

PARTIAL PURIFICATION AND SEROLOGICAL ANALYSIS
OF APHID TRANSMISSIBLE AND NON APHID TRANSMISSIBLE
BEAN YELLOW MOSAIC VIRUS ISOLATES

by

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INTRODUCTION

Bean Yellow Mosaic Virus (*/* : */* : E/E : S/AP) is transmitted by many aphid species (Homoptera: Aphididae) in the nonpersistent manner and readily by sap inoculation. The particles are elongated and flexuous (750 nm long and 15 nm wide) (Bos, 1970). The virus is classified among the Potato Virus Y group (Edwardson, 1974).

Two isolates of Bean Yellow Mosaic Virus (BYMV) have been separated and studied on the basis of transmissibility by Acyrtosiphon pisum (Harris) and Myzus persicae (Sulz.) and on symptom differences in selected herbaceous host plants (Thottappilly et al., 1972). This study revealed that one isolate, Oregon IV BYMV (Ore BYMV), was transmitted by aphid vectors whereas the second isolate, Michigan BYMV (Mich BYMV), was not transmitted by aphid vectors. These isolates were examined for symptom differences among various legume plants and a differential host reaction occurred on Phaseolus vulgaris L. cv. 'Bountiful'. Mich BYMV produced severe chlorotic spots on inoculated leaves followed by systemic symptoms of yellow mosaic on young uninoculated leaves, whereas Ore BYMV produced only mild chlorotic local lesions in response to mechanical inoculation. Loss of vector transmissibility of nonpersistent viruses has been reported several times (Swenson, 1957;

Kamm, 1969; Evans and Zettler, 1970; Thottappilly et al., 1972; Lung and Pirone, 1973) but has not been adequately explained. This research was undertaken to serologically compare Mich and Ore BYMV isolates in order to elucidate the possible reasons for differences between these two isolates in aphid transmissibility.

Prior to serological study, a reliable purification procedure of the isolates needed development. Long flexuous rod potyviruses (potato virus Y group viruses) are difficult to purify because they commonly aggregate (both end to end and side by side), agglutinate, and break (Shepherd and Pound, 1960). Nevertheless, there are several reports on the purification of various viruses in this group. Initial efforts at purification of Ore and Mich BYMV using some of these procedures, yielded preparations with relatively low infectivity and, as supported by electron microscopy, a high percentage of aggregated broken particles and particle fragments. The success of previous attempts utilizing polyethylene glycol (PEG) precipitation for the purification of potyviruses varied on the virus being purified and the buffer selected for the procedure (Steven-son and Hagedorn, 1973; Knesek, Mink, and Hampton, 1974; Sun and Herbert, 1972; Damirdagh and Shepherd, 1970). Considerable effort was spent on recovering the virus from the crude sap, then the virus had to be prevented from aggregating and agglutinating. The present study also reports a purification procedure for two BYMV isolates which yielded preparations with high infectivity.

The partially purified preparations of the two isolates of BYMV also were tested via sucrose density gradient electrophoresis and rate zonal density gradient centrifugation for a possible method of further purification and of determining expedient, reliable isolate differences.

MATERIALS AND METHODS

Virus Isolates: The aphid transmissible isolate of BYMV (Oregon IV BYMV) was originally obtained from J.A. Kamm, Dept. of Entomology, Oregon State University, Corvallis, Oregon, in 1971. The non aphid transmissible isolate of BYMV (Michigan BYMV) was obtained from J.L. Lockwood, Dept. of Botany and Plant Pathology, Michigan State University.

Maintenance of the Virus Isolates: Broadbean, Vicia faba L., served as host plants in maintaining each separate isolate through the course of the research. Older host plants (for both isolates) were replaced with young plants by one of two methods: (i) In the case of Ore BYMV, vector transmission of the virus from the old plant to a young plant occurred through multiple vector acquisition access periods (AAP's) of 2-5 min, and at least a three hour inoculation period (IP) prior to a fumigation with Dibrom (Naled). The plants were returned to the greenhouse for symptom expression. (ii) Since the Mich BYMV is a strictly mechanically transmitted isolate, infective tissue from older source plants was ground in tap water using a sterile mortar and pestle; the inoculum was rubbed with fingers onto carborundum dusted (grit 400) host plants and then washed with cold tap water and returned to the greenhouse for symptom expression.

Source Plants: The source plant used throughout the research to purify the virus isolates and the test plant for the various experiments was the garden pea, Pisum sativum L., cv. 'Melting Sugar'. The plants used as source plants in the purification experiments were mechanically inoculated with crude juice from infected broadbeans. Dusting with carborundum, mechanically inoculating with the virus preparation and rinsing with water prior to return to the greenhouse, was the basic test inoculation procedure used.

Vectors: The vector used in this research was the pea aphid, Acyrtosiphon pisum (Harris) biotype East Lansing (Bath and Tsai, 1969). These were reared on broadbean grown under continuous artificial light at room temperature. In conjunction with earlier comparative transmission work by Thottappilly et al. (1972), 1st instar nymphs were always used in transmission work.

Purification: Both virus isolates were purified utilizing polyethylene glycol (PEG) precipitation and differential centrifugation. Infected tissues were macerated with a Waring blender in buffer and chloroform (ratio 1:2:2) and allowed to sit in ice for 60 min then clarified by low speed centrifugation (5 min at 1,280 g).

The supernatants (SN) were pooled and both 4% PEG and 4% NaCl were mixed into the supernatants for 1 hr. After PEG precipitation, the solutions were centrifuged for 10 min at 10,800 g and the subsequent pellets resuspended thoroughly. The virus solution was dialyzed against buffer at

4.5 C for 12 hr then differentially centrifuged with alternate low speed, high speed centrifugations. After the final (3rd) low speed centrifugation, the partially purified virus was stored in 1 ml increments at 4.5 C.

Electrophoresis: Attempts were made to further purify the partially purified virus using sucrose density gradient electrophoresis. The buffer used was 0.02 M Phosphate Potassium Chloride pH 8, and the sucrose used was Special Enzyme Grade Sucrose (manufactured by Schwarz/Mann, Orangeburg, N.Y.). A linear 10-40% sucrose gradient was formed in the electrophoresis column and the viral preparation was mixed to make a 2.5% viral-sucrose solution that was layered on top of the sucrose gradient in the column.

