THE USES OF METRIZAMIDE IN THE FRACTIONATION OF NUCLEI FROM BRAIN AND LIVER TISSUE BY ZONAL CENTRIFUGATION

A.P. MATHIAS and Coral V.A. WYNTER
Wellcome Laboratories, Department of Biochemistry, University College London, Gower Street, London WC1E 6B7, England

Received 4 April 1973

1. Introduction

Sucrose is the most commonly used solute for density gradient centrifugation. It has two disadvantages, firstly its permeability to cellular membranes and secondly its high viscosity. Although the first may be overcome by the use of polymers such as Ficoll, such materials have the drawback of a high viscosity combined with a low upper limit of the density of their solutions. The disadvantage of solutions of sucrose are most apparent in the separations involving isopycnic centrifugation, for example, the nuclei of brain tissue which requires the use of 2.8 M sucrose [1]. We have investigated the use of a new material which offers the advantages of a considerably higher maximum solution density without the severe problems of viscosity, and which is also biochemically inert.

The compound, which is given the trivial name metrizamide, is *N*-(2,4,6-triiodo, 3-*N* methyl acetyl amino, 5-acetylamino benzoyl) glucosamine (mol. wt. 788.1).

2. Methods

2.1. *Isolation of nuclei*

Nuclei were prepared from rat and rabbit brain as described in [1]. The rabbits were New Zealand Whites weighing 2 kg each. In some experiments the cerebellum was removed taking care to include the parafloculus by cutting the cerebullar peduncles but leaving the pons and medulla oblongata attached to the cerebrum. Nuclei were counted in a Coulter Counter Model F with 100 μm orifice. Nuclei were prepared from livers of Wistar rats as described by Johnston et al. [2].

2.2. *Metrizamide solutions*

Metrizamide was obtained from Nyegaard and Co., A/S, Oslo, Norway, and dissolved by the slow addition of solid metrizamide, stored in the dark, to water with continual stirring and gentle warming. Solutions for brain nuclei contained 1 mM $MgCl_2$ and 1 mM sodium cacodylate buffer pH 6.5. For liver nuclei, the pH of the metrizamide solutions was adjusted to 7.4 at 20° with a dilute solution of $NaHCO_3$. Solutions were stored frozen and in the dark.

Solutions of metrizamide containing 0.32 M sucrose were made by dissolving the appropriate quantity of sucrose in the solutions of metrizamide. Refractive indices were determined with an Abbé refractometer.

2.3. *Density of gradient centrifugation*

Linear gradients of metrizamide were prepared by use of a Technicon pump, as described by Davis et al. [3], in 15 ml tubes of the M.S.E. 6 × 15 ml swinging bucket rotor. Gradients were used shortly after preparation. The sample of nuclei was suspended in a volume of 0.5–0.8 ml. After centrifugation the gradients were unloaded using the M.S.E. gradient recovery system by pumping water to the top of the tube and forcing the contents from the base of the tube. The effluent was monitored at 600 nm in a flow cell (2 mm path length) mounted in a Gilford recording spectrophotometer, and collected manually in 20 drop fractions.

2.4. *Assay*

RNA polymerase was measured by the incorpora-

North-Holland Publishing Company – Amsterdam

tion of [5-^{3}H]UTP as described by Austoker et al. [1]. DNA was estimated by the method of Burton [5] using calf thymus DNA as standard and RNA by the orcinol reaction (Kerr and Seraiderian, [4]) using highly polymerised yeast RNA as standard. Protein was measured by the method of Lowry et al. [6] using bovine serum albumin standard.

2.5. *Microscopy*

Nuclei were examined under oil immersion in the Wild M20 phase contrast microscope. For fixation and staining a drop of solution containing nuclei, from a gradient, was spread by gentle shaking on a microscope slide and allowed to dry. The technique of smearing tended to burst the nuclear membranes. Celloidin Searle Scientific Company) dissolved in ethanol/ether (50:50, v/v) was poured over the slide and allowed to drain to form a thin film. This served to maintain the nuclei on the slide during the subsequent procedures. After a rinse in 70% alcohol and water, the nuclei were fixed in 10% (v/v) formalin containing 0.9% (w/v) saline, 1 mM $MgCl_2$ and 0.05 M sodium cacodylate adjusted to pH 6.5, for 3 min. After a rinse in water, the nuclei were stained for 1 min each with 0.5% cresyl violet, and dehydrated in 70, 90 and 100% (v/v) alcohol each containing 10% (v/v) chloroform to prevent dissolution of the celluloid film. The nuclei were then differentiated in clove oil containing methyl orange for 10 sec, twice rinsed in absolute alcohol, xylene and mounted in Canada Balsam.

