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Potassium Tartrate-Glycerol as a Density Gradient Substrate for Separation of Small, Round Viruses from Human Feces

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Cesium chloride density gradients are frequently used for virus concentration or purification in the preparation of human feces for examination by electron microscopy. Disruption of some of the fecal viruses occurs if they are pelleted from the density gradient in an additional concentration step. This report highlights an important limitation imposed by the use of cesium chloride as a density gradient substrate in attempting to recover small, round, virus-like particles from feces and suggests an alternative substrate which preserves virus morphology without the use of additional protective agents.

In recent years, there have been numerous reports of the detection of small, round viruses (SRV) or virus-like particles in human feces (1–3, 6–13, 15–17). Because of difficulties in propagating most of these SRV in cell cultures, detection and presumptive classification have relied extensively on morphology determination by electron microscopy (EM). Some of these SRV have readily recognizable morphologies, but others have no obvious, identifying surface features (E. O. Caul and H. Appleton, J. Med. Virol., in press). These featureless SRV are usually initially identified by size estimation, but because of inherent magnification errors in electron microscopes, this may be an unreliable method. In addition, some SRV may be present in the feces only in small numbers. To overcome the pitfalls of classification by size alone and to concentrate and purify SRV virus preparations, density researchers have used gradient centrifugation extensively in the investigation of SRV. The density gradient substrate most commonly used is cesium chloride (2, 9, 16).

In the course of our work with SRV-like particles morphologically indistinguishable from the Norwalk agent (6) (these particles are hereafter referred to as SRSV), we observed a marked instability of the morphology of SRSV after they were pelleted from cesium chloride density gradients. A similar disruption of astrovirus morphology after concentration through cesium chloride has also been observed (unpublished data) and has been overcome to some extent by the inclusion of bovine serum albumin as a protective agent in the gradient (T. W. Lee, personal communication).

Positive-density, negative-viscosity gradients made by progressively diluting potassium tartrate with glycerol have been described for the purification of large enveloped viruses and their subviral components (5, 14). Before the present report, this gradient substrate has not been used in the study of the smaller, denser, unenveloped viruses found in feces. The protective effect of glycerol on virus morphology and the efficient retardation of cell membrane fragments by this type of gradient has led us to investigate its suitability as an alternative to cesium chloride as a substrate in the purification of SRV from human feces.

MATERIALS AND METHODS

Viruses. Human parvovirus-like particles and astroviruses from the feces of children suffering from diarrhea and vomiting and SRSV (Norwalk-like) from the feces of an adult involved in a ward outbreak of diarrhea and vomiting were extracted from 20 ml of a fecal emulsion (ca. 10%) in basic Earle medium by precipitation with ammonium sulfate as previously described (4). The precipitated material was suspended in 1 ml of SET buffer (0.1 M NaCl, 0.001 M EDTA, 0.05 M Tris [final pH, 7.2]).

Feline calicivirus strain F9 was propagated in feline embryonic lung fibroblast monolayer cultures and harvested at 24 h postinfection, when cytopathic effects were maximal (infectivity, $10^{7.5}$ 50% tissue culture infective doses per ml). We extracted virus by freezing and thawing the cultures three times before centrifugation at 2,000 rpm to remove cell debris. The virus present in 20 ml of the clarified supernatant fluid was concentrated by precipitation with ammonium sulfate in the same way as were the fecal viruses, and the precipitated deposit was suspended in 4 ml of SET buffer. The infectivity of this concentrated virus suspension was $2 \times 10^{9.5}$ 50% tissue culture infective doses per ml.

