A Comparison of Four Methods Used to Concentrate Rous Sarcoma Virus from Tissue Culture Fluids

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(Accepted 2 July 1976)

SUMMARY

Three methods of pelleting, pelleting followed by Pronase treatment, polyethylene glycol (PEG)-Pronase, and diafilo ultrafiltration (diafiltration) were used to concentrate RSV(RAV-I) from tissue culture fluids. Sucrose-gradient fractions containing virus preparations which had been concentrated by diafiltration or pelleting were heavily contaminated with amorphous debris. This debris was not present in similar, gradient-purified preparations that had been concentrated by the PEG-Pronase or pellet-Pronase methods. Maximum recovery of radiolabelled virus particles and virion-associated RNA-dependent DNA polymerase activity was obtained in gradient fractions containing virus concentrates prepared by the pellet-Pronase and PEG-Pronase methods. Although there were slight differences in recovery by these two methods, the advantages of the PEG-Pronase method make it the preferred method, especially when large volumes of tissue culture fluids are used.

INTRODUCTION

Some of the methods commonly used to concentrate oncornaviruses from tissue culture medium include pelleting, either to the bottom of the centrifuge tube or on to a cushion of a high-density solution in a preparative ultracentrifuge (Robinson, 1967; Bader & Steck, 1969); precipitation with ammonium sulphate (Duesberg et al. 1968; Cardiff et al. 1974); and diafiltration (Rhim et al. 1969). Pelleting by centrifugation is time consuming and may damage virus particles. Precipitation generally requires the slow addition of ammonium sulphate, results in heavy contamination of the concentrates with proteinaceous debris, and may also disrupt some viruses (Robinson & Duesberg, 1968). Concentration by diafiltration also requires extended time intervals, depending on a flow rate that is subject to numerous variables (e.g. type of membrane used, the concentration of proteinaceous supplements in the medium, the volume of fluids, the amount of pressure exerted on the system), and the quantity of fluids which can be concentrated is severely limited by the progressive increase in viscosity of the concentrate as the volume is reduced.

Another method that has had limited application, with some differences in technique, is concentration of oncornaviruses from culture medium with polyethylene glycol (PEG; Green et al. 1970; Bishop et al. 1971; Naso et al. 1972; Syrewicz et al. 1972; Officer et al. 1973; Fidanián, Drohán & Baluda, 1975). The primary advantages of the PEG method are that it is rapid, simple, and inexpensive; it is independent of virus concentration (Yamamoto et al. 1970; Adams, 1973); it can be used with large volumes of fluids; and it is effective
even with highly labile viruses such as the Epstein–Barr virus (Adams, 1973) and foot-and-mouth disease virus (Wagner, Card & Cowan, 1970).

A major disadvantage of the method is that PEG also concentrates proteins from solutions (Polson et al. 1964; Zeppezauer & Brishammar, 1965; Chun, Fried & Ellis, 1967). Virus concentrates prepared by the PEG method from tissue culture medium are highly viscous because of the high protein concentration (Wagner et al. 1970), and this interferes with purification of the virus particles. Our previous studies with RSV(RAV-1) demonstrated that this interference is eliminated by treatment of the concentrates with Pronase (Bronson, Elliott & Ritzi, 1975).

However, the recovery of virus particles by this method relative to recovery obtained by other methods has not been determined. In the present studies, the PEG-Pronase, pellet, pellet-Pronase, and diafiltration methods for concentrating RSV(RAV-1) from culture medium were compared. The results demonstrate that the PEG-Pronase method is a very effective means for concentrating and partially purifying virus preparations.

METHODS

Cell culture and virus. The methods for the propagation of RSV(RAV-1) and the assay for focus forming units (f.f.u.) in secondary or tertiary monolayers of chicken embryo fibroblasts (CEF) have been described (Bronson et al. 1975). Cultures of infected CEF were maintained in Medium 199 (Grand Island Biological Co., Grand Island, New York) supplemented with 2 % heat-inactivated calf serum, 5 % tryptose phosphate broth, 1 mM-L-glutamine, penicillin (100 units/ml), and streptomycin (100 #g/ml: medium A) or in Medium 199 containing 2 % heat-inactivated foetal calf serum, 2 mM-L-glutamine, and antibiotics (medium B).

