

# Purification of Maize Dwarf Mosaic Virus by Continuous-Flow Centrifugation

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## ABSTRACT

Continuous-flow centrifugation with equilibrium-zonal banding was used in the purification of maize dwarf mosaic virus (MDMV). Alterations in conventional techniques of continuous-flow centrifugation were required. The efficiency of the continuous-flow rotor (Beckman B16) was greatest at high flow rates at which only a fraction of the virus sedimented into the gradient during a single passage of extract. Multiple passages through the rotor allowed nearly complete removal (94%) of virus. An average of 89% of virus sedimenting into the rotor was recovered in the virus band from the gradient.

Further purification was achieved by isopycnic-zonal centrifugation in CsCl gradients. No detectable impurities were present in final virus preparations as judged by sedimentation properties and serology. Yields of 5-30  $\mu\text{g}$  of purified virus/g of infected corn leaves were obtained.

The isopycnic density of MDMV-A depended on the composition of the gradient. In CsCl-water gradients the density was 1.300 g/ml; in CsCl gradients containing 0.5 M potassium phosphate, 1 M urea, pH 7.0, it was 1.285 g/ml. The isopycnic density in sucrose was 1.266 g/ml.

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Continuous-flow centrifugation, as developed by Anderson et al. (2), permits convenient recovery of larger amounts of virus than conventional centrifugation. Continuous-flow centrifugation with isopycnic banding (1) has been applied successfully to the purification of viruses of bacterial or mammalian origin (5, 9, 10, 12, 13, 15, 21, 27, 28). To our knowledge, the technique has not been applied to plant virus purification. The technique appeared to have important advantages for the purification of plant viruses, especially those recoverable only in small quantities by conventional centrifugation.

Because of a need for large quantities of purified maize dwarf mosaic virus (MDMV), we explored the possibility of MDMV purification by continuous-flow centrifugation. Although MDMV has a significantly lower sedimentation coefficient ( $S_{20,w}=148-168$ ) (4, 18, 22, 24) than viruses previously purified by continuous-flow centrifugation, calculations using Berman's (6) formulae predicted that the technique would be feasible. Testing this prediction revealed that modifications of the usual procedure for continuous-flow centrifugation were required.

This paper describes the development of a procedure utilizing continuous-flow centrifugation for the rapid and convenient purification of relatively large quantities of MDMV. A preliminary report of this work has been published (16).

**MATERIALS AND METHODS.**—*Preparation of clarified extract.*—Virus for purification was increased either in corn (*Zea mays* L.) inbred Oh28, or sorghum [*Sorghum bicolor* (L.) Moench] 'Sart'. Strains A, D,

and E of MDMV (19) and 13B, a virus similar to MDMV-B (17), were used in the investigation. Plants were inoculated at the two- to three-leaf stage. Leaves with symptoms were harvested 4-8 weeks later. Leaves were homogenized in 0.5 M potassium phosphate, pH 7.0, plus 0.1% thioglycolic acid (1 g tissue/ml of buffer) in a Waring Blendor at room temperature. An equal or half-volume of chloroform was added to the homogenate, which was emulsified in the blender and then centrifuged at 8,000-9,500 rpm for 10-15 min in the GSA rotor of the Sorvall RC2-B centrifuge (Ivan Sorvall Co., Norwalk, Connecticut). The aqueous phase was recovered, filtered twice through glass wool, and used as the clarified extract in continuous-flow centrifugation. The above procedure was followed in all cases unless otherwise stated.

*Assays.*—Samples from purification steps were assayed serologically by the microprecipitin (MP) test [modified from Ball (3)], optically by rate-zonal centrifugation (8), and by infectivity tests. Two-fold dilution steps of antigen, and a 1:8 dilution of MDMV-A antiserum, were used in the MP test. Dilutions were made with physiologically buffered saline (0.85% NaCl, 0.01 M sodium phosphate, pH 7.0). Microprecipitin plates were incubated overnight at 3-6 C before reading. Rate-zonal assays were used for assessing the relative amount, purity, and extent of aggregation of virus. Unless otherwise stated, assays were done in linear percent sucrose gradients prepared in Beckman SW27 rotor (Beckman Instruments, Inc., Palo Alto, California) cellulose

nitrate tubes by successively layering 7.0, 10.0, 10.0, and 5.0 ml of 40, 30, 20, and 10% (w/w) sucrose in 0.5 M potassium phosphate, pH 7.0 [modified from Stace-Smith & Tremaine (26)]. Gradients were kept overnight or longer at 3-4 C before use. Virus samples of 0.3-0.5 ml were layered on gradients. After centrifugation to  $\omega^2 t = 7.3 \times 10^{10}$  rad<sup>2</sup>/sec, ca. 2.5 hr when operated at 27,000 rpm, gradients were scanned at 254 nm by the method of Brakke (7) using an ISCO Model D density-gradient fractionator and UA-2 ultraviolet analyzer (Instrumentation Specialties Co., Lincoln, Nebraska) coupled to an external chart recorder.