Density gradients. A cesium chloride solution was prepared in SET buffer to give a cesium chloride concentration calculated to produce a density gradient with a center density of 1.38 g/cm^3 when centrifuged to equilibrium. A 7-ml portion of this solution was intro-

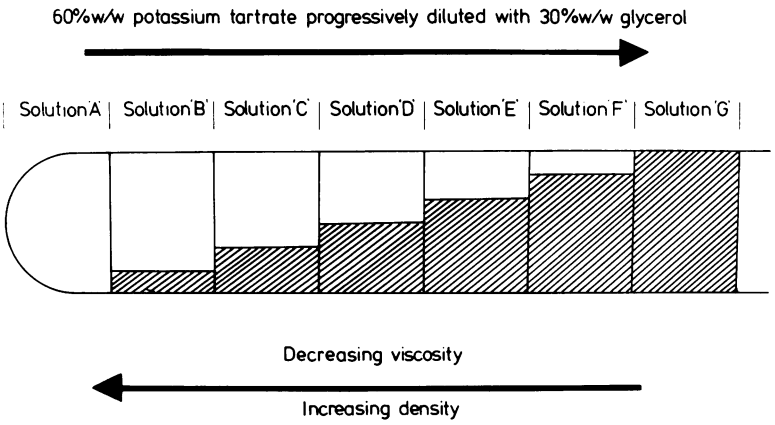

FIG. 1. Diagrammatic presentation of a discontinuous potassium tartrate-glycerol density gradient showing the effects on viscosity and density of progressively diluting 60% (wt/wt) potassium tartrate with 30% (wt/wt) glycerol.

duced into a polycarbonate centrifuge tube (14 ml; Measuring & Scientific Equipment, Ltd.). This portion was overlaid with 0.5 ml of the concentrated virus suspension, and the tube was filled with liquid paraffin and capped. The gradient was centrifuged to equilibrium at 220,000 × *g* for 18 h in an angle-head rotor at 4°C. We collected nine fractions (0.75 ml each) by puncturing the bottom of the tubes. The refractive index of the fractions was measured with a Bellingham and Stanley pocket refractometer, and the density was calculated from a standard curve.

We prepared discontinuous potassium tartrate-glycerol density gradients by progressively diluting 60% (wt/wt) potassium tartrate in SET with 30% (wt/wt) glycerol in SET buffer (Fig. 1). We prepared the gradients in polycarbonate tubes (14 ml) by sequentially overlaying 1-ml amounts of the following stock solutions: solution A, 60% (wt/wt) potassium tartrate in SET buffer; solution B, 50 g of solution A plus 10 g of solution G; solution C, 40 g of solution A plus 20 g of solution G; solution D, 30 g of solution A plus 30 g of solution G; solution E, 20 g of solution A plus 40 g of solution G; solution F, 10 g of solution A plus 50 g of solution G; and solution G, 30% (wt/wt) glycerol in SET buffer.

The complete gradient was overlaid with 0.5 ml of the concentrated virus suspension. The tubes were then filled with liquid paraffin and capped. Centrifugation and fraction collection were as described above.

Assay of virus recovered from gradients. (i) Electron microscopy direct counts. A sample was removed from each fraction, and virus was adsorbed for 30 min onto a 250-mesh Formvar-coated electron microscope grid. The excess fluid was drained off the grid, which was then washed briefly on a drop of distilled water. The grid was stained on a drop of 1.5% phosphotungstic acid (pH 6.5) for 30 s and then blotted dry and examined under an AEI 801 electron microscope at what was indicated to be a magnification of ×63,000. The numbers of virus particles present in four randomly selected grid squares were counted: only particles occurring singularly or in pairs were included. The number of virus particles counted on each grid was then expressed as a percentage of the total number of virus particles counted on all fractions of the gradient.

(ii) Pellet counts. The remainder of each fraction was diluted 10-fold with SET buffer and centrifuged at 49,000 × *g* for 1.5 h at 4°C in a Sorvall SM24 angle-head rotor. The resulting virus pellet was suspended in one drop of distilled water and adsorbed onto an electron microscope grid for 30 min. The grid was washed and stained, and virus was counted as described above.