Virions were radiolabelled by adding 10 µCi of 3H-uridine (25 to 28 Ci/mmole; Schwarz/Mann, Orangeburg, New York) per ml of medium to cultures in 75 cm² Falcon plastic flasks (Bioquest, Cockeysville, Maryland). All harvests were made at 10 to 12 h intervals. Tissue culture fluids in each harvest were pooled, clarified at 13000 g for 15 min in a Sorvall GSA rotor, and stored at -85 °C.

Virus concentration and purification. In order to provide uniform virus preparations for concentration by the four different methods in each experiment, culture fluids were thawed, pooled, uniformly mixed, and divided into four equal volumes. Vol. of 40 to 100 ml of pooled fluids from radiolabelled cultures of infected CEF were mixed with four to sixfold volumes of non-radiolabelled culture fluids. The fluids were concentrated by the following methods: (A) concentration by the PEG-Pronase method has been described (Bronson et al. 1975). Briefly, a 1/10 vol. of 4 M-NaCl and 8 % (w/v) PEG (mol. wt. 5700 to 6700) were added to the culture fluids. The suspension was stirred in an ice bath for 2 to 3 h and pelleted at 8000 g for 15 min in a Sorvall GSA rotor. The supernatant fluids were discarded, with care being taken to remove all of the fluids from the concentrated preparations. Even small volumes of residual fluids containing PEG reduce the effectiveness of Pronase treatment, which may result in significant losses of virus particles during purification of the concentrates.

The pellets were resuspended in 10 ml of TNE (0·01 M-tris-HCl, 0·1 M-NaCl, and 0·001 M-EDTA, pH 7·2). The concentrates were incubated at 37 °C for 30 min with 20 mg of Pronase (B grade, Calbiochem, San Diego, California) and cooled in ice.

In method (B) diafiltration was performed at 4 °C under positive N₂ pressure with a 402 diaflo unit (Amicon Corp., Lexington, Massachusetts) and an Amicon XM-100A or
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XM-300 membrane. After concentration of culture fluids to 8 to 10 ml, the concentrate was removed, and the unit was washed with 5 ml of TNE, which was added to the concentrate.

In method (C) tissue culture fluids were centrifuged at 27000 rev/min for 60 min in the Spinco SW-27 rotor. The supernatant fluid was discarded, and 1 ml of TNE was added to each centrifuge tube. The tubes were placed in an ice bath for approx. 30 min, which assisted in resuspension of the pellets. The pellets were resuspended by repeated ejection from a 1 ml pipette, combined, and diluted to a final volume of 10 ml with TNE (pellet method).

For method (D) tissue culture fluids were centrifuged, and the pellets were resuspended in 10 ml of TNE as described for method (C). The concentrates were incubated with 20 mg of Pronase at 37 °C for 30 min and cooled in ice (pellet-Pronase method).

Concentrates were layered over discontinuous gradients of 12 ml of 20% (w/v) sucrose cushioned by 5 ml of 55% (w/v) sucrose and centrifuged at 27000 rev/min for 2 h in the Spinco SW-27 rotor at 5 °C. The band at the interphase between the 20 and 55% sucrose layers was collected by puncturing the bottom of the tube and diluted to approx. 11 ml with TNE. Virus preparations concentrated by the PEG-Pronase or pellet-Pronase methods were again incubated with 20 mg of Pronase for 30 min at 37 °C and cooled in ice. Concentrates were layered over 23 ml linear gradients of 20 to 55% sucrose and centrifuged 8 to 9 h at 27000 rev/min in the Spinco SW-27 rotor. One ml fractions were collected from the bottom of the tube and were assayed for buoyant density, f.f.u., extinction at 260 nm (E260), and either radioactivity as ct/min or virion-associated RNA-dependent DNA polymerase (reverse transcriptase) activity, as previously described (Bronson et al. 1975). Protein concentration was determined by a modification (Wang & Smith, 1975) of the Lowry procedure (Lowry et al. 1951).