Systemic infectivity assays of samples were done with five dilutions (1:10, 1:30, 1:90, 1:270, and 1:810) in 0.01 M potassium phosphate, pH 7.0. Inoculations were made on 'Rio' sorghum in the two- to four-leaf stage. Ten to 30 plants were inoculated at each dilution. Carborundum (600-mesh) served as abrasive. Inoculum was applied by rubbing leaves with thumb and index finger, beginning with the greatest dilution. Results were recorded 14-19 days after inoculation.

**Continuous-flow centrifugation.**—The Beckman B16 rotor in the Model L4 ultracentrifuge was used for continuous-flow centrifugation. The rotor was loaded with sucrose gradient at 4,000 rpm and accelerated to 40,000 rpm. The rotor contained a 250-ml cushion, 350-ml gradient, and an approximate 150-ml overlay. The cushion and overlay were the heavy and light solutions, respectively, used to form the gradient. The gradient was formed with a Beckman high-capacity gradient pump, Model 141. Clarified virus suspension was pumped through the B16 rotating at 40,000 rpm, by means of a Cole-Parmer Ultramasterflex pump (Cole-Parmer Instrument Co., Chicago, Illinois). The Beckman  $\omega^2 t$  integrator was used to totalize the force. At the appropriate value, the rotor was decelerated to 4,000 rpm or stopped, and contents were displaced with a dense sucrose solution. During unloading, the gradient was scanned at 254 nm with the ISCO ultraviolet analyzer. Percent sucrose readings were determined with a B & L Abbé 3L refractometer (Bausch & Lomb, Rochester, New York) at room temperature. Readings were not corrected for buffer contribution.

**Isopycnic-zonal centrifugation.**—*CsCl*.—Virus-containing B16 gradient fractions were diluted 1:3 with buffer or distilled water, and the virus concentrated by centrifugation (78,480 g, 1 hr). The pelleted virus was suspended in a small volume of 1.0 M urea in phosphate buffer (0.025 M, pH 7.4, or 0.5 M, pH 7.0) to give a 100- to 250-fold concentration relative to clarified extract. Solid *CsCl* was added to the virus suspension at 0 C until the density of the suspension was  $1.30 \pm 0.01$  g/ml. Centrifugation was performed in the SW50L or SW50.1 rotor at 45,000 rpm for 16-20 hr. The virus band was removed by piercing the side of the tube with a sterile syringe and withdrawing the virus band, or by displacing the gradient with dense *CsCl* solution, and collecting the absorption peak. In some cases, stock *CsCl* of density 1.30 g/ml in

urea-phosphate was added to restore the volume and the centrifugation repeated. The virus was separated from *CsCl* by dialysis or high-speed centrifugation.—*Sucrose*.—Linear-percent sucrose gradients were prepared in Beckman SW50.1 cellulose nitrate tubes by successively layering 1.3 ml each of 55, 50, 45, and 40% (w/w) sucrose in 0.5 M potassium phosphate, pH 7.0, and gently mixing. Each solution contained 0.1 mg/ml MDMV-A. The gradients were centrifuged for 48 hr at 45,000 rpm at 20 C and scanned in the ISCO density gradient fractionator. The densities of 0.4 ml fractions were determined gravimetrically at 25 C and the density at the position of the virus peak was considered the buoyant density of the virus.

**Electron microscopy.**—Preparations for electron microscopy were prepared on formvar-coated grids. Grids were stained with a 4% solution of potassium phosphotungstate, pH 7.2, and viewed with a Siemens IA electron microscope (Siemens Inc., Iselin, New Jersey).

