ISOLATION OF VIRAL RNA USING CESIUM CHLORIDE-ETHIDIUM BROMIDE EQUILIBRIUM SEDIMENTATION

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SUMMARY

The effect of ethidium bromide (EB) on the buoyant density of reovirus RNA during equilibrium sedimentation has been investigated. The addition of the dye ethidium bromide was found to reduce the buoyant density of reovirus RNA in a Cs_2SO_4 gradient by a value of 0.13 to 0.15 g/cc, and provided a separation limit of 0.10 g/cc relative to the ρ of marker DNA. Ethidium bromide was found also to reduce the ρ of reovirus RNA to allow this RNA to band on a CsCl gradient. The separation factor between DNA and RNA on a CsCl-EB gradient was found to be 0.23 g/cc, indicating this type of gradient to be highly effective for separating the two types of polynucleotides.

INTRODUCTION

The intercalation model which Lerman (1) proposed as the mode of interaction between acridines and DNA has been extended to explain the interaction between DNA and other antibiotics or drugs, such as chloroquine (2), actinomycin D (3) and ethidium bromide (EB) (4,5,6). These DNA-drug interactions have been found to alter some of the physical properties of DNA resulting in a decrease of the sedimentation coefficient, an increase in viscosity and a lowering of the buoyant density (7,8,9). Numerous studies performed with EB have suggested that the alterations in the physical properties of DNA result from the intercalation of dye molecules between adjacent base pairs of a polynucleotide. In addition, it is the restricted intercalation

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of EB in closed double stranded circular molecules which mediates the separation of linear, nicked circular and closed circular polynucleotides on cesium salt gradients (10,11,12). Despite these observations, there is a paucity of information relative to isolation and buoyant density properties of viral RNAs in the presence of intercalating agents. The present study was performed to assess the utilization of cesium salt-(EB) gradients as a means of isolating and characterizing RNA extracted from virus or from infected cells

MATERIALS AND METHODS

Cells, media and virus: L-929 cells were propagated in Eagle's minimal essential medium (13) as previously described (14), with the exception that 5% fetal bovine serum was employed. Reovirus type 3 (Abney strain) was quantitated by plaque formation on L-929 cell monolayers after 3 to 4 day incubation in an atmosphere of 95% oxygen and 5% CO_2, using a liquid overlay. Identification of the reovirus type was accomplished by a hemagglutination inhibition test using standard antisera purchased from ATCC.

Extraction of RNA from virus: At maximum reovirus replication in L cells, the monolayers were frozen and thawed three times and the virus pelleted at 78,000 x G for 2 hours. Following two washes in phosphate buffered saline (PBS), the crude virus preparation was treated with 100 µg RNAase (1.05 units) and 100 µg DNAase (250 units) for 60 minutes at room temperature. The reovirus was banded twice on a preformed cesium chloride (CsCl) step gradient with intermittent and final dialysis of the virus pool against PBS. RNA was extracted from the purified viruses four times with an equal volume of either water saturated phenol, or phenol equilibrated with 2M NaCl containing 0.01 M EDTA, pH 7.1. Residual phenol was removed by four ether extractions followed by the extensive dialysis for 2 days against three changes of PBS (4°C). These preparations were pervaporated to an approximate volume of 1 ml.

Extraction of RNA from infected cells: A total of 6.0×10^7 L-929 cells,

grown as stationary monolayers were exposed to a multiplicity of 15 pfu/cell of reovirus. Following a 2 hour adsorption period at 4°C the cells were rinsed with 0.15 M NaCl to remove unadsorbed virus. Each monolayer was supplemented with 20 ml of Eagle's medium containing dialyzed serum, 0.5 µg/ml actinomycin D and 5 $\mu C/ml^3$ H uridine. At 24 hours post infection, the medium was removed, and the monolayers were rinsed three times with cold physiological saline, and thoroughly drained. Cells were scraped from the glass, pooled, and cytoplasmic fractions were prepared with NP-40 as described (14). The RNA was extracted as described for purified virus.

