# THE USE OF METRIZAMIDE TO SEPARATE CYTOPLASMIC RIBONUCLEOPROTEIN PARTICLES IN MUSCLE CELL CULTURES: A METHOD FOR THE ISOLATION OF MESSENGER RNA, INDEPENDENT OF ITS POLY A CONTENT

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## 1. Introduction

The separation of cytoplasmic particles on buoyant density gradients of CsCl provides an important tool in their characterisation [1]. The necessity for prior fixation of protein–nucleic acid complexes (to avoid dissociation of protein at the high ionic strength), however, rules it out as a preparative method for most subsequent analyses of the particles. Metrizamide has been described as an alternative material for use in isopycnic centrifugation [2].

This compound has the advantage that since it is not ionic, fixation is avoided and recuperation of the experimental material for further analysis is possible. Metrizamide has been used principally in biochemical studies on chromatin [3]. Its application to studies on liver cell supernatant fractions has been mentioned [4]. We have decided to investigate further the separation of cytoplasmic particles.

Our particular interest is in the separation of cytoplasmic ribonucleoproteins (RNPs) from muscle cell cultures before and after cell differentiation. In particular the RNP containing the 26 S RNA, putative messenger for the large subunit of myosin [5], appears to be an important component in the post-transcriptional control of myosin gene expression [6]. This species, identified in the dividing myoblast and the fused myotube as a peak sedimenting at 26 S after pulse labelling of the cultures, undergoes an increase in stability just prior to fusion. Before its eventual entry into heavy polysomes, this RNA is present as a free cytoplasmic ribonucleoprotein. The present paper describes the use of metrizamide density centrifugation to separate total cytoplasmic RNPs and the 26 S RNP particle from muscle cells.

## 2. Materials and methods

Primary myoblast cultures were prepared from the skeletal muscle of foetal calf, as described previously [6]. All results shown were corrected to represent the optical density or cpm from the equivalent of $10^6$ cells. Cultures were labelled with 100 $\mu$Ci [$^3$H] uridine (20 Ci/mmol; supplied by C.E.A., Saclay, France) for 2 hr in 2.5 ml of medium. Three min before the end of the labelling period 100 $\mu$g/ml of cycloheximide (Sigma Chem. Co.) was added to the medium. The drug was retained in all subsequent buffers and gradients used in the preparation of polysomes. The cells were washed with phosphate-buffered saline (132 mM NaCl, 2.6 mM KCl, 6.5 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$, pH 7.5) and scraped off the surface of the Petri dish in 0.1 ml of polysome buffer (140 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris–HCl, pH 7.5 or 10 mM triethanolamine, pH 7.8). The cells were lysed either by homogenisation in a tight fitting Potter homogeniser or by the addition of 0.5% $NP_{40}$. Nuclei were pelleted by centrifugation at 42 000 rev/min in the S.W. 56 Spinco rotor Maximum resolution in the monosome–trisome region of the gradient was obtained by centrifugation for 1 hr, 10 min. Fractions were collected and absorption at 254 nm measured, using an Isco gradient collector.

Buoyant density centrifugation was carried out in the presence of formaldehyde according to Spirin et al. [1].

Cytoplasmic samples (buffered with triethanolamine) were prepared for analysis on CsCl density gradients by fixation in 6% formaldehyde at 4°C for 2 days. The fixed extracts were analysed on two step gradients containing heavy and light solutions (1.8 ml of each) of density 1.75 and 1.25 g/cm$^3$ respectively, in buffer containing 3 mM $MgCl_2$, 140 mM NaCl, 3 mM triethanolamine pH 7.8, 6% (v/v) formaldehyde. The sample to be analysed, previously dialysed against this buffer, was included in the light solution of CsCl. Centrifugation was for 36 hr at 40 000 rev/min in the S.W. 56 Spinco rotor. Fractions were collected on an Isco gradient collector and analysed for their refractive index and for radioactivity after precipitation with trichloroacetic acid.

