Concentration of Rous Sarcoma Virus from Tissue Culture Fluids with Polyethylene Glycol

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Concentration of Rous sarcoma virus from tissue culture fluids with polyethylene glycol, with and without NaCl or dextran sulfate, resulted in significant and highly variable losses caused by entrapment of virus particles in proteinaceous debris. Treatment of concentrated preparations with Pronase greatly enhanced the recovery of virions. Maximum recovery of virus particles was obtained by the addition of 8% polyethylene glycol and 0.4 M NaCl to tissue culture fluids, followed by Pronase treatment of the concentrated virus preparations.

Polyethylene glycol (PEG), a high-molecular-weight, water-soluble polymer, has been used to concentrate viruses from aqueous suspensions. This method, termed a phase separation rather than a precipitation (22), was initially used in combination with NaCl and another polymer, dextran sulfate (DS), to concentrate preparations of poliovirus (13), bacteriophage, adenovirus, and ECHO virus (15).

Subsequent studies indicated that PEG is equally effective when used only in combination with NaCl to concentrate bacteriophage (22). McSharry and Benzinger (11) reported quantitative recovery of infectious vesicular stomatitis virus with 6% PEG and 0.5 M NaCl and also found that this method partially purified the virus preparations. Maximum recovery of Epstein-Barr virus was obtained at concentrations of 8% PEG and 0.45 to 0.6 M NaCl, and it was concluded that this method is preferable to centrifugation for concentrating this herpesvirus (1). PEG has also been used at various concentrations, and with or without NaCl or DS, to concentrate myxoviruses (10), foot-and-mouth disease virus (20), oncornaviruses (3, 8, 12, 14, 18), and simian virus 40 and polyoma virus (6).

The purpose of this study was to determine the optimum conditions, in terms of the properties and relative recovery of virus particles, for concentrating Rous sarcoma virus (Rous-associated virus-1) [RSV(RAV-1)] from tissue culture fluids with PEG with and without NaCl or DS. The results demonstrate that maximum recovery is obtained with 8% PEG and 0.4 M NaCl if concentrated preparations of RSV(RAV-1) are treated with Pronase.

MATERIALS AND METHODS

Reagents. TNE buffer [0.01 M tris(hydroxy-methyl)aminomethane, 0.1 M NaCl, and 0.001 M ethylenediaminetetraacetate, pH 7.2] and sucrose solutions prepared in TNE were sterilized by filtration (0.45-μm porosity, Millipore Corp.). PEG of molecular weight 5,700 to 6,700 was obtained from General Biochemicals. Ribonuclease-free sucrose and [3H]uridine triphosphate ([3H]dTTP, 42 Ci/mmol) was obtained from New England Nuclear. Pronase (B grade, Calbiochem) was prepared in TNE buffer and was self-digested at 37 C for 90 min prior to storage at −20 C. DS of molecular weight 500, 2-mercaptoethanol, diethyl pyrocarbonate, and deoxyribonucleoside triphosphates (dATP, dCTP, and dGTP) were from Sigma Chemical Co.

Cell cultures and virus. RSV(RAV-1) seed virus was provided by Paul Meyers, Mayo Foundation, Rochester, Minn. Chicken embryo fibroblasts (CEF) were grown in 75-cm² Falcon plastic flasks (BioQuest) in medium 199 (Grand Island Biological Co.) supplemented with 10% tryptose phosphate broth (TPB), 10% fetal calf serum, and antibiotics (100 units of penicillin and 100 μg of streptomycin per ml) (growth medium [GM]). Maintenance medium was medium 199 containing 5% TPB, 2% calf serum, and antibiotics.

Confluent secondary or tertiary monolayers of CEF were infected with 1 ml of virus (approximately 2 × 10⁶ focus-forming units [FFU]) and incubated at 37 C for 1 h on a platform shaker. The inoculum was removed, and 10 ml of GM was added to each flask. After incubation at 37 C for 24 h, the infected cells were subcultured at a density of approximately 7 × 10⁶ cells in 15 ml of GM per 75-cm² flask. The medium was replaced daily and changed to maintenance medium by day 3 or 4.