Sucrose Density Gradient Centrifugation: The rate zonal density gradient centrifugation and analysis was carried out by layering 1 ml of partially purified virus solution onto a 20-50% linear sucrose solution in 0.05 M Potassium monobasic, Sodium dibasic Phosphate buffer pH 8. These gradients were centrifuged for 1 hr at 18,000 rpm in a Beckman SW 27.1 rotor. The gradients were fractionated at a 3.3 ml/min rate and analyzed using a single beam 254 nm ultraviolet analyzer.

Bioassay: The partially purified virus preparations were assayed for infectivity using the systemic hosts, Vicia faba L. (broadbean) and Pisum sativum L. cv. 'Melting Sugar'. These assays conducted at various stages of the purification technique determined the mechanical infectivity of the virus with respect to the purification steps.

Serology: Antisera (As) were produced against each of three groups of antigens. Sera were made against: (i) partially purified samples of Ore BYMV, (ii) partially purified samples of Mich BYMV, and (iii) partially purified samples of healthy pea plant proteins.

In each case, the antigens (Ag) were prepared by exactly the same purification procedure. Each antigen solution (usually 1 ml) was mixed with an equal volume of Freund's incomplete adjuvant and injected at 4 day intervals into the hind leg muscles (IM) of female New Zealand white rabbits. Each rabbit received 6 of these IM injections, then each received a pair of 1 ml intravenous (IV) injections one week apart prior to bleeding. For the IV injections, the antigens used were the same antigens used to prepare the IM injections without the adjuvant added. Seven days after the last IV injection each rabbit was bled from the marginal vein of the ear, the serum separated overnight and given a low speed centrifugation. The serum was stored at -20 C in 0.5 ml and 1.0 ml lots.

Three serological tests were used for three specific but related purposes. The Ring Interface Precipitin test (Ball, 1974) was used to primarily determine the titer of the antibodies (Ab) produced. The Microslide Gel Diffusion test (Aappola and Rochow, 1971) was chosen to show cross reactivity between the two isolates. The SLS (Sodium Lauryl Sulfate, or Sodium Dodecyl Sulfate) Agar Gel Diffusion was chosen to show cross reactivity but it differs from the Microslide Gel Diffusion technique in that

SLS breaks down the migrating virus particles whereas, due to the very low agar concentration in the Microslide technique, whole virus particles diffuse through the gel.

The Ring Interface Diffusion test involves placing serum at a constant 1:2 dilution using Phosphate Buffered Saline (PBS), mixed with 15% glycerin into the bottom of closed glass tubes (6mm X 500mm) and very carefully layering the the various Ag dilutions made with PBS on top. The tubes were incubated at 20 C for 1.5 hr while making frequent observations. After the necessary incubation, the most dilute dilution of Ag used to show a reaction was chosen and mixed with 15% glycerin to be placed in another set of glass tubes with various Ab dilutions layered on top. The titer of the virus is determined in this second part of the experiment, as the most dilute dilution of Ab showing a reaction.

The Microslide Gel Diffusion technique is an agar gel diffusion technique that allows whole virus particles to interact with Ab. The Ag particles diffuse through only a small amount of agar before interacting with the Ab. Two layers of black plastic electrician tape were placed on both ends of a clean microscope slide and a 0.75% Ionagar No. 2, pH 8 was layered between the tape exactly the thickness of the tape. The slides should be preheated to 56 C prior to the addition of agar and stored during incubation in a 100% relative humidity chamber. The Ab was introduced to the center well and the various Ag's were introduced to the peripheral wells.

The SLS Agar Gel Diffusion technique involved making 0.1% SLS-Ag solutions which are placed in the agar wells; The SLS breaks the long viral particle into smaller capsid subunits and these interact with the diffusing Ab. The agar (Ionagar No. 2) was layered into sterile petri plates (15mm X 100mm) and stored at 4.5 C in a 100% relative humidity chamber until used.

Equipment Description: In the purification procedure, the low speed centrifugations were carried out on a Sorvall SS-1 Superspeed centrifuge in a cold room (4.5 C). The ultracentrifuge used was a Beckman Model L-2, and both a Type 30 rotor (for the 80,000 g centrifugations) and a Type 50 Ti rotor (for the 105,000 g centrifugations) were utilized. The sucrose gradient solutions that were ultracentrifuged were done on a Type SW 27.1 rotor. The spectrophotometric analysis of the partially purified preparation was done on a Beckman DB Spectrophotometer powered by a Beckman Hydrogen Lamp power unit. All the equipment used for the sucrose density gradient exercises, either electrophoresis or rate zonal centrifugation analysis was manufactured by Instrumentation Specialties Company (ISCO). The preparative density gradient electrophoresis apparatus was ISCO Model 630. The power supply for electrophoresis was an ISCO Model 490. The rate zonal fractionation was carried out by an ISCO Model D fractionator. The ultraviolet analysis of both the electrophoresis and rate zonal centrifugation was accomplished with an ISCO Model UA-2 (254 nm) analyzer.

RESULTS

Partial Purification: Upon the initiation of purification efforts, 4 buffers were chosen based on their relatively successful use as separation buffers. Since the problems of particle aggregation and/or agglutination and the persistent problems of particle breakage are such severe obstacles, careful attention was given in choosing the most appropriate buffer to suit the project's needs. The basic criterion used to determine the relative suitability of a specific buffer was based on the infectivity of the purified virus in the specific buffer.

Research by Huttinga (1973) with numerous potyviruses suggested using either 0.18 M Phosphate-Citric acid pH 7 containing 0.1% Thioglycollic acid, or 0.18 M Phosphate-Citric acid pH 9. Other buffers used were 0.05 M Sodium Borate pH 8.2 and, 0.05 M Potassium monobasic, Sodium dibasic Phosphate pH 7 (hereafter referred to as 'Phosphate' buffer). These latter buffers were used to purify Pea Seed-borne Mosaic Virus (Stevenson and Hagedorn, 1973) which is also a flexuous rod virus.

When testing these buffers, 10 grams of infected pea tissue were ground in a clean mortar with a pestle along with 20 ml of buffer and 20 ml of chloroform. All 4 buffers were tested simultaneously. The crude scheme of

purification at that time involved, (i) low speed clarification, (ii) PEG precipitation, cnetrifugation at 10,800 g, and resuspension in the original buffer tested, and (iii) mechanical inoculation of pea plants with the viral suspension of each buffer in a dilution series to assay the infectivity of each preparation (Table 1).

Table 1. Retention of infective virus using different buffers at different dilutions*.