(iii) Infectivity assay. Fraction collected from gradients containing feline calicivirus were also assayed for infectivity. From each fraction, a 0.2-ml sample was obtained; we diluted each sample in 10-fold steps to a dilution of 10^{-9} in Eagle medium supplemented with 1% fetal bovine serum, 10% tryptose-phosphate broth, and antibiotics. From each of these dilutions, we took a 0.2-ml sample, which we inoculated into each of two feline embryonic lung fibroblast cell monolayers. These cultures were incubated at 35°C for 4 days.

RESULTS

Figure 2 shows the recovery of feline calicivirus as determined by infectivity assay and EM with cesium chloride and potassium tartrate-glycerol gradients. Coincident peaks of infectivity and virus detection by EM were present only for the grids from the cesium choride gradient examined directly; no virus was detected by EM in the pellets from cesium chloride fractions. Coincident peaks were present in all direct and pellet assays with the potassium tartrate-glycerol gradient.

Figure 3 shows the recovery of parvovirus-like particles, astroviruses, SRSV, and feline calicivirus from pelleted fractions of cesium chloride and potassium tartrate-glycerol gradients. Only the parvovirus-like particles, with a

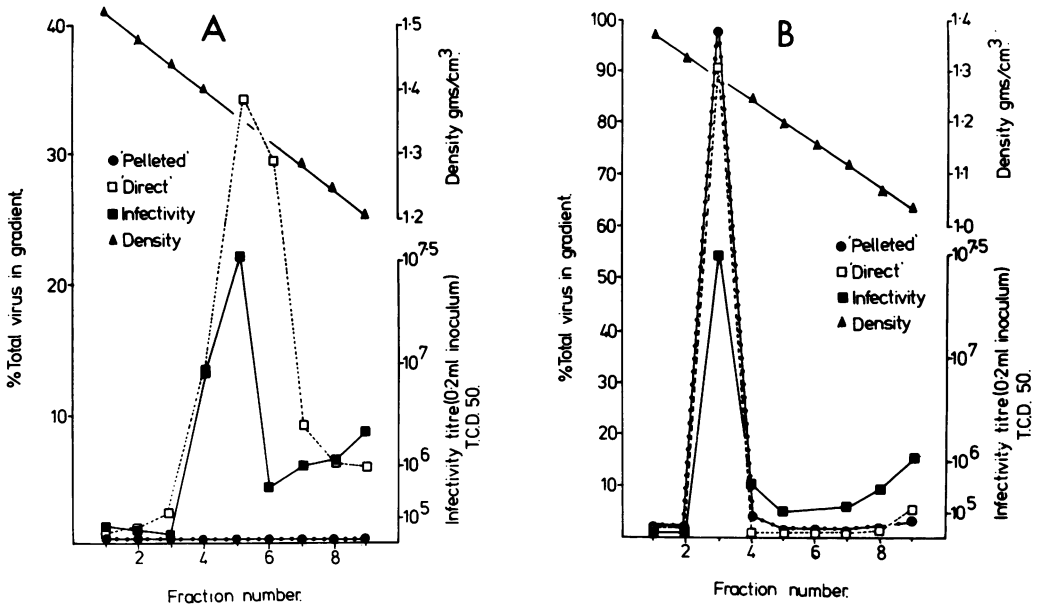

FIG. 2. (A) Recovery of feline calicivirus strain F9 in cesium chloride gradients. Note the loss of virus after it was pelleted from this substrate. Coincident peaks of virus infectivity and detection by direct EM examination of fractions are shown. (B) Recovery of feline calicivirus strain F9 in potassium tartrate-glycerol gradients. Virus was not lost after being pelleted from this gradient. Coincident peaks of infectivity and detection were present irrespective of the method of assay.