FFU assays. Primary CEF were trypsinized and seeded at a density of 1.2 × 10⁶ cells in 5 ml of GM per 60-mm Falcon plastic tissue culture dish. The cell cultures were infected in duplicate within 4 h postseeding by adding 0.1 ml of appropriate virus dilution per dish. At 20 h postinfection, the medium was removed, and the monolayers were overlaid...
with 5 ml of GM containing 0.9% Ionagar no. 2 (Oxoid Division, Consolidated Laboratories, Chicago Heights, Ill.) and incubated at 37 C in a humidified atmosphere of 5% CO₂. Three days later, 2 ml of GM was added to each plate, and 1 ml of GM containing neutral red (1:10,000) was added on day 7. Foci of altered cells were counted 24 h later.

Virus purification. Supernatant culture fluids (10 ml of maintenance medium/flask) were harvested at 12-h intervals starting on day 6 to 8 after subculture of infected CEF and were clarified at 13,000 x g for 15 min prior to storage at -85 C. Virions were radiolabeled by addition of 10 μCi of [3H]uridine per ml to infected CEF cultures at the same time intervals.

Concentration of virions was accomplished by addition of a 1/10 volume of 4 M NaCl in distilled water and 8% (wt/vol) PEG to the clarified culture fluids (PEG method). The suspension was stirred in an ice bath for 3 h and centrifuged at 8,000 x g for 15 min in a Sorvall SS-34 or GSA rotor at 4 C. The pellets were resuspended in a total volume of 10 ml of TNE. Some of the virus suspensions were incubated at 37 C for 30 min with 20 mg of Pronase (PEG-Pronase method) and then cooled in ice.

Concentrates were layered over discontinuous gradients of 12 ml of 20% sucrose cushioned by 5 ml of 55% sucrose and centrifuged at 27,000 rpm for 2 h in a Spinclo SW-27 rotor at 5 C. The band at the interphase was collected by puncturing the bottom of the tube and was diluted to approximately 11 ml with TNE. Virus preparations concentrated by the PEG-Pronase method were again incubated for 30 min at 37 C with 20 mg of Pronase and cooled in ice. Concentrates were layered over 23-ml linear gradients of 20 to 55% sucrose and centrifuged 8 to 9 h at 27,000 rpm in the Spinclo SW-27 rotor. Fractions (1 ml) were collected from the bottom of the tube with the aid of a Buchler piercing unit (Buchler Instruments, Fort Lee, N. J.), and the radioactivity in a 50- or 100-μl sample of each fraction was measured in 10 ml of Beckman Ready-Solv VI. Buoyant density was determined by refractometry, and absorbancy determinations were made at 260 nm in a Beckman Model DB spectrophotometer.

RNA extraction. Appropriate gradient fractions were diluted with TNE and centrifuged at 40,000 rpm for 60 min in the Spinclo SW-40 rotor. The pellets were resuspended in 0.9 ml of TNE containing 0.05% 2-mercaptoethanol and 1% diethyl pyrocarbonate. Viral ribonucleic acid (RNA) was extracted with 1% sodium dodecyl sulfate (final concentration) at room temperature for 5 min and immediately layered over an 11.4-ml linear gradient of 5 to 20% sucrose. Approximately 0.4 ml of sterile mineral oil was added, and the gradient was centrifuged at 40,000 rpm for 2 h in the Spinclo SW-40 rotor. Fractions of 0.5 ml were collected from the bottom of the tube and assayed for acid-precipitable radioactivity retained by membrane filters (Millipore) (4) in 10 ml of Beckman Ready-Solv IV.

