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Electron Microscopy of Viral RNA: Molecular Weight Determination of Bacterial and Animal Virus RNAs

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A method for preparation of single-stranded RNA for electron microscopy determination of molecular weight is reported. The method uses treatment with formaldehyde at elevated temperatures to remove secondary structure and spreading in a protein monolayer from 50% formamide onto a 50% formamide hypophase. Molecular weights were determined for some bacterial and animal viruses, for which conflicting values had been reported earlier. Molecular weights determined by the method, using *Escherichia coli* large subunit rRNA for a standard (1.1×10^6), are as follows: *E. coli* small subunit rRNA, 0.53×10^6; coliphage f2-RNA, 1.3×10^6; Qβ-RNA, 1.55×10^6; and Newcastle disease virus RNA, 5.78×10^6.

Sedimentation analysis and gel electrophoresis have been used commonly to determine RNA molecular weight; however, there is no single equation relating molecular weight to sedimentation constant or intrinsic viscosity for all RNAs, and calculation of molecular weight from sedimentation constants can lead to errors (3). Gel electrophoresis of RNA gives greater resolution of RNAs than sedimentation (2), but electrophoretic mobility of RNA is more dependent on secondary structure than was previously thought (18). Gel electrophoresis of RNA under denaturing conditions has been reported (4, 21, 18) and is useful for RNAs of molecular weight up to 2×10^6 (4), but the relationship between logarithm of molecular weight and mobility of RNA apparently is not linear when several small and large molecular weight RNA standards are used (9).

Determination of molecular weight and topology of double-stranded DNA by electron microscopy has been possible for many years by using the monolayer technique developed by Kleinschmidt and Zahn (14). This technique has been modified by Westmoreland et al. (23) for determination of molecular weight of single-stranded DNA. Methods have been published for determination of molecular weight of single-stranded RNA by electron microscopy (13, 11, 19), but in these earlier papers the relationship between the observed length of RNA and molecular weight was not linear over a broad range of RNA sizes. Wellauer and Dawid (22) recently reported a method for preparation of RNA which shows a linear relationship between length and expected molecular weight of HeLa rRNA and precursor rRNA, although the method does not utilize complete denaturation of the RNA.

We report here a technique for determination of molecular weight of single-stranded RNA which differs from the method reported by Wellauer and Dawid (22). Our method eliminates secondary structure in the RNA and is applicable over a wide range of molecular weight, guanine plus cytosine content, and native duplex content. The method requires only small amounts of RNA for individual determinations. This electron microscope procedure has been used to determine molecular weights of several bacterial and animal virus RNAs for which conflicting values have been reported previously.

MATERIALS AND METHODS

RNA preparation. Bulk *Escherichia coli* RNA was isolated by sodium dodecyl sulfate (SDS)-phenol extraction from *E. coli* B cells (12) and centrifuged on 2.5 to 25% gradients of sucrose in TEK-buffer (0.01 M Tris, 0.001 M EDTA, and 0.1 M KCl, pH 7.4) for 6 h at 40,000 rpm at −3 C in the SB 283 rotor of the IEC B-60 ultracentrifuge. The peaks at 16 and 23*S* were collected and recentrifuged separately as described above. The peak regions were collected, precipitated with ethanol, resuspended in PE-buffer (0.01 M phosphate buffer, pH 6.8, and 0.01 M EDTA), and stored at −70 C.

Coliphage f2 RNA and Qβ RNA were prepared as described (1), and the RNA was further purified by sucrose gradient centrifugation as described above for *E. coli* RNA.

Newcastle disease virus (NDV), Blackburg strain, was grown on chicken chorioallantoic membrane, and

the allantoic fluid was harvested after 66 to 69 h of incubation. Allantoic fluid was clarified by low speed centrifugation, and the virus was sedimented by centrifuging at 30,000 × *g* for 50 min. The RNA was extracted with SDS-phenol and centrifuged on 2.5 to 25% sucrose gradients for 2.5 h at 40,000 rpm in an IEC SB 283 rotor.

Coliphage φX-174 was grown on *E. coli* C according to the procedure of Sinsheimer (20). The phage was concentrated and purified by the method of Yamamoto et al. (24). DNA was extracted with SDS-phenol, and DNA preparations showing contamination by nonviral DNA when screened by electron microscopy were centrifuged on 2.5 to 25% sucrose gradients before use, as described for *E. coli* RNA.