**RESULTS.**—*Banding of MDMV by continuous-flow centrifugation.*—Considerable loss of MDMV may result from clarification of leaf extracts (18, Gordon & Gingery, unpublished). Continuous-flow centrifugation with equilibrium-zonal banding offered the possibility of separation of virus from chloroplastic contaminants without extract clarification and the resulting loss of virus. However, tests showed that this possibility was not feasible for two reasons; (i) chloroplastic contaminants overloaded the gradient and optically

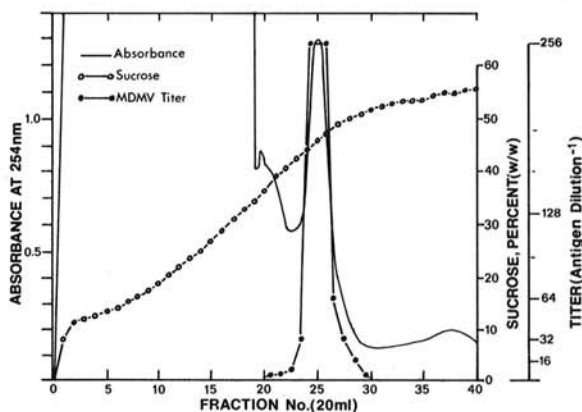


Fig. 1. Continuous-flow centrifugation of a clarified extract containing MDMV-A. A chloroform-clarified extract prepared from 1 kg of MDMV-A-infected tissue homogenized in 1 liter of 0.5 M sodium citrate, 0.5% 2-mercaptoethanol was diluted with two volumes of distilled water to 2.45 liters and passed through the Beckman B16 rotor at a flow rate of 1.5 liters/hr. The rotor contained a 10-55% sucrose gradient in 0.005 M sodium borate, pH 7.8, and was operated at 40,000 rpm and 7 C during sample passage. Centrifugation was continued until  $\omega^2 t = 24 \times 10^{10}$  rad<sup>2</sup>/sec (ca. 3.83 hr). The gradient was displaced at 4,000 rpm and 20-ml fractions were collected. Fractions 19-30 were assayed serologically by the microprecipitin test using MDMV-A antiserum at a dilution of 1:8.

