ISOPYCNIC CENTRIFUGATION OF SHEARED CHROMATIN IN METRIZAMIDE GRADIENTS

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Received 24 April 1973

1. Introduction

Much information basic to the understanding of fundamental processes in cell biology can be obtained by studies of the structure, composition and properties of interphase chromosomes. For a number of reasons, isopycnic sedimentation of chromatin in a suitable medium might appear to offer a good approach to developing a reproducible method of purifying, and possibly fractionating, this complex material. However, the properties of chromatin are such that a suitable medium is not easy to find. Chromatin will band isopycnically in CsCl [1], but only after fixation with formaldehyde since high concentrations of salt rapidly dissociate nucleoproteins [2]. Unfortunately, the reaction of chromatin and formaldehyde is irreversible and material so fractionated is of little use for further study. Chromatin does not band in sucrose gradients, but will band in very dense sucrose–glucose gradients [3]. These gradients are so viscous that only unsheared chromatin ($s_{20,w}$ 30000 to 80000) can be sedimented through them in a reasonable time. This would appear to militate against any fractionation of chromatin which depends on the chromatin being sheared extensively [4, 5]. Chromatin will band in chloral hydrate gradients [6], but these gradients are also viscous and difficult to handle and so may have the same disadvantage as sucrose–glucose gradients.
The recent report on the use of metrizamide gradients for the isopycnic banding of unfixed ribonucleoprotein particles [7] immediately suggested that these gradients might be of use for fractionating chromatin. This paper reports on preliminary studies of the formation of density gradients in metrizamide solutions and of the isopycnic banding of chromatin from mouse livers in these solutions.

2. Materials and methods

Metrizamide (2-(3-acetamido-5-*N*-methylacetamido-2,4,6-tri-iodobenzamido)-2-deoxy-D-glucose) was a generous gift from Nyegaard and Co. A/S, Oslo, Norway. A 58% (w/v) solution was prepared by slowly adding 44 g of metrizamide to 56 ml of 1 mM EDTA, 1 mM Tris-HCl, pH 7.5, with stirring at room temp., followed by filtering through glass-fibre (Whatman GF/A). The pH of the solution was adjusted to 7.5 with 0.1 N NaOH. This stock solution was kept at −20°C; it was diluted with 1 mM EDTA, 1 mM Tris-HCl, pH 7.5, as required.

Nuclei were prepared by a modification of the method of Blobel and Potter [8] and washed with 1% (v/v) Triton X-100 in 0.25 M sucrose, 3 mM $CaCl_2$ [9] which removes more than 90% of the nuclear membranes [2]. Lysed nuclei were prepared by suspending the nuclear pellet in sufficient 1 mM EDTA, 1 mM Tris-HCl, pH 7.5, to give a solution containing 1 mg of DNA/ml, and dialysing the lysate against 200 vol of 1 mM EDTA, 1 mM Tris-HCl, pH 7.5 for 2 hr at 4°C. Chromatin was isolated from the nuclear pellets as described previously [2] except that the nuclei were extracted three times with 0.14 M NaCl, 0.01 M EDTA, 0.05 M Tris-HCl, pH 7.5, then washed once with 0.01 M EDTA, 0.05 M Tris-HCl, pH 7.5. The pellet of chromatin was homogenised in distilled water and dialysed as for lysed nuclei. Both the lysed nuclei and the chromatin preparations were in solution at this stage; they

were then sheared by ultrasonication (Dawe Soniprobe, 1/4 in. tip, 3.5 A at power setting 4) in an ice-bath for periods of 15 sec (alternating with 15 sec cooling periods) for a total time of 2 min.