Extracts of radiolabelled cells. Confluent CEF monolayers in four 75 cm² flasks were incubated for 48 h in medium A containing 10 μCi of 3H-uridine per ml. The culture fluids were discarded, and the monolayers were washed with medium A, trypsinized, and pelleted and washed twice with TNE. The cells were resuspended in 15 ml of TNE, frozen and thawed twice, and sonicated for 5 min in a Branson Model D-50 Sonogen (Branson Instruments, Inc., Stamford, Connecticut). The extract was clarified at 13000 g for 15 min and stored at −85 °C.

RESULTS

Recovery of virus particles

Volumes of 250 ml of culture fluids (medium A) were mixed with 40 ml of fluids from radiolabelled cultures for each method of concentration. Assays of gradient fractions indicated that maximum recovery of radioactivity in the ct/min peak fraction was generally obtained with preparations concentrated by the pellet-Pronase method (Fig. 1). These concentrates yielded sharp, coinocnings of radioactivity and E260 at a buoyant density of 1.14 to 1.15 g/ml. Similar results were obtained with preparations concentrated by the PEG-Pronase method, although recovery of radioactivity in the ct/min peak fraction of the sucrose gradient in four separate experiments, each employing volumes of 250 to 480 ml of culture fluids, varied from approx. 80 to 105% of that obtained with the pellet-Pronase method (Fig. 1b).

Concentrates prepared by the pellet method produced much broader profiles of radioactivity and E260 at a buoyant density range of 1.14 to 1.17 g/ml (Fig. 1c). Although recovery of radioactivity in the ct/min peak fraction was only 50 to 60% of that obtained with the pellet-Pronase method, the total recovery of ct/min in the gradient was very similar to total
recovery obtained by the pellet-Pronase method. In addition, the $E_{260}$ profile was much higher in the ct/min peak fractions from the gradient containing the pelleted preparation, indicating the presence of non-radiolabelled material.

Concentration by diafiltration produced severe and variable losses of virus particles. These concentrates were highly viscous, and this interfered with the collection of the material banding at the interphase of the discontinuous sucrose gradient. After a few drops were released from the puncture in the bottom of the tube, the high viscosity greatly reduced the flow rate and caused extensive mixing by creating a vortex that pulled material from the 20% sucrose layer. Such preparations yielded high background levels of radioactivity and $E_{260}$ in nearly all fractions of the linear sucrose gradients, with a slight peak of radioactivity at a buoyant density range of 1.14 to 1.17 g/ml (Fig. 1d). The recovery of virus particles in these preparations was slightly enhanced when smaller volumes (100 to 200 ml) of culture fluids were used, but in none of these experiments did recovery of radiolabelled virus particles at a buoyant density range of 1.14 to 1.17 g/ml exceed 40% of that obtained by the pellet-Pronase method.

Assays for f.f.u. in the gradient fractions reflected the results obtained with assays for radioactivity and $E_{260}$ (Fig. 1). However, the profile of virus bioactivity did not coincide with the $E_{260}$ and radioactivity profiles but was located in the high density region of these profiles. Similar results have been reported with avian and murine oncornaviruses (Robinson, 1967; Duesberg et al. 1968; Manning, Schaffer & Soergel, 1972). It has been suggested that the lower bioactivity in the low buoyant-density region of the radioactivity profile might be caused by incomplete RNA and virus production or by an intermediate step in
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Fig. 2. Reverse transcriptase activity in gradient fractions containing purified RSV(RAV-I) that had been concentrated from 260 ml of medium by: (a) the pellet-Pronase method; (b) the PEG-Pronase method; (c) the pellet method; and (d) diafiltration. Gradient fractions were assayed for buoyant density (●—●), E260 (○—○), and endogenous reverse transcriptase activity, expressed as ct/min, resulting from incorporation of 3H-TMP into acid-insoluble product (∆—∆—∆).

virus degradation (Manning et al. 1972). The results obtained in the present study, however, do not justify speculation concerning the lack of coincidence between the bioactivity profile and the radioactivity and E260 profiles.

