ISOLATION OF CYTOPLASMIC NON-RIBOSOMAL RIBONUCLEOPROTEIN PARTICLES (INFORMOSOMES)

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1. Introduction

There is substantial evidence that in eukaryotic cells, messenger-like RNA (mRNA)** is always found in association with specific proteins as nucleoprotein particles called "informosomes" by Spirin et al. [1, 2]. Informosomes could be characterized in cell nuclei [3–6] and in the cytoplasm [7–11]. Due to the scarcity of ribosomal-like particles in nuclei, nuclear informosomes could be isolated in a rather pure form [3–6]. Cytoplasmic informosomes, however, are mixed with a dense population of ribosomal particles. Thus, only cytoplasmic informosomes with sedimentation coefficients well below 40 S can be separated accurately from ribosomes by sedimentation velocity gradient centrifugation. These informosomes are only a small fraction of the total informosomes. Due to their lower density, informosomes, either free or detached from polysomes, can also be separated by isopycnic sedimentation in CsCl density gradients [7]. But in this case, previous "fixation" of the particles with formaldehyde is necessary in order to prevent dissociation of proteins from the nucleic acids [12]. Informosomes isolated in this way are difficult to analyse further, nor can they be tested for their activity in protein synthesis. Thus new methods are needed for an efficient separation of native informosomes from other cytoplasmic particles such as ribosomes or membrane fragments.

We found that in the case of the mouse plasmocytoma, informosomes, centrifuged to equilibrium in a sucrose density gradient, have much lower buoyant densities than in CsCl. But concentrated sucrose solutions are highly viscous and require very long centrifugation times to approach sedimentation equilibrium. We have replaced the solvent water by heavy water (D_2O) and have thus obtained sucrose solutions of densities above 1.30 with sucrose concentrations not higher than 2 M. This density range was sufficient to isolate an informosome fraction almost free of ribosomes and membranes.

2. Methods

2.1. *Labelling of cytoplasmic ribonucleoprotein particle constituents*

2.1.1. Labelling of rRNA

Plasma cell tumors RPC 5 grown on Balb/c mice were used. Tumor-bearing mice were injected intraperitoneally with 0.2 mCi/mouse of [5-^{3}H]uridine and killed 24 hr later. The homogeneous labelling of rRNA thus obtained served as a tracer for this RNA and for ribosomes.

2.1.2. Labelling of dRNA, phospholipids and phosphoprotein

The same mice received, 3.5 hr before sacrifice, 100 μg of actinomycin D, and 3 hr before sacrifice, 2 mCi/mouse of [^{32}P]orthophosphate. As we have shown [13], under these conditions actinomycin completely inhibits rRNA synthesis, but there is a residual synthesis of dRNA and tRNA. Thus ^{32}P radioactivity is found in these RNA's, and also in phospholipids

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** *Abbreviations:*
rRNA: ribosomal RNA; dRNA: DNA-like RNA; mlRNA: messenger-like RNA; tRNA: transfer RNA; DOC: sodium deoxycholate; TCA: trichloroacetic acid.

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and phosphoproteins. The respective parts accounted for by each of these compounds can be easily determined.

Occasionally, dRNA was also selectively labelled for a short time (10 min) following intratumoral injection of tritiated uridine (1 mCi/tumor).

2.2. *Determination of radioactivity*

As a rule, radioactivity was measured by the method of Mans and Novelli [14], using a scintillation spectrometer Intertechnique ABAC Sl 40, programmed for $^{3}H/^{32}P$ double label counting. The ^{32}P labelled compounds were successively determined by a subtraction method. The radioactive material on filter papers (2 X 4 cm) were immersed in cold 10% TCA and washed 3 times in cold 5% TCA. After the first count of the dried papers, they were extracted 2 times with a chloroform–methanol mixture (2:1, v/v) at 54°. After a second count, the rehydrated filter papers were treated with 5% TCA heated at 95° during 15 min. The last count corresponds to [^{32}P]phosphoprotein [15]; subtraction of the third from the second count corresponds to [^{32}P]actinomycin D resistant RNA; subtraction of the second from the first count corresponds to [^{32}P]phospholipid.