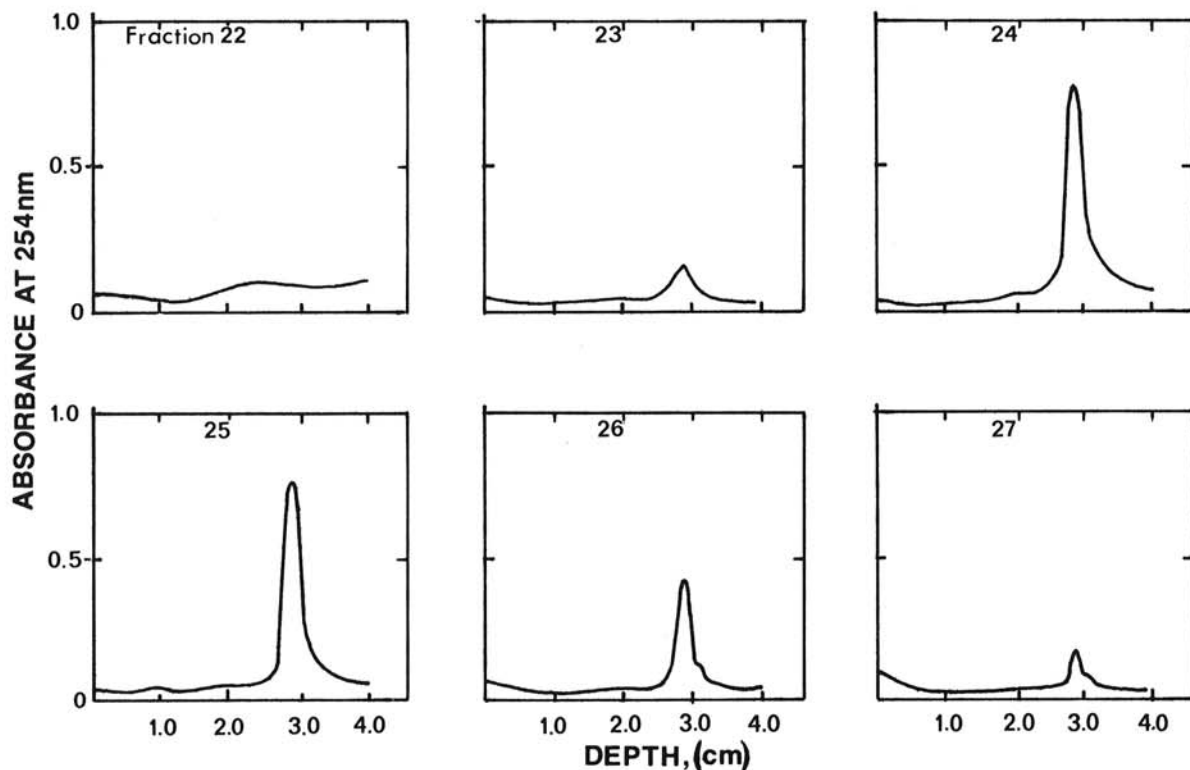


Fig. 2. Sedimentation characteristics of peak fractions after continuous-flow centrifugation of MDMV-A. Fractions 22-27 from Fig. 1 were concentrated by centrifugation in the Beckman Type 30 rotor at 30,000 rpm for 90 min. Pellets were suspended in 0.025 M potassium phosphate, 1 M urea, pH 7.4, and clarified by centrifugation at 10,000 g for 15 min. The supernatants were analyzed on 10-40% sucrose gradient columns in 0.005 M sodium borate, pH 8.7. The Beckman SW27 rotor was centrifuged at 12,000 rpm until  $\omega^2 t = 7.3 \times 10^{10}$  rad<sup>2</sup>/sec (ca. 14 hr). Gradient columns were scanned at 254 nm with an ISCO Model UA-2 ultraviolet analyzer.

obscured the virus band, and (ii) the contaminants accumulated in the rotor lines causing blockage. A similar problem of rotor line blockage had been reported previously (21) in purification of influenza

virus from allantoic fluid. In our experiments, chloroform clarification consistently circumvented these difficulties.

An experiment was done to determine whether continuous-flow centrifugation could effectively concentrate MDMV-A from a large volume of clarified leaf extract and separate the virus from the contaminants (Fig. 1). The virus zone occupied a volume of about 100 ml, which represented a 25-fold concentration. The ultraviolet-absorbing contaminants remained above, and largely separated from the virus. Rate-zonal analysis of fractions 22-27 from the continuous-flow gradient revealed that MDMV-A sedimented typically without much aggregation or contamination (Fig. 2).

*Efficiency of MDMV-A removal from clarified extracts.*—Removal of virus from the clarified extract during passage through the rotor under the above conditions was inadequate. Effluent samples from the rotor contained appreciable quantities of virus as determined by serological assay. The fraction of virus removal expected under these conditions was calculated by the formula of Berman (6) to be approximately 0.63.

For total removal, Berman's formula (6) predicted a flow rate of 1.0 liter/hr, but when tested

TABLE 1. Relationship of flow rate to hold-up volume of continuous-flow rotor<sup>a</sup>

Flow rate (liters/hr)	Hold-up volume (ml)
6.43 <sup>b</sup>	93
6.21 <sup>b</sup>	89
5.75	91.2
3.87	83.9
2.08	70.7
0.89	32.6
0.47	17.5

<sup>a</sup> The Beckman B16 continuous-flow rotor was accelerated to 40,000 rpm while pumping solution through at the high flow rate. Hold-up volumes were determined by injecting RNA solution into the feed line and monitoring the effluent line for the emergence of material with optical density at 254 nm. The volume of effluent from the time of introduction of RNA to the peak of absorbance was considered the hold-up volume.

<sup>b</sup> These values are from two additional experiments.

experimentally only about 50% of the virus was removed, as estimated by serological assay. Recalculation using the observed value of 50% removal at 1 liter/hr predicted 490 ml/hr would give complete removal. However, experimentally only about 75% of the virus was removed at this flow rate.

The failure of the rotor to perform at theoretical efficiency with low flow rates was investigated by using low molecular weight RNA as a marker to determine the rotor hold-up volume. A relationship between flow rate and hold-up volume of the rotor was observed (Table 1). Although this relationship at low flow rates was not predictable, lower flow rates always resulted in smaller hold-up volumes. The reason for this is not known. It is conceivable that all four sectors of the rotor were not being utilized equally at lower flow rates. At 6 liters/hr a hold-up volume of about 90 ml was consistent. The rotor is most efficient at the large hold-up volume, and conditions were modified to increase the amount of virus removed at the higher flow rate. These modifications were; (i) an increase of the operating temperature to 20 C; (ii) use of buffer as the light solution in gradient formation; and (iii) multiple passages of the extract through the rotor. The first and second modifications increased the sedimentation rate of the virus. The third increased the dwell time of the virus in the rotor. For these conditions it was calculated that 36, 59, 83, and 97% of the virus would be removed from the extract following 1, 2, 4, and 8 passages through the rotor, respectively. Experimentally, an estimate of virus removal after these passages of extract was obtained by rate-zonal assays (Fig. 3). Combined data from three experiments were plotted by least squares analysis and estimates of 27, 47, 74, and 94% removal were obtained for the first, second, fourth, and eighth passages, respectively. The regression coefficient was  $0.987 \pm 0.005$ . The agreement between experimental (94%) and theoretical (97%) values for the removal of virus after eight passages through the rotor was reasonably good. Berman's formula (6) used in the calculation of the theoretical value is based on assumptions which do not fully represent our conditions; consequently, the theoretical value is an approximation.

