THE MOLECULAR WEIGHT AND OTHER BIOPHYSICAL PROPERTIES OF BROMEGRASS MOSAIC VIRUS

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ABSTRACT Data are presented which show that bromegrass mosaic virus has a particularly low molecular weight and nucleic acid content. A molecular weight of 4.6×10^6 was calculated from the sedimentation coefficient, $s^\circ_{20,w} = 86.2S$, the diffusion coefficient, $D_{20,w} = 1.55 \times 10^{-7}$ cm²/sec., and an assumed partial specific volume, $\bar{v} = 0.708$ ml/gm. The virus has a ribonucleic acid content of 1.0×10^6 atomic mass units. Electrophoresis experiments showed that the virus is stable in 0.10 ionic strength buffers in the pH range 3-6. Breakdown of the virus was observed outside this pH range. Some characteristics of the breakdown products are described.

INTRODUCTION

Investigations concerning the structure of viruses may help explain such phenomena as protein–nucleic acid interaction, genetic coding, and virus synthesis. Important considerations in choosing a virus for such investigations include the quantity of virus material obtainable, relative ease of purification, and stability. Bromegrass mosaic virus (BMV, *Marmor graminis* McK.), one of the spherical viruses, is well suited in these respects for studies concerning virus structure.

In addition, BMV has several interesting physical and chemical characteristics. We recently pointed out in a preliminary report (1) that it has the lowest molecular weight of any known virus (4.6×10^6) and contains a quantity of nucleic acid smaller than that of any other known virus (1×10^6 atomic mass units). It has been shown (2) by means of x-ray scattering that the virus is about 280 A in diameter and has a central cavity more than 100 A in diameter. Also, its isoelectric point is quite high—pH 7.9.

The purpose of this paper is to describe the isolation and purification of the virus, to present in detail the evidence which leads to the conclusion that BMV and its ribonucleic acid are unusually small and to describe electrophoretic properties of the virus and conditions under which it is degraded.

MATERIALS AND METHODS

Purification Procedures. BMV was given to us by Dr. H. H. McKinney, United States Department of Agriculture, Beltsville, Maryland, as isolate No. 66 of the American Type Culture Collection. Moore barley was employed as the systemic host. Young barley plants were inoculated by rubbing the leaves with a dilute virus solution containing carborundum. The plants were grown in the greenhouse during the winter (average temperature approximately 20°C) and in the field during the summer (average temperature approximately 30°C). The barley leaves were harvested when symptoms of the disease appeared which was usually about 10 days after inoculation. Harvested leaves were kept frozen until needed.

Juice from infected leaves was expressed with a meat grinder, adjusted to pH 4.8 with glacial acetic acid, and allowed to stand 12 hours at 4°C. The precipitated plant proteins were removed by low-speed centrifugation. The supernatant solution was centrifuged at 30,000 RPM for 120 minutes in the No. 30 rotor of a Spinco model L preparative centrifuge. The virus pellets were resuspended in distilled water or an appropriate buffer, and this material was clarified by centrifuging for 10 minutes at 5000 RPM. These cycles of high and low speed centrifugation were repeated three times. Yields of about 1 gm per liter of plant juice were obtained. Somewhat higher yields are obtainable by increasing the time of the high speed centrifugation. Light brown pigment in the final virus pellets could not be removed by means of any of several standard protein fractionation procedures including those described by Steere (3).

Analytical Procedures. Sedimentation coefficient determinations were made in the 12 mm cell of a Spinco model E analytical ultracentrifuge operated at 35,600 RPM. The temperature of all of the runs was 23.3°C. The buffer was pH 6.0 and 0.10 ionic strength of which 0.08 was contributed by sodium chloride and 0.02 by sodium phosphates.