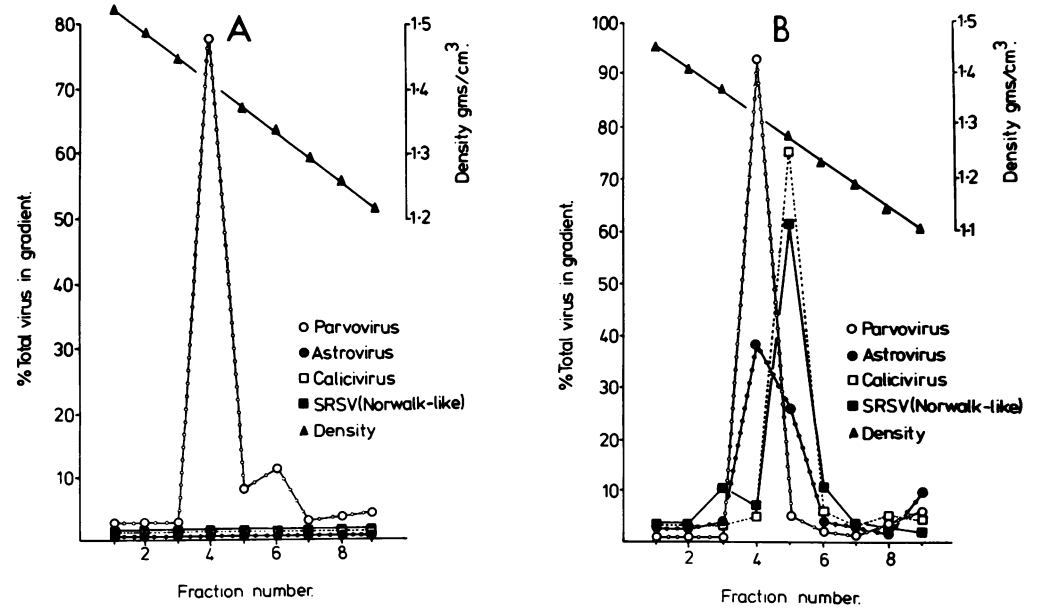

FIG. 3. (A) Effect of pelleting parvovirus-like particles, astrovirus, calicivirus, and SRSVs in cesium chloride gradients. Only the parvovirus-like particles survived this procedure. (B) Effect of pelleting parvovirus-like particles, astrovirus, calicivirus, and SRSV in potassium tartrate-glycerol gradients. All of the viruses survived the pelleting procedure in this gradient.

