# Serology, Physical Properties, and Purification of Unaggregated Infectious Maize Dwarf Mosaic Virus

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## ABSTRACT

A method is given for rapid purification of unaggregated, infectious maize dwarf mosaic virus-B (MDMV-B) using a nonionic detergent and without use of organic solvents. Yields of purified virus varied from 0.5-2.8 mg/100 g infected tissue.

None of the antisera produced against virus purified by utilization of chloroform, detergent, or urea reacted with healthy corn juice in liquid precipitin tests. However, the antisera produced precipitin lines in agar diffusion plates against healthy corn juice.

MDMV was very sensitive to RNase. Infectivity was destroyed within 30 sec by 1 μg/ml RNase in 1:10 diluted juice buffered with phosphate and containing 0.02 M 2-mercaptoethanol. Virus remained infectious in 1:10 diluted juice up to 24 hr and for 2.5 years in frozen dried leaves. MDMV-B had a 280/260 ratio of 0.82, and a sedimentation constant of 167 S.

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*Additional key words:* RNase susceptibility, serology.

Although several procedures for the purification of maize dwarf mosaic virus (MDMV) or the related sugarcane mosaic virus (SCMV) have been reported, we have not been able consistently and routinely to purify infectious MDMV with these methods (14, 17, 18, 19). Another MDMV purification procedure published (21) while this investigation was in the final stages was not tried.

Snazelle et al. (21) reported difficulties with published procedures. During the 1968, 1969, and 1970 annual meetings of the North Central States Cooperative Regional Project on Dwarfing Virus Diseases of Corn, aggregation, insolubilization, loss of infectivity of the virus, and contamination of the virus with host proteins were reported as problems encountered in attempts to purify MDMV. Damirdagh & Shepherd (8) have produced an excellent treatment of the problems involved in purification of the viruses in the potato virus Y group. Purification of MDMV is especially difficult to achieve because the virus is relatively unstable (17, 22).

MATERIALS AND METHODS.–*Virus source.*–Maize dwarf mosaic virus (A and B strain) were obtained from naturally infected sweet corn in Nebraska in 1967. Serology (antiserum supplied by R. E. Ford, Ames, Iowa), host range, and symptoms were used to identify the virus. Virus was maintained in mechanically inoculated sweet corn (*Zea mays* L. 'Goldencross Bantam') grown in the greenhouse.

*Virus purification.*–Plants with marked symptoms were harvested 10 days to 6 weeks after inoculation. Leaf tissue was ground in cold 0.1 M TAC extraction buffer (3 ml/g leaf tissue) for 3 min at high speed in a Waring Blendor. The TAC buffer consisted of 0.1 M Tris [tris(hydroxymethyl) amino methane], 0.05 M citric acid, 0.8% polyvinyl pyrrolidone (PVP, avg mol wt 40,000), and 0.2% 2-mercaptoethanol (2-ME), and was adjusted to pH 8.2 with 1,3-propane diamine (∿0.4%, practical grade). Juice was expressed through muslin and stirred with a magnetic stirrer, while 1 ml of 2.0 M $CaCl_2$ and then 2 ml of 2.0 M $K_2HPO_4$ were added for each 50 ml juice. The resulting mixture was centrifuged for 10 min at 8,500 rpm in a Servall GSA rotor. The supernatant liquid was recovered, and Triton X-100 (alkylphenoxypolyethoxyethanol, Rohm and Haas, Philadelphia, Pa.) as a 20% solution in distilled water, and polyethylene glycol, MW 6000 (PEG 6000), were added to make final concentrations of these compounds of 0.5 and 6%, respectively. The solution was stirred for 2-3 hr at room temperature, and the precipitate that formed was subsequently removed by a 10-min low-speed centrifugation at 8,500 rpm in the GSA rotor. Pellets were taken up in sufficient cold 0.01 M TACm buffer (0.01 M Tris, 0.005 M citric acid, 0.01 M 2-ME, and 0.1% Triton X-100) to provide a final volume one-twentieth that of the juice. The pH of the buffer was adjusted to 7.2 with 0.2% 1,3-propane diamine. The resulting suspension was stirred briefly to dissolve clumps, then centrifuged for 10 min at 9,000 in a Servall SS-34 rotor to remove insoluble matter. The light green-yellow, opaque supernatant liquid was layered on 10 ml of 30% decolorized sucrose (6) dissolved in 0.01 M TACm buffer in 30-ml tubes of the Spinco No. 30 rotor. A high-speed centrifugation for 1 hr at 28,000 rpm (68,000 *g*) was sufficient to provide pellets of the virus. The supernatant liquid was not tested for residual virus. The clear, transparent to light-green pellets were dissolved in 0.01 M