Reverse transcriptase

Assays for reverse transcriptase activity were conducted in duplicate with 25 μl samples of gradient-purified preparations of RSV(RAV-I). The results of these assays indicated that the maximum recovery of reverse transcriptase activity was obtained with the gradient-purified concentrate prepared by the PEG-Pronase method, although the activity was only 5 to 10 % greater than that obtained with the concentrate prepared by the pellet-Pronase method (Fig. 2). Two additional experiments using volumes of 260 and 400 ml of culture fluids yielded very similar results.

The fraction with maximum reverse transcriptase activity in duplicate gradients containing RSV(RAV-I) concentrated by each method from 260 ml of medium A was mixed with the fraction on either side of it. The six combined fractions were diluted with TNE and centrifuged at 40000 rev/min for 60 min on to a 1 ml cushion of 55 % sucrose in a Spinco SW-40 rotor. The material that banded on the cushion was collected by puncturing the bottom of the tube and assayed for protein concentration and reverse transcriptase activity (Table 1). The results indicate that (i) the incorporation of 3H-TMP per microgram of protein was greatest in the gradient-purified concentrate prepared by the PEG-Pronase method and (ii) Pronase treatment did not affect the reverse transcriptase activity of
Table I. *Endogenous reverse transcriptase activity per microgram of protein in gradient-purified preparations of RSV(RAV-I)*

<table>
<thead>
<tr>
<th>Method</th>
<th>Gradient fractions†</th>
<th>Protein concentration (µg/ml)</th>
<th>³H-TMP incorporated per µg protein‡ (ct/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pellet-Pronase</td>
<td>14, 15, 16</td>
<td>175</td>
<td>9907</td>
</tr>
<tr>
<td>PEG-Pronase</td>
<td>14, 15, 16</td>
<td>159</td>
<td>11006</td>
</tr>
<tr>
<td>Pellet</td>
<td>12, 13, 14</td>
<td>245</td>
<td>4792</td>
</tr>
<tr>
<td>Diafiltration</td>
<td>13, 14, 15</td>
<td>170</td>
<td>4910</td>
</tr>
</tbody>
</table>

* Method used to concentrate RSV(RAV-I) from tissue culture fluids.
† Combined gradient fractions are from gradients shown in Fig. 2 and from gradients in a duplicate experiment, and they were prepared as described in the text.
‡ Assays for reverse transcriptase activity were done twice in triplicate with 25 µl samples of each virus preparation. One pmol represents approx. 42 000 ct/min of ³H-thymidine triphosphate.

RSV(RAV-I), but that it decreased greatly the amount of contamination of the virus preparations with protein without reverse transcriptase activity.

**Cell debris in purified virus preparations**

Concentration of vesicular stomatitis virus with PEG yielded quantitative recovery of virions, and it also partially purified the virus concentrates (McSharry & Benzinger, 1970). We conducted similar experiments with 300 ml volumes of non-radiolabelled culture fluids from infected CEF, each mixed with 3 ml of an extract of radiolabelled cells. The results indicate that (i) concentrates prepared by the PEG-Pronase method yielded approx. 200 ct/min/µl ml at a buoyant density of 1.10 to 1.15 g/ml in sucrose; (ii) the pellet-Pronase method gave 400 to 500 ct/min/µl ml at a similar buoyant density; (iii) the pellet method yielded a maximum of 1536 ct/min/µl ml at a density of 1.155 g/ml; and (iv) diafiltration produced 400 to 1650 ct/min/µl ml throughout the gradient. Therefore, the least amount of radiolabelled cell debris was found in purified concentrates prepared by the PEG-Pronase method, although Pronase treatment itself reduced significantly the amount of radiolabelled debris.

