.

treatment seemed to result in the least amount of aggregation and impurity. The objective was to increase virus yield and to reduce host constituents prior to DG centrifugation.

It was anticipated that virus-particle aggregation due to disulfide linkages could be prevented by clarifying CE in the presence of beta-mercaptoethanol. It was also expected that the addition of EDTA to chelate Mg^{++} ions, would facilitate dissociation of ribosomes into their subunits, thus making it possible to separate them from virus particles.

The effects of incorporating 0.01M beta-mercaptoethanol and/or 0.01M EDTA during BYMV extraction on infectivity and absorbancy profiles of final BYMV preparations were therefore evaluated (Table 24, Figure 14). Equal amounts of BYMV-infected C. amaranticolor leaves was homogenized in 0.05M diPO_4, 0.1M KCl, pH 7.5 plus the respective compound to be evaluated. Partially purified virus preparations were obtained by ultracentrifugation of the PEG extract in Figure 10. Partially purified virus preparations from each of four tests were then diluted 1/5 and bioassayed on C. amaranticolor.

The partially purified virus preparation was DG centrifuged (10-40% w:v sucrose gradient) at 8,000 x g for 10 minutes and then at 49,300 x for 90 minutes in an SW 25.1 rotor. The DG columns were fractionated and scanned photometrically. The fractions were concentrated by ultracentrifugation (100,300 x g for 90 minutes),

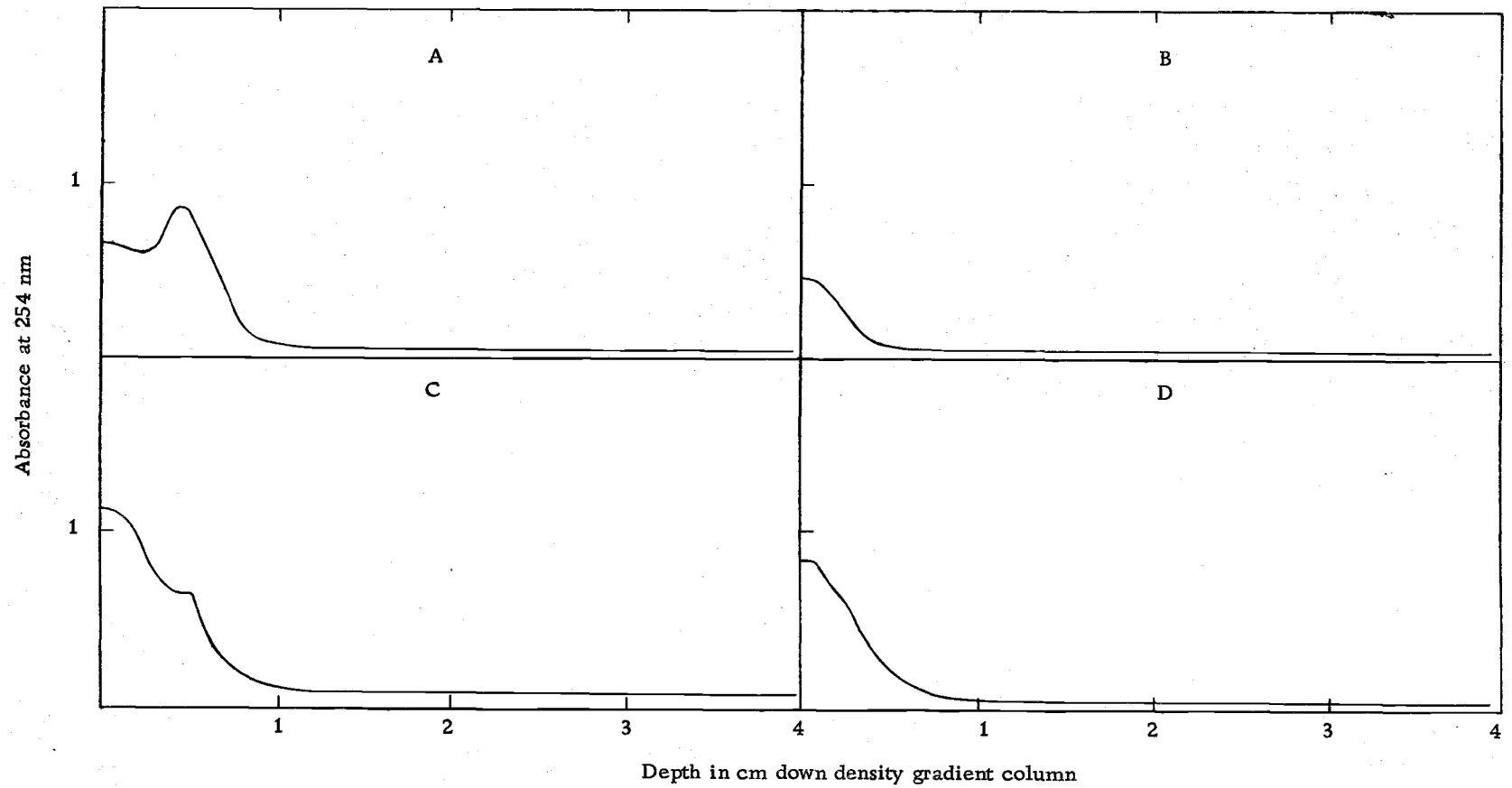


Figure 14. The effect of selected chelating and reducing agents on the absorbance profiles of partially purified virus preparations. A) 0.05M $diPO_4$ with 0.1M KCl, pH 7.5 buffer. B) Buffer with 0.01M ethylenediamine tetraacetic acid (EDTA). C) Buffer with 0.01M beta-mercaptoethanol. D) Buffer with 0.01M EDTA and 0.01M beta-mercaptoethanol.

resuspended in the respective compound being tested, and bioassayed on C. amaranticolor.

Infectivity results for the partially purified virus samples (Table 26) indicated that EDTA, beta-mercaptoethanol, or a combination of these two reduced infectivity relative to that of the buffer alone. Absorbancy profiles from these experiments (Figure 14) suggested that these compounds reduced the quantity of host protein in the final preparation. However, this decrease in host protein was accompanied by decreased infectivity (Table 26). Therefore, these compounds were not used in the preparation of the partially purified BYMV.