2.3. *Preparation of cytoplasmic fractions*

Tumors were homogenized in 4 volumes of ice-cold standard buffer: 0.05 M triethanolamine-HCl buffer (pH 7.6), 0.15 M KCl, 0.004 M Mg acetate, 0.006 M β-mercaptoethanol, 1.1 M sucrose, in a Potter-Elvehjem homogenizer and centrifuged at 20,000 *g* for 15 min in a Spinco R 65 rotor. The resulting supernatant (S_{20}) was then layered on the discontinuous D_2O sucrose gradient as described in the legend to fig. 1. Other fractionation schemes, such as sucrose gradients, D_2O-sucrose gradients and CsCl gradients are also described in the legends to figures.

3. Results

Fig. 1a shows that the ^{32}P extractible with chloroform–methanol (phospholipids) was found mainly in fractions A, B and C, reflecting the high amount of membranous material in these fractions. The ribosomal particles, identified by the long labelled tritiated rRNA they contained, mostly sedimented to the bottom (fraction E). It is interesting that only a minor part of the ribosomes were bound to the membranes (fractions B and C).

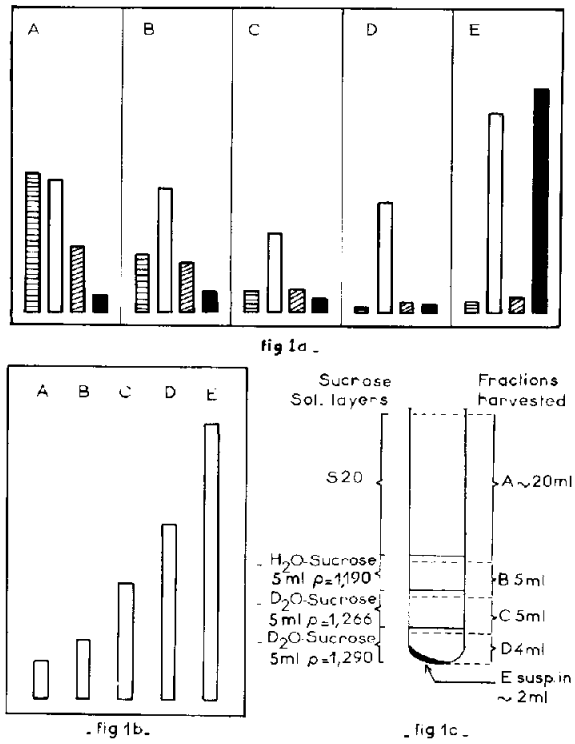

Fig. 1. Fractionation of cytoplasmic extract (S_{20}) on a discontinuous D_2O-sucrose gradient. D_2O-sucrose solutions contain the concentrations of salts indicated for the standard buffer solution in Methods. Layers of D_2O-sucrose and of H_2O-sucrose are superposed as described on the schematic view (1c). 15–20 ml of S_{20} are layered on top of the gradient and centrifuged during 20 hr, at 4° and at 50,000 rpm (180,000 *g* av) in the Spinco rotor 60 Ti. After centrifugation, indicated volumes are carefully removed with a syringe, and aliquots of each fraction were processed for radioactivity measurement. a) Tumor-bearing mice were injected with 0.1 mCi tritiated uridine. After 20 hr-labelling, they received 100 μg actinomycin D and 0.5 hr later 2 mCi [^{32}P]orthophosphate. Mice were sacrificed after 3 hr-labelling by ^{32}P (24 hr by tritiated uridine). ▤: ^{32}P radioactivity from phospholipids; □: ^{32}P radioactivity from actinomycin D-resistant RNA (1a), or ^{3}H-radioactivity from fast-labelled RNA (1b); ▨: ^{32}P radioactivity from phosphoproteins. ■: ^{3}H radioactivity from long-term labelled rRNA. b) Tumor-RNA is labelled by intratumoral injection of 2 mCi of tritiated uridine. c) Schematic representation of the subfractionation procedure of S_{20}.