**Effects of type of medium supplements**

Routine lot analyses indicate that the protein concentration in calf serum is approx. twice that in foetal calf serum (J. Petrusl, GIBCO, personal communication). We previously reported that large amounts of protein in concentrates prepared by the PEG method interfered with virus purification (Bronson et al. 1975). This interference was also discernible in the present studies when viruses were concentrated by the diafiltration method or, to a lesser extent, by the pellet method. Therefore, these experiments were repeated with concentrates prepared by all four methods using culture fluids from infected CEF maintained in medium B, to reduce protein concentration.

In three experiments, concentrates prepared from 250, 280, and 345 ml of medium B by the pellet-Pronase and PEG-Pronase methods yielded results similar to those obtained by these methods with virions produced by cells in medium A. Preparations concentrated by the pellet method yielded sharper and more uniform radioactivity and E₂₆₀ profiles, with recovery of radioactivity in the ct/min peak fraction increased to approx. 75% of that obtained with the Pronase-treated preparations. Concentrates prepared by diafiltration were still highly viscous, however, and the recovery of radiolabelled virions at a buoyant density of 1.15 to 1.17 g/ml was not enhanced significantly.
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DISCUSSION

We previously reported some variations in the use of PEG to concentrate RSV(RAV-1) from tissue culture medium and demonstrated that optimum results were obtained by treating the concentrates with Pronase (Bronson et al. 1975). However, the efficiency of the technique as a method of virus concentration was not determined. The present studies were undertaken to compare the recovery of RSV(RAV-1) from sucrose gradients containing virus concentrates prepared by four different methods.

Virus concentrates prepared by dialfiltration were highly viscous, and severe losses occurred during attempts to purify the concentrates by centrifugation in sucrose gradients. In addition, the dialfiltration method is very time consuming; e.g., concentration of 300 ml of medium B containing RSV(RAV-1) to approx. 10 ml with the Amicon 402 dialflo unit required 8 to 9 h, and concentration of a similar volume of tissue culture medium A required as long as 16 h. Therefore, under the conditions employed in these studies, dialfiltration is not a satisfactory procedure for concentrating RSV(RAV-1) for subsequent purification by sucrose gradient centrifugation, even if the culture fluids contain only a low concentration of proteinaceous supplements.

Virus concentrates prepared by the pellet method from medium A yielded broad radioactivity and $E_{260}$ profiles, with maximum values at a buoyant density of 1.15 to 1.16 g/ml in sucrose gradients. When this method was used with concentrates prepared from medium B, these profiles were slightly sharper and more uniform and were located at a buoyant density of 1.14 to 1.15 g/ml. The slight shift in buoyant density may result from the amount of protein present in the virus concentrates, as suggested in earlier experiments (Bronson et al. 1975). However, examination by electron microscopy revealed large amounts of debris in all gradient-purified preparations of RSV(RAV-1) concentrated by the pellet method (unpublished data). The presence of debris is also reflected in the high $E_{260}$ profile (Fig. 1c and 2c), by the relatively low reverse transcriptase activity per microgram of protein (Table 1), and by the radioactivity from labelled cell debris in these gradient fractions.

The recovery of radioactivity in the peak fractions from gradients containing virus concentrates prepared by the PEG-Pronase method was similar to that obtained by the pellet-Pronase method. However, in three separate experiments, reverse transcriptase activity in the gradient fractions containing virus concentrates prepared by the PEG-Pronase method was approx. 5 to 10% greater than the activity obtained in similar preparations concentrated by the pellet-Pronase method. These differences probably reflect the greater amount of radiolabelled cell debris in preparations concentrated by the latter method.

Although the recovery of virus particles by the PEG-Pronase and pellet-Pronase methods was approx. equivalent, the PEG-Pronase method has the advantages of being rapid and gentle and also produced less contamination of virus concentrates with cell debris. Obviously, the PEG-Pronase method is applicable only to viruses that are not adversely affected by treatment with this enzyme. With this exception, the PEG-Pronase method is a very effective technique for concentrating, and partially purifying, viruses from cell-culture media.

We thank H. Asmussen and K. Peterson for excellent technical assistance and Judith Gunn Bronson, M.S., for editing the manuscript. This study was supported by Grant CA 13095-03 from the National Cancer Institute.
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(Received 3 March 1976)