Metrizamide solutions were centrifuged under conditions similar to those previously used for gradients of CsCl and NaI [10]; a fixed-angle rotor was used rather than a swing-out rotor because of the increased capacity and resolution obtainable [11]. The solutions, with or without chromatin, were adjusted to the initial density required and 5 ml portions, overlaid with paraffin, were centrifuged in 10 ml polyallomer tubes in the aluminium 10 X 10 fixed-angle rotor of the MSE Superspeed 50 centrifuge at 5°C. The rotor was allowed to come to rest without braking, the gradients were unloaded by upward displacement with Fluorochemical FC43 (Measuring and Scientific Equipment Ltd., Crawley, Sussex) and 0.25 ml fractions were collected. The refractive index (from which density was calculated) of each fraction was measured; 500 μg of bovine serum albumin was then added to a portion of each fraction followed by 5 ml of ice-cold 0.6 N $HClO_4$. Acid-insoluble material was pelleted by centrifugation at 1500 *g* for 10 min, then washed twice by resuspension in 5 ml portions of 0.6 N $HClO_4$ to remove metrizamide. RNA and DNA were separately extracted from the acid-insoluble pellet and assayed by reaction with orcinol and diphenylamine, respectively [12].

3. Results

Metrizamide is very soluble in water and readily forms dense solutions of quite low viscosity; for example, a 56% (w/v) solution of metrizamide has a density of 1.30 g/cm^3 and a viscosity (20°C) of 8 cp. In this it is markedly superior to sucrose, with which a solution of density 1.30 g/cm^3 has a viscosity (20°C) of 81 cp. Sucrose–glucose solutions have even higher viscosities (for example, 1240 cp at 20°C for a solution of density 1.376 g/cm^3 [3]).

The effects of speed and time of centrifugation, and of the initial density of the metrizamide solution, on the density gradient obtained are summarised in figs. 1–3. As occurs with other materials which form a density gradient in a centrifugal field the gradients formed in metrizamide solutions are dependent on both

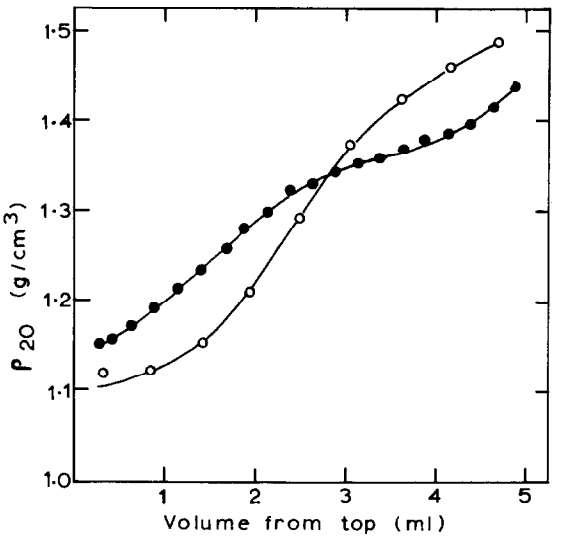

Fig. 1. The effect of speed of rotation on the formation of a metrizamide gradient. Tubes containing 5 ml of 58% (w/v) metrizamide solution (density 1.312 g/cm^3) were centrifuged for 68 hr at 5°C at 35000 rpm (●-●-●) or 45000 rpm (○-○-○).

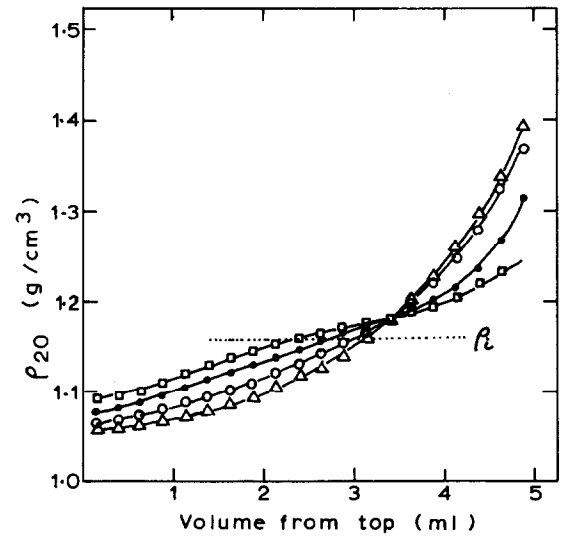

Fig. 2. The effect of time of centrifugation on the formation of a metrizamide gradient. Tubes containing 5 ml of 29% (w/v) metrizamide solution (density 1.158 g/cm^3) were centrifuged at 35000 rpm at 5°C for 14.5 hr (□-□-□), 22.5 hr (●-●-●), 40 hr (○-○-○) and 68 hr (△-△-△).