All virus and cellular preparations were treated with 10 to 50 μg of DNase per ml for 30 min at 25 C prior to SDS-phenol extractions.

Electron microscopy. Two preparative methods were used for electron microscopy of nucleic acids.

(i) **Aqueous method.** The nucleic acid was suspended in 1 M ammonium acetate and 0.01% cytochrome *c* (Sigma Chemical, horse heart, type III) at a concentration of 1 to 2 μg/ml and was spread from wet glass slides onto a water hypophase contained in the cover of a 60 by 15-mm disposable plastic petri dish. The protein-nucleic acid monolayer was picked up on carbon-coated copper or platinum specimen supports, dehydrated in absolute ethanol, and air-dried. The specimens were rotary-shadowed with uranium oxide at a 7° angle, 8 cm from the metal source.

(ii) **Formaldehyde-formamide method.** Stock solutions: Before use, formaldehyde (Baker; reagent grade, 37%) was diluted to 33% with 10× PE-buffer and placed in a boiling water bath for 10 min. Formamide (Matheson, Coleman, and Bell; 99%) was crystallized at 0 C and stored at −20 C. Cytochrome *c* stock solution contained 1 mg of cytochrome *c* per ml of 1 M ammonium acetate. Cytochrome *c* stock solution and PE-buffer were filtered through 0.45-μm pore diameter membrane (Millipore Corp.) filters before use.

Denaturation of nucleic acid. A nucleic acid solution containing about 10 μg of RNA per ml, 5 to 10 μg of φX DNA per ml (for an internal standard), 4% formaldehyde, 0.01 M phosphate buffer, pH 6.8, and 0.01 M EDTA was heated to 65 C for 15 min and then chilled in ice.

Preparation of protein-nucleic acid monolayers. To prepare 50 μliters of the nucleic acid spreading solution, the following components were mixed in order (at 0 C): 15 μliters of PE-buffer, 5 μliters of denatured nucleic acid solution, 25 μliters of formamide, and, immediately before spreading, 5 μliters of cytochrome *c* stock solution. The nucleic acid solution was spread from 50% formamide-washed slides onto a hypophase of 50% formamide, pH 5.4. All grids were picked up within a 5-min interval after addition of cytochrome *c* to avoid clumping of the RNA. The hypophase should be prepared within a half-hour before use and the pH adjusted with glacial acetic acid. Grids were prepared and shadowed as in the aqueous procedure.

Specimens were observed with a Siemens Elmiskop IA, and magnification was calibrated with a 2,160 line/mm carbon grating replica. Photographs were taken at 20,000× and magnified to 100,000× with a photographic enlarger. Molecules were measured with map measurers (Tacro or Dietzgen).

RESULTS

Figures 1 and 2 show examples of ribosomal and viral RNAs prepared by aqueous and formaldehyde-formamide methods. Figures 1A, B, E, and F and 2A and B demonstrate the differences between the "folded" appearance of single-stranded RNAs prepared by the aqueous method and the extended, easily measured RNA seen in the formaldehyde-formamide preparations. It can be seen in Fig. 1B-F and 2B that molecular length increases with molecular weight.