As previously illustrated (Figure 8A), DG centrifugation was used to achieve good separation of the virus from most of the host protein. An additional experiment was performed to more clearly define the zone of greatest BYMV concentration and purity following DG column centrifugation. This experiment was carried out as previously described (Figure 10), except that columns were subdivided into 2 ml fractions. The virus in each fraction was concentrated by ultracentrifugation (100,300 x g for 90 minutes); the resultant pellets were resuspended in 0.02M diPO_4, 0.1M KCl, pH 7.5, and bioassayed on C. amaranticolor. The ultraviolet absorption profiles (Figure 15) of the 2 ml fraction 12-16 mm below the meniscus of the DG column indicated that it contained the most highly purified suspension of monodispersed BYMV obtained during this study. The DG columns showed

Table 25. Ultraviolet absorbance of infectious BYMV[1] fractions after zonal rotor centrifugation of sucrose clarified supernatant.[2]

Fraction No.	Dilution	As_{260}	As_{280}	260/280
19	1/16	0.85	0.51	1.66
21	1/16	0.63	0.358	1.75
23	1/8	0.29	0.12	2.4
25	1/8	0.035	0.01	3.5

[1] Isolate 724.

[2] This supernatant was prepared by homogenization of BYMV-infected C. amaranticolor tissue in 30 percent w:v sucrose in 0.05M $diPO_4$, 0.1M KCl, 0.005M $MgCl_2$, 1 gm phenyl thio urea/l of buffer, pH 7.3. The crude extract was followed by three successive higher steps of low-speed centrifugation.

Table 26. Infectivity of partially purified virus[1] when treated with β-mercaptoethanol and/or EDTA.

Test	Treatment	Infectivity (LL/100 sq. cm)
A	buffer[2], control	41.1
B	buffer with 0.01M EDTA	36.6
C	buffer with 0.01 M β-mercaptoethanol	15.0
D	buffer with 0.01M EDTA with 0.01M β-mercaptoethanol	26.8

[1] Isolate 724 of BYMV.

[2] Buffer was 0.05M $diPO_4$, 0.1M KCl, pH 7.5.

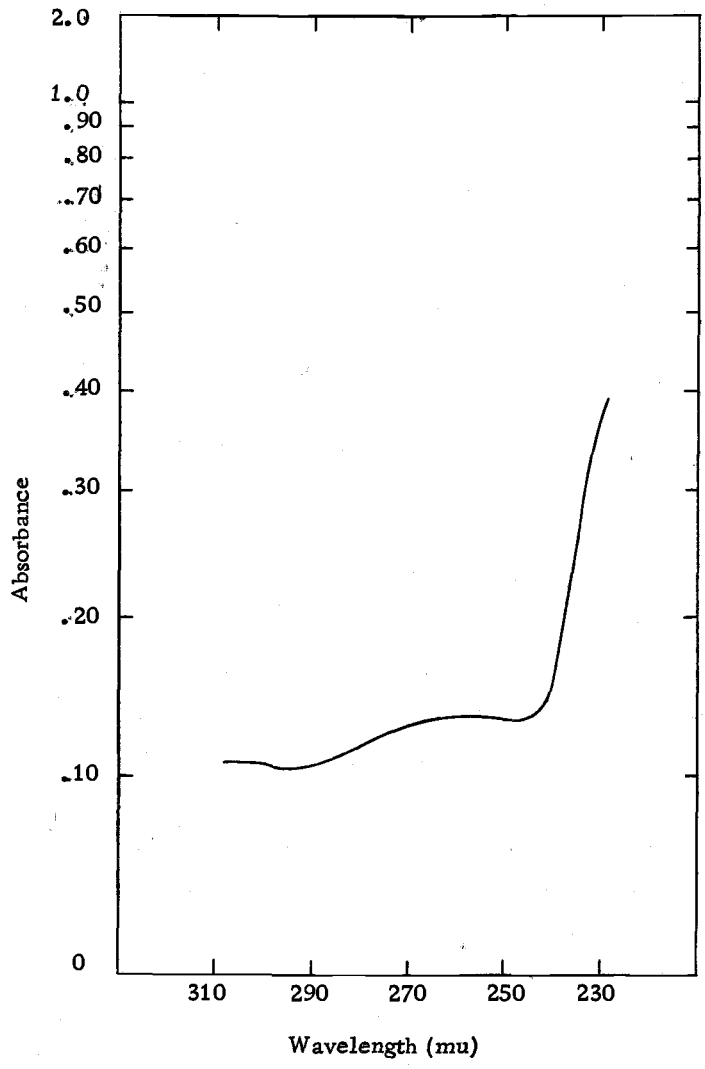


Figure 15. Ultraviolet absorption spectrum of a partially purified BYMV sample after density gradient (DG) centrifugation. This sample corresponded to the fraction 12-16 mm below the meniscus of the DG column (Table 27).

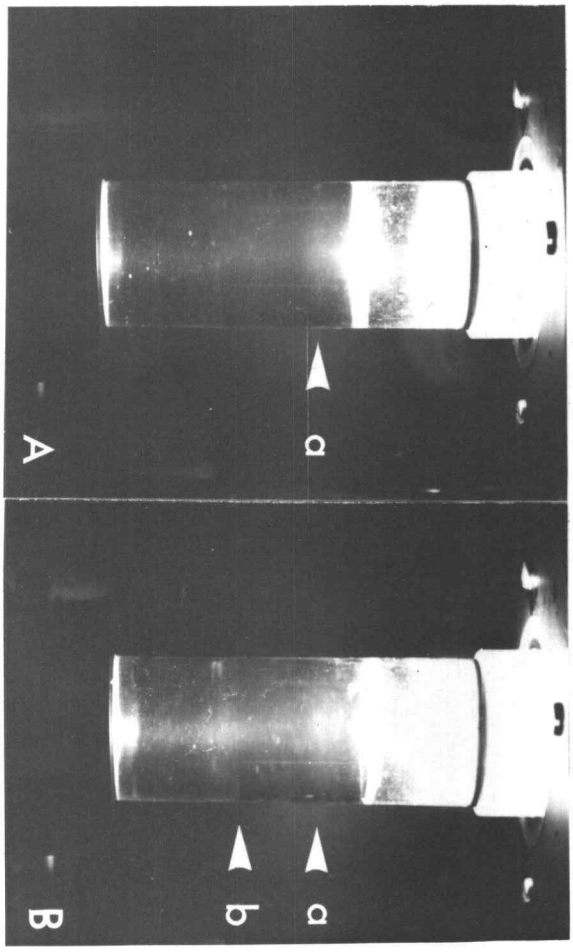
two light-scattering bands, but only one band was formed when a comparable preparation from a healthy host was used (Figure 16). Extracts from both healthy and diseased plants produced light-scattering bands 4-6.5 mm below the meniscus (Figure 16B-a), while only infectious extracts produced banding 14.5-22.5 mm below the meniscus. This very faint lower band contained most of the BYMV (Figure 16B-b).

Electron Microscopy

Loss of BYMV upon initial low-speed centrifugation of CE from systemically-infected plants was attributed to the association of the BYMV particles with sub-cellular constituents. Electron micrographs of virus particles within the chloroplasts of infected Bountiful bean (Figure 3) provided evidence for association between BYMV and cellular organelles.