Diffusion measurements were carried out in an 11 ml quartz Tiselius cell in a Spinco model H electrophoresis-diffusion apparatus. The buffer was the same as that used in the sedimentation experiment. A sharp boundary was created between the solvent and the virus solution, and the change in concentration of the solute as a function of distance and of time was followed by means of the Rayleigh interference optical system. The temperature of the water bath was held constant at 2°C and diffusion was allowed to proceed for 5 days. Apparent diffusion coefficients were calculated according to the method of Longs-

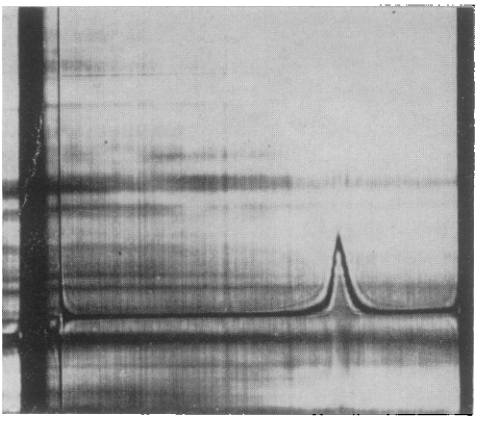

FIGURE 1 Sedimentation pattern of bromegrass mosaic virus in 0.10 ionic strength phosphate buffer + NaCl (pH 6.0) after 16 minutes at 35,600 RPM. Sedimentation is from left to right.

worth (4) from photographs of the Rayleigh fringe patterns taken at selected time intervals.

Electrophoresis experiments in 0.10 ionic strength buffers were carried out in an 11 ml Tiselius electrophoresis cell at 2°C in the same instrument. Virus preparations were dialyzed 48 hours with two or three changes of buffer for all experiments except the pH 9.5 experiment in which the period of dialysis was only 17 hours. The current was 12 or 16 ma. The time interval over which electrophoresis proceeded varied from 1 to 10 hours. The pH of the buffers used was measured at 22°C and conductivity measurements were made at 0°C. Mobilities were calculated from photographs of the schlieren patterns.

Ultraviolet absorption measurements were obtained with a Cary model 11 recording spectrophotometer.

RESULTS

1. *Sedimentation.* BMV sediments as a single boundary in the analytical ultracentrifuge (Fig. 1). Occasionally a trace of a second component sedimenting at a higher rate than the virus is observed. We have been unsuccessful in isolating this material, and assume it is a dimer of the virus. The sedimentation coefficient of BMV was determined from five sedimentation runs at virus concentrations of 0.12, 0.25, 0.37, 0.49, and 0.99 per cent. Fig. 2 shows the resulting plot of sedimentation

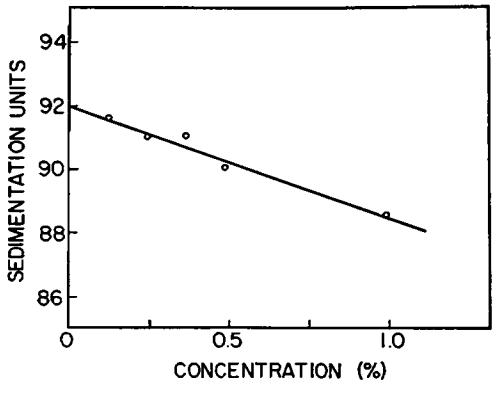

FIGURE 2 Sedimentation coefficients as a function of virus concentration. Extrapolation to infinite dilution in buffer gives $s^0 = 92.0S$.

coefficient *versus* concentration, which gives 92.0S upon extrapolation to infinite dilution. This was corrected to the value expected in water at 20°C. Thus $s^\circ_{20,w} = 86.2S$.

2. *Diffusion.* The diffusion coefficient of BMV was measured at a virus concentration of 0.42 per cent. Fig. 3 shows a plot of apparent diffusion coefficient *versus* the reciprocal of time. Extrapolation to infinite time gives a diffusion coefficient of 8.62×10^{-8} cm²/sec. This value was corrected to that expected in water at 20°C. Thus $D_{20,w} = 1.55 \times 10^{-7}$ cm²/sec.