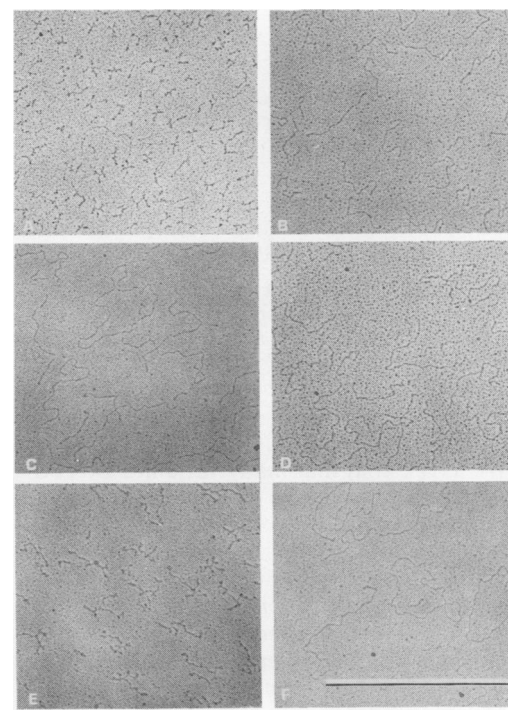

FIG. 1. *E. coli and coliphage RNAs prepared by aqueous and formaldehyde-formamide methods. (A) E. coli 16S rRNA prepared by aqueous method; strands are of a uniform size and folded. (B) E. coli 16S rRNA prepared by the formaldehyde-formamide method; molecules are extended and easily measured. (C) E. coli 23S rRNA prepared by formaldehyde-formamide method. Molecules are twice as long as 16S rRNA; there is some contamination by 16S rRNA molecules. A circular φX-174 DNA molecule can be seen. (D) Coliphage f2 RNA prepared by formaldehyde-formamide method. (E) Coliphage Qβ-RNA prepared by aqueous method. There is some uniformity in secondary structure patterns of the folded molecules. (F) Qβ-RNA prepared by formaldehyde-formamide method. Magnification line, 1 μm.*

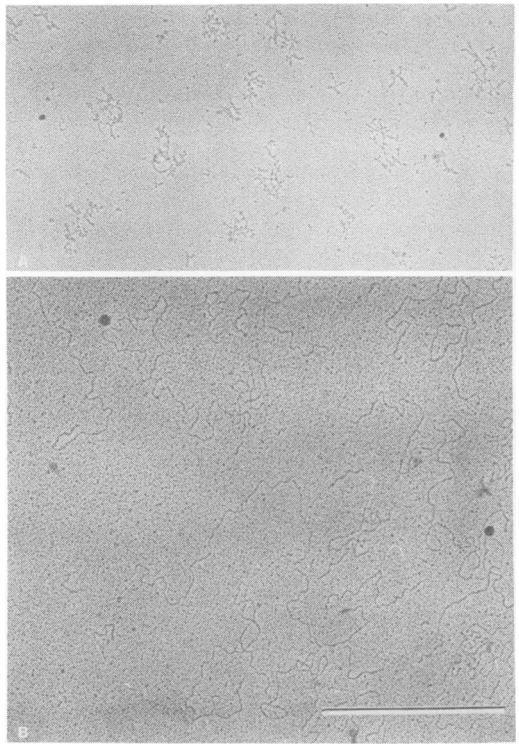

FIG. 2. *(A) NDV RNA prepared by aqueous method; strands are folded and several smaller molecules are visible. (B) NDV RNA prepared by formaldehyde-formamide method; several long NDV RNA molecules and φX-174 circular DNA molecules are visible.*

We have found that many variables affect the length and appearance of the RNA, and all variables must be controlled as rigidly as possible to attain uniform results. Temperature, heating time, formaldehyde concentration, formamide concentration in hyper- and hypophase, and salt concentration all affect the length of RNA, and different brands of formamide can affect the contrast of the RNA in electron microscope preparations. Each new lot number of formamide is tested with RNA standards (*E. coli* rRNA) to check for changes in linear density. Occasional grids show excessively "bumpy" or stretched molecules, and these grids are discarded. The causes for these appearances are not known, but they have also been reported for other electron microscope methods for single-stranded nucleic acids (19). There was less than 10% variation in strand length from grid to grid, but φX-174 single-stranded circular DNA was included as an internal standard in all formaldehyde-formamide preparations.

Figures 3A–E give the length distributions of the tested RNAs prepared by the formaldehyde-formamide procedure. *E. coli* 16*S* rRNA has an average length (± standard deviation [SD]) of 0.38 ± 0.06 μm; 23*S* rRNA, 0.77 ± 0.08 μm; f2 RNA, 0.93 ± 0.1 μm; Qβ RNA, 1.11 ± 0.11 μm; and NDV RNA, 4.13 ± 0.49 μm. All measureable molecules 0.1 μm or longer in each photograph were scored. The number average length was determined by using molecules within two standard deviations of the modal length. All of the RNAs show a single major peak, except NDV RNA. For NDV RNA the main concentration of strand lengths shows a major peak at 4 μm and a second smaller peak at 4.3 μm which is within the standard deviation. It cannot be determined whether the distribution is due to the preparative technique for electron microscopy or to fragmentation of the RNA during isolation. Aqueous preparations of gradient-purified NDV RNA show fragments as seen in Fig. 2A, and the aqueous technique should introduce fewer breaks than the formaldehyde-formamide procedure. It is possible that the presence of RNase inhibitors during the purification of large RNAs would give a better distribution of molecular lengths.