Viral RNA-dependent deoxyribonucleic acid polymerase. The endogenous deoxyribonucleic acid polymerase (reverse transcriptase) assay was modified from the method of Garapin et al. (7). The standard incubation mixture contained 2% (vol/vol) 2-Mercaptoethanol; 0.1% (vol/vol) Nonidet P-40; 100 mM tris(hydroxymethyl)aminomethane, pH 8.1; 10 mM MgCl₂; 0.08 mM each of DATP, dCTP, and dGTP; and 4.8 μM [3H]TTP. A 25-μl portion of this mixture was added to 25 μl of virus suspension and incubated at 42 C for 60 min. Each sample was co-precipitated in ice for 10 min with 50 μl of yeast RNA (1 mg/ml) by the addition of 0.5 ml of 5% trichloroacetic acid containing 0.08 M Na₃P₂O₅. Precipitates were collected on Whatman GF/A glassfiber filters (immersed in saturated Na₃P₂O₅ prior to use) and washed eight times with 5 ml of 5% trichloroacetic acid containing 0.08 M Na₃P₂O₅ and once with 5 ml of 80% ethanol. Dried filters were counted in 10 ml of BBOT-toluene (8 g/liter) in plastic scintillation vials.

Electron microscopy. Virus particles were pelleted at 40,000 rpm for 60 min in the Spinclo SW-40 rotor. Pellets were resuspended in 0.45 ml of TNE, centrifuged in LKB capsules over a cushion of undiluted glycerol at 44,000 rpm for 60 min in the Spinclo SW-65 rotor, fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), and postfixed in 1% OsO₄ in the same buffer. The pellets were treated with aqueous uranyl acetate, dehydrated through a graded ethanol series, and embedded in Epon 812. Thin sections were cut with a diamond knife on an LKB ultramicrotome and collected on carbon-Formvar-coated grids. The sections were stained with uranyl acetate and lead citrate and were examined with a Siemens Elmiskop I electron microscope.

RESULTS
Concentration of RSV(RAV-1) with PEG. The recovery of radiolabeled virions concentrated with PEG was examined by centrifugation of virus preparations on sucrose gradients. Supernatant fluids from radiolabeled cultures were mixed with approximately fourfold volumes of nonradioactive culture fluids to provide sufficient material for absorbancy determinations. In preliminary experiments, final concentrations of 10% PEG (wt/vol) and 0.5 M NaCl were used (6, 8) with 200 to 400 ml of culture fluids. Assays of gradient fractions revealed highly variable but significant levels of radioactivity (approximately 25 to 75% of the total counts per minute) and a high absorbancy profile at a buoyant density range of 1.20 to 1.24 g/cm³, with another peak at a density of 1.15 to 1.16 g/cm³. Electron microscopic examination of samples in the high-density region of the gradients revealed large aggregates of virus particles enmeshed in amorphous material, and virus particles in a background of similar material were found at a density of 1.15 to 1.16 g/cm³. PEG has been reported to be effective in concentrating proteins from solutions (5, 9, 16, 23), and it was considered probable that proteinaceous material from medium supplements may

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be responsible for aggregation and subsequent losses of RSV(RAV-1). Therefore, 0.1-ml samples from the high-density gradient fractions were treated with 25 μg of Pronase at 37 C for 30 min. Examination of these samples by electron microscopy indicated that most of the aggregated material was dispersed by the Pronase treatment.

**Optimum concentration of PEG and NaCl.** The concentrations of PEG and NaCl were varied to determine the optimum conditions for the recovery of infectious virions in the pellets. In addition, some of the samples were treated with Pronase to determine whether such treatment might have adverse effects on focus-forming activity. In all experiments, the amount of PEG added was corrected for volume changes of the samples caused by the addition of a 1/10 volume of NaCl solution. The pellets obtained from 50-ml portions of a pool of supernatant culture fluids were each resuspended in TNE. All samples, in a final volume of 5 ml, were prepared in duplicate, and one of the samples was incubated at 37 C for 30 min with 2 mg of Pronase per ml.

Maximum and equivalent recovery of infectious virions was obtained by Pronase treatment of samples concentrated with 8, 10, or 12% PEG and NaCl concentrations of 0.4 and 0.5 M. Less than 10<sup>5</sup> FFU/ml remained in the supernatant fluids after pelleting of these samples (Fig. 1). Six percent PEG appeared to be a transitional concentration, as recovery of infectious virions varied from approximately 20 to 100% of recovery obtained with the higher concentrations. Recovery of FFU in pellets that were not treated with Pronase was extremely variable, probably as a result of aggregation of virions in these samples, and the titers obtained in two experiments are so meaningless that these data are not presented. However, recovery of FFU in the Pronase-treated samples was as much as 50-fold greater than recovery in samples not treated with Pronase.

