ISOPYCNIC CENTRIFUGATION OF MAMMALIAN METAPHASE CHROMOSOMES IN METRIZAMIDE

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1. Introduction

Isopycnic centrifugation is a powerful separation technique, but when applied to metaphase chromosome research it has been technically frustrating. High concentrations of salt rapidly dissociate metaphase chromosomes, and MacGillivray, et al. [1] show that high concentrations of salt dissociate nucleoproteins. Chromatin will band isopycnically in CsCl after fixation with formaldehyde [2], but the reaction is irreversible and the material of little use thereafter. Obviously, formaldehyde treated chromosomes are equally useless. We have used sucrose, Ficoll, and fructose as the support media for isopycnic banding of metaphase chromosomes, but at the density required to band the chromosomes (ρ=1.31 g/cm^3) the viscosity of the sugar solutions is so high as to make their use prohibitive. Chromatin has been shown to band in chloral hydrate gradients [3], but these gradients are very difficult to handle and are also quite viscous which again make them unsuitable for banding metaphase chromosomes. In order to circumvent these problems an elaborate system of nonaqueous gradients was developed by Stubblefield and Wray [4] for buoying chromosomes. These organic gradients accomplish their objective of isopycnically banding metaphase chromosomes (ρ=1.36 g/cm^3) in a low viscosity media. However, the technical problems in preparing these gradients, the solvent properties of the organic chemicals and especially their ability to extract certain proteins from the chromosomes make this system amenable only to certain specified applications. The introduction of iodinated density gradient media for biological separations represents a significant advance over previous technology. The isopycnic centrifugation of chromatin in metrizamide [5,6] solutions leads to the obvious extension of its use for isopycnic banding of metaphase chromosomes.

2. Materials and general methods

Mitotic cells from Chinese hamster ovary (CHO) and HeLa were collected from monolayers previously treated with 0.06 μg/ml Colcemid [7]. Metaphase chromosomes were prepared by the method of Wray and Stubblefield [8], and purified according to Wray [9]. Examination of the metaphase chromosomes by electron microscopy was performed on a Phillips 200 electron microscope after critical point drying according to Anderson [10]. DNA and protein were measured by the methods of Burton [11] and Lowry [12]. Chromosomal proteins were prepared for polyacrylamide gel electrophoresis by dissolving chromosomes in 3% sodium dodecyl sulfate (SDS) (BDH Chemicals, Ltd., Poole, England), 0.062 M Tris, (Sigma, St. Louis, Mo.) pH 6.8, at 100°C for 10 min. The amount of protein in the sample was assayed by spectrophotometric assay [13] after which β-mercaptoethanol (Eastman Organic) was added and the samples reheated. Chromosomal proteins were analyzed on 9.5% polyacrylamide slab gels using a Tris-glycine buffered SDS system [14]. Gels were stained with 0.05% Coomassie blue in methanol: acetic acid: water 40:7.5:52.5 (v/v/v) and detained by diffusion in 7.5% acetic acid. Metrizamide was purchased from Nyegaard and Co., (Oslo, Norway).

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3. Specific methods and results

Major sources of difficulties during these studies were divided between problems inherent with working with metaphase chromosomes and problems associated with the isopycnic banding of the chromosomes. The particular problem endogenous to metaphase chromosome research is their very annoying tendency to stick to each other and to the sides of the centrifuge tubes.

To circumvent many of the wall effects associated with conventional centrifugation techniques, chromosomes were mixed in 0.75 M metrizamide layered on the bottom of a preformed gradient and allowed to rise until they reached their buoyant density. To further minimize wall effects and chromosomes sticking together, tubes have been treated with 1% dimethyldichlorosilane (PCR, Inc., Gainesville, Florida) in carbon tetrachloride, and chromosomes have been suspended in buffer containing NDA [(2-naphthyl 6, 8 disulfonic acid) disodium salt, Eastman Organic)]. Time and speed of centrifugation also turned out to be very important as very short centrifugation times at relatively high speeds tend to minimize wall effects. The optimal conditions that we have established for isopycnic buoying of metaphase chromosomes in metrizamide are:

(1) Treat 15 ml Corex centrifuge tubes for 5 min with 1% dimethyldichlorosilane in carbon tetrachloride.