Buffer	Virus dilution**			
	SN	1/10	1/100	1/1000
P.C.A.	12	3	3	0
P.C.A. + Thio-acid	13	1	0	0
Borate	25	4	1	0
K-Na-Phos.	66	29	4	2

*Dilutions were made with the buffers involved in the exercise, and plants were dusted with carborundum prior to mechanical inoculation.

**Each test at each dilution involved the inoculation of 96 single plants.

After this preliminary work testing these buffers, the 0.05 M Phosphate pH 7 buffer was tested for retention of infective BYMV through two stages of ultracentrifugation. These experiments involve the same crude purification scheme used in the previous buffer tests, with the addition of a 2 hr 80,000 g ultracentrifugation: After resuspension of the pellets in their appropriate buffer, a 10 min 1,280 g low speed centrifugation, and finally the pooled

supernatants were ultracentrifuged at 105,000 g for 2 hr. These final pellets were resuspended and mechanically inoculated onto pea to test for infectivity (Table 2). This viral extract of BYMV after the differential centrifugation also was assayed for nucleic acid and protein content by spectrophotometric analysis. Based upon three analyses, a 260:280 (nm) ratio of 1.75 for the nucleic acid and protein was determined.

Table 2. Retention of infective BYMV through 2 stages of ultracentrifugation.

Buffer	Plants infected with BYMV after mechanical inoculation			
	SN	1/10	1/100	1/1000
0.05 M Potassium monobasic, Sodium dibasic Phosphate pH 7	12/15	4/15	2/15	0/15

After these preliminary observations, it became evident that the problems of long flexuous rod aggregation and agglutination were more acute than originally anticipated. Through trial and error, it became clear that virus remained in suspension, and did not tend to aggregate as easily if buffer pH was altered to be slightly more alkaline. The virus pellet from the PEG centrifugation step resuspended more easily and the virus stayed in suspension longer if the buffer used was at pH 8 rather than pH 7. This was macroscopically evident after the dialysis of the

viral suspension, since the preparation dialyzed against pH 8 buffer showed more of a cloudy suspension with very little sediment than the preparation dialyzed at pH 7 (which had a good deal of sediment in the dialysis bag).

To confirm the suspicions about the role of pH with virus suspension, a purification preparation was carried out using 0.05 M Phosphate buffer pH 7. The preparation after PEG centrifugation and resuspension was divided into 4 equal portions for dialysis. Each preparation was dialyzed against the same Phosphate buffer at a different pH. After dialysis, each preparation was centrifuged at 1,280 g for 5 min and both the supernatant and the pellet from each preparation were assayed for infectivity in a dilution series experiment of mechanical inoculation onto peas. These results clearly show that the buffer at pH 8 retains more infective BYMV in the SN after the dialysis centrifugation and thus less infective virus is pelleted out (Table 3). After the BYMV preparation underwent 2 cycles of ultracentrifugation, the particles became increasingly difficult to resuspend. This problem of virus aggregation, especially after high speed ultracentrifugation, was diminished considerably with the addition of 1 M Urea and 0.1% 2-Mercaptoethanol to the 0.05 M Phosphate buffer pH 8 used to resuspend the ultracentrifuged pellets of Tobacco Etch Virus (TEV) (Damirdagh and Shepherd, 1970). To determine whether this application of work with TEV could in fact be of use to BYMV purification, preparations were made to compare the addition of these chemicals in

Table 3. Effect of pH on virus retention in 0.05 M Potassium monobasic, Sodium dibasic Phosphate buffer after dialysis.

0.05 M Potassium monobasic, Sodium dibasic Phosphate buffer		Dilution end point series*			
		1X	1/10	1/100	1/1000
pH 6.5	Pellet	8	1	0	0
	SN	0	0	0	0
pH 7.0	Pellet	9	3	0	0
	SN	0	0	0	0
pH 7.5	Pellet	4	0	0	0
	SN	3	0	0	0
pH 8.0	Pellet	2	0	0	0
	SN	8	2	1	1

*Each test for each separate dilution involved inoculating 12 single plants.