**Concentration of RSV(RAV-1) by PEG and PEG-Pronase methods.** A comparison was made of the recovery, by sucrose gradient centrifugation, of radiolabeled virions concentrated by the PEG and PEG-Pronase methods. Pooled supernatant culture fluids were divided into two equal volumes prior to concentration, or a concentrated preparation was divided into two equal volumes, with one volume (approximately 10 ml) used for each method.

Radiolabeled virions concentrated by the PEG-Pronase method yielded a sharp, prominent peak of radioactivity, coincident with the peak of absorbancy at 260 nm, at a buoyant density of approximately 1.14 g/cm<sup>3</sup> in sucrose (Fig. 2A). An additional absorbance peak was found at the top of the gradient which was primarily caused by Pronase and membranous debris.

Conversely, preparations concentrated by the PEG method yielded the variable results described above, and in none of these experiments did recovery of radioactivity in the peak fraction (buoyant density range of 1.15 to 1.16 g/cm<sup>3</sup>) exceed 35% of recovery obtained with the PEG-Pronase method (Fig. 2B).

Murine oncornaviruses have been concentrated from culture fluids with 6% PEG and 50 μg of DS per ml but without NaCl (12, 18). All solutions used for purification and storage of the viruses also contained 50 μg of DS per ml. However, our attempts to duplicate this procedure with 250 to 400 ml of culture fluids containing RSV(RAV-1) produced losses similar in magnitude to losses encountered with the PEG method. Maximum recovery of radioactivity, at a buoyant density of 1.16 g/cm<sup>3</sup>, was obtained when 0.4 M NaCl was used with 6% PEG and 50 μg of DS per ml. But the radioactivity profile was broad and did not coincide with the absorbancy profile, suggesting the presence of unlabeled debris, and recovery of radioactivity in the peak fraction was only 40 to 75% of that obtained by the PEG-Pronase method.

**Electron microscopy.** Electron microscopic
examination of the fractions from the 1.23- to 1.24-g/cm³ region of gradients containing preparations concentrated by the PEG method revealed virus particles entrapped in amorphous debris (Fig. 3B), and a background of debris was also observed in samples from fractions with a buoyant density of 1.15 to 1.16 g/cm³ (Fig. 3C). Large amounts of similar debris were also found in the counts per minute peak fractions (density of 1.15 to 1.16 g/cm³) from gradients containing virus preparations concentrated with PEG and DS or with PEG, NaCl, and DS (Fig. 3D). Examination of samples from preparations concentrated by the PEG-Pronase method and banded at a buoyant density of approximately 1.14 g/cm³ revealed large numbers of virus particles essentially free of this amorphous material, although some membranous debris was found in these fractions (Fig. 3A). Much of the membranous debris was removed by passage of these fractions through membrane filters (Millipore; 0.45-μm porosity) that were pretreated with GM to reduce losses caused by adsorption of the virus to the membranes (19). These results support the interpretation that the losses obtained with preparations of RSV(RAV-1) concentrated by the PEG method, with or without DS, occur through entrapment of virus particles in proteinaceous debris.

Viral RNA. Figure 4 shows representative results obtained by velocity sedimentation of RNA extracted from RSV(RAV-1) that had been concentrated by the PEG-Pronase method and banded at a buoyant density of 1.14 g/cm³. The RNA is located as a sharp, prominent peak of radioactivity in the 60 to 70S region of the gradient and comprises 70 to 75% of the total radioactivity in the gradient.

Reverse transcriptase activity. Assays for reverse transcriptase activity were conducted on gradient fractions containing virus preparations concentrated by either the PEG method or the PEG-Pronase method. Maximum incorporation of [3H]TTP coincided with maximum absorbancy at a buoyant density of 1.145 g/cm³ in gradients containing preparations concentrated by the PEG-Pronase method (Fig. 5), but maximum reverse transcriptase activity and absorbancy with PEG preparations were located in fractions near the bottom of the gradient. A slight peak of enzyme activity, corresponding to 25 to 30% of the activity obtained with preparations concentrated by the PEG-Pronase method, was located at a density of 1.15 g/cm³ (D. L. Bronson, unpublished data).