speed and time of centrifugation (figs. 1 and 2). However, the gradient forms more slowly than it does in,

for example, solutions of CsCl in which equilibrium gradients are established in 18–24 hr [13]. Indeed, under the conditions explored so far, formation of an equilibrium gradient in metrizamide has not been observed, indicating that the rate of diffusion of metrizamide is very much slower than its rate of sedimentation. The rate at which a given shape of gradient is formed in a metrizamide solution also appears to be dependent on the initial concentration of metrizamide (fig. 3), probably due to the viscosity of solutions of metrizamide which is higher than that of salt solutions of the same density.

Sheared preparations of chromatin readily band isopycnically in metrizamide gradients. Fig. 4A shows the distribution of DNA and RNA along the density gradient after centrifugation of a preparation of lysed nuclei in metrizamide solution (ρ_i 1.182 g/cm^3) at 35000 rpm for 44.5 hr. The DNA shows two distinct peaks, the larger at 1.20 g/cm^3, the smaller at 1.24 g/cm^3. After making allowance for the overlap between them (assuming the peaks are symmetrical) the ratio between light and heavy DNA is estimated to be about 4:1. RNA also shows two peaks, the lighter one coincident with the heavier DNA peak at 1.24 g/cm^3 while the heavier one bands at 1.28 g/cm^3.

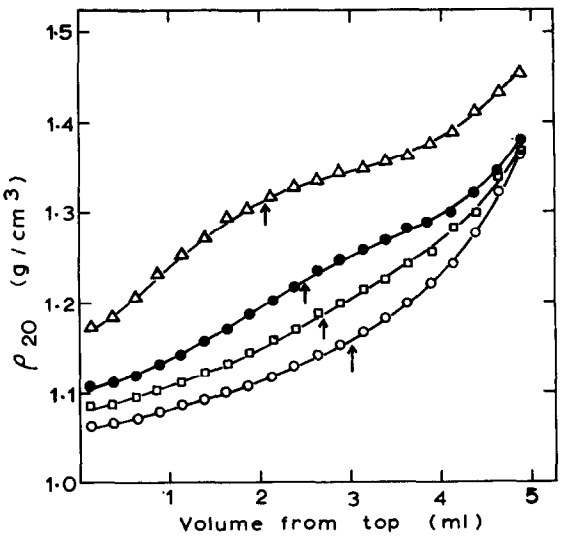

Fig. 3. Effect of initial density (arrows) on the formation of a metrizamide gradient. Tubes containing 5 ml of metrizamide solution of initial density 1.158 g/cm^3 (○-○-○), 1.186 g/cm^3 (□-□-□), 1.224 g/cm^3 (●-●-●) and 1.312 g/cm^3 (△-△-△) were centrifuged at 35000 rpm for 40 hr at 5°C.