Table 1 summarizes the distribution data and compares molecular weights of these RNAs determined by the formaldehyde-formamide

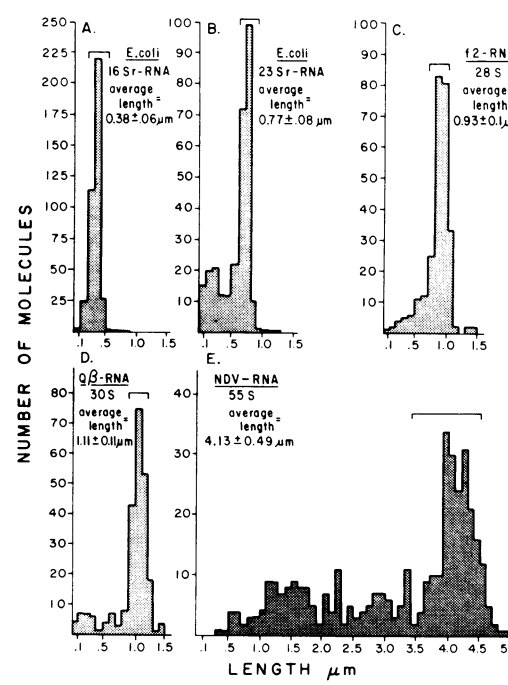

FIG. 3. *Length distributions of RNAs. Molecules within brackets were used for calculating average length.*

TABLE 1. *Molecular weights of RNAs determined by the formaldehyde-formamide method compared with those determined by other methods*

Nucleic acid	No. avg. length (μm)	Wt. avg. length (μm)	Modal length	SD	Coefficient of variation	No. avg. mol. wt.[a]	Mol. wt. determined by other methods[b]
E. coli 16S rRNA	0.38	0.38	0.4	0.06	0.157	0.53×10^6	0.56×10^6 A (16) 0.56×10^6 B (18)
E. coli 23S rRNA	0.77	0.77	0.8	0.08	0.104	1.1×10^6	1.1×10^6 A (16) 1.13×10^6 B (18)
Coliphage f2 RNA	0.93	0.97	0.9	0.10	0.108	1.3×10^6	1.0×10^6 (MS2) C (17) 1.1×10^6 (R17) C (10) 1.22×10^6 (MS2) B (18) 1.3×10^6 (R17) D (4)
Coliphage Qβ RNA	1.11	1.11	1.1	0.11	0.099	1.55×10^6	0.9×10^6 C (17) 1.4×10^6 B (18) 1.5×10^6 D (4)
NDV RNA	4.13	4.13	4.0	0.49	0.119	5.78×10^6	2.3×10^6 E (15) $3\text{–}4 \times 10^6$ (Sendai virus) F (9) 6.3×10^6 E (7) 6.8×10^6 G (6)
Coliphage φX-174 DNA	1.58	1.58	1.6	0.12	0.076		

[a] Determined by using a linear density of $1.4 \times 10^6/\mu m$, calculated from a molecular weight of 1.1×10^6 for *E. coli* 23S rRNA (16).

[b] A, sedimentation viscosity/light scattering; B, urea gel electrophoresis; C, light scattering; D, formaldehyde gel electrophoresis E, Me_2SO-sucrose rate sedimentation; F, formamide gel electrophoresis; G, electron microscopy of nucleocapsid. MS2 and R17 are subgroup names of group I RNA bacteriophages.

procedure with molecular weights determined by other methods. The linear density of $1.4 \times 10^6/\mu m$ was calculated by using a molecular weight of 1.1×10^6 for *E. coli* 23S rRNA (16). It can be seen that the 16S rRNA is one-half the length of the 23S rRNA as expected from molecular weights determined by sedimentation (16). Also f2 RNA is shorter than Qβ RNA as expected from sedimentation coefficients and electrophoretic mobilities (4). NDV RNA molecular weight is within the range predicted for paramyxovirus RNAs from sedimentation coefficients in sucrose gradients (7) and from electron microscope measurement of nucleocapsid length (6), but it is greater than that predicted recently by sedimentation in dimethyl sulfoxide (Me_2SO)-sucrose gradients (15) or electrophoresis in formamide gels (9).

