THE USE OF METRIZAMIDE AS A GRADIENT MEDIUM FOR ISOPYCNIC SEPARATION OF RAT LIVER CELLS

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1. Introduction

Separation of intact cells by means of isopycnic gradient centrifugation is a potentially powerful technique, the application of which has been somewhat limited by the lack of suitable gradient materials. At the densities required for cell separation (1.05–1.16 g/cm^3) most available substances (Ficoll, sucrose, albumin etc.) form solutions of undesirably high osmolality or viscosity.

A new gradient material, Metrizamide (2-(3-acetamido-5-*N*-methylacetamido-2,4,6-triiodobenzamido)-2-deoxy-D-glucose), has been successfully utilized in the separation of nuclei [1], mitochondria and lysosomes [2] and various subcellular particles and macromolecules [3–5]. Metrizamide gives dense solutions of low viscosity and osmolality, and would appear particularly well suited for the separation of intact cells. The present communication documents that the heterogeneous cell population from rat liver can be partially resolved by isopycnic banding in Metrizamide gradients.

2. Materials and methods

Suspensions of intact cells were prepared from the livers of lightly fasted, male Wistar rats (270–300 g) as previously described [6–8]. The initial cell suspension, a mixture of parenchymal and non-parenchymal cells, was prepared in a slightly hypotonic (260 mosM) buffer, pH 7.6 at 37°C (7.14 g NaCl, 0.5 g KCl, 0.18 g $CaCl_2 \cdot 2\,H_2O$, 2.4 g HEPES, 5.5 ml 1 M NaOH and H_2O ad 1000 ml) and used without further purification.

Metrizamide gradients containing liver cells were prepared by the gradient formation method of Stone [9]. Four solutions containing 30% (w/v), 20%, 15% and 5% Metrizamide were made by mixing the cell suspension with varying amounts of slightly hypotonic (260 mosM), buffered 30% (w/v) Metrizamide, pH 7.6 at 37°C (30 g Metrizamide, 0.24 g HEPES, 0.05 g KCl, 0.018 g $CaCl_2 \cdot 2\,H_2O$. 5.5 ml 0.1 M NaOH and H_2O ad 100 ml). 2 ml portions of these conditions were layered on top of each other (4 × 2 ml = 8 ml) in centrifuge tubes of 13 ml capacity; the tubes were then carefully turned to a horizontal position and left for 90 min at 4°C. The tubes were subsequently turned upright, and the diffusion-generated gradients were centrifuged at 4°C for 20–60 min in a Beckman SW 40 rotor at 3000–10 000 rpm; within these limits there were no detectable differences in the cellular distribution pattern. The gradient was fractionated manually (drop counting) by siphoning from the bottom of the tube, and a differential cell count of each fraction was done with a microscope and a Bürker chamber. The density of the fractions was determined with a Hilger refractometer. The linear relationship between density and refractive index was not disturbed by the presence of cells.

The densities of aqueous solutions of Metrizamide and Ficoll were determined with the refractometer, with a density hydrometer, or by weighing in constriction pipettes, all methods giving the same result. Viscosities were determined with Ostwald viscosimeters (Jena Glaswerkschott & Gen., Mainz), and osmolalities with a Knauer type M osmometer.

Incubation of parenchymal cells and metabolic analyses were performed as previously described [10].

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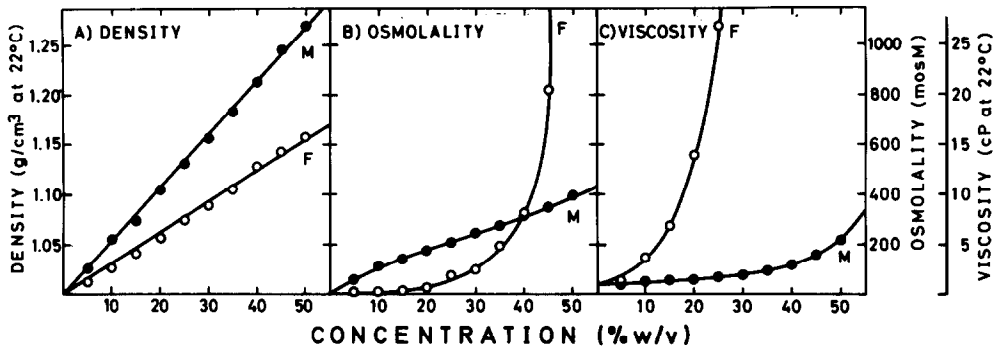