Banding of the virus in the gradient with these modifications appeared satisfactory (Fig. 4). Infectivity in gradient fractions corresponded to serological titer and ultraviolet absorbance peaks. Unlike the banding of virus shown in Fig. 1, more virus was distributed through the lighter gradient fractions (21-28), possibly because the rotor was stopped before unloading. Cline et al. (9) observed a similar phenomenon with respiratory syncytial virus in that at least 1% of the virus captured in continuous-flow centrifugation was detected in gradient fractions outside the virus band. The temperature of the extract during these experiments reached 28 C which caused no apparent problem as judged by the banding characteristics of virus in rate-zonal assays.

#### Recovery of virus following continuous-flow

*centrifugation.*—In order to estimate the virus recovery after continuous-flow centrifugation, virus in clarified extracts before and after eight passages through the Beckman B16 rotor, and virus recovered from the gradient were concentrated by centrifugation. The virus concentration of each sample was determined by rate-zonal analysis. Virus from the zone was diluted to correct for the concentration resulting from continuous-flow centrifugation. The recoveries (virus in zone/virus in extract before passage minus virus in effluent after eight passages) were 65 and 97% for MDMV-A in two experiments, respectively, 107% for MDMV-E, and 86 and 90% for 13 B.

*Isopycnic banding in CsCl and sucrose.*—As shown in Fig. 2, virus concentrated from the zone in the continuous-flow gradient by centrifugation gave a

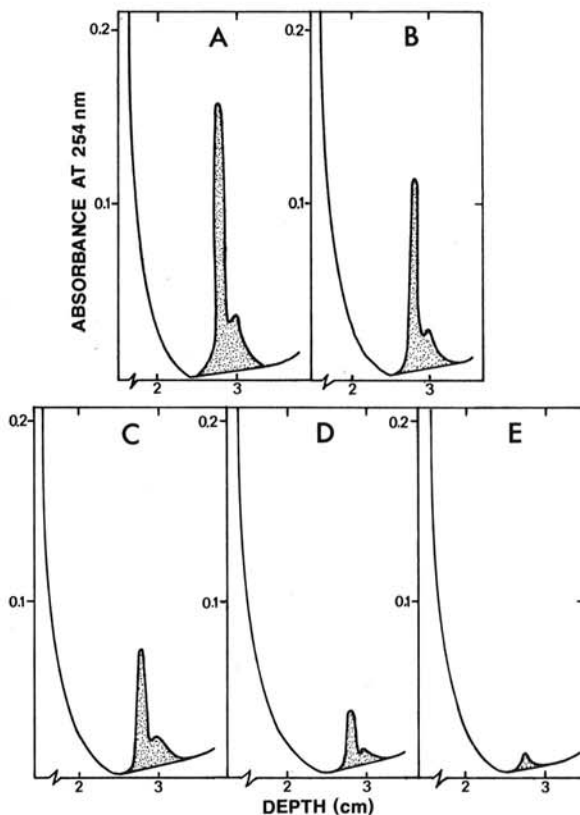


Fig. 3. Absorbance profiles of sucrose density-gradient columns following rate-zonal centrifugation of a clarified extract of MDMV-D. A) Not passed through the B16 rotor, B) after one, C) two, D) four, and E) eight passages. The B16 rotor was operated at 40,000 rpm, 20 C, and a flow rate of 6.29 liters/hr. Virus from the extract and effluents was concentrated 20-fold by centrifugation before rate-zonal centrifugation. Virus samples (0.5 ml) were layered on 10-40% sucrose gradient columns in 0.5 M potassium phosphate, pH 7.0. The Beckman SW27 was centrifuged at 27,000 rpm until  $\omega^2 t = 7.3 \times 10^{10}$  rad<sup>2</sup>/sec (ca. 2.5 hr). Gradient columns were scanned at 254 nm with an ISCO Model UA-2 ultraviolet analyzer. The stippled area beneath the peak represents the relative virus concentration.