Electron micrographs of dip preparations from cross-section cuts of leaves from systemically-infected plants also showed fibrous masses of virus-like particles (Figure 4). The relatively small loss of infectivity after a low-speed centrifugation of CE from C. amaranticolor suggested that the virus particles might not be bound to organelles or inclusions in this host. To test this possibility dip preparations were made from cross-sections through BYMV-induced local lesions on C. amaranticolor leaves and examined by electron

Figure 16. Faint light-scattering bands from density-gradient centrifugation of a partially purified BYMV preparation. A) A faint band 4-7.5 mm below the meniscus was observed in the partially refined extract from healthy plants. B) The partially purified virus was prepared by concentrating the virus plus host proteins in the polyethylene glycol extract as previously described (Figure 10). Two light-scattering bands: a) 4-7.5 mm from the meniscus corresponding to most of the host constituents, and b) 14.5-22.5 mm below the meniscus corresponding to the most infectious DG fractions.

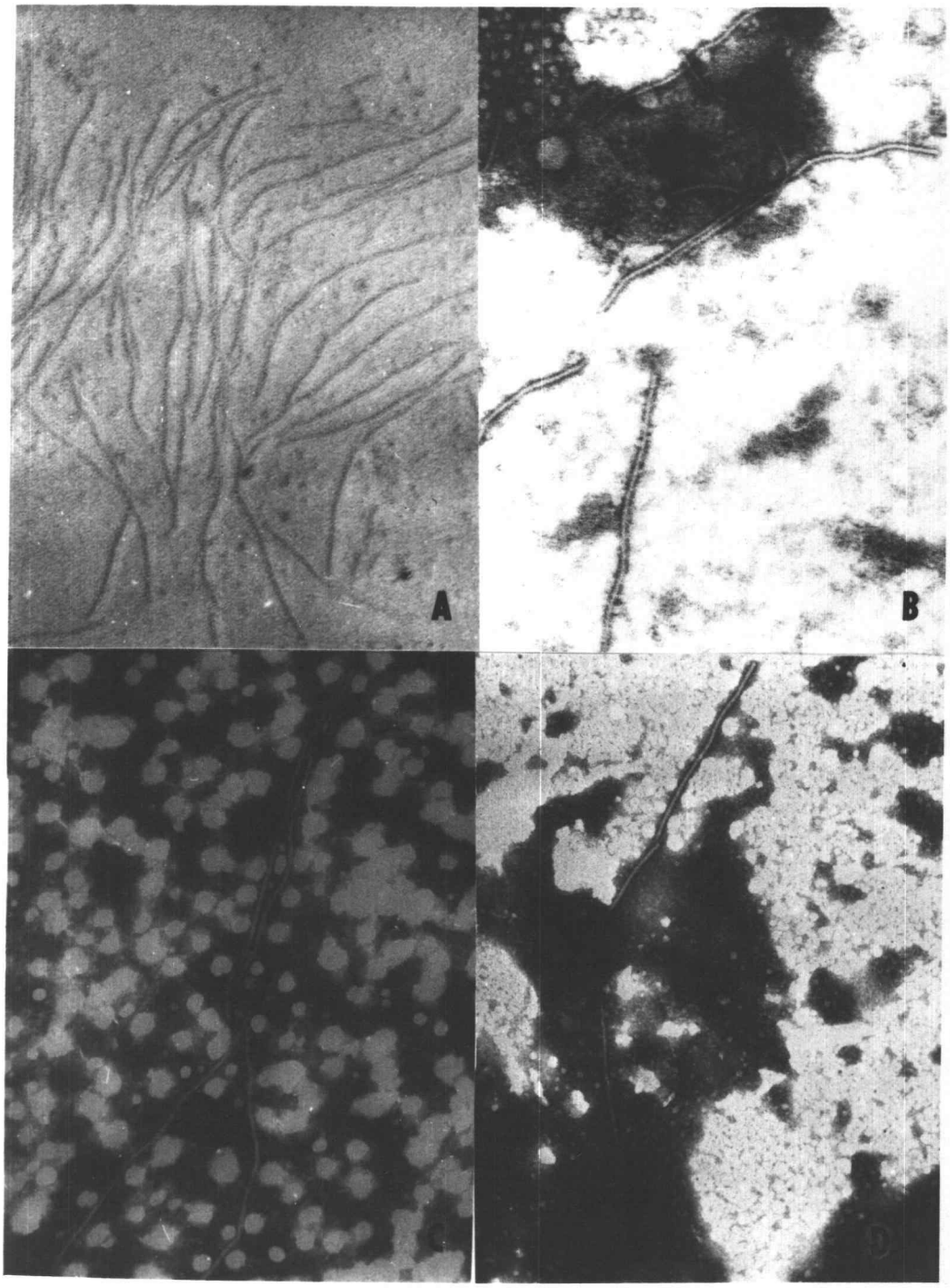

A
a
B
b
a

microscope. Dip preparations were made by submerging dissected lesions into a droplet of one percent formalin positioned on a Formvar-coated 400-mesh copper grid. The cut tissue was held in the droplet one minute and then slowly withdrawn. The droplet was allowed to remain two minutes and was then drawn off completely with a piece of filter paper. The virus particles were negatively stained with 2 percent phosphotungstate, pH 6.9 for two minutes.

BYMV particles from local lesions were fairly uniformly distributed, and occurred in moderate numbers. The particles were free of observable membranes and did not occur as intracellular inclusions (Figure 17B).

Dip preparations were made (as described previously for C. amaranticolor leaves on p. 107) from Trifolium pratense L. (red clover) systemically-infected with BYMV. Monodispersed BYMV particles occur in abundance in this host, which is used for culturing isolates of BYMV (Figure 17A). BYMV from local lesions in C. amaranticolor (Figure 17B) also occurred as monodispersed particles in about one-tenth the concentration of the dip preparation from red clover. Particles from a partially purified preparation from the concentrated PEG extract (Figure 17C) occurred in a nonaggregated state and appeared to occur in about half the numbers that were seen in the dip preparation from the local lesions. BYMV particles from the DG fraction 12-16 mm below the meniscus (Figure 17D) in a DG

Figure 17. BYMV particles from infected tissues and partially purified preparations. A) Dip preparations from BYMV-infected Trifolium pratense L. Shadow cast with 80-20 platinum and palladium. Magnification: 50,635 x. B) Dip preparations from BYMV-induced local lesions on Chenopodium amaranticolor. Negatively stained in 2% phosphotungstate, pH 6.9. Magnification: 60,967 x C) BYMV particles extracted from polyethylene glycol pellets as described on p. 82 paragraph 3 (also see Figure 10). Negatively stained. Magnification: 73,017 x. D) BYMV particles in partially purified preparations from the density-gradient (DG) fraction 12-16 mm below the meniscus in the DG column. Negatively stained. Magnification: 60,944 x.