The single-stranded DNA of φX 174 does not have the same linear density as single-stranded RNA prepared by the formaldehyde-formamide method, as would be expected. The molecular weight of φX DNA would be 2.2×10^6 if the linear density of RNA were used. However, the molecular weight of φX DNA is usually taken to be 1.7×10^6 (20); thus the linear density would be $1.06 \times 10^6/\mu m$. Since the distribution, standard deviation, and coefficient of variation of φX DNA were similar to those of RNAs prepared by the same method, it seemed acceptable to use it as an internal standard, since no circular or otherwise easily differentiable RNA was available. RNA lengths were corrected relative to the lengths of the internal standard φX 174 DNA molecules, although there was no significant effect on modal or average length, standard deviation, or coefficient of variation, using these corrections.

DISCUSSION

Several different methods previously reported to denature RNA were tested for preparation of single-stranded RNA for electron microscope determination of molecular weight. Only the combination of heating the RNA in formaldehyde for denaturation and addition of formamide before spreading for increased contrast and extension gave uniform results and molecular weights in agreement with those from other methods. Heating the RNA in 4% formaldehyde at 70 C for 5 or 10 min gave good results for smaller RNAs, but was not sufficient for larger RNAs such as 55S NDV RNA. Heating the RNA in 4% formaldehyde at 65 C for 15 min was sufficient to remove secondary structure in the RNAs tested, which had guanine plus cytosine contents ranging from 47% for NDV (8) to 54% for *E. coli* 16S rRNA (17). It is important to control temperature, heating time, and formamide concentration and to use an internal

standard to obtain uniform results with this method. The resolution of the method is sufficient for RNAs as small as 4S tRNA if higher magnifications are used (Y. Y. Chi and A. R. Bassel, unpublished data), and use with large RNAs seems to be limited more by the steps involved in purification of the RNA, which produce "hidden" breaks, than by the preparative method for electron microscopy. Perhaps addition of a RNase inhibitor which does not interfere with the spreading procedure would improve the length distributions for large RNAs.

The values for f-2 RNA and Qβ-RNA are in agreement with those reported by Boedtker (4), using formaldehyde gels, and these values seem more reasonable than the early low values of 1 to 1.1 $\times$ 10^6 determined by light scattering (10, 17). We think that that the value of 5.8 $\times$ 10^6 for NDV RNA fits well with the molecular weight of 6.3 $\times$ 10^6 obtained by sucrose gradient or Me_2SO-sucrose gradient sedimentation by Duesberg (7) and with electron microscope measurements of nucleocapsid length reported by Compans and Choppin (6). The value of 2.3 $\times$ 10^6 from Me_2SO-sucrose gradient sedimentation reported by Kolakofsky and Bruschi (15) seems too small to code for the known virus functions, since virion proteins alone total 353,000 (5), requiring approximately 3.5 $\times$ 10^6 of RNA for coding. The value of 3 to 4 $\times$ 10^6 for Sendai virus RNA, whose RNA cosediments with NDV RNA in sucrose gradients (15), reported by Duesberg and Vogt (9) using formamide gels is in question because they were unable to place their standard RNAs on a linear logarithm molecular weight-mobility calibration curve. This value would also appear low for coding for viral functions and perhaps indicates that formamide gels may not be accurate for determining molecular weights of large RNAs.

We are currently investigating the genome structure and molecular weight of avian myeloblastosis RNA by using these electron microscope techniques, since the tumor virus RNA components are believed to fall within the size range of the RNAs tested in this report.