Effects of serum and TPB on the buoyant density of purified RSV(RAV-1). RSV(RAV-1) concentrated by the PEG-Pronase method consistently banded at a buoyant density of 1.14 to 1.15 g/cm³, whereas virions concentrated with PEG or PEG and DS yielded a peak at a density of approximately 1.15 to 1.16 g/cm³. Although these differences are slight, these results suggest that Pronase treatment may be responsible for the shift in buoyant density.

To examine this possibility, purified virus preparations that banded at a buoyant density of 1.14 g/cm³ after concentration by the PEG-Pronase method were uniformly mixed and then divided into three samples of equal volume. One sample was diluted with TNE, and the two other samples were each mixed with 20 ml of calf serum and 5 ml of TPB. The preparations were centrifuged at 27,000 rpm for 60 min in the SW-27 rotor, and the pellets were resuspended in 10 ml of TNE. One sample pelleted in calf serum and TPB was incubated at 37 C for 30 min with 20 mg of Pronase, and the three
Fig. 3. Electron micrographs of RSV(RAV-1). Tissue culture fluids (300 ml) containing RSV(RAV-1) were used for each method of concentration. (A) Virions were concentrated from fluids by the PEG-Pronase method and banded at a buoyant density of 1.134 to 1.15 g/cm³ in sucrose. (B and C) RSV(RAV-1) was concentrated by the PEG method. The samples were taken from gradient fractions 1, 2, and 3 (buoyant density of 1.233 to 1.244 g/cm³) (B) and from gradient fractions with a buoyant density of 1.147 to 1.163 g/cm³ (C). (D) RSV(RAV-1) was concentrated with 6% PEG (wt/vol), 50 μg of DS per ml, and 0.4 M NaCl and banded at a buoyant density of 1.153 to 1.67 g/cm³. ×30,000.
CONCENTRATION OF RSV

dium supplements. These data indicate that pelleting purified RSV(RAV-1), concentrated by the PEG-Pronase method, in the presence of proteinaceous medium supplements results in a slight increase in the buoyant density of the virions.

Fig. 4. Velocity sedimentation of RSV(RAV-1) RNA. Virions were labeled with 3H[uridine, concentrated by the PEG-Pronase method, and banded at a buoyant density of 1.142 g/cm³ in sucrose. Viral RNA was sedimented in a 5 to 20% linear sucrose gradient, and fractions were assayed for acid-precipitable counts per minute. The position of 35S-labeled 28S ribosomal RNA is indicated by the arrow.

Fig. 5. Reverse transcriptase activity in gradient fractions containing RSV(RAV-1). Virus particles were concentrated from 300 ml of tissue culture fluids by the PEG-Pronase method and purified as described in the legend to Fig. 2. Gradient fractions were assayed for buoyant density (●), absorbance at 260 nm (○), and reverse transcriptase activity in duplicate 20-µl samples (△).

preparations were rebanded in linear sucrose gradients.

Radiolabeled RSV(RAV-1) pelleted in TNE, or in calf serum and TPB followed by Pronase treatment, banded at a buoyant density of 1.14 to 1.15 g/cm³, whereas samples pelleted in calf serum and TPB banded at a buoyant density of 1.15 to 1.16 g/cm³, and comparatively high absorbancy at 260 nm readings were obtained for these fractions (Fig. 6). However, recovery of radiolabeled virions was enhanced in the preparations pelleted in calf serum and TPB, possibly because of the formation of a more compact pellet with debris from the proteinaceous me-
DISCUSSION

PEG separation is a rapid, inexpensive, and gentle method for concentrating viruses from small to relatively large volumes of culture medium. In addition, PEG does not cause changes in the pH. These properties are particularly suited for concentrating labile viruses (1, 20). However, concentration of proteins from medium supplements also occurs and can interfere with recovery of viruses during purification procedures. Wagner et al. (20) obtained optimum recovery of foot-and-mouth disease virus in concentrates prepared with 6% PEG when serum was omitted from the medium during virus propagation. The presence of serum in the medium produced concentrates that were difficult to dissolve completely, resulting in a loss of foot-and-mouth disease virus which was attributed to entrapment of the virus in proteinaceous debris. In the present study, this interference was demonstrated by the variable results obtained by concentration of RSV(RAV-1) by the PEG method and was resolved by treating the concentrated preparations with Pronase.