A
B

column also appeared nonaggregated and appeared to occur in about half the numbers that were present in the concentrated PEG extract sample.

Size distributions of BYMV particles from C. amaranticolor local lesions and DG fractions were compared (Figure 18) to determine (i) the distribution of particle lengths of BYMV in the local lesions, and (ii) the distribution in the partially purified preparation from the DG centrifugation fraction 12-16 mm below the meniscus of the DG column. Although the modal length of BYMV particles from the local lesions was 750 nm, 70 percent of the particles were below this length (Figure 18A). Modal particle lengths for partially purified virus was 667 nm, with 56 percent of the particles exceeding this length. Thus, it appeared that the 750 nm flexuous BYMV particles underwent some fragmentation during partial purification.

Partial Characterization of BYMV

Ultraviolet Absorption Spectrum

As previously noted the most highly purified BYMV preparation occurred 12-16 mm below the meniscus of the DG column (Table 27). The criteria used to determine the relative purity of this DG fraction were (i) infectivity (Table 27), (ii) the ultraviolet absorption spectrum (Figure 15), (iii) the 260/280 ratio of 1.152 (Table 27). The ultraviolet absorption spectrum of BYMV was thus based on the properties of this

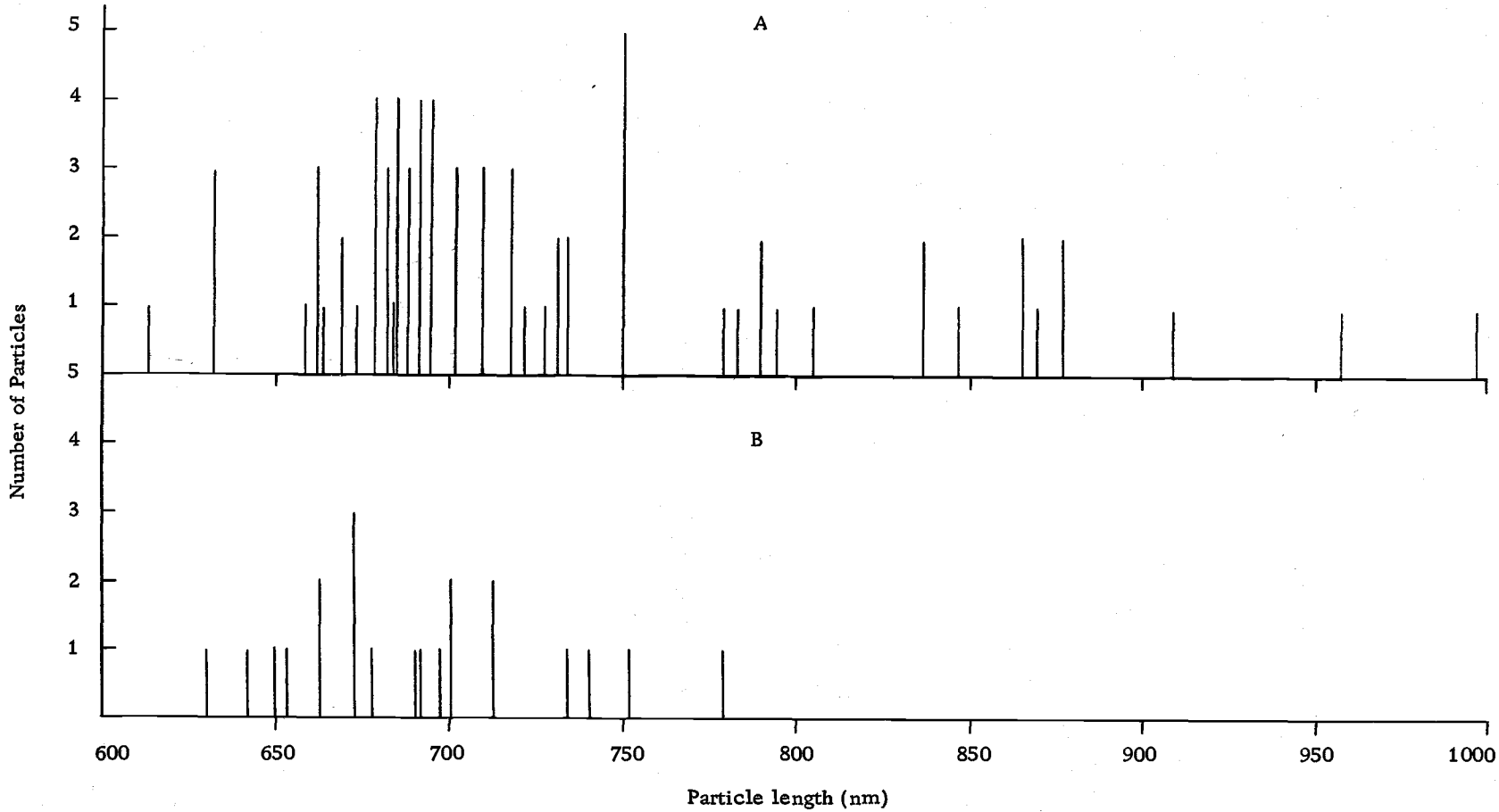


Figure 18. Particle length distribution of BYMV. A) Particles from local lesions in BYMV-infected C. amaranticolor. B) Particles from density gradient (DG) centrifugation fraction 12-16 mm below the meniscus of the DG column.

Table 27. Infectivity[1] and 260/280 ratio of fractions collected from density-gradient tubes after centrifugation.

Fraction No.	Distance from meniscus (mm)	Infectivity (LL/100 sq. cm)	260/280
2	4- 8	0.00	1.25
3	8-12	0.16	1.14
4	12-16	0.431	1.152
5	16-20	15.03	1.0
6	20-24	0.62	1.5
7	24-28	0.0	1.0
8	28-32	0.0	1.08
9	32-26	0.0	1.06

[1] Isolate 724 of BYMV.

Table 28. Migration rates for the schlieren pattern produced by a partially purified BYMV preparation.

	x(cm)	r[1](cm)	ln r	xF = x (4.75)
1.	$21,155 \times 10^{-4}$	6.7048	0.90286	1.0048
2.	$21,486 \times 10^{-4}$	6.7206	0.90524	1.0206
3.	$21,733 \times 10^{-4}$	6.7342	0.90707	1.0342

[1] r = xF + 5.7 cm.

fraction. The ultraviolet absorption spectrum of this partially purified virus (Figure 15) had a broad peak between 252 and 261 nm, and a minimum of 242 nm.

