PREPARATIVE CENTRIFUGATION OF *ARTEMIA SALINA* RIBOSOMES IN HIGH DENSITY SUCROSE GRADIENTS

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1. Introduction

Isopycnic centrifugation is often used as an analytical method for the identification and characterization of ribonucleoproteins [1,2] but it is neglected as a preparative purification method. This is due to the fact that the density media such as CsCl and metrizamide are inadequate for preparative purposes. Isopycnic centrifugation in CsCl necessitates prior fixation of particles with formaldehyde [1] or glutaraldehyde and renders the biological macromolecules useless for the majority of subsequent experiments. Metrizamide, a derivative of glucose, is often used for radioactive materials [2–6] but not for non-radioactive ribonucleoproteins because of its strong ultraviolet absorption [7]. Several other density gradient media have been described including chloral hydrate [8], Urographin [9], Ficoll [10], colloidal-silica [11] and sucrose in D_2O [12]. None of them has been used successfully for the large scale preparation of ribonucleoproteins. The use of sucrose is limited as a consequence of the high viscosities of dense sucrose solutions. However, a study of isopycnic centrifugation in sucrose revealed that this problem is only a minor disadvantage and that this technique is very useful for the separation and purification of cytoplasmic ribonucleoproteins [13].

Ribosomes prepared by differential centrifugation and subsequent zonal sucrose gradient centrifugation are still contaminated by other cytoplasmic structures as evidenced by hybridisation with poly([^{3}H]U) [13] and electron microscopy.

We have investigated the sedimentation of *A. salina* ribosomes in high density sucrose gradients and its application to the preparation of pure ribosome solutions.

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid; PMS, postmitochondrial supernatant

2. Materials and methods

Cryptobiotic embryos of the brine shrimp *A. salina* [14] from San Francisco Bay were treated with 5% NaClO solution at room temperature for 10 min, extensively washed with distilled water, dried by suction and homogenized in a precooled mortar in the presence of a minimal volume of 150 mM sucrose in Hepes buffer (pH 7.6), containing 20 mM Hepes, 70 mM KCl, 9 mM Mg acetate and 1 mM dithiothreitol. The homogenate was filtered through cheese cloth and centrifuged twice at 20 700 × *g* for 30 min in a Beckman JA20 rotor at 4°C to obtain the postmitochondrial supernantant (PMS).

Crude ribosomes were obtained by differential centrifugation of PMS in the Beckman R60 rotor at 60 000 rev./min and 4°C for 50 min; the pellets were redissolved in Hepes buffer.

The isopycnic sucrose density gradients were fractionated by pumping the solutions from the bottom of the tubes through a diam. 1.5 mm stainless steel needle. Appropriate tubings were used to overcome the difficulties of the high viscosity of dense sucrose solutions.

The $A_{260\ nm}$ of the gradient fractions was measured with a Zeiss PMQ III spectrophotometer. The

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densities of the fractions were determined by weighing in calibrated micropipettes.

Macromolecular structures, present in the fractions obtained after isopycnic or zonal centrifugation, were visualized with a JEOL-100B transmission electron microscope. Samples were negatively stained with a 2% solution of uranyl acetate in distilled water.

Ribosomes were examined in an MSE Analytical Ultracentrifuge, equipped with an automatic photoelectric scanning device, using double sector cells and absorption optics at 260 nm and 280 nm. Boundary sedimentation of unfixed and formaldehyde fixed ribosomes was carried out in Hepes buffer. Density gradient equilibrium centrifugation of fixed ribosomes in CsCl solutions was performed at 25°C and 45 000 rev./min for at least 16 h.

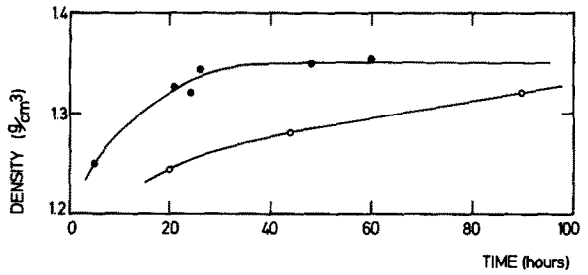

Fig.2. Density position of ribosomes as a function of centrifugation time. Crude ribosomes prepared by differential centrifugation were loaded on a 70%, 50% and 30% (w/w) sucrose layer system and centrifuged at 50 000 rev./min and at 4°C in the R60 (•) or at 30 000 rev./min in the R35 (○) rotor.