We have shown that in mouse plasmocytoma cells, actinomycin D blocks the synthesis of rRNA, but

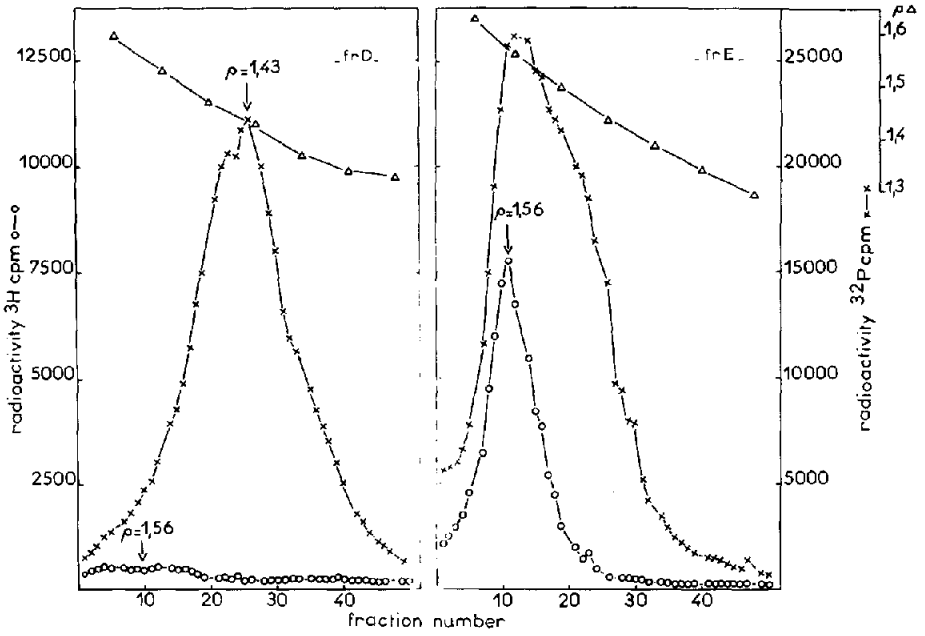

Fig. 2. Isopycnic sedimentation in CsCl. Linear CsCl gradients were preformed with densities between 1.25 and 1.65 g/ml. Cytoplasmic fractions are fixed with formaldehyde at 1% final concentration, layered on top of the gradient in a volume of 0.2 to 0.5 ml, and centrifuged during 14 hr at 10° and at 45,000 rpm in the Spinco rotor SW 65. Two drop fractions are recovered for radioactivity measurement. Fr. D and fr. E are, respectively, fraction D and fraction E obtained as indicated in fig. 1. (o—o—o) ^{3}H-radioactivity from long-term labelled rRNA. (x—x—x) Acid insoluble and chloroform-methanol extracted ^{32}P radioactivity corresponding to dRNA. (Δ—Δ—Δ) Density of CsCl solution.

allows a consistent level of dRNA and tRNA synthesis to take place [13]. The ^{32}P label, which can be attributed to the actinomycin D resistant RNA, was found in all fractions. The upper regions of the gradient (A, B and part of C) contain the labelled tRNA. The distribution of true dRNA can be seen after a very short label of 10 min by tritiated uridine (fig. 1b).

Our attention was focused on fraction D, which seems to contain very little phospholipid (membranous) or ribosomal material, but a large amount of dRNA. The ribosomal pellet, called fraction E, will serve as a reference fraction during the study of fraction D. Fraction E contained the highest amount of the dRNA, but this dRNA could not be separated from the ribosomes.