Determination of Sedimentation Coefficient

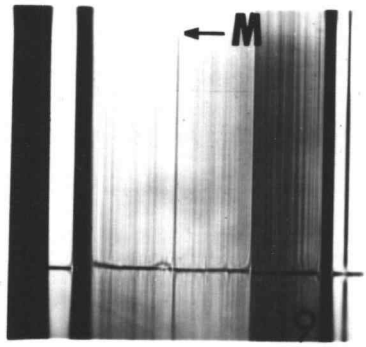
A faint light-scattering band was formed 14.5-22.5 mm below the meniscus after DG centrifugation at 8,000 x g for 10 minutes, and then at 49,300 x g for 90 minutes (Figure 16B-b). The 8 mm width of this band and the dispersion of virus (Table 27) within this region precluded the use of Brakke's (18) DG centrifugation method as a means of estimating sedimentation coefficient.

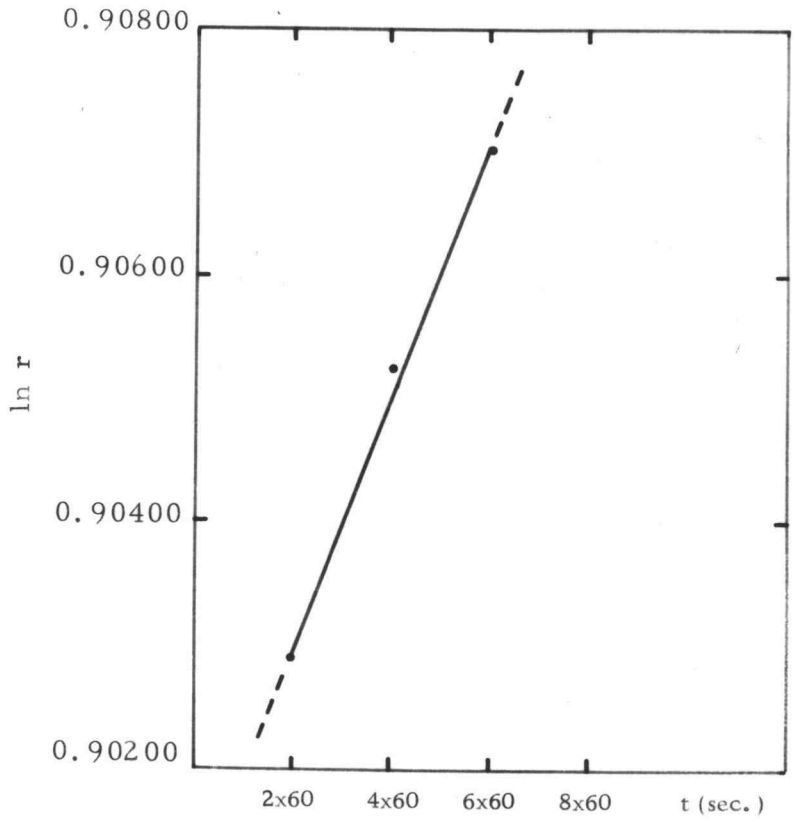
Determination of the sedimentation coefficient was thus attempted by analytical centrifugation of the partially purified BYMV preparation. In this experiment the partially purified virus was prepared as previously described (Figure 10), except that the DG fraction 14.5-22.5 mm below the meniscus (Table 27) was used. This fraction was collected, and the virus was concentrated by ultracentrifugation (100,300 x g for 90 minutes). The pellet was resuspended in 0.02M diPO_4, 0.1M KCl, pH 7.5.

Analytical centrifugation was performed in a Spinco Model E analytical ultracentrifuge using schlieren optics. A 30 mm double sector cell was used in the AN-J rotor. The sedimentation coefficient determined from the schlieren pattern (Figure 19) was calculated as follows:

Figure 19. Schlieren pattern for a partially purified BYMV preparation centrifuged in a Spinco Model E ultracentrifuge. The virus was in 0.02 diPO_4 with 0.1M KCl, pH 7.5. The photograph was taken four minutes after the rotor reached 3000 rpm. Movement is from right to left. M is the meniscus.

Figure 20. A graph showing a plot of ln r versus time (sec.). The slope of the line obtained was equal to sw^2.


M


0.90800
0.90600
0.90400
0.90200
ln r
2x60
4x60
6x60
8x60
t (sec.)

Since $dr/dt = sw^2 r$, then by integration

$$\int_{r(t_o)}^{r(t)} dr/r = \int_{t_0}^{t} sw^2 dt$$

and thus $\ln (r/r_o) = sw^2 t$, or $\ln r = sw^2 + \ln r_o$. By plotting the values (Table 28) of ln r versus t, a straight line was obtained (Figure 20), the slope of which equaled sw^2. The value for sw^2 was 1.756×10^{-5} sec^{-1}.

Since $w = 2(\pi)(rps) = 2(\pi)(3000/60) = \pi(100) = 3.14 \times 10^2$, $w^2 = 9.82 \times 10^4 sec.^{-2}$. Thus sw^2 = slope = $1.756 \times 10^{-5} sec^{-1}$.

$s = slope/w^2 = 1.756 \times 10^{-5} sec^{-1} / 9.86 \times 10^4 sec^{-2}$

$= 1.7809 \times 10^{-10} sec.$

$s = 1,780.9 \times 10^{-13}$ sec. at 7^oC.

Thus s (specific viscosity of water at 7^oC/specific viscosity at 20^oC) $= S_{20,w} = (1,780.9 \times 10^{-13} sec)(1.7971/1.5608) = 2050.5$ S.

Purcifull's (67) sedimentation coefficient value of 154 S for tobacco etch virus (a 750 nm flexuous virus) as well as S values derived for other anisometric viruses (6, 30) indicate that a sedimentation coefficient value of 2050.5 S is 12 to 15-fold to large for BYMV monomers. Such an S value suggests that BYMV particles became aggregated into polymers. Infectious fractions from gel filtration

experiments (Figure 11) suggested that only dimers (first) and monomers (second) passed through the column. Their concentrations however, would have been too low to produce visible peaks during analytical centrifugation with conventional optics.

V. DISCUSSION

A survey of the literature dealing with BYMV purification revealed that there has been little change in purification methods in the last decade. The author was unable to achieve BYMV purification by exhaustive use of reported techniques. This failure to purify BYMV with standard techniques indicated that improved methods were needed. The nature of this problem was examined in preliminary tests to increase the virus yield. Failure of these preliminary tests to elucidate the limiting factors indicated that detailed studies would be required.