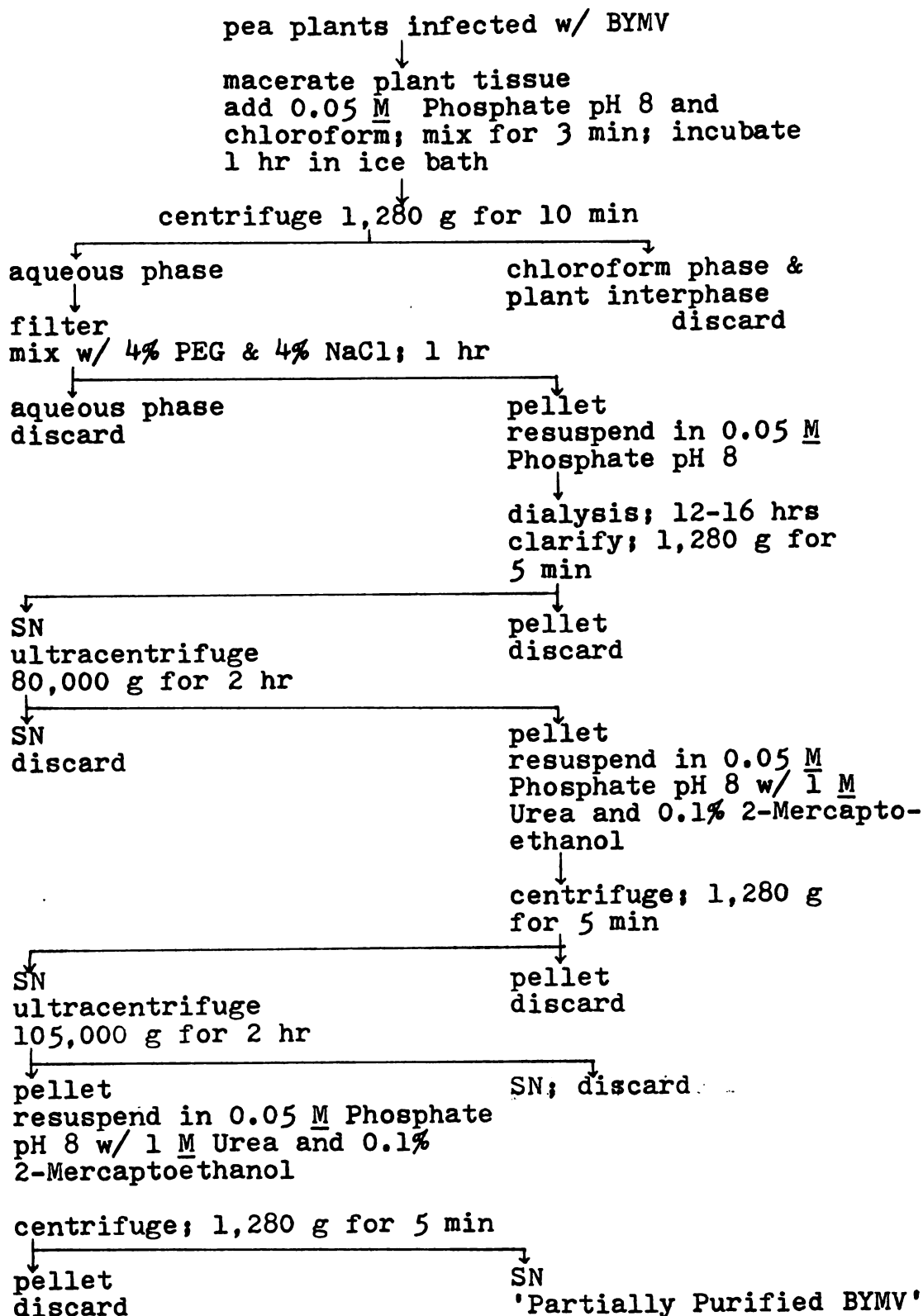
virus retention. The BYMV was prepared as defined by the crude purification technique except that after the first ultracentrifugation the pellets were resuspended in different test solutions. The pellets were resuspended in either of 3 buffers: (i) 0.05 M Phosphate buffer pH 8, (ii) 0.05 M Phosphate buffer pH 7.5 with 0.1% 2-Mercaptoethanol, or (iii) 0.025 M Phosphate buffer pH 7.4 with 1 M Urea and 0.1% 2-Mercaptoethanol. They were further differentially centrifuged (1,280 g for 5 min, then 105,000 g for 2 hr) and the final pellet was taken up in the suggested 0.025 M Phosphate buffer pH 7.4 with 1 M Urea and 0.1% 2-Mercaptoethanol. These three suspensions were each layered onto separate 20-50% linear sucrose gradients and centrifuged at 18,000 rpm (Beckman SW 27.1) for 1 hr, then analyzed with a UV analyzer (254 nm) for detection and concentration differences of the virus in each preparation (Figure 1). During the analyzation, 2 ml aliquots at two stages (indicated with arrows) of each run were set aside and used to mechanically inoculate broadbeans to determine the infectivity of this preparation. The results (Figure 1) showed that: (i) The sample most likely containing the virus is the large peak. Through infectivity bioassays, the large peak of the preparation made in 0.025 M Phosphate buffer pH 7.4 with 1 M Urea and 0.1% 2-Mercaptoethanol (Figure 1, C) remained as the only infective sample. (Each sample was mechanically inoculated onto 4 broadbeans, and all the samples were not infective except this sample which infected 3 of 4 plants). (ii) The

absorbtion spectra showed varying amounts of virus were retained and resuspended. Both preparations of Phosphate buffer plus the additional chemicals (Figure 1, B and C) resuspended more material than the control solution (Figure 1, A), yet only the virus preparation made with Urea and 2-Mercaptoethanol (Figure 1, C) added in the buffer to resuspend the pellets retained its mechanical infectivity. With the evidence that the addition of Urea and 2-Mercaptoethanol to the buffers used to resuspend the ultracentrifuged pellets was advantageous, the purification procedure for BYMV was finalized. The entire procedure is modeled in Table 4.

Electrophoresis: Initially, the power running the column was 50 mA and 200 V (the amperage was held constant and the voltage was allowed to fluctuate with changing resistance of the column gradient), and held for 2-3 hrs. Since the separation was poor, after consultation (J.V. French, personal communication) the power was altered to 10 mA (constant) and 35 V (variable), with the electrophoresis continuing for 16 hrs. The altered power change yielded fair separation yet none of the samples taken from the column were infective (Figure 2). Though this electrophoresis clearly showed differences between preparations of Mich and Ore BYMV, since all the samples taken were not infective, another method of sucrose density gradient separation was employed.

Density Gradient Centrifugation: The method of Clarke (1974) to separate isolates of PEMV was tried with BYMV.

Table 4. Partial purification of BYMV



The sucrose density gradient tubes were made into a 20-50% linear gradient and the virus was mixed into ultimately a 2.5% virus-sucrose preparation and layered on the gradient. The gradients were centrifuged at 18,000 rpm (Beckman SW 27.1) for 1 hr, then analyzed on the ultraviolet analyzed (254 nm). The results were inconclusive. Prior to incorporating Urea and 2-Mercaptoethanol into the buffer, the virus preparation remained infective yet would not separate and layer in the gradient (Figure 3). Longer ultracentrifuge runs and higher speed trials were ineffective at attaining better separation.

Serology: The serological tests used to help differentiate these isolates of BYMV gave the following results.

The Ring Interface Precipitin test showed that the antisera produced for each of the three antigens to be of very low titer (Table 5). The controls of this indicated

Table 5. Serology Results: Titer of antibodies of various antigens as determined by the Ring Interface Precipitin Test.

Antigen	Serum dilution (w/ PBS)						
	0	1/2	1/4	1/8	1/16	1/32	1/64
Ore BYMV	+	+	+	+	+	+(?)	+
Mich BYMV	+	+(?)	-	-	-	-	-
Healthy plant material	-	-	-	-	-	-	-

that the antisera, although low in titer, was not contaminated with host plant material. The same purification

technique was (described above) was used for all the antigen preparation. Though the purification technique was suited to yield infective virus, it was assumed that many host plant proteins would in fact remain in all the preparations. The purification and preparation of healthy pea plant antigen, to react against both isolates of BYMV, would help to tell the amount of host plant protein that was purified along with the virus. Since the procedure for the preparation of the healthy pea plant antigen was exactly the same as the virus preparation (and in fact, host plant antigen was always prepared side by side along with either isolate as it was purified), the healthy pea plant antigen could be used in serological tests to assay the host pea plant contamination of the virus antigen preparations. The healthy host plant antigen appeared as a very clear solution (rather than the densely opalescent color of Mich BYMV, or the slightly opalescent color of Ore BYMV) and the results show that no antisera was produced against the healthy pea plant antigen.

The Microslide Gel Diffusion test showed that the particles in Ore BYMV preparation could diffuse through the gel to interact with the antisera. In this case, the titer of the Mich BYMV preparation was too low to be detected by this technique. Basically, this procedure replicates the Agar Gel double diffusion test except that the Microslide Gel diffusion relies on whole particle diffusion rather than relying upon SLS to degrade the particles and allow them to diffuse.