Table 1

The effect of metrizamide on the chemical composition and RNA polymerase activity of rabbit brain nuclei.

Suspension medium	RNA/DNA	Protein/ DNA	*RNA polymerase activity
50% Metrizamide	0.66	4.60	3.10
2.4 M sucrose	0.56	4.20	2.92

* pmoles [^{3}H]UMP/pg DNA incorporated.
Nuclei from rabbit brain were suspended in 2.4 M sucrose or 50% metrizamide containing 0.32 M sucrose for 3 hr at 8° the suspensions were diluted with an equal vol. of 0.32 M sucrose and the nuclei collected by centrifugation (2.6 × 10^6 g/min). The analytical results are the means of 5 experiments. The S.E.M.'s represent 7.5% of the mean value for DNA analysis, 10.2% for RNA and 9.9% for protein.

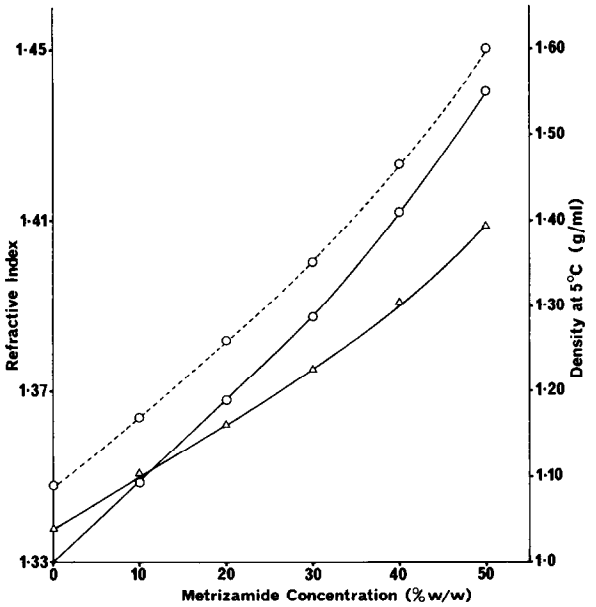

Fig. 1. The relationship between concentration of metrizamide and the refractive index and density of its aqueous solutions. Density was determined at 5°, and the refractive index at 20°. (o—o—o) Refractive index of metrizamide alone; (o--o--o) refractive index of metrizamide containing 0.32 M sucrose; (△—△—△) density at 5° of metrizamide solutions containing 0.32 M sucrose.

3. Results and discussion

3.1. *Suitability of metrizamide for density gradient centrifugation*

Primary requirements for a satisfactory material for density gradient centrifugation are that it should not cause loss of essential components from the particles which it is proposed to examine nor should there be any deleterious effect on the enzymic and other biological properties of these particles. To investigate the usefulness of metrizamide in these respects, rabbit brain nuclei were suspended in 50% w/w metrizamide containing 0.32 M sucrose for 3 hr at 8°. The nuclei were recovered by centrifugation, analysed chemically and the activity of RNA polymerase determined, using, as a standard of comparison, nuclei that had been suspended in 2.4 M sucrose (table 1). The data show that less material was lost from nuclei resuspended in metrizamide and the activity of RNA polymerase better preserved than is the case for nuclei maintained

Table 2
Comparison of nuclear fractions centrifuged first in sucrose gradient followed by a metrizamide gradient.

Sucrose (M)	Fraction	% Composition N	A	O
	A	60	30	10.0
	B	60.6	31.1	8.1
	C	18.2	36.4	45.5
2.0 | 2.3 | 2.4 | 2.5 | 2.6 | 2.8	D	29	4.4	92.9
	A1	3.1	18.6	78.4
↑ ↑ ↑ ↑	A2	23.0	17.7	59.3
A B C D	A3	62.2	30.0	7.8
	A4	72.8	26.4	0.8
	B1	30.2	24.1	45.6
	B2	57.0	33.6	9.4
Metrizamide (% w/v)	B3	68.2	26.4	5.4
	B4	81.6	16.1	2.3
	C1	2.2	12.8	85
25 | 33 | 35 | 36 | 36.5 | 37	C2	6.6	11.4	82
	C3	33.6	38.0	28.4
↑ ↑ ↑ ↑	C4	84.4	15.6	0
1 2 3 4	D1	1.9	4.7	93.4
	D2	3.4	5.1	91.5