3. *Partial Specific Volume.* A partial specific volume of 0.708 ml/gm for the virus was calculated from an assumed partial specific volume of 0.550 ml/gm for RNA (5), a partial specific volume of 0.751 ml/gm for protein calculated from

an amino acid analysis, and a nucleic acid content of 21.4 per cent (to be discussed below).

4. *Molecular Weight.* The molecular weight of BMV was calculated from the Svedberg equation. From the sedimentation and diffusion coefficients at a virus concentration of 0.42 per cent and the partial specific volume, the molecular weight was found to be 4.6×10^6.

5. *Frictional Ratio and Water of Hydration.* A frictional ratio, f/f_0, of 1.27 was calculated for BMV. It is known from electron microscopy and x-ray

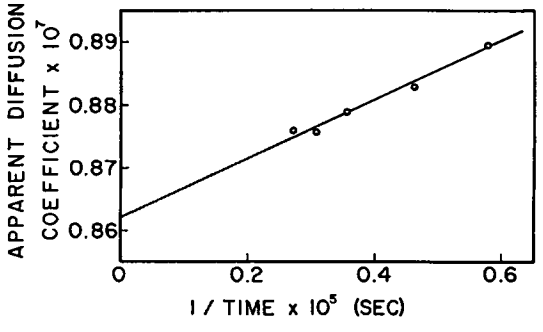

FIGURE 3 Extrapolation of apparent diffusion coefficients as a function of the reciprocal of time. The value of the diffusion coefficient corresponding to infinite time is 0.862×10^{-7} cm²/sec.

scattering studies that the virus is nearly spherical (2). Under the assumption that the virus is spherical in solution and the deviation of f/f_0 from unity is due to hydration, a value for the water of hydration equal to 0.75 gm water/gm of nucleoprotein was calculated.

6. *Ultraviolet Spectrum and Absorbancy Index.* An ultraviolet spectrum of a purified sample of BMV in pH 6.0 buffer (0.10 ionic strength) at a concentration of 0.21 mg/ml is shown in Fig. 4. The curve has a maximum at 258 mμ and a minimum at 238 mμ. The 260/280 ratio, *i.e.* the ratio of the absorbancy at 260 mμ to that at 280 mμ, is 1.7. It may be be seen that the amount of light-scattering in the region of 310 mμ and beyond is quite small, indicating that the preparation was

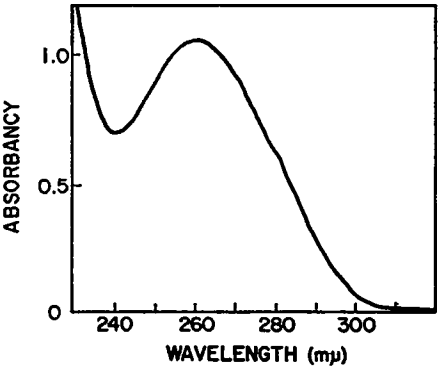

FIGURE 4 Ultraviolet absorption *versus* wavelength for a 0.21 mg/ml concentration of bromegrass mosaic virus.

substantially free of aggregated material which could contribute to light-scattering.

The absorbancy index of the virus was determined from a measurement of the refractive index increment of a solution (to give the nucleoprotein concentration) and a corresponding measurement of the absorbancy at 260 mμ. The measured absorbancy of the virus solution was corrected for the effect of light-scattering. This correction factor was 1.4 per cent. The specific refractive increment was taken to be 1.8×10^{-4} ml/mg (6). The absorbancy index of BMV was found to be 5.08 cm^2/mg.