The Agar Gel diffusion test showed primarily the same results as the Microslide Gel Diffusion tests, in that the titer of the healthy antisera and the virus antisera were so low to be detected by the test. This test also showed cross-reactivity between Ore BYMV antigen and Mich BYMV antisera. The results (Figure 4) showed that the Ab produced against Ore BYMV did react together with the Ore BYMV Ag (Figure 4, A), the Ab produced against the Mich BYMV Ag did not react, but reacted with the Ore BYMV Ag (Figure 4, C), and the healthy plant antisera did not react with any antigens tested.

Symptom Expression: In the course of the maintenance of the virus isolates, the symptom expression of each isolate appears to have changed on the garden pea, P. sativum cv. 'Melting Sugar'. Also due primarily to the selection of each isolate in its most preferred means, the vector transmission rate of Ore BYMV has increased slightly (Kukorowski, unpublished data). Compared with the original Ore BYMV material after 3 inoculations with aphids, the Ore BYMV material after 12 inoculations with aphids had about a 5% increase in the vector transmission rate. This is significant if one considers that more than likely, each isolate exists as a combination of many su isolates of the virus, and the expression of the virus, either through vector transmissibility rates or symptom severity or symptom expression, depends on most likely the dominant sub isolate (Kamm, 1969). Thus, the virus sub isolates that are involved

somehow with vector transmission are selected out each time. In the case of Mich BYMV, the virus was maintained throughout the experiment via mechanical inoculation, and it changed symptoms from a yellow-green systemic mosaic to a light green-dark green systemic mosaic, but records on rate of mechanical transmissibility were not kept.

DISCUSSION

Purification: There have been accounts and methods developed for purifying long flexuous rod viruses (Huttinga, 1973; Andrew Grannet, personal communication; Stevenson and Hagedorn, 1973), but each of these methods proved inadequate for the criteria that I established. The purification procedure had to retain the virus in its infective state, and unfortunately the methods suggested by Huttinga and Grannet, while producing abundant particles, did not yield infective virions.

The PEG/Differential Centrifugation purification method that was utilized had many shortcomings. Hopefully these defects can be worked out of the purification scheme, but the resultant partially pure preparation substantiated the criteria. New designs of PEG purification, Density gradient-PEG precipitation by Knesek et al. (1974), may yield more pure preparations with higher concentrations. Of course, the infectivity of these methods will need to be extensively tested. The purification procedures that were investigated (Huttinga, 1973; Grannet, personal communication) gave such poor infectivity that alternate methods had to be found. As it occurred, a method of PEG precipitation coupled with differential centrifugation eventually proved to be the most successful method; as it was very

very similar to Huttinga's (1973) purification method with the difference relating to buffer selection and the use of PEG precipitation. Both BYMV isolates purified much better in phosphate buffer than anything else attempted. Considerable effort was spent searching for the buffer to give the most infective preparation, yet in retrospect, though the buffer selection was important (as it turned out, Huttinga's method failed with the buffers he suggested, but succeeded with other buffers), critical inspection of infectivity of each step in the purification procedure should be stressed more fully.

The purification procedure used for BYMV turned out to be very successful, in comparison to the other methods tried. The procedure gave good infectivity, but based on transmission electron microscope inspection, it was evident that most particles of the preparation were broken. It was distressing that the purification technique did not give full particles, yet these results very pointedly asked the question, "How much of the particle is necessary for infectivity?" Surely, the coat protein portion of the virion is not necessary for infection, because it has been shown that naked RNA can be infective (Matthews, 1966). In this purification technique either the low molecular weight BYMV RNA is separated from the coat protein sometime during the purification and is purified along with the remaining broken particles or the RNA inside the broken particles remaining at the end of the purification scheme have all the information necessary for replication of the entire

intact virus particle. Each isolate, either in crude sap or partially purified, produced characteristic symptoms on P. vulgaris cv. 'Bountiful', P. sativum cv. 'Melting Sugar', and V. faba L. (Figure 5).

Electrophoresis: It was hoped that Density Gradient Electrophoresis would be of great use to help solve the dilemma of BYMV differential transmission. The technique was chosen for two reasons, (i) since virus particles are polar, they would be attracted at various locations in an electrophoretic field, and (ii) the sucrose density gradient electrophoresis was thought to be a "gentle" method of separating virus from host plant material. It was also hoped that further electrophoresis could separate various protein components of the virus. The electrophoresis technique had a serious shortcoming, however, in that with prolonged usage, necessary for good separation, the samples of virus material became non-infective. Separation of the different isolates was possible (Figure 2), but since none of the samples were infective, the use of the electrophoresis column to improve purification of BYMV was discarded.

Density Gradient Centrifugation: The separation of the virus preparation in the sucrose density gradient was not satisfactory. At its best, the technique showed some separation of virus. But either most of the long flexuous rods did not migrate at all into the sucrose (and became moderately separated in the top layers of sucrose) or the virus particles aggregated together and pelleted out

completely after centrifugation.

The problems with both sucrose density gradient techniques could possibly be worked out, but probably represent much too frustrating a venture for expedient results. With Ore BYMV in the early tests with electrophoresis, I attained a sample that was infective, yet the separation of material (virus from host plant) did not occur. It appears that as the virus gradually separated from the host plant material it became non-infective. After the rate zonal centrifugation, it was learned that Urea and 2-Mercaptoethanol added to the buffer helped prevent aggregation and agglutination of the virus particles. However, the virus also appeared to be too bouyant to enter the gradient, even after lengthier ultracentrifugation or higher speeds.

Serology: The antisera produced against all 3 antigens yielded disappointingly poor titers of antibodies. Because it is more difficult to purify long flexuous rods, the poor results of Mich BYMV antisera production indicate that, that particular isolate should be purified and serological experiments conducted in another buffer that would help produce a higher titer of antibodies (Clarke, 1974). The purification scheme was originally thought quite adequate in selecting for the virus and voiding any extraneous host plant material, and this view was supported when the healthy host plant antisera was produced with such a low titer (as compared with the titer of Ore BYMV antisera). The antisera produced against Ore BYMV yielded a workable titer, but without the necessary information that should be supplied

from the Mich BYMV antisera, very little cross reactivity information can be deduced. It can be said that the Ore BYMV antigen does in fact cross react with the Mich BYMV antisera, as should be expected, yet the diffuse nature of the band tells nothing of the relatedness of the two BYMV's.