Nuclei from rat cerebrum minus the cerebellum were fractionated on a discontinuous sucrose gradient as indicated to give fractions (A–D. The nuclei from each fraction were collected after dilution and sedimentation at 2.6×10^6 *g*/min and serum on a discontinuous metrizamide gradient to give fractions A_1–A_4 etc. The percentage composition of each fraction was calculated after identification of the nuclei by phase contrast microscopy.

in 2.4 M sucrose. Prolonged storage of nuclei in concentrated metrizamide solutions had little effect on the appearance of the nuclei under phase contrast microscopy. Although impairment of RNA polymerase was not observed in nuclei stored in metrizamide, the addition of this compound during assay of RNA polymerase caused an inhibition of the enzyme. In the presence of 5% (w/w) metrizamide the activity was reduced to 50% of the original level. With 10% metrizamide the activity was reduced to 25%. However this residual activity was not further affected by doubling the concentration of metrizamide. It is possible that the activity resistant to metrizamide represents form B of RNA polymerase.

3.2. *Fractionation of rat and rabbit brain by isopycnic centrifugation*

Preliminary experiments indicated that solutions of pure metrizamide were not suitable for fractionation of heterogeneous populations of nuclei. Accordingly the effects of the additions of small amounts of sucrose were investigated. It was found that a final concentration of 0.32 M sucrose was sufficient for most purposes.

The variation of refractive index and density with concentration of metrizamide is shown in fig. 1. A 50% solution of metrizamide containing 0.32 M sucrose has a density of 1.4 g/cc which is greater than the density of nuclei in solutions of sucrose, namely 1.29–1.35 g/cc [1, 7]. In most experiments with brain tissue, nuclei were prepared from the cerebrum alone, to eliminate the problems of distinguishing the nuclei of the small granule cell neurons from the oligodendroglial nuclei. A suspension of nuclei prepared from rats weighing 90 g, was layered on a gradient of metrizamide ranging from 32.8% to 39.2%, linear with respect to length. The underlay was 41.5% (1 ml) and the overlay consisted of a layer of 23.8% over another layer of 29.2% (both 0.7 ml). Centrifugation was at 24,000 rpm for 3 hr (7.2×10^9 w^2t).

The light scattering profile and the identification of the nuclei in the fractions from the effluent of the rotor tube is shown in fig. 2. The neuronal nuclei are located predominantly in the most dense regions of the gradient and are concentrated in fractions 6–11 spaning a range of density from 1.275 to 1.283 g/cc. The oligodendroglial nuclei are found at the lighter end of the gradient forming a broad peak at density 1.266 g/cc. The sedimentation behaviour of the brain nuclei in solutions of metrizamide, contrasts sharply with that in solutions of sucrose in which the oligodendroglial nuclei band at approx. 1.348 g/cc and the neuronal nuclei at 1.287 g/cc. The separation of neuronal and astrocyte nuclei was not markedly improved by introduction of steps into the gradient.

A detailed survey of the separation of nuclei from rabbit cerebellum, which was complicated by the difficulty of distinguishing between granule cell nuclei and oligodendroglial nuclei even after staining with cresyl violet and counterstaining with methyl orange, showed that the fractionation was inferior to that obtained with the rat nuclei although partial resolutions were achieved.