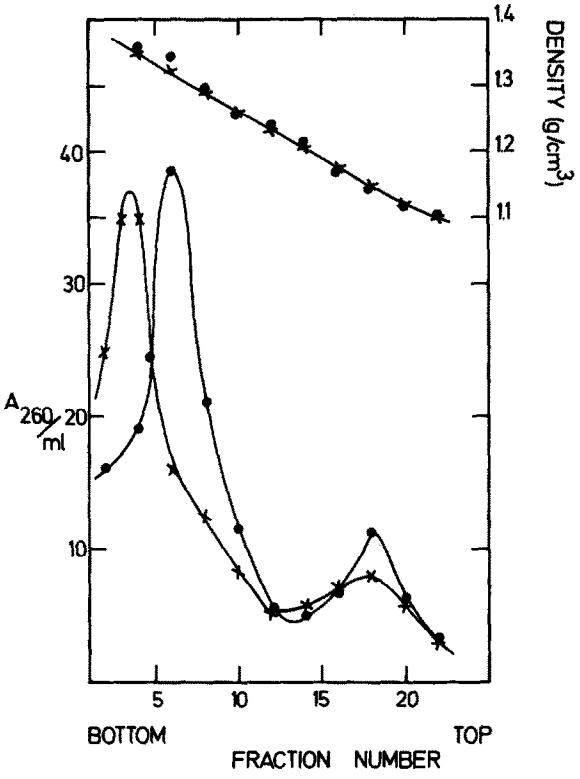

Fig.1. Density gradient and absorbancy profile as a function of centrifugation time. Crude ribosomes were loaded on a 70%, 50% and 30% (w/w) sucrose layer system in the R60 rotor and centrifuged at 50 000 rev./min and 4°C for 21 h (•) and 49 h (X), respectively.

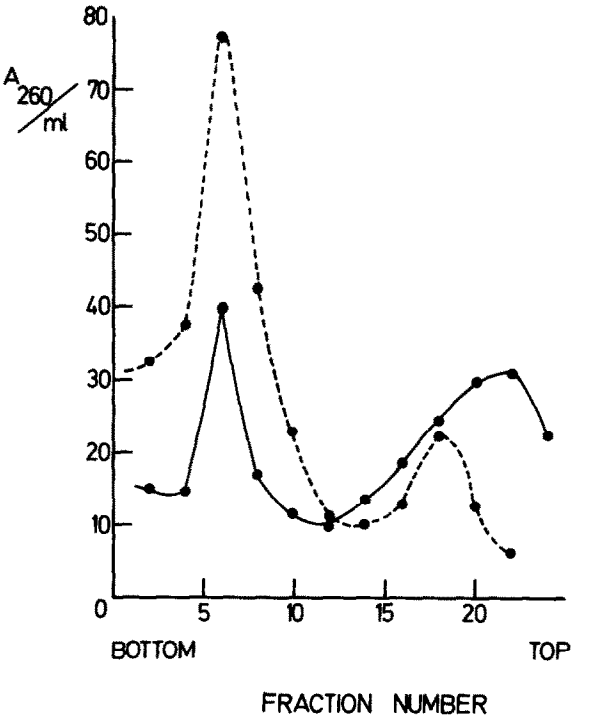

Fig.3. Absorbancy profiles after centrifugation of undiluted postmitochondrial supernatant (——) and of crude ribosomes (– – –) on a 70%, 50% and 30% (w/w) sucrose layer system in the R60 rotor for 20 h at 50 000 rev./min and at 4°C.

3. Results

Preparative sucrose density gradients were centrifuged in the Beckman R60 rotor (8 X 38 ml) or in the R35 rotor (6 X 94 ml) at 4°C at 50 000 and 30 000 rev./min, respectively. The centrifugation tubes were filled by layering on top of each other three 9 ml (R60) or 22 ml (R35) volumes of 70%, 50% and 30% (w/w) sucrose in Hepes buffer and 11 ml (R60) or 28 ml (R35) of crude ribosomes or postmitochondrial supernatant.

The density distribution of the gradients becomes independent of time after a centrifugation of 21 h in the R60 at 50 000 rev./min (fig.1); practically a linear density gradient is obtained, assuring the same resolution throughout the gradient.

The isopycnic centrifugation in sucrose clearly allows collection of the ribosomes in a sharp band (fig.1). After centrifugation for 21 h in the R60 rotor they have almost reached their equilibrium position and are already separated from other macromolecules with different buoyant densities. Measurement of the density position as a function of centrifugation time in the R60 and the R35 indicated that the equilibrium density is close to 1.36 g/cm^3 (fig.2). Routinely only the high density part of the R60 density gradients is fractionated after centrifugation for 21 h at 4°C to collect the banded ribosomes. As demonstrated in