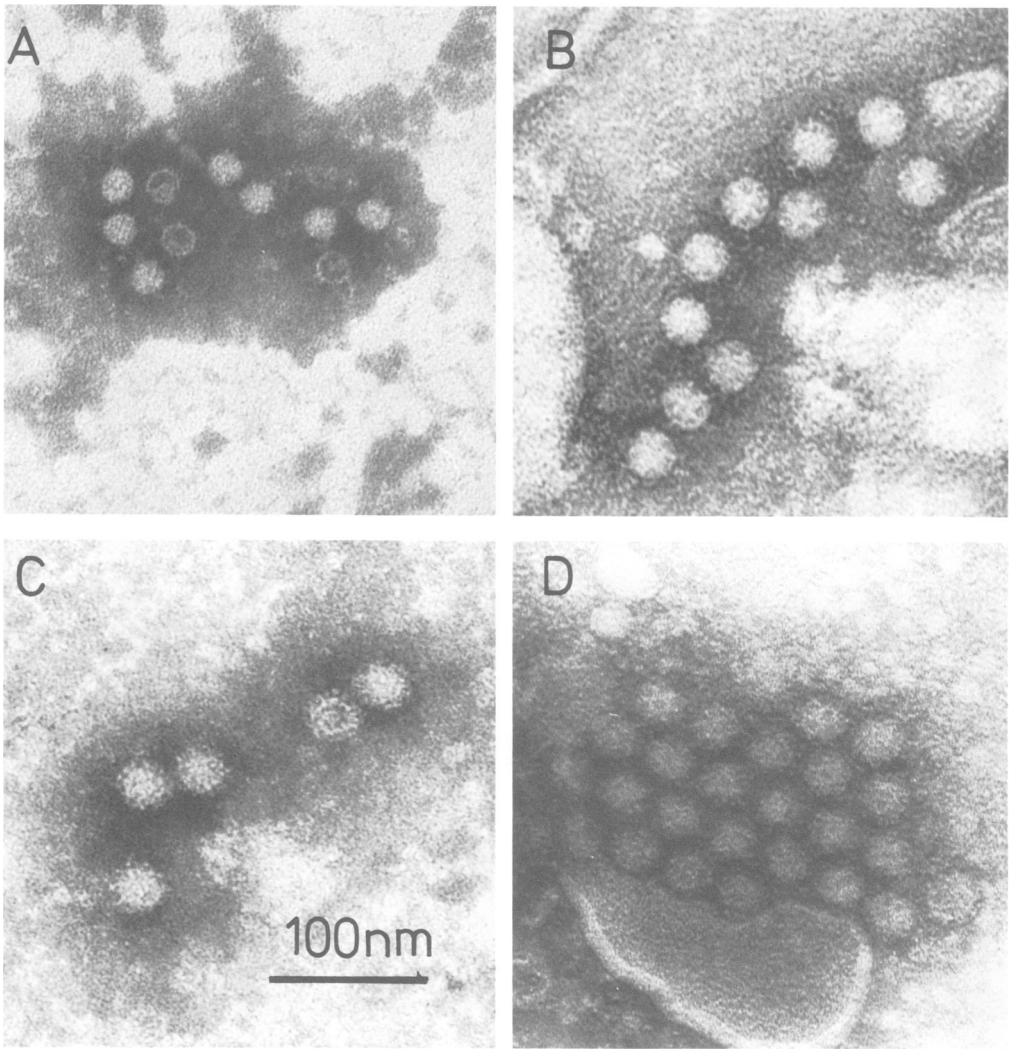

FIG. 4. The morphologies of parvovirus-like particles (A), astroviruses (B), SRSV (C), and feline calicivirus strain F9 (D) after recovery from a potassium tartrate-glycerol gradient. In each case, the morphology of the virus was unimpaired.

peak density of 1.40 g/cm^3, were recovered from the cesium chloride gradient. All sample viruses were recovered from the potassium tartrate-glycerol gradient: the peak density for the astrovirus and parvovirus-like particles was 1.32 gm/cm^3, and that for the SRSV and the feline calcivirus was 1.28 g/cm^3. The morphologies of all of the viruses recovered from the potassium tartrate-glycerol gradients were unimpaired (Fig. 4).

DISCUSSION

The results of the study clearly show that the critical factor in retaining the structure of several SRV from unprotected cesium chloride density gradients is the avoidance of the physical trauma associated with pelleting the virus from the gradient as an additional concentration step. Since these SRV are often present in the feces in only small numbers, this additional concentration is often necessary to allow detection by EM. There is no risk incurred in concentration if potassium tartrate-glycerol is used as the gradient substrate.

Additional benefits of using potassium tartrate-glycerol as a density gradient substrate are low cost, negligible toxicity, and reduction in centrifugation time due to the gradient being preformed.

This study also demonstrated that unprotected cesium chloride density gradients were not ideal for preparing feces for the detection of

some SRV by EM. In dual infections caused by parvovirus and SRSV (Norwalk-like) or astrovirus, experience has shown that the use of an unprotected cesium chloride density gradient does not permit the detection of SRSV, caliciviruses, or astroviruses if the virus is concentrated from the gradient by pelleting. This experience agrees with our findings. These observations indicate that when dual virus infections occur, parvovirus-like particles may appear, because of their stability after centrifugation of fractions from unprotected cesium chloride gradients, to be more important as causal agents of human gastroenteritis than is presently justified. This limitation on the use of unprotected cesium chloride gradients can be overcome if fractions are dialyzed against phosphate-buffered saline (10) rather than centrifuged. However, this additional dialysis step need not be carried out if potassium tartrate-glycerol is used as the gradient substrate.

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