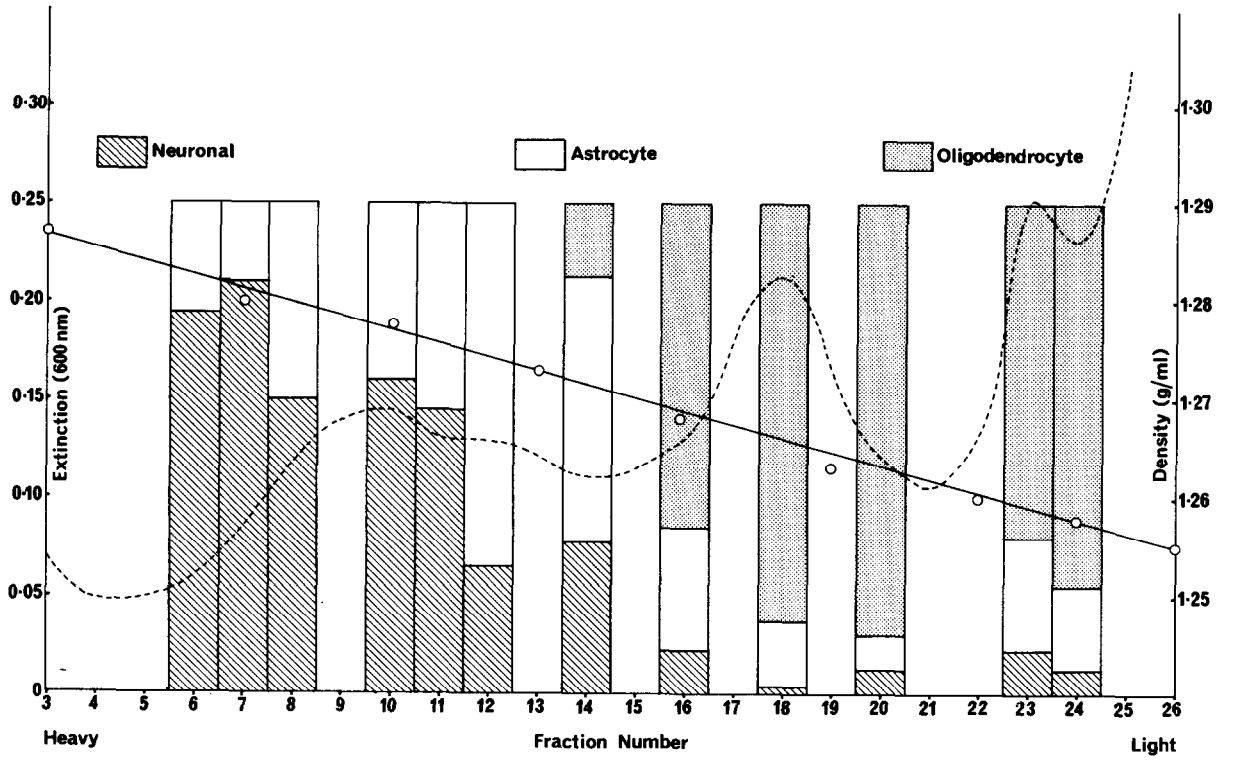

Fig. 2. Distribution of nuclei from rat cerebrum sedimented to isopycnic positions in a linear gradient of metrizamide containing 0.32 M sucrose. The percentages of the nuclei of different classes in each fraction are shown by histograms in which the nuclei are represented as follows: ▨ Neuronal nuclei; □ astrocytic nuclei; ▩ oligodendroglial nuclei. The peak in fractions 23–24 is mainly damaged nuclei. (– – – –) Extinction at 600 nm; (——) density of gradient.

If rat brain nuclei that had been centrifuged in discontinuous gradients were recovered and then centrifuged in a discontinuous sucrose gradient according to the method of Austoker et al. [1] they no longer exhibited the normal pattern of fractionation in the sucrose gradient. The nuclei from each of the zones in the metrizamide gradient gave a major peak at the interface between the 2.5 and 2.6 M sucrose which is the position normally occupied by the first of the oligodendroglial zones. After the second sedimentation the nuclei retained their characteristic appearance. It may be concluded that the exposure to metrizamide has effected a change in the buoyant density of the different types of nuclei that is not reversed when the nuclei are transferred to solutions of sucrose.

The results of an experiment in which the nuclei were first fractionated on a sucrose gradient and secondly on a metrizamide gradient, are shown in table 2. The nuclei found at the interface between 36 and 36.5% (w/w) metrizamide were neuronal nuclei comparatively free of other types, and the nuclei at the top of the metrizamide gradient were predominantly oligodendroglial nuclei.

3.3. *Fractionation of rat liver nuclei*

The use of metrizamide in separations based on sedimentation rate was illustrated by the fractionation of rat liver nuclei in a linear gradient ranging from 11.2–33.5% (w/w) metrizamide. After 1 hr at 600 rpm two peaks were observed (fig. 3). The slower running peak contained diploid nuclei with the diploid stromal nuclei concentrated at the trailing edge and the diploid parenchymal nuclei in the leading fractions. The second peak was composed predominantly of tetraploid parenchymal nuclei. The separations were very similar to those obtained in solutions of sucrose (Johnston et al. [2]).