Fig. 1. Physico-chemical properties of Metrizamide and Ficoll. The substances were dissolved in H_2O at concentrations ranging from 5 to 50% (w/v), and density (A), osmolality (B) and viscosity (C) of the solutions were measured at room temperature (approximately 22°C) as described in the text. •—•, Metrizamide (M); o—o, Ficoll (F).

Metrizamide was a generous gift from Nyegaard & Co. A/S, Oslo, Norway. Ficoll was purchased from Pharmacia, Uppsala, Sweden; other chemicals were of reagent grade, obtained from various sources.

3. Results

Ficoll (polysucrose) has been used for separation of many different cell types [11–15]. Fig. 1 compares the physicochemical properties of Ficoll and Metrizamide. The relationship between concentration and density is essentially linear for both substances (fig. 1 A), and the densities required for cell separation (up to 1.16) can be obtained with both. However, the cellular demand for an isotonic medium (270–300 mosM) sets a practical upper limit to the concentrations which can be used. It can be seen that both Metrizamide and Ficoll reach this limit approximately at 40% (w/v) concentration (fig. 1 B), where the density of Metrizamide is 1.21 and that of Ficoll only 1.13. In buffered solution the osmotic pressure of the buffering agent (and other salts, nutrients etc.) will necessitate the use of even lower concentrations.

The shapes of the osmolality/concentration curves reveal an important difference between Metrizamide and Ficoll. None of the curves are linear, but Metrizamide deviates much less from linearity than Ficoll (fig. 1 B). This means that if a linear density gradient is made by mixing an isotonic Ficoll solution with an isotonic buffer (or medium), the gradient will be isotonic only at the top and in the bottom, and hypotonic in the middle region. Conversely, a linear density gradient of Metrizamide will tend to be somewhat hypertonic at the middle, but the deviation from isotonicity will be much smaller than with Ficoll.

Another distinct advantage of Metrizamide solutions is their low viscosity. The viscosity of a Ficoll solution increases as a nearly exponential function of the concentration (fig. 1 C), and in a 40% (w/v) solution the viscosity of Ficoll [16] is nearly 100 times that of Metrizamide. The low viscosity of Metrizamide permits the cells to travel rapidly to their equilibrium position in a gradient.

In order to assess the suitability of Metrizamide for isopycnic gradient separation of cells, an attempt was made to analyse the density distribution of cells from rat liver. The liver is a heterogeneous population of parenchymal and non-parenchymal cells (Kupffer cells and others), and by perfusing the rat liver with collagenase under physiological conditions [6–8] a suspension of intact cells, of which 10–20% are non-parenchymal [8], can be prepared.

Metrizamide gradients were generated by diffusion at 4°C according to the method of Stone [9]. This method is particularly suitable for cell separation, since the cells can be mixed directly into the gradient prior to centrifugation without the use of roller pumps or stirrers which might damage the cells. It is advantageous to have the cells distributed throughout the gradient prior to centrifugation, since aggregation phenomena at interfaces or in concentrated thin layers can