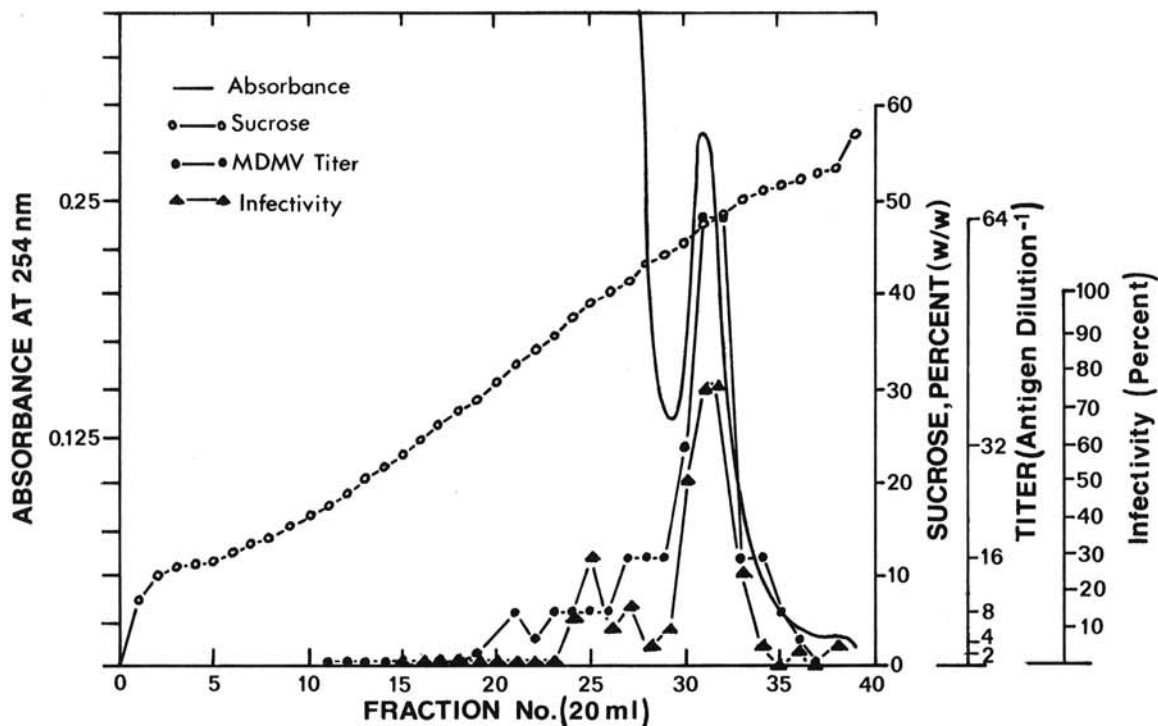


Fig. 4. Banding of MDMV-D by continuous-flow centrifugation with multiple passages. Virus-containing clarified extract (1,480 ml) was successively passed through the Beckman B16 rotor (40,000 rpm, 20 C) eight times at a flow rate of 6.62 liters/hr. The rotor contained a 0-52% sucrose gradient in 0.5 M potassium phosphate, pH 7.0, and was operated at 40,000 rpm until  $\omega^2 t = 27.6 \times 10^{10}$  rad<sup>2</sup>/sec (ca. 4.4 hr). The gradient was displaced after stopping the rotor. The infectivity of fractions 15-38 was quantitatively assayed on *Sorghum bicolor* (L.) Moench, 'Rio'. The infectivity shown is the percent of plants infected at a dilution of 1:270. Fractions 11-37 were quantitatively assayed by the microprecipitin test.

single sedimenting band in rate-zonal analysis. However, isopycnic banding of virus in CsCl resulted in visible bands above and below the virus band (Fig. 5A). Recentrifugation of an aliquot of the virus zone showed only a single peak, indicating a high degree of purity (Fig. 5B).

The buoyant density of MDMV-A in CsCl depended on the dissolving medium. The buoyant density of MDMV in unbuffered CsCl, pH 7.0, was 1.300 g/ml; in CsCl dissolved in 0.5 M potassium phosphate, 1.0 M urea, pH 7.0, it was 1.285 g/ml.

The buoyant density of MDMV-A in sucrose in 0.5 M potassium phosphate, pH 7.0, was 1.266 g/ml after isopycnic-zonal centrifugation. After continuous-flow centrifugation, MDMV banded at 1.254 g/ml, indicating that it had not reached isopycnic density.

*Yield and properties of purified virus.*—Based on an extinction coefficient for tobacco etch virus of 2.4/cm for a 0.1% solution at 261 nm (20), virus yields after CsCl centrifugation ranged from 15-30  $\mu$ g/g of tissue for MDMV-A and 5-20  $\mu$ g/g for MDMV-D, -E, and 13B. However, there was an appreciable loss of infectivity following isopycnic banding in CsCl.