Metrizamide (2-(3-acetamido-5-*N*-methylacetamido-2,4,6-triodobenzamido)-2-deoxy-D-glucose) (supplied by Nyegaard and Co., Norway) gradients were prepared as 40% (w/v) solutions in polysome buffer containing 10 mM β-mercaptoethanol. Samples to be analysed were included in the solution. Centrifugation was at 8°C for 68 hr at 40 000 rev/min in the S.W. 56 Spinco rotor. Fractions were collected at constant time intervals, using a peristaltic pump. They were analysed for their refractive index and for radioactivity after precipitation with trichloroacetic acid.

In experiments where RNA was extracted and analysed for its size on sucrose gradients and for its content in poly A containing RNA, the procedures were as described previously [6].

## 3. Results

Preparations of subunits, free ribosomes and polysomes from myoblast cultures were centrifuged on metrizamide density gradients as described in the Methods section. In initial experiments these gradients were prepared in buffer containing zero magnesium [2,3]. The banding positions of the different particles under these conditions were as indicated in the first column of table 1. The difference in density was not sufficient, particularly in the case of the subunits and RNPs, to give a separation of the particles. It was only when the concentration of magnesium in the gradient was increased that a better separation could be achieved. As indicated in the second column of table 1, the principal effect of increasing the $Mg^{2+}$ concentration was to increase the apparent density of all the particles examined except the RNPs. In particular the 60 S subunit now no longer bands with the RNPs, but at a higher density. The result, therefore, of including $Mg^{2+}$ in the metrizamide gradient is to implement the separation of cytoplasmic particles and especially to facilitate the isolation of uncontaminated RNPs. The type of separation obtained for a total cytoplasmic extract of myoblasts, labelled with [$^3$H] uridine and centrifuged on metrizamide under the conditions described in the Methods section, is shown in fig.1. In order to obtain a separation between RNPs and the 40 S subunits, it is important that the gradient be as shallow as possible in this region. This form of gradient was obtained most reproducibly under the conditions described, rather than with preformed gradients. The form of gradient assumed varies with the conditions of centrifugation, probably because the high viscosity of metrizamide results in an unstable equilibrium. Identification of the particles was verified by fixation of the labelled peaks obtained on metrizamide, and recentrifugation on caesium chloride density gradients (table 1, right hand column).

Table 1

| Particle | $p$ (g/cm$^3$) Metrizamide | | $p$ (g/cm$^3$) CsCl |
|---|---|---|---|
| | 0 $Mg^{2+}$ | 3 mM $Mg^{2+}$ | |
| 40 S | 1.220 | 1.235 | 1.49 |
| (40 S newly synthesized) | (1.230) | (1.260) | (1.46) |
| 60 S | 1.215 | 1.320 | 1.57 |
| 80 S | 1.255 | 1.305 | 1.56 |
| Polysomes | 1.240 | 1.350 | 1.53 |
| RNP | 1.210 | 1.205 | 1.39 |

Table 1 shows the buoyant density of different cytoplasmic particles when centrifuged to equilibrium after fixation on caesium chloride (right hand column) and on metrizamide made up in a buffer containing 0 mM $Mg^{2+}$ and 3 mM $Mg^{2+}$ (left hand columns). The experimental procedure was as described in the Methods section.

The isopycnic density on caesium chloride, which corresponded exactly to that obtained without prior passage on metrizamide, indicates that the latter compound does not damage the particles. Furthermore, subsequent extraction and analysis of the RNA indicated that it was still intact. As Birnie et al. [2] have

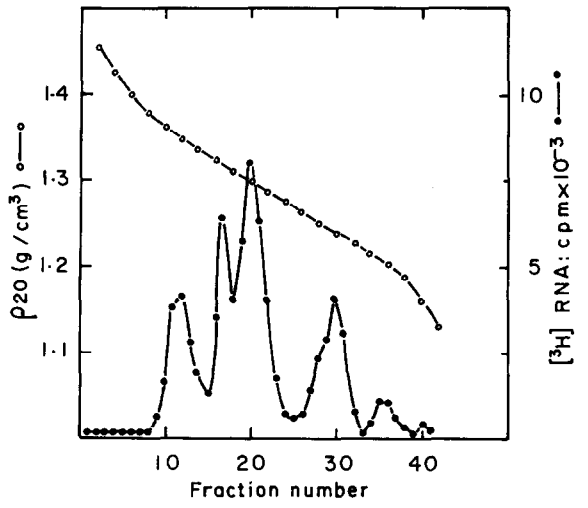


Fig.1. Total cytoplasmic extracts, pulse-labelled with [$^3$H]-uridine, were prepared and centrifuged on metrizamide as described in the Methods section. Figure 1 shows the distribution of TCA precipitable radioactivity in such a density gradient. The material was obtained from myoblast cultures just prior to cell fusion.