TACm buffer. Insoluble material was removed by centrifugation for 10 min at 9,000 rpm in the SS-34 rotor. The opalescent supernatant solution was layered on linear 100-400 mg/ml sucrose density gradients (6). Gradients were buffered with 0.01 M TACm buffer and equilibrated overnight at 3 C before use. Centrifugation was for 2 hr at 23,000 rpm in a Spinco SW 25.1 swinging bucket rotor. Centrifuged gradients were scanned photometrically at 254 nm with an ISCO density-gradient fractionator coupled to an external recorder, or the bands either were removed and dialyzed or centrifuged for 1 hr at 28,000 rpm in the No. 30 rotor to remove the sucrose. Time required for purification (up to removal of virus bands from density gradients) was 7-8 hr.

*Serology.*—Rabbits were injected for 4 consecutive weeks with a total of 4-5 mg MDMV-B purified by removal of green pigments with chloroform (five rabbits) as used by Shepherd (19), or by dispersion of green pigments by urea (two rabbits) as used by Damirdagh & Shepherd (8). Five rabbits were injected with virus purified according to the procedure described above. All antigen was emulsified with Freund's complete adjuvant and deposited intramuscularly and intradermally. Rabbits were sacrificed 6-7 weeks after the initial injection.

High pH gel-diffusion plates (10) were prepared by combining equal volumes of autoclaved 1% agar (lot No. 02-106, Baltimore Biological Laboratory, Baltimore, Md.) and an unautoclaved solution consisting of 0.2 M dibasic ammonium phosphate, 1%