7. *RNA Content and Molecular Weight.* The RNA content of BMV was determined from the absorbancy indices of the virus nucleoprotein, RNA, and protein. The absorbancy index of the RNA at 260 mμ was determined from a nucleic acid analysis of the virus. The relative molar base composition of the RNA was found by acid hydrolysis to be adenine 27.8, quanine 18.6, cytosine 24.5, and uracil 29.2. The contribution of each base to the absorbancy index at 260 mμ was calculated from the base composition of the RNA. The absorbancy index of the RNA at 260 mμ was calculated to be 32.8 cm^2/mg. This value was corrected for hypochromicity in the following way. It has been demonstrated that spherical RNA viruses show a hypochromic effect due, presumably, to ordered configuration of the RNA. The absorbancy at 260 mμ, after alkaline hydrolysis, was increased by a factor of 1.48 for bushy stunt virus (7), 1.48 for turnip yellow mosaic virus (7), 1.57 for

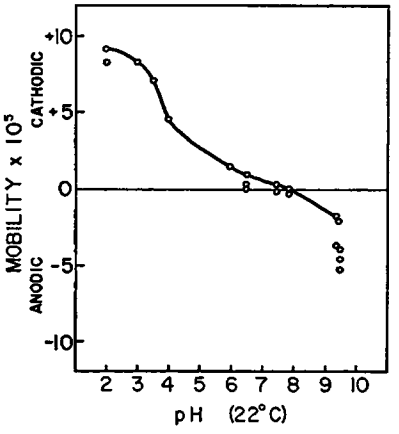

FIGURE 5 Electrophoretic mobility of bromegrass mosaic virus at 0.10 ionic strength as a function of pH.

southern bean mosaic virus (7), and 1.46 for wild cucumber mosaic virus (8) (assuming for the first three viruses a 2.5 per cent correction for absorption due to protein). These four typical spherical RNA viruses have very nearly the same hypochromicity. Their average, 1.50, was assumed in correcting the absorbancy index of BMV RNA. Thus the absorbancy index of BMV RNA at 260 mμ, corrected for hypochromicity, is 21.8 cm^2/mg. The protein absorbancy index was taken to be 0.5 cm^2/mg (6). From the absorbancy indices of the virus nucleoprotein, RNA,

and protein, the RNA content was calculated to be 21.4 per cent.[1] Thus the RNA content of the virus is 1.0×10^6 atomic mass units.

8. *Electrophoretic Properties.* The electrophoretic behavior of BMV was investigated at several pH values between 2 and 10 in 0.10 ionic strength buffers. The mobilities and the buffers used at the various pH values selected for study are listed in Table I. The electrophoretic results are plotted in Fig. 5 as mobility *versus*

TABLE I

MOBILITIES OF COMPONENTS OCCURRING IN ELECTROPHORESIS OF BROMEGRASS MOSAIC VIRUS AS A FUNCTION OF pH

*p*H	Buffer	Component	Mobility $\times 10^5$
	0.1 ionic strength		$cm^2\ volt^{-1}\ sec.^{-1}$
2.0	Glycine-HCl	1	+9.10
		2	+8.34
3.0	Glycine-HCl		+8.28
3.5	Glycine-HCl		+7.10
4.0	Acetate		+4.46
6.0	Cacodylate		+1.41
6.5	Cacodylate	1	+0.925
		2	+0.362
		3	+0.196
7.5	Cacodylate	1	+0.246
		2	−0.131
7.9	Tris-acetate	1	−0.265
		2	−0.008
9.4	Glycine-NaOH	1	−3.72
		2	−1.94
9.5	Glycine-NaOH	1	−5.41
		2	−4.81
		3	−4.06
		4	−2.22

pH. The virus migrated as a single boundary in the Tiselius cell at pH values between 3 and 6. Fig. 6 is a photograph of a schlieren pattern of BMV taken during electrophoresis at pH 4.0 in acetate buffer. Migration is from left to right. This picture was taken 207 minutes after migration from the original boundary position indicated by the vertical line at the tail of the arrow. The virus peak remained a single sharp peak throughout the entire experiment.