CONCLUSION

It is reasonable to expect that each isolate of BYMV used in this experiment could have substantial differences. However, the only concrete known information on the isolates appears as transmission differences amongst aphids and symptom differences in bean plants (P. vulgaris cv. 'Bountiful') (Thottappilly et al., 1972). It was hoped that a serological identification of the reason for differential transmission could be achieved. The serological experiments showed only that the isolates are related, but could not determine the degree of relatedness. With better antisera production, better results may exist in explaining more possible isolate differences. However, it is clear that even very specific serological analysis may be insufficient to explain differential transmission. Many pitfalls exist in the pathway of adequately explaining non persistent virus differential transmission. If in fact a "transmission factor" is necessary for the transmission of the Ore BYMV (Govier and Kassanis, 1974), then even the most specific serological tests will not add to the evidence for differential transmission. This "transmission factor" (thought to be a low molecular weight protein) has been shown to aid the transmission of Potato Virus Y (PVY), but unfortunately,

with BYMV no evidence yet has shown the occurrence of a "transmission factor". It is often assumed many times that there must be some differences between the isolates that determine their transmissibility. This may indeed be the entire answer, but the vector may play more than just a passive role in virus transmission. Although still just an infant theory, the thought that these viruses may be transmitted via vector regurgitation remains quite plausible, and if such a biological phenomenon, of regurgitation, were to provide clues for unlocking this differential transmission mystery, then less emphasis would have to be placed upon virus composition.

These two isolates were shown in density gradient electrophoresis experiments to have a common migrating component, but each isolate had a separate different component which migrated at a different rate than the common component (Figure 3). Though it may seem presumptuous to call attention to such differences as possible differential transmission differences, one has to keep in mind that both isolates when placed on the electrophoretic column were in a partially purified state and the fact that the Ore BYMV loses its vector transmission infectivity after the very first purification step (macerating the tissue) seems to emphasize these differences as more likely to be other isolate differences. The situation of protein mobility differences of Ore BYMV and Mich BYMV may be the necessary clue to explain differential transmissibility in BYMV, as was the case in PEMV (Clarke, 1974). However, Cauliflower Mosaic Virus (Lung and Pirone,

1973) investigations showed that a non transmissible isolate migrated on an agarose-acrylamide gel intermediate of two transmissible isolates.

BYMV proved to be a very difficult virus to use to answer such an intriguing question about differential transmission. As a long flexuous rod it was difficult to purify and then keep in an infective state. The virus tended to aggregate and agglutinate which prohibited rate zonal centrifugation as a good method of further purifying the virus. Density gradient electrophoresis separated the virus, but made it non-infective. For such long flexuous rod viruses, the sucrose density gradient methods should really not be utilized since the methods are too harsh on maintaining workable virus. A better serological analysis should be made to better determine any more isolate differences or similarities between the two isolates, yet one should be wary of serological answers to biological phenomenon questions. The best or only answer to properly answer this differential transmission problem may be to rather look more closely at any interactions, including feeding, between the virus and its vector.

LIST OF FIGURES

Figure 1. Ultraviolet analyzation* of partially purified Ore BYMV samples resuspended after ultracentrifugation in different buffers: Effects of these buffers on virus retention and infectivity**.

- A.) 0.05 M Phosphate buffer pH 8
- B.) 0.05 M Phosphate buffer pH 7.5 with 0.1% 2-Mercaptoethanol
- C.) 0.025 M Phosphate buffer pH 7.4 with 1 M Urea and 0.1% 2-Mercaptoethanol

* 254 nm

** 2 ml aliquot samples taken at two positions in the sucrose density gradient as marked by the arrows

RELATIVE ABSORBANCE AT 254 nm.