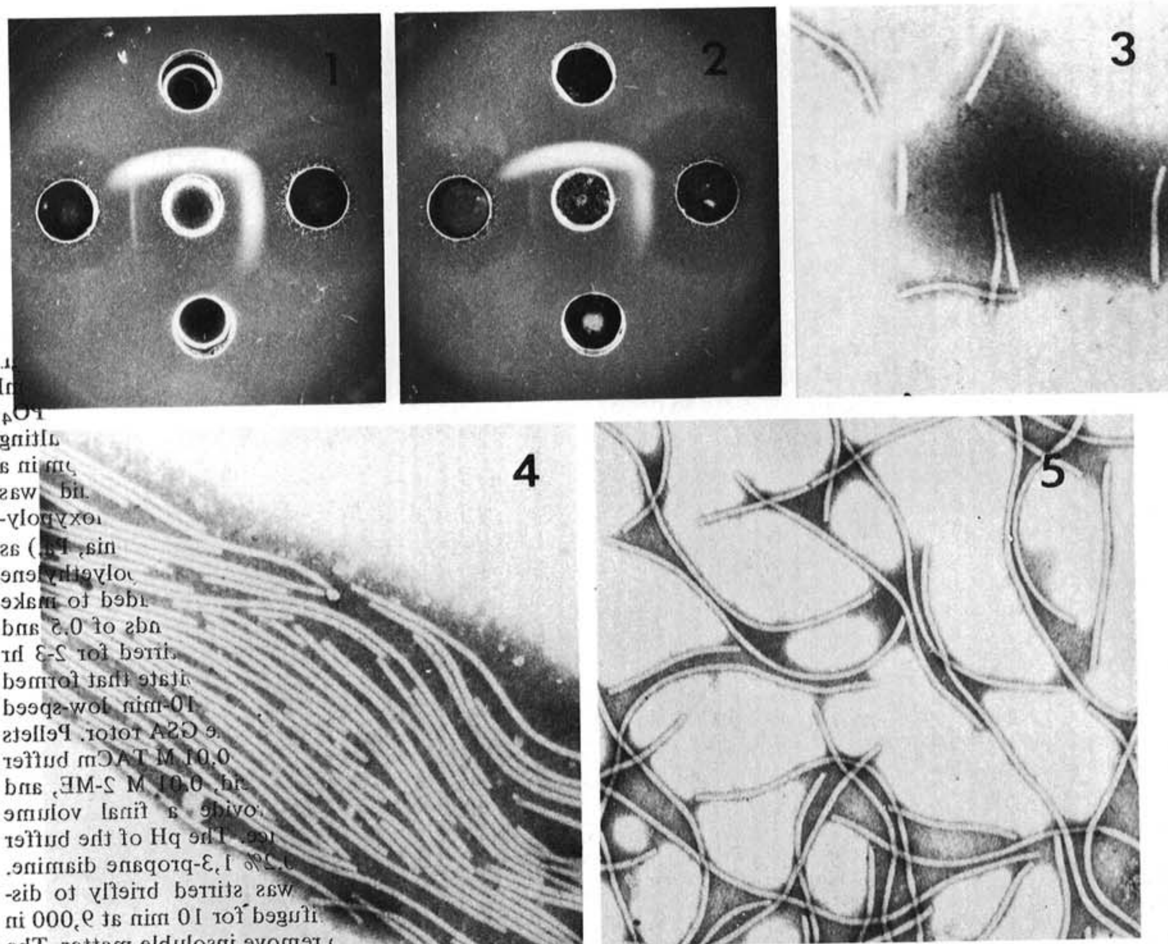



**Fig. 1-5. 1)** High pH agar gel diffusion plate. Center well: antiserum against maize dwarf mosaic virus (MDMV) purified by utilization of chloroform to remove green plant material. Clockwise from top well: 1 mg/ml purified MDMV, crude juice of MDMV-infected corn, high-speed pellet of healthy plant material isolated with virus purification method, and crude juice of healthy corn. **2)** High pH agar gell diffusion plate. Center well charged with antiserum prepared against MDMV purified according to the "detergent" method described in text. Other wells charged as for Fig. 1. **3)** Negatively stained preparation of short virus particles found in front of the main peak from a density-gradient centrifugation of MDMV-B (× 52,000). **4)** Aggregated virus as found in the pellet of density-gradient centrifugation columns (× 52,000). **5)** Unaggregated virus from the main peak of density gradient columns (× 52,000).

1,3-propane diamine, and 0.2% Igepon T-73 (active ingredient 28-30% *N*-methyl-*N*-oleoyl taurate). The results were recorded after incubating the plates overnight (18 hr) at 37 C.

*RNase susceptibility.*—Systemically infected leaves, 10-14 days after inoculation, were ground in 10 X their weight of 0.01 M $K_2HPO_4$ which contained 0.02 M 2-ME, or in 0.01 M $K_2HPO_4$. Both extracts then were adjusted with NaOH to pH 7.2. The juice was strained through muslin and aliquots were used in the following tests. Bovine pancreatic ribonuclease (Sigma Chemical Co., St. Louis, Mo.) was added to the juice to make 1 μg/ml. The flask was briefly swirled, and sweet corn seedlings were brush-inoculated with the juice. The plants were then immediately rinsed with water. Approximately 30 sec elapsed between RNase addition and rinsing. The same juice was also used as inoculum 10, 20, 40, and 60 min later. Controls consisted of plants inoculated with untreated juice 60 min after extraction. The experiment was repeated 4 times for MDMV-B, and was performed once for MDMV-A. Results were read 14 days after inoculation.

*Extinction coefficient determination.*—Purified virus was concentrated by high-speed centrifugation, and the clear pellets were dissolved in TACm buffer without 2-ME or detergent. Residual sucrose was removed by dialysis against TACm buffer without detergent or 2-ME for 2 days with two changes of 4 liters. Virus was dried at 90 C for 16 hr.