Electrophoresis experiments at pH values 6.5 and above revealed two or more components. Two components were observed at pH 2. Degradation studies carried out so far suggest that the points through which the lines are drawn represent the undegraded virus. The additional boundaries observed above pH 6 and below pH 3 are believed to represent breakdown products of the virus. Under the solution conditions employed the isoelectric point of the virus is pH 7.9.

[1] Dr. W. C. Burger has kindly informed us that he has determined from phosphorus analysis that the RNA content of BMV is about 20 per cent.

9. *Alkaline pH Degradation Studies.* Dialysis of BMV preparations against 0.10 ionic strength buffers at pH values between 7.5 and 10 resulted in breakdown of the virus with formation of precipitate after about 48 hours. For example, a sample of BMV was dialyzed against glycine-NaOH buffer of pH 9.4. The precipitate observed after 48 hours dialysis formed a white opaque pellet upon low-speed centrifugation which did not redissolve in distilled water or in pH 5 acetate buffer. An appreciable amount of the precipitate redissolved in glycine-HCl buffer of pH 2. Analytical ultracentrifugation of this solution at 35,600 RPM showed

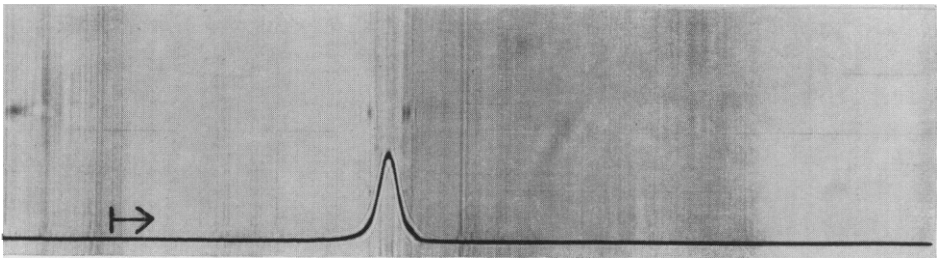

FIGURE 6 Electrophoretic pattern (descending boundary) of bromegrass mosaic virus at pH 4.0 in 0.10 ionic strength acetate buffer after 207 minutes at 4.1 volts/cm. Migration is from left to right. The original boundary position is indicated by the vertical line at the tail of the arrow.

a single peak remaining near the meniscus. An ultraviolet spectrum of this solution gave a 260/280 ratio of 1.39, while an ultraviolet spectrum of the original supernatant solution, from which the precipitate had been removed, showed the usual BMV nucleoprotein curve with a 260/280 ratio of 1.65. Analytical ultracentrifugation of the supernatant solution resulted in a single peak of 81.0S (uncorrected). An electrophoresis experiment was carried out on this supernatant solution. Two schlieren boundaries were observed (Fig. 5, pH 9.5), although precipitate continued to form during the experiment. A clue to the identity of one boundary was obtained. The presence of the precipitate interfered with transmission of ultraviolet light through the cell, and this resulted in an underexposed region on the photographic negative of the schlieren pattern. The border of this region intersects the higher mobility boundary. Thus, it is evident that the high mobility peak represents the precipitate. The slower moving peak is believed to be undegraded virus.

An attempt was made to study breakdown of the virus under high pH conditions before precipitate formation. Precipitation at pH 9.4 was not observed, as mentioned before, until after approximately 48 hours' dialysis. Therefore, a sample of virus was dialyzed against pH 9.5 glycine-NaOH buffer for only 17 hours. No precipitate was visible after this period of dialysis. Analytical ultracentrifugation of this solution resulted in three relatively small peaks, 26.3S, 46.7S, and 57.2S, trailing a large 80.0S peak. Four boundaries were observed during electrophoresis of this solution

(Fig. 5, pH 9.5). The low mobility peak remained separate from the three high mobility peaks, and resembled in shape the single peaks observed during electrophoresis of the virus between pH 3 and 6. The three high mobility peaks were not resolved and diffused rapidly during the experiment. Two samples, *A*, the low mobility component, and *B*, consisting of the remaining high mobility components, were withdrawn from the Tiselius cell with a syringe. Analytical ultracentrifugation experiments were run on the two samples. Sample *A* sedimented as a single 85.0S peak and resembled in shape the 80.0S peak observed in the original sample before electrophoretic separation. Sample *B* appeared to contain the slower sedimenting peaks observed in the original sample. Their sedimentation coefficients were 27.6S, 47.3S, and 61.6S.