Density Gradient Centrifugation: Up to one ml of the RNA preparations was mixed with three ml of CsCl or Cs_2SO_4 in 0.01 M tris pH 7.4. Ethidium bromide (Calbiochem) was added to a final concentration of 100 µg/ml and finally 0.5 ml mineral oil was layered on top. The preparations were centrifuged at 185,000 x G for 40 to 44 hours (15°C) in the Beckman 50.1 rotor. Following centrifugation, the tubes were examined under ultraviolet light and the position of the bands noted. Fractions were collected and the refractive index determined using an Abbe-3L refractometer. A 10 µl portion of each fraction was assayed for radioactivity in the Beckman-LS-100 scintillation counter and the density profile of the gradient was determined using the equations of Vinograd and Hearst (15).

Electron Microscopy: Portions from selected gradient fractions were prepared for electron microscopy by the method of Wetmur et al (16). Accordingly, RNA mixed with cytochrome C and floated on a hypophase of .15 M ammonium acetate (17) was picked up on formvar coated grids, stained with uranyl acetate (16) and/or rotary-shadowed with platinum-palladium at an incident angle of 6°. Grids were examined in a Hitachi Hu-11B electron microscope. Magnification calibration of the electron microscope was determined with a 28,800 lines per inch grating replica. The RNA molecules were photographed and contour lengths measured with a Minerva map curvimeter.

RESULTS

The banding characteristics of polydeoxyribonucleotides (Micrococcus lysodeikticus DNA) and polyribonucleotides (Reovirus RNA) were compared using a Cs_2SO_4-EB gradient. Following centrifugation the band positions were marked and then the gradients were fractionated. The distribution of radioactive M. lysodeikticus DNA indicated a peak fraction with a ρ of 1.36 g/cc. The interpolation of the band position (intersection of cross bar on the gradient profile) gave a value of 1.37 g/cc. The band of RNA, extracted from purified reovirus, had a ρ of 1.46 g/cc (Figure 1B).

Experiments were subsequently performed to determine the utility of Cs_2SO_4-EB equilibrium sedimentation for the isolation of viral RNA from cytoplasmic extracts of infected cells. Reovirus infected L cell monolayers exposed to ^3H uridine and actinomycin D, were extracted as described, centrifuged and monitored for U.V. fluorescent bands and distribution of radioactivity. The interpolation of the band position indicated a ρ value of 1.48 g/cc which corresponded with the peak of radioactivity found in the fraction with a ρ of 1.47 g/cc (Figure 1C). Two additional bands of nucleic acid were observed in the gradient by U.V. fluorescence. The ρ of these bands which did not contain detectable radioactivity corresponded to 1.37 and 1.32 g/cc, values more closely corresponding to DNA.

The ρ of reovirus RNA is 1.61 g/cc (18) in Cs_2SO_4. The ρ values obtained in this study indicated EB was effective in lowering the ρ of these molecules to 1.46 g/cc.

The banding characteristics of reovirus RNA and M. lysodeikticus DNA were compared using a CsCl-EB gradient. The material employed was unlabeled RNA extracted from purified reovirus, and ^3H thymidine labeled M. lysodeikticus DNA. Analysis of the gradients indicated a peak of radioactivity in a fraction with a ρ of 1.60 g/cc, while the interpolation of the band position gave a ρ 1.58 g/cc for the DNA. A band of weakly fluorescent material with no detect-