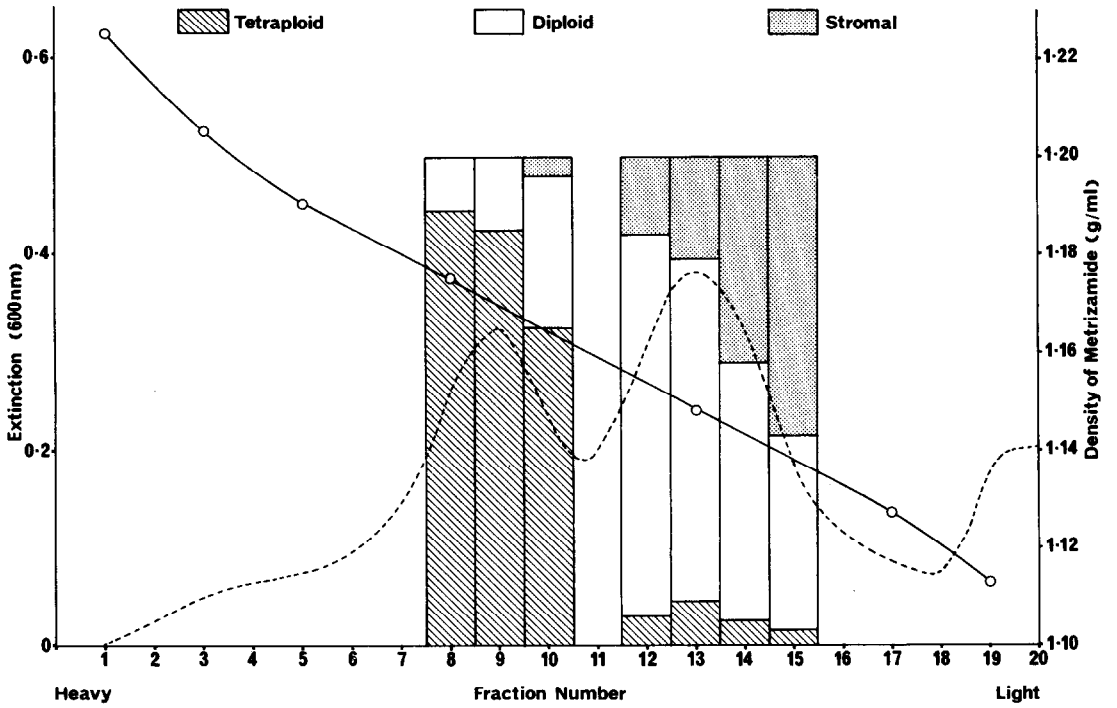

Fig. 3. Distribution of various classes of rat liver nuclei after centrifugation in a linear gradient of metrizamide for 1 hr at 600 rpm. ▨ Tetraploid parenchymal nuclei; □ diploid parenchymal nuclei; ▩ diploid stromal nuclei. The nuclei were recovered from the fractions and identified according to the criteria defined by Jonhston et al. [7]. (– – – –) Extinction at 600 nm; (——) density of gradient.

3.4. *Conclusions*

These experiments have demonstrated that metrizamide because of its inertness towards biological materials is suitable for use in their fractionation. Because it can form solutions of higher density and lower viscosity it has advantages over sucrose in certain circumstances.

Acknowledgements

This work has been supported by the Science Research Council. We are grateful to Nyegaard and Company for samples of metrizamide.

References

[1] J. Austoker, D. Cox and A.P. Mathias, Biochem. J. 129 (1972) 1139.
[2] I.R. Johnston, A.P. Mathias, F. Pennington and R. Ridge, Biochem. J. 109 (1968) 127.
[3] G.A. Davis, R.J. Santen and B.W. Agranoff, Anal. Biochem. 11 (1965) 153.
[4] S.E. Kerr and K. Seraidarian, J. Biol. Chem. 159 (1945) 211.
[5] K. Burton, Biochem. J. 62 (1956) 315.
[6] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[7] I.R. Johnston, A.P. Mathias, F. Pennington and D. Ridge, Nature 220 (1968) 668.
[8] I.R. Johnson and A.P. Mathias, in: Subcellular components: preparation and fractionation, ed. G.D. Birnie (Butterworths and Co., London, 1972) p. 53.