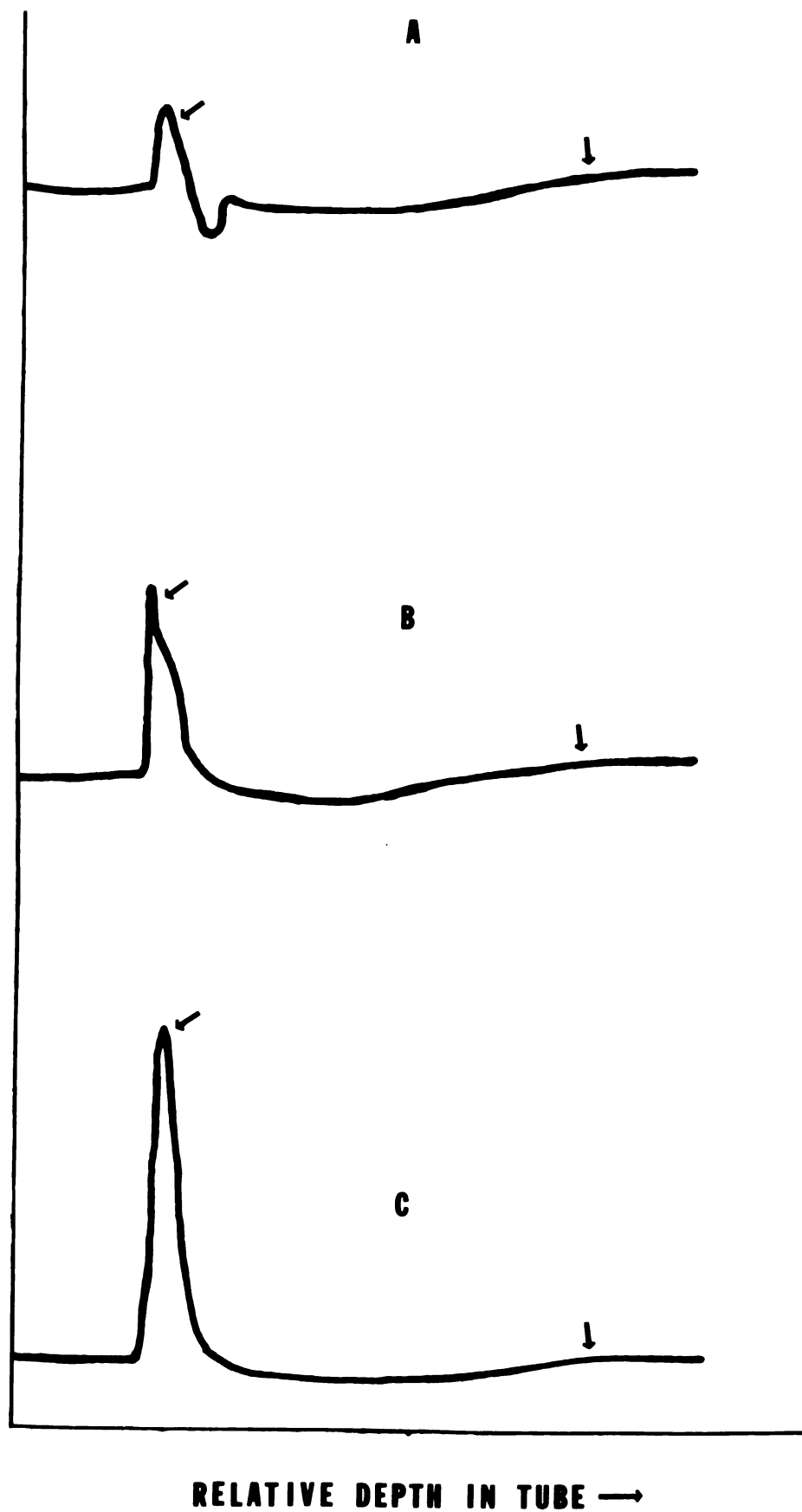


Figure 2. Ultraviolet analyzation* of partially purified BYMV isolates after density gradient electrophoresis: Differential separation of different components of each isolates**.

A.) Mich BYMV

B.) Ore BYMV

* 254 nm

** samples taken at numerous locations along each curve yielded no infectivity when mechanically inoculated onto either broadbean or pea

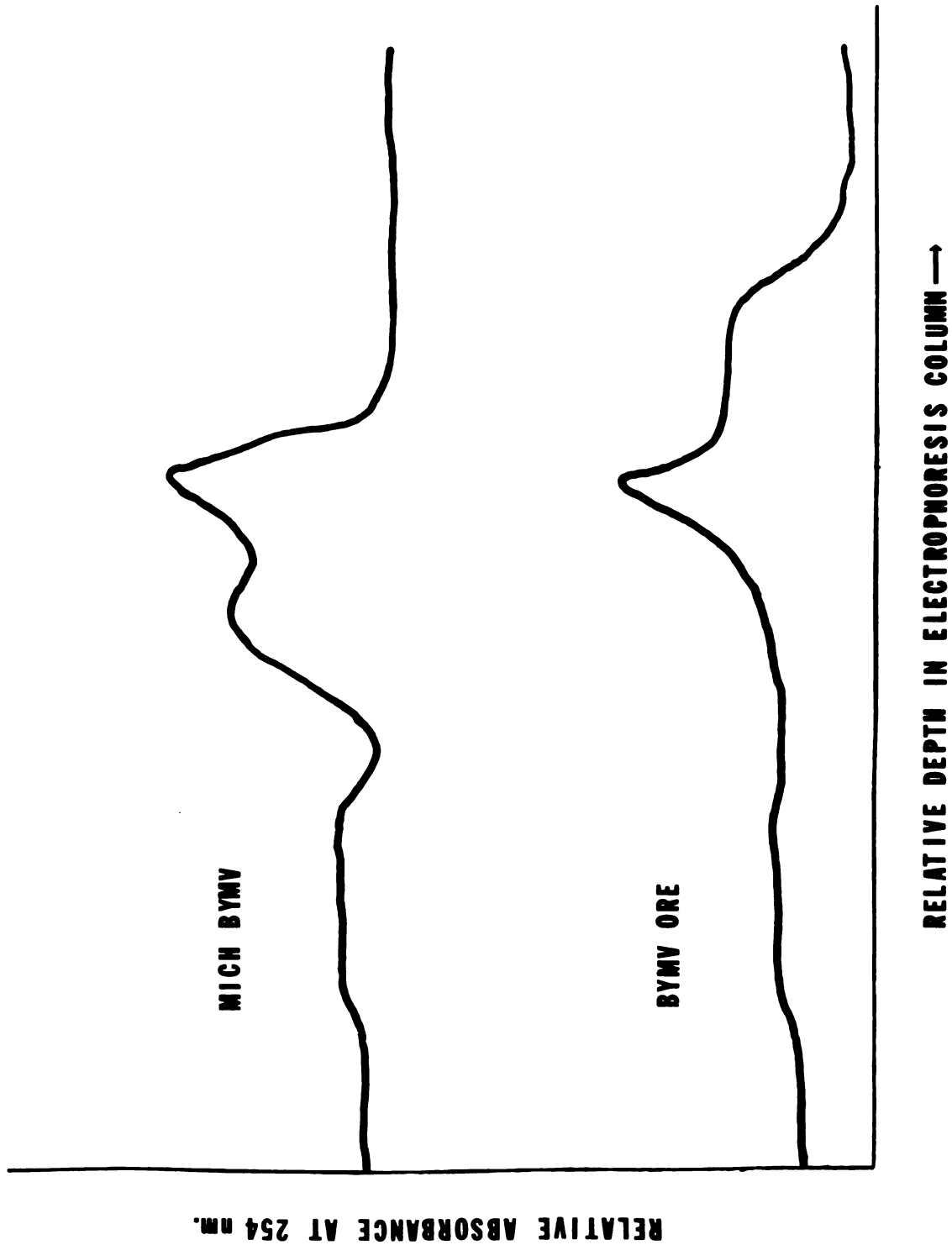


Figure 3. Ultraviolet analyzation* of partially purified BYMV isolates and healthy pea given rate zonal centrifugation**.