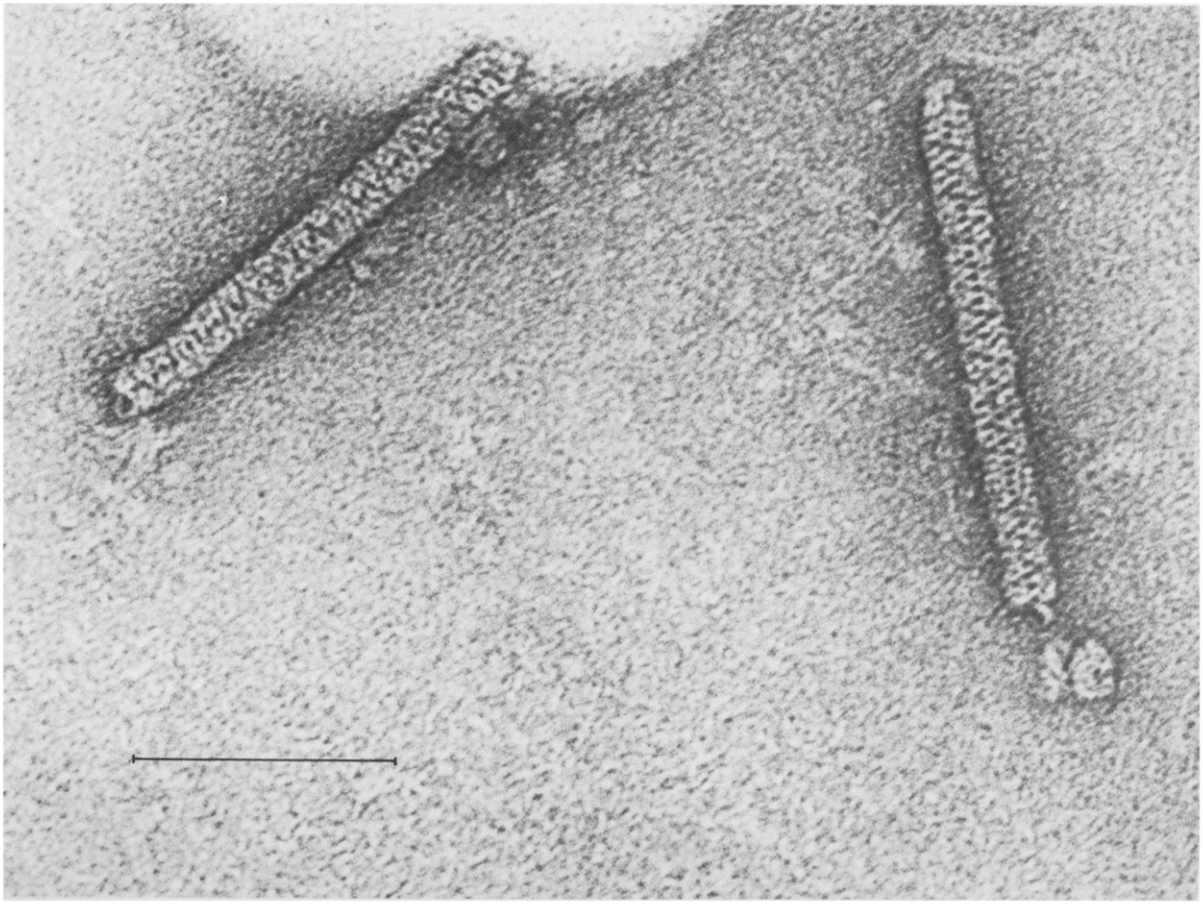

Fig.4. Electron micrograph of regular, rodlike structures present in *A. salina*. The particles were negatively stained with 2% uranyl acetate. The mark represents 1000 Å.

fig.3 concentrated ribosome solutions can be purified without loss of resolution. Indeed while isopycnic centrifugation of postmitochondrial supernatant already resulted in ribosome fractions of 40 A_{260} units/ml, concentrations of at least 80 A_{260} units/ml can be obtained if crude ribosome solutions are used without significant peak broadening (12 A_{260} units = 1 mg [15]) (fig.3).

Electron microscopy of particles present in the ribosome-band showed not only ribosomes but also low concentrations of regular, rodlike structures [16] (fig.4); the fact that they were present in fractions of densities around 1.3 g/cm^3 suggests that they are nucleoprotein particles.

Ribosome fractions obtained by isopycnic centrifugation in sucrose were further purified by zonal centrifugation. They were mixed with equal vol. 20% polyethylene glycol 6000 in Hepes buffer [17]. The precipitate was collected by low speed centrifugation, redissolved in Hepes buffer and layered on a 15–50% (w/w) sucrose density gradient in a Beckman Ti14 zonal rotor for a separation based on differences in sedimentation velocity. Electron microscopy of the resulting ribosome fractions showed the absence of rodlike structures; the latter were located in the gradient fractions corresponding with faster sedimenting material [15]. The purity of the ribosomes finally obtained was also confirmed by other techniques. Analytical boundary sedimentation and density gradient centrifugation in CsCl showed one sharp boundary and one symmetrical peak, respectively; the A_{260}/A_{280} was >1.9. No polydispersity in diffusion coefficient was revealed by photon correlation spectroscopy.

4. Discussion

Isopycnic centrifugation in sucrose can be used for the isolation of ribosomes from the postmitochondrial supernatant. The method avoids the artificial adsorption of proteins to ribosomes which may occur when they are pelleted from the PMS by high centrifugal forces [18]. Because of the difference in buoyant density of ribosomes and other ribonucleoprotein complexes, these particles will be separated even though they may have the same sedimentation coefficient [13], which makes their separation impossible by procedures which are only based on sedimentation velocity. The R60 rotor is preferred because the volume of PMS that can be processed is large enough (8 X 11 ml); owing to the lower rotor speed, longer centrifugation times are required with the R35 rotor.

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