*Sedimentation constant determination.*—The sedimentation constant of MDMV-B was determined in a linear-log gradient (7) of sucrose dissolved in TACm buffer. Sedimentation markers used were tobacco mosaic virus (190 S), brome mosaic virus (79 S), and southern bean mosaic virus (115 S).

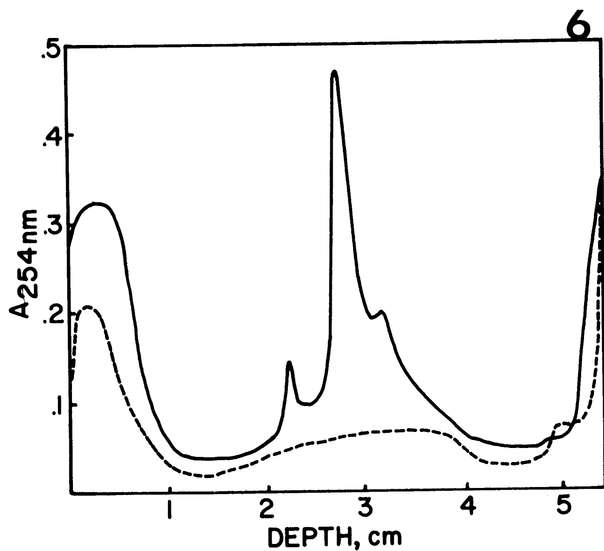
*Negative staining.*—Stain was prepared as reported by Ball & Brakke (1), and consisted of a mixture of three parts neutral 2% (w/v) potassium phosphotungstate and one part 1% (w/v) vanadatomolybdate.

RESULTS.—*Virus purification.*—Virus purified by removal of green pigments with chloroform still contained host proteins. Homologous antiserum gave a precipitin line against healthy corn juice in gel diffusion plates (Fig. 1). Antiserum produced against virus purified by the method reported here did not produce a line against healthy corn juice or only a faint one (Fig. 2). Five rabbits were injected with MDMV purified with the method reported here. Of the five rabbits, two produced antisera without detectable antibodies against healthy host protein; three antisera contained antibodies to healthy host protein, but only to plants older than approximately 4 weeks. No precipitin lines were obtained in agar diffusion plates with juice from 2-week-old healthy plants. In contrast, all antisera to virus purified by removal, or dispersion of green pigments with chloroform or urea, respectively, contained antibodies to healthy juice of plants of all ages. Urea at 0.5 M in 0.1 M TAC extraction buffer was tested as a dispersing agent of host proteins because urea, in another buffer system (8), has been used successfully to purify MDMV-A. Urea, however, was unsatisfactory for the purification of MDMV because unlike Triton X-100, it failed to disperse adequately the green plant material so that it could be separated from the virus.

Calcium phosphate was used to remove most green material according to an in situ formation scheme reported by Brakke (5), except that the calcium chloride was added first to avoid a rise in pH that resulted when the sequence was otherwise. Preformed calcium phosphate was not tested. MDMV-B was not adsorbed by calcium phosphate under the conditions of purification. However, Sehgal (17) found that partially purified MDMV was strongly absorbed by calcium phosphate gels.

The Triton X-100 purification method gave good yields of infectious MDMV-B almost free of healthy plant antigens. Comparable preparations from healthy plants yielded a small gelatinous pellet before density gradient centrifugation, but the pellet did not contain long flexuous rods typical of MDMV. The antisera did not give rise to gel precipitin lines against this preparation. Virus yields varying from 0.5 to 2.8 mg MDMV-B from 100 g tissue were not correlated with length of infection. The virus was unstable at a pH higher than 7, and at high salt concentrations; pH levels below 7 were not tested. The initial pH of the homogenate was kept at 8, however. Homogenation in 0.1 M TAC buffer at lower pH levels resulted in lower yields. The use of sodium bromide and potassium tartrate density gradient columns resulted in denatured virus.