In contrast to the results of Syrewicz et al. (18) and Naso et al. (12), the use of DS with PEG did not yield greater recovery of RSV(RAV-1) than was obtained with the PEG method. The reasons for this discrepancy may be differences in purification procedures and our use of PEG of lower molecular weight, which yields solutions of lower viscosity (5, 22), with lower concentrations of proteinaceous supplements in the medium.

Assays of infectivity in resuspended pellets of RSV(RAV-1) prepared with different concentrations of PEG and of NaCl indicated that maximum recovery was obtained with 8% PEG and 0.4 M NaCl after Pronase treatment of the preparations (Fig. 1). The lower, variable rates of recovery of FFU in preparations concentrated with PEG are probably caused by aggregation. These results suggest that Pronase treatment has no significant adverse effects on the focus-forming activity of RSV(RAV-1), as recovery of FFU in these preparations was as much as 50-fold greater than recovery in preparations concentrated by the PEG method.

Velocity sedimentation of viral RNA extracted from purified virions concentrated by the PEG-Pronase method indicated that very little degradation of viral high-molecular-weight RNA occurred during concentration, purification, and extraction procedures (Fig. 4). However, incubation of murine oncornaviruses for prolonged intervals at 37 C resulted in "nicking" of viral high-molecular-weight RNA that could only be detected by denaturing the RNA with dimethylsulfoxide (2, 21). Conversely, Scheele and Hanafusa (17) found no alterations or "nicks," as determined by polyacrylamide gel electrophoresis of denatured viral RNA, in the high-molecular-weight RNA from RAV-2 that had been incubated with 20 μg of Pronase per ml at 37 C for 30 min. The authors concluded that the heterogeneity of denatured viral RNA results mainly from degradation that occurs during characterization of the RNA as 60 to 70S. These data suggest that concentration of RSV(RAV-1) by the PEG-Pronase method may not alter the viral high-molecular-weight RNA, but additional experiments are needed to examine this possibility.

The buoyant density in sucrose of avian oncornaviruses is generally reported to be 1.15 to 1.16 g/cm³, even after incubation of concentrated preparations at 37 C for 30 min with 20 μg of Pronase per ml (17). Preparations of RSV(RAV-1) concentrated by the PEG method, with or without DS, contained large amounts of proteinaceous debris and yielded a peak of radioactivity at a density of 1.15 to 1.16 g/cm³. Virus preparations concentrated by the PEG-Pronase method contained little or no proteinaceous debris and banded at a density of approximately 1.14 g/cm³. However, when pelleted in the presence of proteinaceous medium supplements and rebanded in sucrose, these purified particles shifted to a density of 1.15 to 1.16 g/cm³. These results suggest that the buoyant density of RSV(RAV-1) is affected by the proteinaceous debris in concentrated preparations.

Virus preparations concentrated by the PEG-Pronase method were treated twice with Pronase at 37 C for 30 min. With volumes of supernatant fluids of as much as 500 ml, the second Pronase treatment was not necessary for maximum recovery of radiolabeled virions (D. L. Bronson, unpublished data). However, some proteinaceous debris was present in these gradient-purified preparations. The need for the second Pronase treatment depends on the volume of fluids employed, the concentration of proteinaceous supplements in the tissue culture medium, the concentration of PEG used for separation, and the objectives of the experiment. Some viruses may be adversely affected by Pronase treatment, and optimum conditions would have to be determined for different viruses. However, the amount of proteinaceous debris in gradient-purified preparations of RSV(RAV-1) that were concentrated by the PEG-Pronase method is greatly decreased, and such preparations may be particularly suited for studies of immunological, enzymatic, or subviral component properties.
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