Electron micrographs of virus after purification by continuous-flow centrifugation revealed particles morphologically typical of MDMV (Fig. 6). Little

contaminating material was seen. In microprecipitin tests, antiserum to purified MDMV-A did not produce a visible precipitate when mixed with protein from healthy corn tissue purified by the method of Fribourg & de Zoeten (14). Purified virus preparations were milky white and strongly birefringent.

The ultraviolet absorption spectrum for virus was typical of a nucleoprotein (Fig. 7). In contrast to Sehgal & Jean (23) and in agreement with Jones & Tolin (18), a shoulder at 290 nm was present indicating tyrosine and tryptophan in the viral protein. This has been confirmed by amino acid analysis (Gingery, unpublished). The 260/280 ratio of  $1.27 \pm 0.02$  for MDMV-A differed from the value of 1.18 reported by Jones & Tolin (18) and Sehgal & Jean (23).

Some aggregation of the virus was evident from the double peaks usually seen in rate-zonal assays at all stages of purification (e.g. Fig. 3). Urea incorporated in resuspending buffer as recommended by Damirdagh & Shepherd (11) did not appreciably decrease aggregation.

**DISCUSSION.**—Since MDMV has a slower sedimentation rate than viruses previously purified by continuous-flow centrifugation, a longer dwell time in the rotor was required to obtain comparable removal of virus from the extract. Using a lower flow rate to

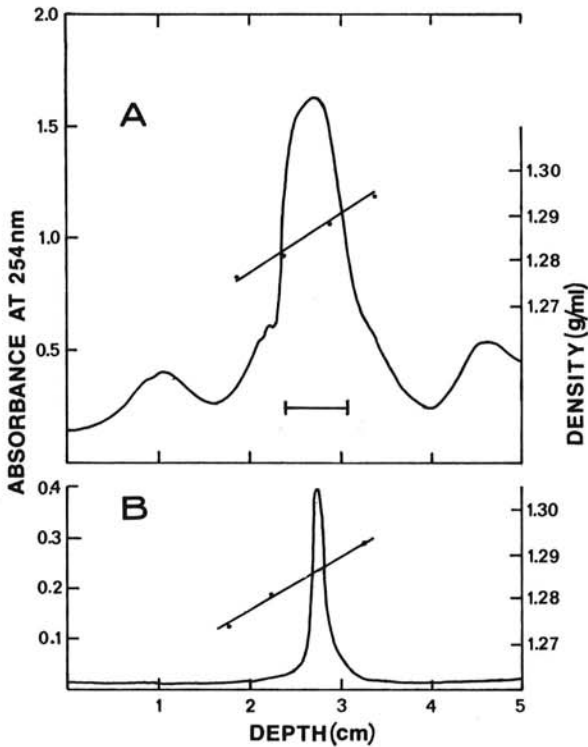


Fig. 5. Isopycnic-banding of MDMV-A in CsCl gradients. A) Following continuous-flow centrifugation, the virus zone was centrifuged at 30,000 rpm for 90 min, resuspended in 0.5 M potassium phosphate, 1.0 M urea, pH 7.0, and adjusted to  $\rho=1.28$  with solid CsCl. Centrifugation was carried out in the SW50.1 rotor for 16 hr at 45,000 rpm and 10 C. The gradients were scanned at 254 nm. The virus zone (denoted by bar) was collected and an aliquot recentrifuged in CsCl. B) The density of individual fractions was determined gravimetrically at 25 C.

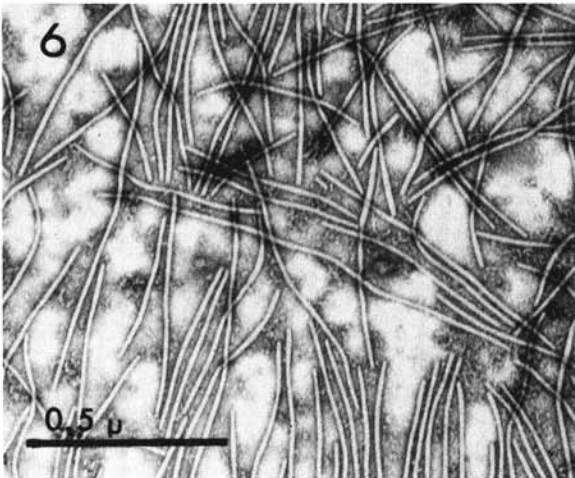


Fig. 6. Electron micrograph of MDMV-A. Negatively stained preparation of the virus zone after continuous-flow centrifugation ( $\times 52,300$ ).