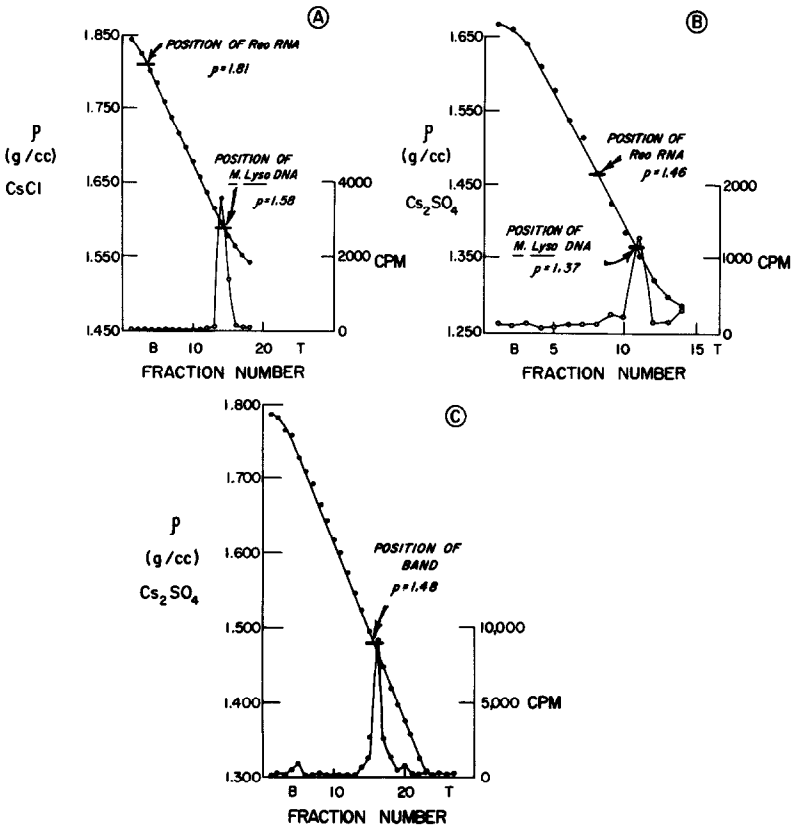

Figure 1 Banding characteristics of reovirus RNA in Cs_2SO_4-EB and CsCl-EB gradients.

The horizontal bar indicates the position of U.V. fluorescent bands of RNA or marker DNA relative to the density gradient profile.

A. Position of RNA extracted from reovirus in CsCl-EB (ρ=1.81 g/cc). DNA from M. lysodeikticus was used as marker.

B. Position of RNA extracted from reovirus in Cs_2SO_4-EB (ρ=1.46 g/cc).

C. Position of RNA extracted from reovirus infected L-929 cells 24 hrs. postinfection in Cs_2SO_4-EB. (ρ=1.48 g/cc).

able radioactivity was observed near the bottom of the gradient with a corresponding ρ of 1.81 g/cc (Figure 1A).

In each experiment U.V. fluorescent fractions from gradients were examined by electron microscopy. The mean values of contour length of

Table 1. Gradient fractions corresponding to the banded RNA from Figure 1C. Molecules of RNA were examined by electron microscopy, photographed and measured to determine mean values of contour lengths.

RNA Source	Mean Contour Lengths (μ)		
	Small	Medium	Large
Reovirus Fig. 1A CsCl gradient	0.290± .08	0.589± .039	1.010± .151
Reovirus Cs_2SO_4 gradient	0.274± .065	0.584± .070	1.020± .054
L cells infected with reovirus Cs_2SO_4 gradient	0.285± .064	0.566± .045	0.970± .123

molecules were determined from histograms with the number of molecules measured ranging from 100 to 200. A tabulation of the histogram data showing a trimodal size distribution of the molecules is presented (Table 1).

DISCUSSION

Cesium chloride equilibrium sedimentation has been effectively employed to isolate and characterize DNA obtained from highly diverse sources. However, since the ρ of RNA usually exceeds the solubility limits of CsCl, the use of CsCl equilibrium gradient centrifugation has not been feasible unless exceptionally high temperatures were employed (19,20). Previous studies have indicated EB interacts with DNA, presumably by intercalating between adjacent base pairs in a polynucleotide (4,5), resulting in a decrement of the ρ of the molecule. The present study demonstrates the effectiveness of EB in lowering the ρ of reovirus RNA sufficiently to permit banding in Cs_2SO_4 (Figure 1B) and also in CsCl (Figure 1A) gradients. The RNA extracted from reovirus infected L-cells also banded at a ρ in Cs_2SO_4 gradients (Figure 1C) equivalent to that of purified reovirus RNA thus sug-

gesting successful isolation of viral RNA from intracellular pools. Further evidence of the viral nature of this material was obtained through electron micrographs (Table 1) which verified the trimodal distribution of the molecules as the values for the size distribution of the segments of the reovirus genome previously published. (2)

It is interesting to note the wide separation between double stranded DNA and double stranded RNA which is achieved by using a CsCl-EB gradient as compared to a Cs_2SO_4-EB gradient. The degree of separation is striking considering the marker DNA has a G+C of 72 mole percent (22.) These data indicate that cesium salt-EB equilibrium centrifugation is a potentially effective means of isolating and characterizing double stranded RNA extracted from virus or infected cells.

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