Triton X-100 purified virus gave only one main light-scattering zone after density-gradient centrifugation (Fig. 6). Photometric scanning showed only one ultraviolet absorbing zone in the central part of the gradient, part of which resulted from the buffer (dashed line). The small peaks in front and after the main virus peak were not always present to the same extent. The peak in front of the main virus band consisted of short unstable rods (Fig. 3). When the material represented by the total peak was collected, dialyzed, and subjected to a second density gradient 24 hr later, the shorter rods, unlike the longer particles, were not present anymore. However, aggregation losses were severe and most virus was present in the pellet of the gradient tube. The concentration of the shorter virus rods varied from experiment to experiment. The long virus particles (main peak) were infectious by themselves, so that the presence of shorter particles is attributed to breakage during purification or incomplete coating. The small peak after the main peak possibly represents a dimer. The recovery of added absorbance units in the virus zone was low (∿30%), and some virus apparently sedimented in spite of the presence of detergent in the gradient column. Electron microscopy showed that virus was present in the pellet in aggregated form (Fig. 4). The pellet material was infectious, but was discarded after attempts to bring most of the virus into solution failed. Virus of the main peak consisted of long flexuous rods (Fig. 5) which were infectious at a concentration of 0.1 μg/ml (Table 1).



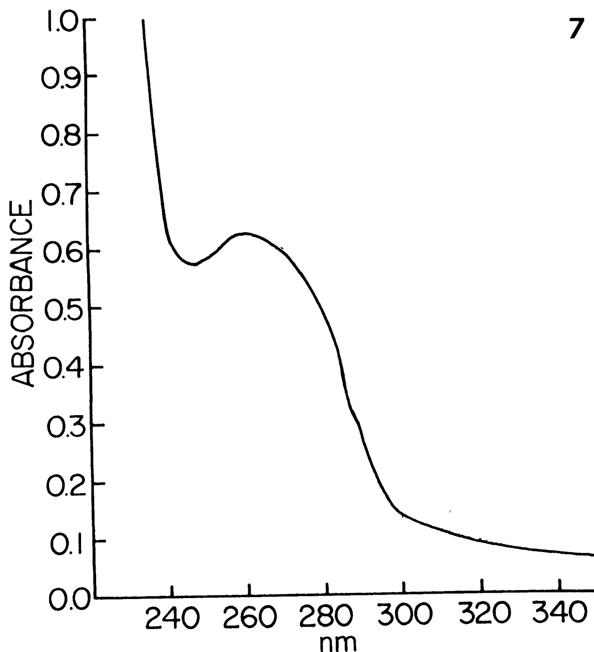




**Fig. 6-7. 6)** Ultraviolet scanning pattern of maize dwarf mosaic virus-B strain (MDMV-B) sucrose density-gradient column (solid line). The dashed line is the absorbance of a gradient column centrifuged after addition of buffer instead of virus solution. Some of the absorbance near the meniscus resulted from components or impurities in the buffer. Reasons for the uneven baseline in the rest of the gradient are unknown. **7)** Ultraviolet absorption spectrum of purified MDMV-B.

MDMV-B absorption spectra had a slight hump at 290 nm in all scans of preparations from 26 purifications (Fig. 7). Thus, tyrosine and tryptophan are probably present in the viral protein. This is in contrast to MDMV-A where the 290-nm hump has not been observed (18). The presence of at least tyrosine in MDMV-B was already known from iodination experiments with I-125 (*unpublished data*). The 280:260 ratio of MDMV-B (not corrected for light-scattering) was 0.82 (average of 21 purifications with min 0.80 and max 0.86). MDMV-A has a reported value of 0.85 after correction for light-scattering (18).

*Serology.*—Chloroform-purified, infectious virus gave several indications of having a high degree of purity: clear, gelatinous high-speed pellets were obtained after high-speed centrifugation of density-gradient zones; normal spectra were observed for a long flexuous virus with maxima at 259 nm (as in Fig. 7); reaction of the virus antiserum against healthy corn juice was not detected in liquid (drop) precipitin tests. However, it was necessary to develop another purification procedure for serological purposes after it was found that antiserum produced by injection of rabbits with MDMV purified by using chloroform or urea gave lines against healthy juice in agar double diffusion plates (Fig. 1).