Electron microscopy of sample *A* showed particles similar in size and shape to BMV. Electron micrographs of sample *B* revealed particles . . . inhomogeneous in size and shape. It was concluded that sample *A* contained undegraded virus or protein shells and sample *B* contained degraded virus particles. In both the pH 9.4 and 9.5 experiments the slower migrating boundaries, believed to represent the undegraded virus, have nearly the same mobilities. It is suggested that the particles of sample *B*, the breakdown products of the virus, aggregate and in time form the precipitate which is observed after 48 hours' dialysis.

Free nucleic acid was not identified in the electrophoresis experiments. All three boundaries observed at pH 6.5 migrated towards the cathode, whereas free nucleic acid should have a high anodic mobility at this pH. The breakdown particles may consist of protein with nucleic acid still attached and probably migrate at different rates than the virus because of exposure of charges which had been previously hidden. Further characterization of these breakdown components and experiments at higher pH values will be required in order to draw more definite conclusions regarding the steps in alkaline degradation of BMV.

DISCUSSION

Of particular interest is the value obtained for the molecular weight of RNA in BMV. It was pointed out by Frisch-Niggemeyer (9) that for a variety of plant and animal viruses the absolute amount of RNA seems to be the same with an average molecular weight of 2×10^6 (approximately 6,000 nucleotides). BMV RNA contains about 3,000 nucleotides. Only broad bean mottle virus (BBMV) contains a comparably small quantity of RNA—about 3,400 nucleotides (10). When methods have been elaborated for the determination of base sequences of nucleic acids, BMV RNA or BBMV RNA may prove useful for base sequence analysis.

The calculated molecular weight of the protein portion of BMV is 3.6×10^6. Preliminary experiments suggest that BMV contains subunits of molecular weight of approximately 20,000. The number of protein subunits would then be about 180. Assuming the subunits are identical, the coding ratio would be 17 or less.

We are very much indebted to Dr. W. C. Burger for discussing with us his studies of bromegrass mosaic virus.

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REFERENCES

1. BOCKSTAHLER, L. E., and KAESBERG, P., *Nature*, 1961, **190**, 192.
2. KAESBERG, P., *in* Proceedings of the First National Biophysics Conference, New Haven, Yale University Press, 1959, 244. ANDEREGG, J., and KAESBERG, P., unpublished data.
3. STEERE, R. L., *in* Advances in Virus Research, (K. M. Smith and M. A. Lauffer, editors), New York, Academic Press, Inc., 1959, **6**, 1.
4. LONGSWORTH, L. G., *J. Am. Chem. Soc.*, 1952, **74**, 4155.
5. COHEN, S. S., and SCHACHMAN, H. K., *Virology*, 1957, **3**, 575.
6. ENGLANDER, S. W., and EPSTEIN, H. T., *Arch. Biochem. and Biophysics*, 1957, **68**, 144.
7. SCHLESSINGER, D., *J. Molecular Biol.*, 1960, **2**, 92.
8. YAMAZAKI, H., and KAESBERG, P., *Biochim. et Biophysica Acta*, 1961, **51**, 9.
9. FRISCH-NIGGEMEYER, W., *Nature*, 1956, **178**, 307.
10. YAMAZAKI, H., and KAESBERG, P., *Proc. Nat. Acad. Sc.*, 1961, **47**, 979.