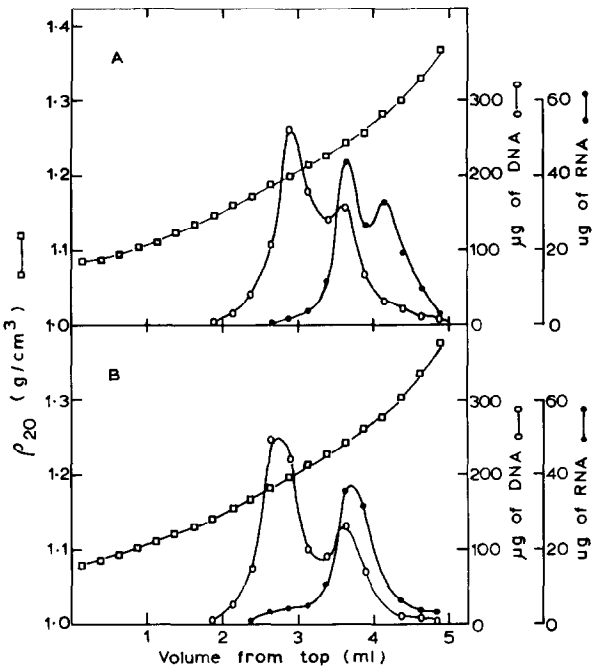

Fig. 4. Isopycnic banding of (A) lysed nuclei and (B) isolated chromatin in metrizamide gradients. Tubes containing 5 ml of 36% (w/v) metrizamide solution (density 1.186 g/cm^3) and either (A) 50 A_{260} units of lysed nuclei or (B) 50 A_{260} units of chromatin were centrifuged at 35000 rpm for 44.5 hr at 5 °C. (□-□-□), density (g/cm^3); (○-○-○), DNA (µg/fraction); (●-●-●), RNA (µg/fraction).

A similar distribution of DNA is observed when isolated chromatin is centrifuged in metrizamide (fig. 4B). The two peaks of DNA have densities of 1.19 and 1.24 g/cm^3 and the ratio between them (allowing for overlap) is 2.6:1. However, in contrast to preparations of lysed nuclei, chromatin preparations show only one RNA peak, coinciding with the smaller and denser peak of DNA at 1.24 g/cm^3.

4. Discussion

Metrizamide is a potentially useful compound for isopycnic sedimentation experiments because it is chemically quite inert, it is non-ionic and it forms solutions which, while much denser than can be obtained with sucrose, are of relatively low viscosity. The spontaneous formation of density gradients in solutions of metrizamide in a centrifugal field is another useful at-

tribute of this new material. It is no disadvantage that the shape of the gradient formed is dependent on the initial concentration of metrizamide as well as the time and speed (and presumably also temperature) of centrifugation since this allows the final form of the gradient to be chosen simply by judicious manipulation of the conditions of centrifugation.

It has now been shown that sheared preparations of unfixed chromatin can be banded isopycnically in metrizamide gradients. This suggests that these gradients will be of considerable use for purifying and characterizing chromatin. In contrast to other reported methods for isopycnic centrifugation of chromatin [1,3,6] the non-ionic nature of metrizamide gradients means that the chromatin need not first be fixed with formaldehyde, while their low viscosity means that sheared preparations of chromatin can be sedimented to equilibrium under conditions in which degradation by endogenous proteases and nucleases is minimized.

The banding density of chromatin is much lower in metrizamide gradients than in sucrose–glucose [3] or chloral hydrate [6] gradients. This was not unexpected since ribonucleoprotein particles band at 1.28 g/cm^3 in metrizamide [7] although the density of ribosomes in sucrose is 1.43 g/cm^3 [14]. Whether the two peaks of DNA seen in metrizamide gradients represent heterochromatin and euchromatin is a question still to be answered. The ratios between the light and heavy fractions and the coincidence of an RNA peak with the heavier one is suggestive though it has still to be demonstrated that this RNA is actually associated with the material containing the DNA.

The appearance of RNA-containing peaks at densities of 1.24 g/cm^3 and 1.28 g/cm^3 in preparations of lysed nuclei, but of the lighter one only in isolated chromatin, is also of interest. The method of preparing nuclei ensures that the nuclear membrane is removed together with perinuclear ribosomes [2] so that neither RNA peak consists of ribosomes. The method of isolating chromatin has been reported to remove nucleolar and nucleoplasmic ribonucleoprotein particles [15], suggesting that the heavier of the RNA-containing bands consists of one, or both, of these species of particle. It is, therefore, of possible significance that the heavier RNA-containing band has the same density (1.28 g/cm^3) as the heavier of the two species of rapidly-labelled ribonucleoprotein particles isolated from rat liver cytoplasm [7].

Acknowledgments

This work was supported by grants to the Beatson Institute from the Medical Research Council and Cancer Research Campaign, and by a generous gift from Nyegaard and Co. A/S, Oslo, Norway. The technical assistance of Mr. R. McFarlane is gratefully acknowledged.

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