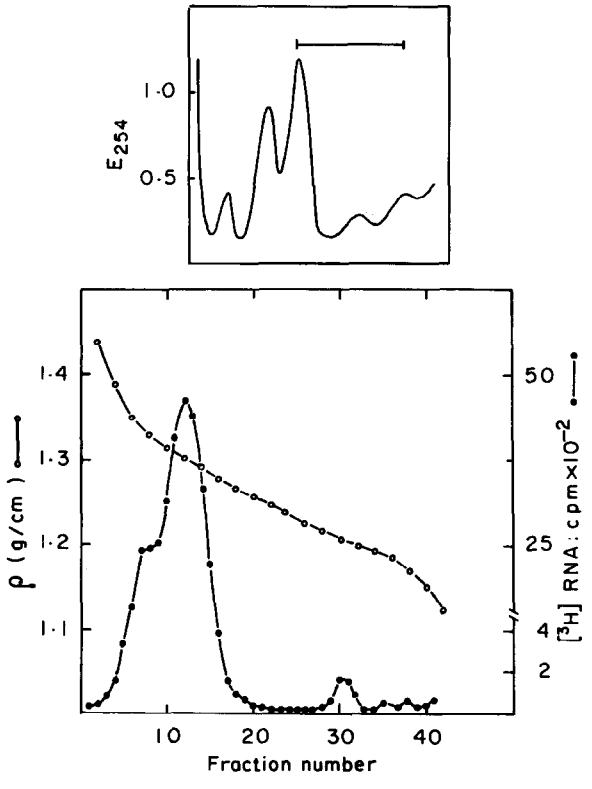


Fig.2. Cytoplasmic extracts of cells pulse-labelled with [$^3$H]-uridine were centrifuged on a polysome gradient, the absorption at 254 nm recorded, and the monosome–trisome region of the gradient selected as indicated in the upper diagram. This material was then centrifuged on metrizamide and the resultant distribution of TCA precipitable material is shown in the lower diagram in fig.2. The experimental details were as described in the Methods section. The cells were taken at a stage just prior to fusion.

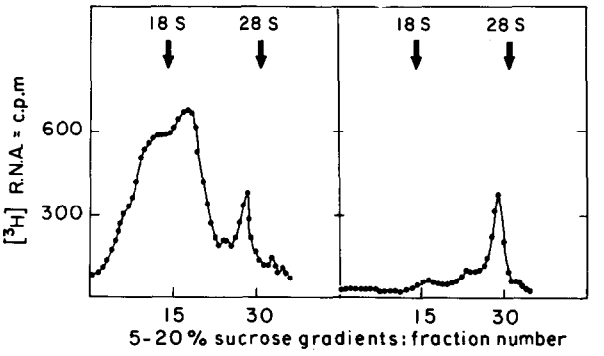


Fig.3. Total RNP was prepared as in fig.1 and the preselected heavy RNP region as in fig.2. In both cases cells just prior to fusion were used. RNA was extracted from these two fractions and analysed on sucrose gradients as described in the Methods section. The distribution of RNA from the total RNP fraction is shown in the left of fig.3, that from the preselected RNP on the right. The arrows indicate the position of 18 S and 28 S ribosomal RNA in the gradients.

already shown, metrizamide does not have a deleterious effect on proteins. Some batches of metrizamide liberated iodine in solution and to avoid oxidation of proteins a reducing agent was added routinely to all metrizamide solutions.