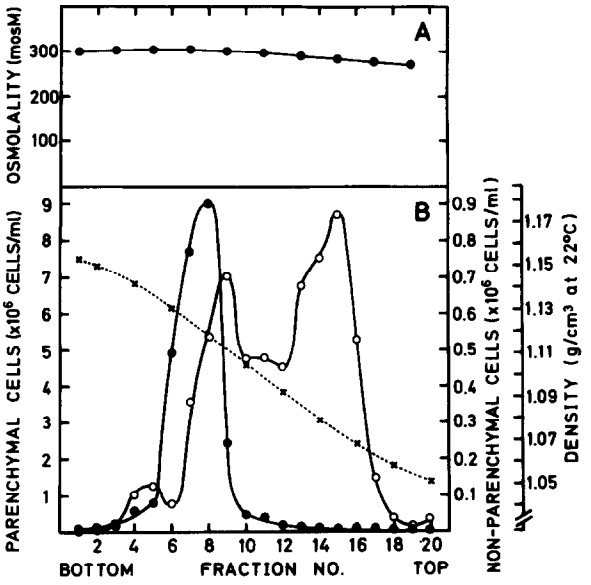

Fig. 2. Distribution of rat liver cells in a Metrizamide density gradient. Freshly prepared rat liver cells were incorporated into an 8 ml Metrizamide gradient (density 1.05–1.16 g/cm^3), and centrifuged for 20 min at 5000 rpm. The osmolality, density and cell content of the fractions were measured as described in the text. A, osmolality (•—•). B, density (x—x), parenchymal cells (•—•) and non-parenchymal cells (○—○). Notice 10-fold scale difference.

thereby be avoided.

As reported by Stone [9], the diffusion-generated gradients were essentially linear (fig. 2B). The osmolality was maintained between 262 and 304 mosM, i.e. a deviation from the normal rat plasma value (283 mosM, cf. ref. [17]) of ± 7% (fig. 2 A).

The liver cells showed a characteristic distribution pattern in the Metrizamide gradient (fig. 2 B). The parenchymal cells banded as a fairly homogeneous peak (mean density = 1.12 g/cm^3). The non-parenchymal cells, on the other hand, distributed heterogeneously with the majority of the cells lighter than 1.10 g/cm^3. A reproducible major peak was found at a density of 1.08, whereas a second major peak in the region of the parenchymal cells (1.11–1.12) was more variable. The possibility of aggregation and trapping artefacts in this region, where the concentration of cells is high, must obviously be considered. Cellular densities may also be artificially changed by the attachment of extracellular debris (fragments of damaged cells etc.). However, free non-parenchymal cells with no visible attachments were found throughout the distribution region indicated, and it may provisionally be assumed that the pattern shown largely represents the true density distribution of these cells.