Antisera elicited by Triton X-100 purified virus still had some detectable antibodies against healthy plant juice, but not against a concentrated preparation from healthy plants in double diffusion plates (Fig. 2). The concentrated preparation from healthy plants was prepared by the Triton X-100 purification procedure used for MDMV without the final density-gradient centrifugation. Instead, the first high-speed

TABLE 1. Infectivity of MDMV-B-infected corn leaf juice and purified virus after indicated treatment

| Treatment | Juice dilution[a] | Virus concentration[a] | Fraction of plants infected |
|---|---|---|---|
| MDMV + 1 μg/ml RNase[b] | 1/10 | | 1/159 |
| MDMV in crude juice[c] | 1/10 | | 77/89 |
| | 1/100 | | 37/78 |
| | 1/1,000 | | 6/95 |
| | 1/10,000 | | 3/94 |
| Semipurified virus[c] | | 100.0 μg/ml | 159/168 |
| | | 10.0 μg/ml | 131/176 |
| | | 1.0 μg/ml | 30/175 |
| | | 0.1 μg/ml | 5/174 |
| Purified virus[d] | | 10.0 μg/ml | 31/35 |
| | | 1.0 μg/ml | 16/34 |
| | | 0.1 μg/ml | 2/40 |

[a] Dilutions with 0.01 M phosphate buffer pH 7.2 containing 0.02 M 2-ME except that in two of four experiments in which RNase was added 2-ME was not added. MDMV = maize dwarf mosaic virus.

[b] Combined results of four experiments. Inoculation was completed within 30 sec after addition of RNase. The possibility that the one infected plant resulted from greenhouse contamination cannot be eliminated, although controls were free of infection.

[c] Result of one experiment after trend was established in others. Virus purified by centrifugation through sucrose cushion only and not placed on density gradients.

[d] Virus from a final density-gradient preparation, concentrated with a 1-hr high-speed centrifugation and dissolved in 0.01 M phosphate, pH 7.2, with 0.02 M 2-ME.

pellet was suspended in a small volume of buffer and used for agar diffusion tests.

All antisera against MDMV-B reacted in liquid (drop) precipitin tests against juice of infected plants, but not against juice of healthy plants. The highest dilution of antisera tested which still gave a visible precipitate was 1/512 for all three antisera. Liquid precipitin tests were performed under paraffin oil (20).

Agar diffusion plates had a pH of 9.1. MDMV broke down extensively and gave a prominent line due to the virus (Fig. 1, 2). At pH 7.2, in phosphate-buffered gel diffusion plates, only a weak line due to the virus was observed from the whole virus well. The alkaline treatment of MDMV occurs in high pH agar plates directly in the well, and no pretreatment with alkali, as performed by Wagner & Dale (23), is necessary. In high pH plates, crude juice or virus can be assayed directly in the plates without any additional treatment.

*Infectivity and storage of purified virus.*—Crude sap (1:10, w/v) inoculated at hourly intervals lost infectivity after 22-24 hr. The number of virus particles in the juice, observed by electron microscopy of negative stained preparations, did not diminish visibly even after 48 hr. The time necessary for purification influenced the specific infectivity of the final preparations. Initially, the pellet from the PEG 6000 precipitation was usually left to stir overnight at 4 C, and virus was further purified the following day. This virus was infectious at a concentration as low as 50-60 μg/ml. RNA prepared from virus left to stir overnight was not infectious (15). In an effort to increase infectivity, the PEG 6000 precipitate was stirred only 15 min before low-speed clarification and layering on 30% sucrose. Viral RNA prepared from these high-speed pellets was infectious. Triton X-100 purified virus, when freshly prepared, was infectious to 0.1 μg/ml; and 0.1 μg/ml was about as infectious as 1/10,000 diluted juice from plants (Table 1).

No difference, other than infectivity, could be detected between infectious and noninfectious virus. RNA prepared from either showed no detectable difference, and sedimented at the same rate in density gradients (15). Ultrathin sections of pelleted noninfectious virus stained as if RNA were present. Identical ultraviolet scanning patterns were obtained.