Centrifugation on metrizamide gradients was used to isolate total cytoplasmic ribonucleoprotein particles (fig.1) and also to separate those particles present in the monosome–trisome region of a polysome gradient (fig.2), since it is in this region that the RNP containing the 26 S RNA is present [6]. Cells in logarithmic growth and stationary phase cells just beginning to differentiate were labelled with [$^3$H]-uridine and either the total cytoplasm, or the monosome–trisome region selected from a polysome gradient, was centrifuged on metrizamide. The RNP material from these gradients was recuperated, the

RNA extracted, and the counts in total and poly A containing RNA measured. As shown in fig.3, analysis of the RNA after separation on sucrose gradients [6] confirmed that the labelled RNA from total cell RNPs was heterodisperse while that isolated as in fig.2 sedimented mainly as a peak at 26 S.

Five experiments, of the type shown in table 2, indicated that after a 2-hr label 80% ± 6% of the messenger RNA present in free ribonucleoprotein particles contains poly A. This figure is rather higher in stationary cultures, at the beginning of differentiation, than in logarithmically growing myoblasts. When ribonucleoproteins containing predominantly 26 S RNA are analysed it is found (table 2) that at least 95% of this pulselabelled RNA contains poly A.

## 4. Discussion

Metrizamide gradients used under the conditions described provide a means of separating cytoplasmic ribonucleoprotein particles. The important technical points are the form of the gradient and the concentration of divalent cations. The effects of $Mg^{2+}$ ions in the gradient are not due to dissociation or reassociation of the particles under the salt conditions described, as shown by their subsequent density on caesium chloride. It is probable that since metrizamide is non-ionic the degree of hydration of the particle plays a role in determining its apparent density. The effect of different degrees of hydration on the behaviour of DNA molecules in metrizamide has already been discussed [2]. Under conditions of zero $Mg^{2+}$ in the gradient, the 60 S particle (protein:RNA, 1.00:1.24) appears less dense than the 40 S subunit (protein:RNA, 1.0:1.02). This correlates with the higher density of protein (main band 1.45) on metrizamide compared with RNA (1.17). In the presence of $Mg^{2+}$ the apparent density of the 60 S subunit becomes much higher, while that of the 40 S increases slightly. Similarly the apparent density of ribosomes and polyribosomes also increases. One explanation for this is the greater binding of magnesium to these particles with concomitant increase in their water of hydration.

Table 2

| Source of RNA | [$^3$H]RNA: cpm. | |
|---|---|---|
| | log growth | allignment/ early fusion |
| RNP from total cytoplasm: | | |
| Total RNA | 3500 | 2900 |
| Poly A containing RNA | 2600 | 2300 |
| RNP containing 26 S: | | |
| Total RNA | 325 | 280 |
| Poly A containing RNA | 310 | 260 |

Table 2 shows the results of analysis of the RNP fraction isolated by centrifugation on metrizamide, either prepared from total cytoplasm as in fig.1 or from a preselected region of a polysome gradient (26 S region) as in fig.2. Total RNA was estimated as TCA.precipitable material on glass fibre filters; poly A containing RNA, as material hybridising to poly U glass fibre filters, as described in the Methods section. The error in this latter estimation is of the order of 6%. The results obtained were for RNPs from cultures in logarithmic growth (right hand column) and in early fusion (extreme right hand column).

Since prior fixation is not necessary, particles recuperated from metrizamide gradients are susceptible to biochemical analysis. We have made use of this to analyse RNA from free ribonucleoprotein particles in order to estimate the proportion of messenger RNA present in this form which contains poly A. In dividing myoblasts this figure is of the order of 80% of pulse-labelled RNA. Part of the remaining 20% must be due to histone messenger RNA, which lacks poly A [7,8]. In stationary cells, where histone synthesis is virtually zero, this figure is of the order of 15%. From these results we can conclude that most pulse-labelled RNA present in free RNP particles contains poly A. The fraction of such RNA without poly A is rather lower than 30% figure reported most recently by Penman et al. [9] in HeLa cells. However this RNA was polyribosome associated whereas our results concern free RNP RNA.

The equivalent figures for pulse-labelled 26 S RNA indicate that this contains 90–100% poly A when in the form of free ribonucleoprotein in both exponentially growing cells and in those undergoing differentiation. This is important since our conclusions on the synthesis and stability of 26 S RNA during muscle cell differentiation apply only to poly A containing RNA [6].

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