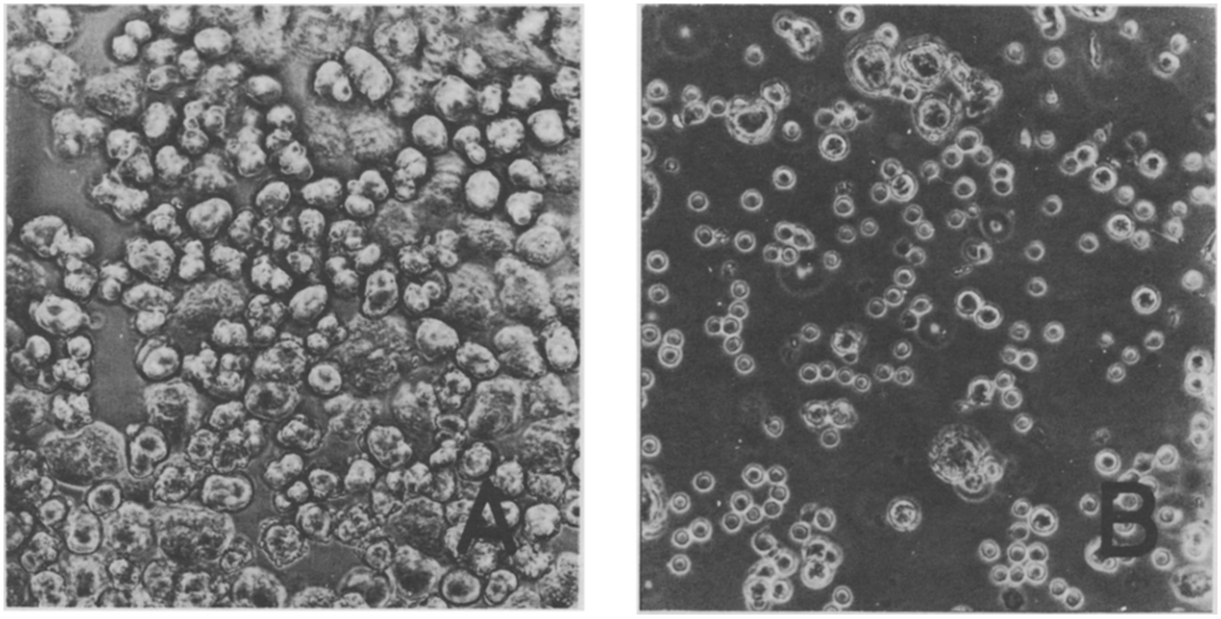

Fig. 3. Cells recovered from a Metrizamide gradient of the type shown in fig. 2. A, fraction 8, mostly parenchymal cells. B, pooled fractions 14 and 15, mostly non-parenchymal cells. Phase contrast, × 225.

Table 1
Direct and indirect effects of Metrizamide on gluconeogenesis and glycolysis in isolated parenchymal rat liver cells

	Gluconeogenic rate (μmoles glucose/g per hr)		Glycolytic rate (μmoles lactate/g per hr)	
	No substrate	10 mM Fructose	No substrate	10 mM Fructose
A) Direct incubation ± MA				
No addition	12.7 ± 0.3	140.5 ± 4.1	0.0 ± 0.0	89.3 ± 1.4
10% Metrizamide	14.7 ± 0.9	80.5 ± 1.6	0.0 ± 0.0	55.0 ± 1.9
B) Preincubation ± MA				
No addition	6.1 ± 0.1	142.2 ± 0.8	0.0 ± 0.0	122.7 ± 0.8
10% Metrizamide	5.7 ± 0.0	146.4 ± 1.0	1.8 ± 0.4	91.4 ± 2.9

In the first experiment (A), purified parenchymal liver cells were incubated for 60 min with or without 10 mM fructose (initial concentration) in the presence or absence of 10% Metrizamide. In the second experiment (B), a crude, initial cell suspension was preincubated for 60 min in the presence or absence of 10% Metrizamide; the parenchymal cells were then purified [8] and simultaneously washed free of Metrizamide. The purified cells were incubated for 60 min with or without 10 mM fructose. The table values show the net formation of lactate or glucose during the final incubation period; each value is the mean ± S.E. of four cell samples. MA = Metrizamide.

Damaged cells of all types, which have a density higher than 1.16, were found mainly as a pellet in the bottom of the tube. However, a few damaged cells were also found in the gradient, particularly in the parenchymal peak, indicating that some distribution artefacts may occur.

Cells recovered from the gradient were structurally intact (excluding Trypan Blue) and exhibited a normal morphology (fig. 3).

In order to see whether exposure to Metrizamide affected cellular metabolism, its effect on fructose utilization by parenchymal cells [10] was tested. Cells incubated in the direct presence of 10% Metrizamide metabolized fructose at a lower rate than did the control cells (40% depression of both the glycolytic and the gluconeogenic rate). Cells washed free of Metrizamide, however, converted fructose to glucose at a normal rate, and their glycolytic rate was reduced only by 25% (table 1).

4. Discussion

Several attempts have been made in the past to separate liver cells by isopycnic gradient centrifugation [11,15, 18–20]. However, these previous studies were done with damaged cells or hypertonic gradients, yielding results different from those obtained in the present study. The use of isotonic Metrizamide gradients has now made it possible to show that the parenchymal rat liver cells form a reasonably uniform population of dense cells (mean density = 1.12 g/cm^3), whereas the non-parenchymal cells have a heterogeneous density distribution (mostly lighter than 1.10 g/cm^3). The favourable physico-chemical properties and relative biological inertness of Metrizamide would seem to make this substance ideal for the gradient centrifugation of other cell types as well.

Acknowledgements

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