It must be emphasized that the densities of the D_2O-sucrose layers are critical for optimal separation of free informosomes. Slight deviations result in higher contamination by ribosomal or membranous material.

Equilibrium sedimentation of fraction D on a CsCl density gradient showed that the ^{32}P corresponding to the dRNA label had a buoyant density of a mean value of 1.4 with a relatively broad distribution of densities, whereas ribosomal particles were virtually absent (fig. 2a). The bulk of the ribosomes appeared in fraction E in a density zone at $\rho = 1.56$ g/ml. This fraction also contained dRNA particles with densities between 1.45 g/ml and 1.60 g/ml (fig. 2b).

Samples of the fractions D and E were sedimented in preformed continuous D_2O-sucrose gradients of densities between 1.15 g/ml and 1.34 g/ml as described in the legend to fig. 3. The highest sucrose concentration was 2 M. After centrifugation at 4° and 200,000 *g* for 48 hr, particles with sedimentation constants above 30 S had reached their density equilibrium. A very low amount of long-labelled RNA was found in the gradient of fraction D. The dRNA labelled with ^{32}P after injection of actinomycin D had a rather

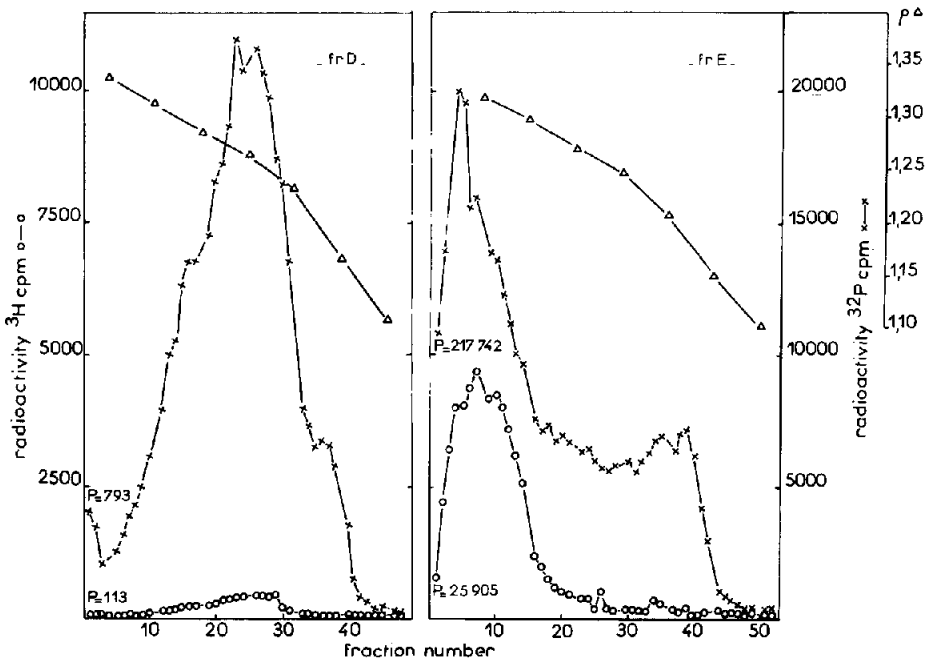

Fig. 3. Isopycnic sedimentation in continuous D_2O–H_2O sucrose gradient. Linear D_2O–H_2O sucrose gradients are preformed using two solutions: for the denser, the sucrose is dissolved in D_2O at 2 M; for the lighter, the sucrose is dissolved in H_2O at 1.5 M. Salt concentrations are the same as in the buffer solution described in Methods. Densities are between 1.15 and 1.34 g/ml. 0.2–0.5 ml fractions from discontinuous D_2O sucrose gradient (fig. 1) are centrifuged during 40 hr at 4° and at 50,000 rpm in the Spinco rotor SW 65, in order to approach density equilibrium. Two drop fractions are recovered for radioactivity measurement. (o–o–o) ^{3}H-radioactivity from long-term labelled rRNA. (x–x–x) Acid insoluble and chloroform–methanol extracted ^{32}P radioactivity corresponding to dRNA. (△–△–△) Density of gradient solution.