- A.) Control (healthy pea plant protein)
- B.) Mich BYMV
- C.) Ore BYMV

* 254 nm

** extracts layered onto a 20-50% linear sucrose gradient and centrifuged at 18,000 rpm (Beckman SW 27.1) for 1 hr

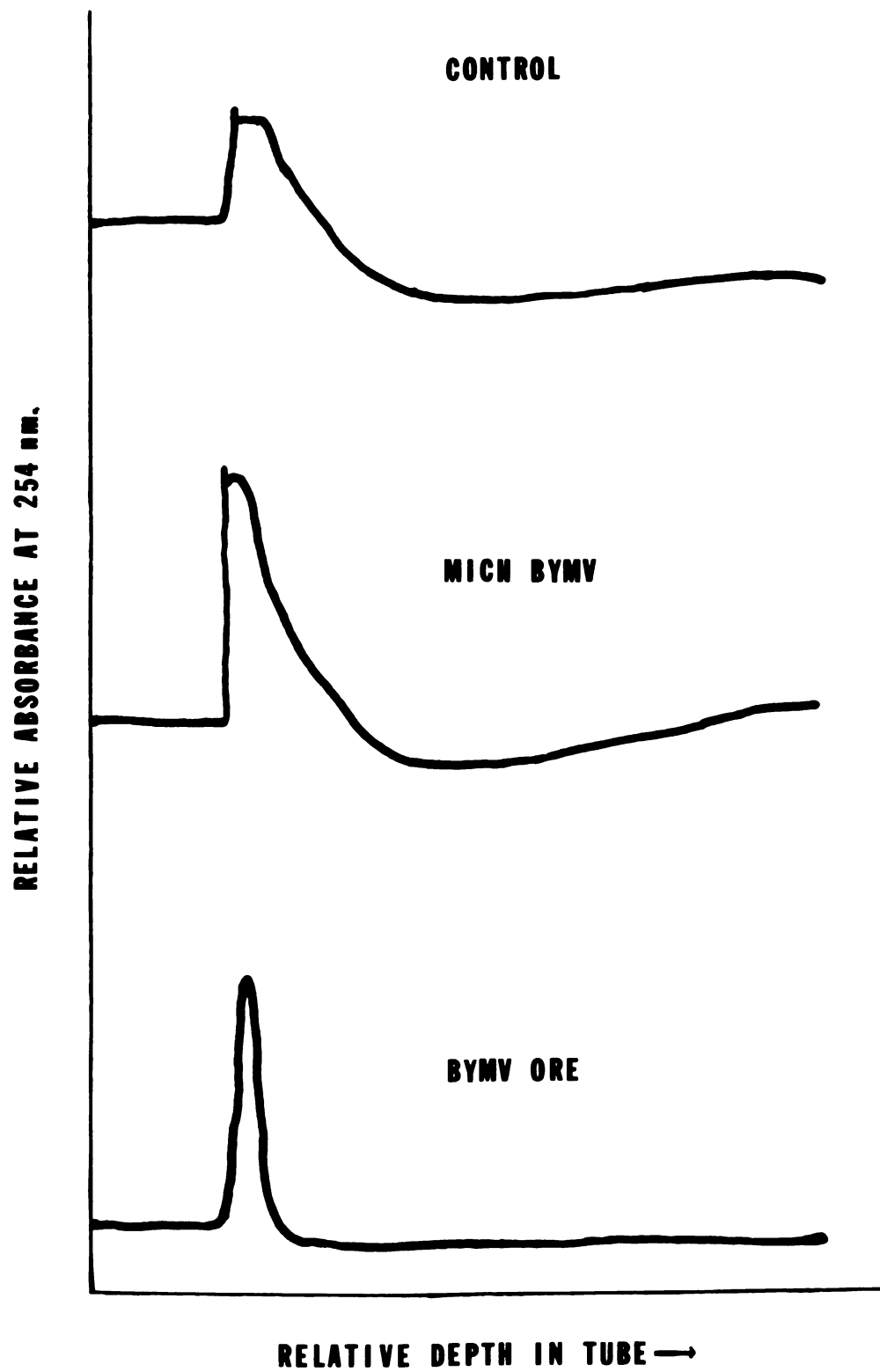


Figure 4. Serology results (cont.)

II. Results of Microslide Gel Diffusion tests: Precipitin band patterns using Mich BYMV and Ore BYMV

D. Reaction of: TAs (antisera against Ore BYMV) against 3 antigens at either 0 or $\frac{1}{2}$ dilutions

Antigen	Agar well (dilution)	
Mich BYMV	3 (0)	6 ($\frac{1}{2}$)
Ore BYMV	2 (0)	5 ($\frac{1}{2}$)
healthy pea protein	1 (0)	4 ($\frac{1}{2}$)

E. Reaction of: NTAs (antisera against Mich BYMV) against 3 antigens at either 0 or $\frac{1}{2}$ dilutions

Antigen	Agar well (dilution)	
Mich BYMV	3 (0)	6 ($\frac{1}{2}$)
Ore BYMV	2 (0)	5 ($\frac{1}{2}$)
healthy pea protein	1 (0)	4 ($\frac{1}{2}$)

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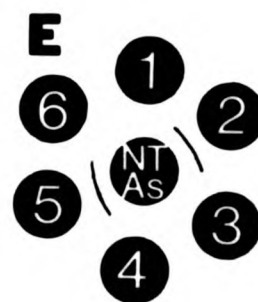
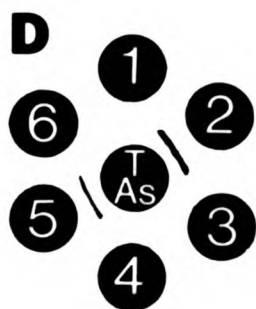
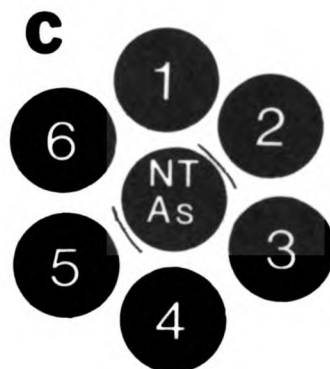
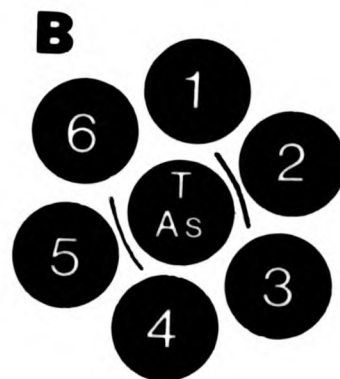
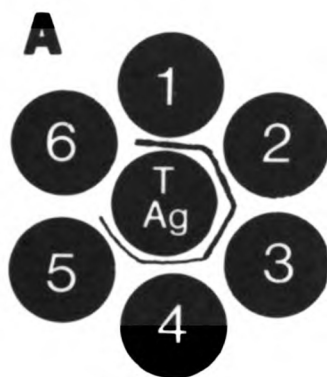


Figure 5. BYMV symptom expression on host legume plants.

- A.) Broadbean, Vicia faba L., uninfected
- B.) Broadbean, V. faba L., showing systemic, mosaic Ore BYMV infection
- C.) Garden pea, Pisum sativum cv. 'Melting Sugar', showing systemic, mosaic Ore BYMV infection
- D.) Bean, Phaeseolus vulgaris cv. 'Bountiful', showing systemic, mosaic Mich BYMV infection

A



B



C



D



LIST OF REFERENCES

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