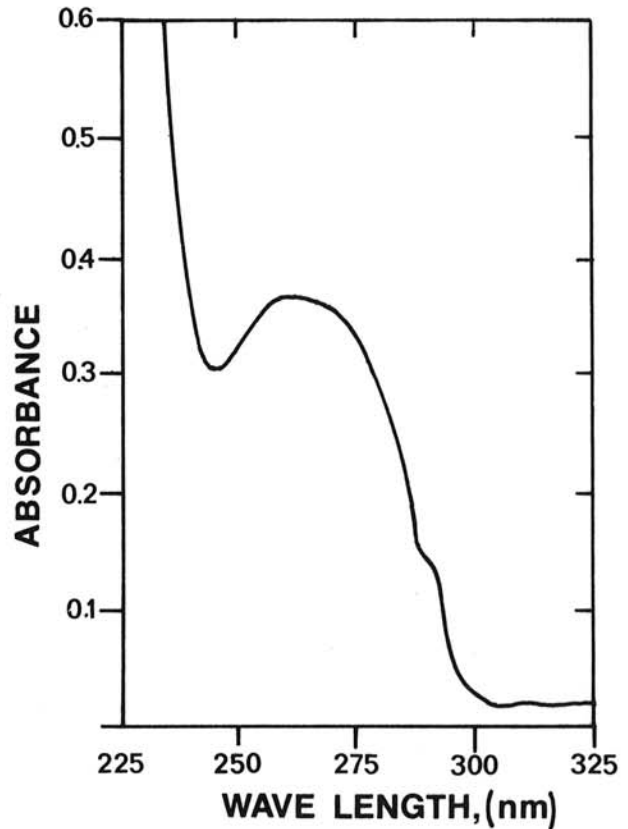


Fig. 7. Ultraviolet absorption spectrum of MDMV-A in 5% sucrose in 0.005 M sodium borate, pH 7.8. The spectrum was obtained with a Unicam SP .800 A spectrophotometer.

increase the dwell time was unsuccessful. A higher flow rate with multiple passages successfully increased dwell time. Under these conditions, the highest flow rate will provide the maximum efficiency, because an increase in flow rate does not decrease proportionately the fraction of virus removed as seen if one plots Berman's (6) equation 42.

Contaminants in virus preparations following continuous-flow centrifugation (Fig. 5) were not detected by rate-zonal centrifugation. This may have been because the contaminants sedimented at the same rate as the virus or at a significantly higher rate causing them to pellet to the bottom of the tube. These possibilities were not investigated. The purity of virus preparations after continuous-flow and isopycnic-zonal centrifugation in CsCl was good. No contaminants were detected by serology, and both rate-zonal and isopycnic-zonal centrifugations revealed no absorbance outside of the virus zone. The efficiency of this purification procedure in recovering MDMV-A from infected tissue (15-30  $\mu\text{g/g}$ ) was comparable to other procedures for which data are available [9.6-23.6  $\mu\text{g/g}$ , (18); up to 18.4  $\mu\text{g/g}$  (25)].

The density values for MDMV-A of 1.285 and 1.300 g/ml are both significantly lower than 1.3245

g/ml reported by Sehgal & Jean (23) for another isolate of MDMV. The discrepancy probably resulted from the different conditions used in the determinations or possibly from density differences between the two isolates.

The critical parameters for successful application of continuous-flow centrifugation to virus purification are the  $s$  and  $\rho$  values of the virus. The  $s$  value determines the efficiency of virus captured by the rotor. The  $s$  value is probably not limiting since the fraction sedimenting into the gradient is not proportional to  $s$  (6, equations 42 and 44). For example, satellite virus with an  $S_{20,w}$  of 50, one-third that of MDMV, theoretically will be 86% removed after eight passages under our conditions. Of course, viruses with higher  $s$  values will be removed more efficiently. The second parameter,  $\rho$ , determines the banding position in the gradient. It is desirable, but perhaps not essential, that the virus density in sucrose be sufficiently different from that of host contaminants to afford good separation in the continuous-flow centrifugation step. Our findings with MDMV, and the fact that many plant viruses have densities greater than MDMV, lead us to predict that most viruses banded near or at their isopycnic points would be well-separated from contaminants. Clarification prior to extract passage should enhance separation.

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