broad distribution in a density range between 1.24 and 1.29 g/ml (fig. 3a). Fraction E, which is the ribosomal-polysomal pellet, taken off from the discontinuous D_2O-sucrose gradient, had not completely sedimented to the bottom. A moderate amount of long-labelled ribosomes, and also of ^{32}P dRNA was found in the density zone above 1.29 g/ml; a low amount of dRNA with a lower density (fig. 3b), was detached from heavier particles during the manipulation and sedimented in the lighter density zone.

Velocity-gradient sedimentation (fig. 4) showed that the informosomal-type particles of fraction D had sedimentation constants extending from 40 S, where they predominate, up to more then 100 S in the polysomal zone (fig. 4a). In fraction E, the dRNA was particularly abundant in the zone between 200 S and 300 S and seems to be linked to polysomes (fig. 4b).

4. Discussion

In this paper we have described a method for the isolation of informosomes based on D_2O sucrose gradient sedimentation to the density equilibrium. Relatively high KCl concentrations were added to the buffer solution, in order to minimize non-specific protein adsorption to particles [16].

Using this method, it has been possible to separate native cytoplasmic informosomes for physiological and biochemical investigations without using detergents.

The informosomal-type fraction is found in a layer of density 1.29 g/ml in the D_2O-sucrose gradient and has a density in CsCl of 1.4. Free ribosomal particles are recovered in the pellet of the D_2O-sucrose gradient, whereas membranes and membrane-bound ribosomes

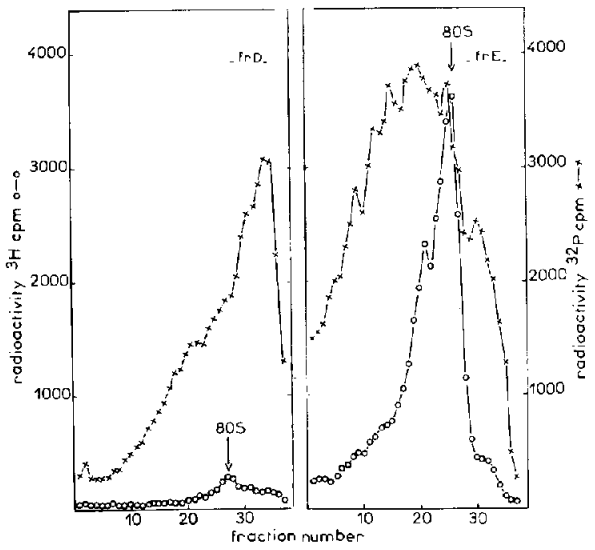

Fig. 4. Sucrose gradient centrifugation of cytoplasmic particles. Linear density sucrose gradients with concentrations between 10 to 30% are established and 0.1 to 0.2 ml of cytoplasmic fractions are centrifuged at 4° during 30 min and at 60,000 rpm in the Spinco rotor SW 65. Two drop fractions are recovered for radioactivity measurement. (o—o—o) ^{3}H-radioactivity from long-term labelled rRNA. (x—x—x) Acid insoluble and chloroform—methanol extracted ^{32}P radioactivity corresponding to dRNA.

do not enter into the layer of 1.29 g/ml density.

It is obvious that the buoyant density of the informosomes is significantly higher in concentrated CsCl than in sucrose solutions. This fact can clearly be attributed to the state of solvation of the particles, and may be related to water activity [17]. Moreover, one must take in account that D_2O–H_2O exchanges are occurring in the solvation layer of the particles, so that their densities would be slightly higher than in water. This effect has been shown by Beaufay et al. [18] for mitochondria and lysosomes centrifuged through D_2O-sucrose gradients.

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