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LITERATURE CITED

1. Bassel, B. A., and S. Spiegelman. 1967. Specific cleavage of Qβ RNA and identification of the fragment carrying the 3'-OH terminus. Proc. Nat. Acad. Sci. U.S.A. **58**:1155-1161.
2. Bishop, D. H. L., J. R. Claybrook, and S. Spiegelman. 1967. Electrophoretic separation of viral nucleic acids on polyacrylamide gels. J. Mol. Biol. **26**:373-387.
3. Boedtker, H. 1968. Molecular weight and conformation of RNA, p. 429-458. *In* L. Grossman and K. Moldave (ed.), Methods of enzymology, Vol. 12B. 429-459.
4. Boedtker, H. 1971. Conformation independent molecular weight determinations of RNA by gel electrophoresis. Biochim. Biophys. Acta **240**:448-453.
5. Choppin, P. W., H. Klenk, R. Compans, and L. A. Caliguiri. 1971. The parainfluenza virus SV-5 and its relationship to the cell membrane. Perspect. Virol. **7**:127-156.
6. Compans, R. W., and P. W. Choppin. 1967. The length of the helical nucleocapsid of Newcastle disease virus. Virology **33**:344-346.
7. Duesberg, P. H. 1968. Physical properties of Rous sarcoma virus RNA. Proc. Nat. Acad. Sci. U.S.A. **60**:1511-1518.
8. Duesberg, P., and W. Robinson. 1965. Isolation of the nucleic acid of Newcastle disease virus (NDV). Proc. Nat. Acad. Sci. U.S.A. **54**:794-800.
9. Duesberg, P. H., and P. K. Vogt. 1973. Gel electrophoresis of avian leukosis and sarcoma viral RNA in formamide: comparison with other viral and cellular RNA species. J. Virol. **12**:594-599.
10. Gesteland, R. F., and H. Boedtker. 1964. Some physical properties of bacteriophage R-17 and its RNA. J. Mol. Biol. **8**:496-507.
11. Granboulan, N., and K. Scherrer. 1969. Visualization in the electron microscope and size of RNA from animal cells. Eur. J. Biochem. **9**:1-20.
12. Hayashi, M., and S. Spiegelman. 1961. The selective synthesis of informational RNA in bacteria. Proc. Nat. Acad. Sci. U.S.A. **47**:1564-1580.
13. Highton, P. J., and M. Beer. 1963. An electronmicroscopic study of extended single polynucleotide chains. J. Mol. Biol. **7**:70-77.
14. Kleinschmidt, A. K., and R. K. Zahn. 1959. Über Desoxyribonucleinsäure-Molekeln in Protein-Mischfilmen. Z. Naturforsch. B **14**:770-779.
15. Kolakofsky, D., and A. Bruschi. 1973. Molecular weight determination of Sendai RNA by dimethyl sulfoxide gradient sedimentation. J. Virol. **11**:615-620.
16. Kurland, C. G. 1960. Molecular characterization of RNA from *E. coli* ribosomes. I. Isolation and molecular weights. J. Mol. Biol. **2**:83-91.
17. Overby, L., G. H. Barlow, R. H. Doi, M. Jacob, and S. Spiegelman. 1966. Comparison of two serologically distinct RNA bacteriophages. II. Properties of the nucleic acids and coat proteins. J. Bacteriol. **92**:739-745.
18. Reijnder, L., P. Sloof, J. Sival, and P. Borst. 1973. Gel electrophoresis of RNA under denaturing conditions. Biochim. Biophys. Acta **324**:320-333.
19. Robberson, D., Y. Aloni, G. Attardi, and N. Davidson. 1971. Expression of the mitochondrial genome in HeLa cells. VI. Size determination of mitochondrial ribosomal RNA by electron microscopy. J. Mol. Biol. **60**:473-484.
20. Sinsheimer, R. L. 1959. Purification and properties of bacteriophage phi-X 174. J. Mol. Biol. **1**:37-42.
21. Staynov, D. Z., J. C. Pinder, and W. B. Gratzer. 1972. Molecular weight determination of nucleic acids by gel electrophoresis in non-aqueous solution. Nature N. Biol. **235**:108-110.

22. Wellauer, P. K., and I. B. Dawid. 1973. Secondary structure maps of RNA: processing of HeLa ribosomal RNA. Proc. Nat. Acad. Sci. U.S.A. **70**:2827-2831.
23. Westmoreland, B. C., W. Szybalski, and H. Ris. 1969. Mapping of deletions and substitutions in heteroduplex DNA molecules of bacteriophage lambda by electron microscopy. Science **163**:1343-1348.
24. Yamamoto, K., B. Alberts, R. Benzinger, L. Lawhorne, and G. Treiber. 1970. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. Virology **40**:734-744.