The consistent loss of virus during the initial low-speed centrifugation of crude extract from Bountiful var. bean and Vicia faba (systemically-infected hosts) led the author to look for other host types. An alternative to systemically-infected hosts (referred to as systemic hosts) was Chenopodium amaranticolor which produces only local lesions when inoculated with any of several BYMV isolates. Infectivity tests showed that this host yielded BYMV which remained suspended during 34,000 x g centrifugation. It was apparent that the factors limiting the extraction of virus from the systemic hosts were not present in this host. Although no cytological or ultrastructural work was done on the BYMV-induced local lesions, inclusion structures were not detectable in dip preparations from localized leaf

lesions. This apparent lack of inclusions (which may tie-up the virus in systemic hosts) in the sites of infection could explain the high yield of virus extracted from C. amaranticolor.

In their paper on BYMV partial purification, Bancroft and Kaesberg (4) emphasized the need of a high-yielding BYMV host and virus isolate in order to purify and concentrate BYMV. It is felt that the combination of BYMV isolate 724 and the local lesions host, C. amaranticolor, represents a significant fulfillment of these requirements.

Attempts to purify BYMV included the ether-carbon tetrachloride clarification method (11, 97). Use of this method resulted in a reduction in infectivity of the clarified extract and instability of the virus during subsequent steps toward purification. It is felt that the BYMV isolates used in the present study were simply not stable in ether, although rigorous precautions were not taken to remove hydrogen peroxide which may have been present. The fact that some infectivity survived ether treatment indicated that if traces of peroxide were present, this factor alone was not responsible for virus loss.

The author's most likely explanation for the reported successful use of the ether-carbon tetrachloride clarification method by Bercks and Wetter (11, 97) is that their isolates of BYMV were stable in this solvent. That this might constitute a special virus characteristic is suggested by the lack of success of American plant virologists in

duplicating this work.

The most effective single clarification step in the partial purification involved the use of PEG. The coacervate PEG 6000 selectively precipitated the BYMV particles leaving most of the host material in solution. The advantage of PEG clarification in the purification of flexuous viruses is being realized by an increasing number of workers (7, 79).

Duplication of the successful separation of host proteins from BYMV by zone electrophoresis as described by van Regenmortel (93) was not possible in this study. There are two probable reasons for this. First, van Regenmortel (93) was unable to obtain an opalescent zone when he used samples purified from V. faba plants; thus, he reasoned that the virus concentration was too low in V. faba. When he used Phaseolus vulgaris, however, he obtained a band which "...moved as a weakly opalescent zone 4.5-6 cm from the origin and was separated from the normal bean antigens...". Thus, van Regenmortel observed only a weakly opalescent band. It is possible that the different host, virus isolate, and a smaller electrophoresis column used in this study resulted in too low a concentration of virus to form a band. Second, it would appear that the isolate of BYMV which van Regenmortel (94) used probably remained nonaggregated throughout the purification procedure, and also had the characteristics of yielding a high concentration of extractable BYMV from P.

vulgaris.

Further attempts at purification utilizing beta-mercaptoethanol and EDTA to prevent particle aggregation were made. It was anticipated that aggregation between particles due to disulfide (-S-S-) linkages might be prevented by the reducing agent beta-mercaptoethanol. EDTA was used to chelate the Mg^{++}, which would result in the dissociation of the ribosomes into their subunits (15) which in turn would facilitate the separation of host ribosomal nucleoproteins from the virus. The use of EDTA, however, caused a sacrifice in infectivity for the sake of purity. It is felt that further information of the effect of EDTA and other chelating agents on the stability of BYMV is necessary before such agents can be fully utilized for purifying this virus.

Although density-gradient (DG) centrifugation was the best method of obtaining sufficiently pure BYMV, aggregation of the particles limited complete characterization. Infectivity data for the various DG fractions showed that the most infectious fraction corresponded to the lower light-scattering band. However, the DG fraction which gave an ultraviolet absorption spectrum and 260/280 ratio typical of purified anisometric viruses was slightly above this zone. It is interesting to note that Damirdagh and Shepherd (28) obtained two bands upon DG centrifugation of tobacco etch virus (TEV). They found that the lower band consisted of end-to-end aggregates of TEV, while the upper band consisted of more disperse particles. This probably

corresponds with distribution of BYMV particles in DG columns of the present study except that monomerous BYMV particles were too dilute to cause visible banding.

The sedimentation coefficient of the most infectious DG fraction (lower band) was 2050.5 S. This value which was much higher than Purcifull's (68) value of 154 S for TEV also indicated substantial aggregation of partially purified BYMV.

Whether the main type of aggregation was lateral or end-to-end is unknown. If the aggregation was mainly of the lateral type, the use of urea to weaken hydrophobic banding (28) could be effective. The infectivity of crude BYMV extract was increased in 0.1-1.0M urea; thus, the use of this agent to prevent lateral aggregation may be feasible for purification. Beta-mercaptoethanol, incorporated to prevent lateral aggregation by blocking disulfide linkage between oxidized sulfhydryl groups, was shown to decrease the infectivity of partially purified BYMV. Since Damirdagh and Shepherd (28) have found ethanol, dioxane, and formamide to be ineffective in preventing aggregation of TEV, the use of these agents to prevent aggregation of BYMV would not seem promising. If the particle aggregation were primarily end-to-end, the problem could be more critical since the above agents apparently would not prevent this type of aggregation.

In attempts to determine sedimentation coefficient for BYMV the refractive index gradient maximum indicated a very low virus

concentration. The single, small virus peak may have actually represented a continuous distribution of sedimentation velocities of variously sized virus aggregates.

A study which time did not permit, but which may have promise, involves use of a zonal rotor to concentrate large volumes of PEG extracts by ultracentrifugation. The resultant resuspension of the high-speed pellet could then be purified by rate-zonal DG centrifugation in a zonal rotor. This procedure could yield sufficiently large amounts of infectious, purified BYMV for thorough characterization. However, the most urgent problem prerequisite to such purification would appear to be prevention of virus-particle aggregation.

In retrospect, certain decisions which were made during this study now seem to need reconsideration. For instance, the decision to use Tris-HCl buffer in the sucrose clarification method was based on the observation that when a BYMV sample was ultracentrifuged, the resultant high-speed pellet was much more readily soluble in Tris-HCl than in $diPO_4$ buffer. However, Tris-HCl buffer resulted in a very green, impure BYMV preparation. The discovery that $diPO_4$ with 0.1M KCl was an excellent buffer for resuspension of BYMV high-speed pellets indicated that massive efforts with sucrose clarification would have been more productive using this buffer.

Also, the use of KBr for centrifugation in high-salt concentrations was obviously made with the assumption that Br^- ions were not

deleterious to BYMV. This experiment should have been tried with KCl on the more likely assumption that high concentrations of KCl would not inactivate the virus.