Purified noninfectious MDMV-B could be stored for months at 4 C in the presence of 0.01 M sodium azide or a few drops of ethylene dichloride without breakdown of virus particles as periodically assessed with a negative stain preparation in the electron microscope.

Purified MDMV-B could not be frozen without denaturation of the virus, nor could virus be dialyzed against distilled water without irreversible virus precipitation. MDMV-B was best stored in frozen leaves at -20 C; although the leaves became dry during storage the virus remained infectious for at least 2.5 years.

MDMV A and B were exceedingly sensitive to pancreatic bovine RNase. As little as 1 μg/ml destroyed all infectivity of virus in crude juice within 0.5 min (Table 1). MDMV-A was as sensitive to RNase as MDMV-B. Juice of MDMV-A infected plants was diluted 0.10 with 0.01 M phosphate buffer, pH 7.2, containing 0.02 M 2-ME, and inoculated to sweet corn seedlings before and after treatment with 1 μg/ml RNase (final concentration) for 1 min. Eighty-two of 86 untreated control plants were infected, and 0 of 100 plants inoculated with RNase-treated juice were infected. All other experiments were performed with MDMV-B only (Table 1).

Virus incubated with RNase for 10, 20, 40, or 60 min was not infectious. 2-Mercaptoethanol (ME) at 0.02 M did not prevent virus from becoming noninfectious when treated subsequently with RNase. However, 2-ME increased infectivity of juice from diseased plants, so it was routinely added. Sehgal (17) reported an increase in infectivity of juice treated with reducing agents. Apparently more factors than simply attack by RNase are involved in rendering virus noninfectious.

*Determination of extinction coefficient.*—MDMV-B was purified for dry weight determination with the Triton X-100 method. Virus was concentrated by high-speed centrifugation after purification. Pellets were dissolved in TACm buffer pH 7.2 without 2-ME or detergent, and residual sucrose was removed by dialysis. The dialyzed virus had a maximum absorption at 259 nm and a coefficient of extinction of 2.7 for a 1-mg/ml solution (2 determinations, 2.68 and 2.74).

The actual value of the coefficient could be lower than that reported. Three of five rabbits injected with similarly purified virus still showed a small reaction against host protein.

*Sedimentation constant.*—The sedimentation constant for MDMV-B was 167 ± 6 (average of 10 determinations on three preparations of virus in two centrifugations in the SW 41 rotor). This agrees with the sedimentation constant found for MDMV by Shepherd (19), but differs from other sedimentation constants reported, 155 S (2) and 148 S (17).

DISCUSSION.—It has been shown that an anionic detergent (4, 11, 16) can disperse plant viruses. Although the use of a detergent for plant virus purification was mentioned by Brakke in 1959 (4), detergents have been used only occasionally (4, 11, 13, 16). MDMV-B was dispersed by the use of nonionic detergent, Triton X-100. The precipitation of MDMV by PEG 6000 was not prevented by the detergent. This detergent has been successfully used in the purification of TMV from zinnia and spinach (13). TMV could not be purified otherwise from these plants because it aggregated with plant organelles. The presence of Triton X-100 throughout all steps of the purification procedure, except the first one, did not prevent losses due to aggregation. No other steps than those reported here have been taken to reduce aggregation losses as purification progressed, and there probably are substantial losses. However, the final yield from 500 g of tissue used in each attempt was always in milligram amounts, and sufficient for the determinations made here.

It was important to purify the virus as rapidly as possible to retain infectivity. Even a 24-hr period between grinding of the tissue and further purification of a semipurified preparation was enough to reduce the infectivity by a factor of 500 (equal infectivity of 50 μg/ml 24-hr-old virus and 0.1 μg/ml 10-hr-old virus).

Few plant viruses are known to be susceptible to RNase, and then only after prolonged incubation (9) or with larger amounts of RNase (3, 12). MDMV susceptibility to RNase has been reported previously (17), but not for times of less than 1 hr.

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