The use of other species of Chenopodium may possibly yield higher titers of extractable virus as either local lesion or systemic hosts. Also, the high yield of extractable virus from V. faba roots might have been worthy of further study. The possible use of hydroponics to produce large masses of roots, plus the development of a homogenizing medium to prevent quinone formation would have made purification from V. faba an interesting possibility.

The potato virus Y group of viruses includes 19 different but morphologically and serologically related plant viruses. Of this group, only four viruses have been purified sufficiently to permit in vitro characterization. These four viruses are potato virus Y (30), tobacco etch virus (68), turnip mosaic virus (82), and maize dwarf mosaic virus (6). Unfortunately, the purification procedures used for these four viruses may not be applicable to the other members of the potato virus Y group. Therefore, in order to characterize the remaining 15 viruses, a new and gentle purification method needs to be developed. The author's objective was to develop a purification procedure for one of the most difficult members of the potato virus Y group, namely, BYMV. It was hoped that this purification procedure could, with slight modifications, be applied to the purification of the

remaining viruses of this group. The significance of the present study is not only that an improved purification method for BYMV has been developed, but hopefully that an important step toward the eventual purification and characterization of a group of viruses has been taken.

VI. SUMMARY

1. The difficulty encountered in purifying BYMV was attributed to the loss of most of the virus upon the initial low-speed centrifugation of the crude extract (CE). Thus, the first problem was recognition of the reasons for this loss of virus. This loss was thought to be due to the tying-up of virus particles inside chloroplasts, or in naturally occurring inclusions, or to the binding of the virus to sub-cellular membranes.
2. Experiments were designed to dissolve the membranes or to break the binding forces involved in these inclusions.
   a. The green chloroplast fraction from CE of systemically-infected hosts was highly infectious. The hypothesis that BYMV occurred inside chloroplasts of infected hosts was tested. Rupture of the chloroplasts resulted in decreased infectivity.
   b. The use of anionic, nonionic, and cationic surfactants for releasing the adsorbed virus, or disrupting the inclusions resulted in no appreciable increase in infectivity.
   c. The methods used to dissolve microsomal membranes resulted in decreased infectivity, when used to dissolve membranes in infectious CE.
   d. Moderate concentrations (0.1M) of KCl increased the amount

of extractable virus.

e. Use of KBr for centrifugation in high salt concentration resulted in an inactivation of the virus. The virus also became noninfectious when exposed to CsCl.

f. Use of zone electrophoresis to separate the virus particles which may have been adsorbed to the membranes was unsuccessful.

g. Use of organic solvents to dissolve the membranes to which virus particles may have been adsorbed resulted in denaturation or decreased infectivity of the virus.

3. The failure of a variety of clarification methods to increase the amount of virus extractable from a systemic host led the author to consider the use of a local lesion host for purification. The assumption involved was that if inclusions did not occur in local lesion infection hosts, then the amount of unbound and extractable virus would be much greater than from systemically-infected hosts.

a. A comparison of the amount of virus extractable from systemic host and from the local lesion host, C. amaranticolor, showed that much more virus could be extracted from the latter.

b. Relatively little virus was lost upon low-speed centrifugation of crude sap when C. amaranticolor was used as the host.

c. Infectivity of the partially purified virus from C. amaranticolor was greater than that from the best systemic host.

d. At least 43 percent of the virus present in CE from infected C. amaranticolor was recovered in the high-speed pellet.

4. The BYMV isolate yielding the most partially purified virus was 724.

5. The selection of C. amaranticolor as the virus production host posed the problem of finding the best homogenizing schedule for this host.

a. Re-extraction from the pulp increased the amount of virus from infected tissue by 30 percent.

b. The best homogenizing medium for extracting BYMV from this host was 0.05M diPO_4, 0.1M KCl.

c. The best extraction with this buffer was obtained within the pH range 8.5-9.0.

d. Various reagents were used in an attempt to prevent aggregation upon homogenization. Beta-mercaptoethanol and urea increased CE infectivity slightly, while EDTA and thioglycollic acid decreased it slightly.

6. Preliminary clarification was achieved by low-speed centrifugation followed by moderate-speed centrifugation. Further clarification was attempted by selective coagulation or precipitation methods.

a. Clarification by freezing resulted in loss of virus, while heating resulted in excessive melanin formation.

b. Clarification by selective precipitation of host protein with saturated $(NH_4)_2SO_4$ resulted in a sharp loss of infectivity.

c. Clarification by selective coagulation of host materials using the organic solvents, ether and carbon tetrachloride, resulted in a loss of infectivity.

d. Attempts to selectively precipitate the virus by acidification were unsuccessful. Partial precipitation of virus occurred at all pH values tested.

e. Clarification by extracting in homogenizing medium containing 0.45M sucrose was evaluated. Upon DG centrifugation this technique gave a highly infectious, partially clarified virus. However, the use of Tris-HCl as the buffer resulted in an apparent complex between the chlorophyll pigment and the virus.

f. Clarification by selective adsorption of host materials was evaluated. The use of bentonite resulted in a sharp decrease in infectivity without an appreciable decrease in noninfectious host material. The use of Celite did not decrease infectivity, but failed to clarify CE at the concentrations used.

g. Differential precipitation of the virus by use of polyethylene glycol (PEG) 6000 was evaluated. Four percent PEG with 0.1M KCl precipitated the virus and left most of the

noninfectious host protein in solution. Virtually all of the virus in the infectious, partially clarified supernatant was precipitated and recovered by the PEG treatment.

7. The efficacity of the bentonite, sucrose, ether-CCl_4, and PEG clarification methods was re-evaluated. The absorbancy profiles and the infectivity distribution after a DG centrifugation indicated that selective precipitation by PEG resulted in less host constituents and in a narrower virus distribution in the centrifuged DG column than the other three treatments.

8. PEG treatment was superior to other clarification treatments, and was better than any combination of these treatments with PEG.

9. A flow diagram of the partial purification procedure (Figure 10), summarizes the schedule used to obtain a highly infectious, partially purified BYMV sample.

10. Four, gentle techniques were attempted for purifying BYMV from the partially purified sample.

    a. Gel filtration (utilizing sepharose 2B) of the partially purified virus resulted in a separation of the virus from the noninfectious host material. The difficulty encountered was that the virus was substantially diluted during elution. This dilution resulted in a very low yield of virus.

    b. Electrophoresis of the highly infectious, concentrated PEG

extract resulted in a banding of noninfectious host material, but the virus failed to band and appeared to migrate throughout the column. Thus there was a separation of the virus from the host material, but again substantial dilution of the virus occurred.

c. The use of a zonal rotor to purify and concentrate BYMV was not efficacious by the method tested. CE was clarified by the sucrose clarification method. The virus migrated into a relatively narrow band during centrifugation but was accompanied by large quantities of host protein.

d. DG centrifugation of concentrated, infectious, PEG extract yielded the most highly purified BYMV sample. The principal problem encountered with purified BYMV preparation was the aggregation of virus particles as they became gradually more pure.

11. Electron microscopy showed that the modal length of the BYMV particles in the local lesions was 750 nm. The modal length of the most highly purified sample of monodispersed BYMV (the fraction 12-16 mm below the meniscus of the density gradient column) was 680 nm, i.e., 9.6 percent shorter than that of the virus in the local lesions. The most highly purified sample of monodispersed virus appeared to contain numerous fragmented particles.

12. The ultraviolet absorption spectrum and the 260/280 ratio of 1.152 were typical of a purified, flexuous virus.

13. Attempts were made to determine the sedimentation coefficient by analytical centrifugation. A sedimentation coefficient value of 2050.5 S for the most infective fraction (16-20 mm below the meniscus) from the DG column suggested that the final preparation of virus was substantially aggregated.

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APPENDICES

APPENDIX I

Glossary

BYMV-infected C. amaranticolor leaves: refers to C. amaranticolor leaves densely covered with BYMV-induced local lesions.

CE: refers to crude extract, which is the plant homogenate resulting from grinding the plant tissue in the homogenizing media and squeezing the ground pulp through cheese cloth.

CFI: Chloroplast fraction, infectious, refers to the chloroplast rich portion of the resuspended low-speed pellet from infectious crude extract. CFI is highly infectious when bioassayed.

$DiPO_4$: refers to diphosphate buffer, which was made from an appropriate combination of K_2HPO_4 and KH_2PO_4.

DG: is an abbreviation for density-gradient.

Local lesions (LL): refers to chlorotic, spots on the leaves resulting from localized infection.

Low-speed supernatant: refers to the supernatant liquid obtained by a low-speed centrifugation.

Low-speed pellet: refers to the pellet resulting from a low-speed centrifugation.

Moderate-speed supernatant: refers to the supernatant liquid obtained by a moderately high speed centrifugation.

Pellet: refers to the portion of the aqueous suspension which was brought down to the bottom of a tube upon centrifugation.

$P\text{-}HS_2$: refers to the second high-speed pellet resulting from one cycle of differential centrifugation. The differential centrifugation was performed as follows: ultracentrifugation (100,300 x g for 90 minutes), followed by resuspension of the pellet and low-speed centrifugation (10,000 x g for 15 minutes) of the resuspension.

$P\text{-}HS_3$: refers to the third high-speed pellet resulting from differential centrifugation of the resuspension from the second high-speed pellets.

Virus production host: refers to the host used to culture the virus for extraction and purification.

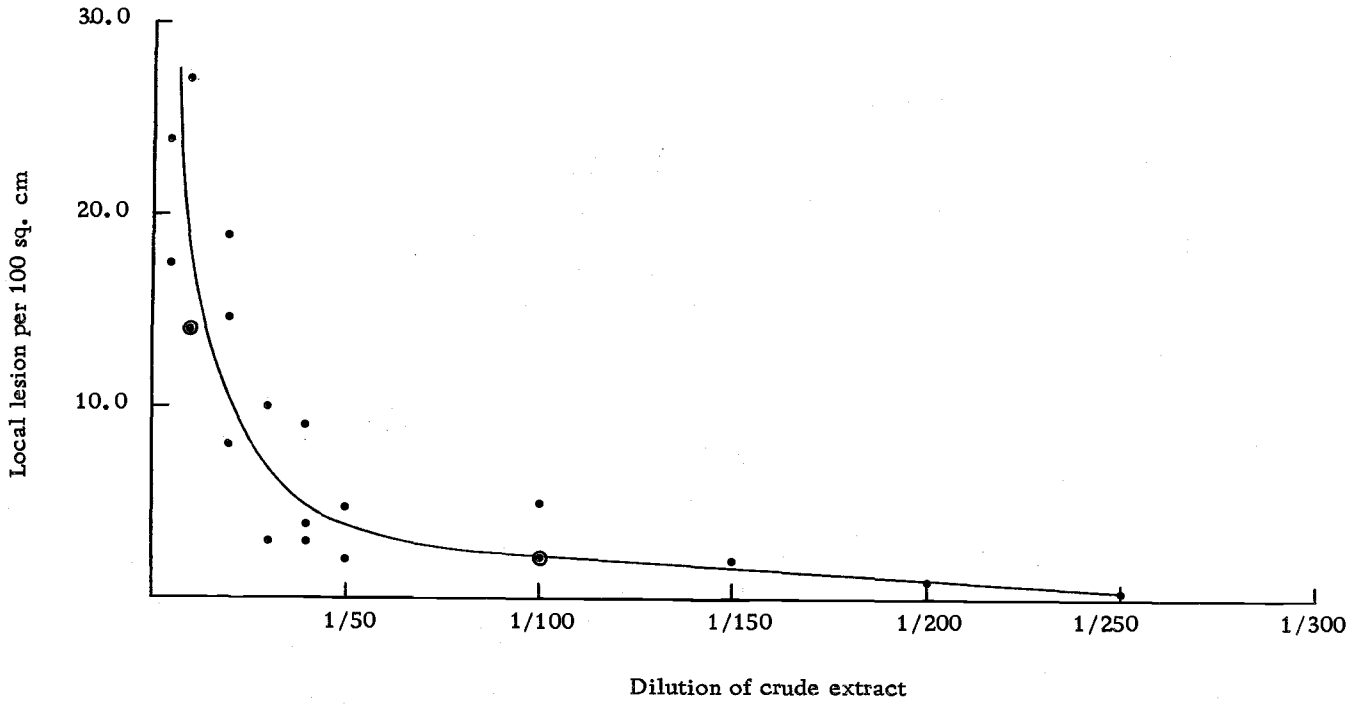
v:v: refers to a volume per volume combination, e.g., ml per ml.

w:v: refers to a weight per volume combination, e.g., gm per ml.

w:w: refers to a weight per weight combination, e.g., gm per gm.

Appendix II. Dilution end point of crude extract from Chenopodium amaranticolor infected with BYMV. C. amaranticolor leaves covered with BYMV-induced local lesions were homogenized in w:3v of 0.05M diPO_4 with 0.1M KCl, pH 8.7. This homogenate was diluted in 0.01M diPO_4 with 0.1M KCl, pH 8.7. The bioassay was on C. amaranticolor, and it was done by the latin square method.

This data was acquired over a one year period. The purpose in showing this graph was to give the reader an indication of the effect that dilution had on the number of local lesions obtained.


30.0
20.0
10.0
Local lesion per 100 sq. cm
1/50
1/100
1/150
1/200
1/250
1/300
Dilution of crude extract