(2) Dry and bake the silicone on the tube at 140–160° F for 24 h.

(3) Suspend chromosomes in 1 ml of chromosome isolation buffer containing 0.75 M metrizamide and 5 mM NDA and place the suspension in the Corex tube.

(4) Layer on the chromosome suspension a preformed 10 ml 0.35 M to 0.75 M metrizamide-buffer gradient containing 5 mM NDA.

(5) Centrifuge at 16 300 *g* (10 000 rev/min) for 10 min in an HB-4 (swinging bucket) rotor in a Sorvall RC-2B centrifuge.

(6) The gradient is fractionated from the top in 0.25 ml aliquots.

Under these conditions chromosomes buoy in a rather sharp and obvious band. There is minimal adherence to the walls of the tubes and most chromosomes do not stick together. The morphology of normal isolated chromosomes when examined with phase contrast light microscopy (fig.1a) and electron microscopy (fig.1b) shows a structure with very good detail and excellent fine structure. Chromosomes exposed to metrizamide (figs.1c and 1d) in comparison cannot be distinguished from normal controls. Thus, after the nagging technical problems have been eliminated, the metrizamide system for isopycnically buoying chromosomes seems to be ideal. There is, however, one significant deterrent to its general use. The price of metrizamide is such that many experiments are simply too expensive for most laboratories to attempt.

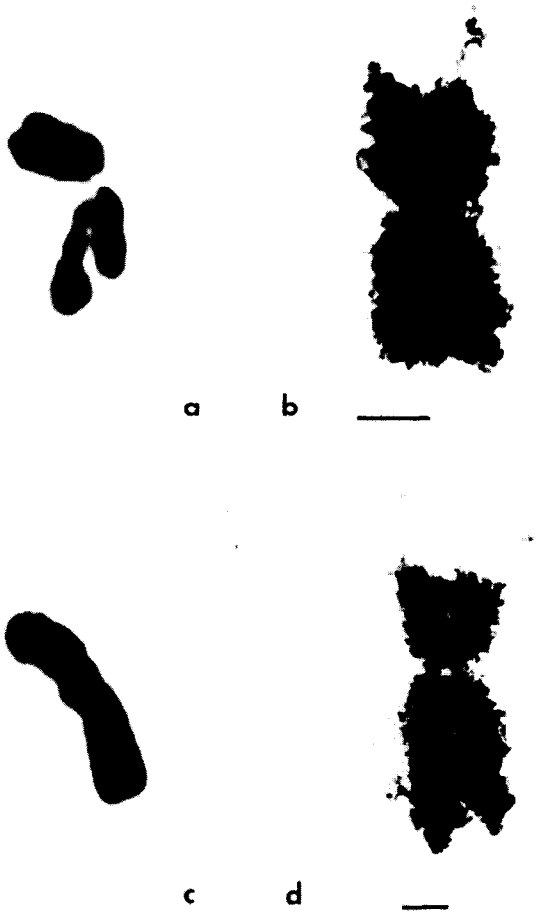

Fig.1. (a) Phase contrast light micrograph of control isolated chromosome. × 800. (b) Electron micrograph of control isolated chromosome. × 18 130. Bar length is 0.5 μm. (c) Phase contrast light micrograph of isolated chromosome exposed to metrizamide. × 800. (d) Electron micrograph of isolated chromosome exposed to metrizamide. × 10 560. Bar length is 0.5 μm.

Table 1

Relative composition of isolated chromosomes			
Treatment	DNA	Protein	Protein/DNA
Control	100	180	1.80
Control	100	209	2.09
Control	100	220	2.20
Metrizamide	100	218	2.18

The relative composition of isolated chromosomes is shown in table 1. The ratio of protein to DNA is about 2.2:1.0 for both control chromosomes and chromosomes exposed to metrizamide.

Fig.2 shows that when chromosomes have been doubly labeled with both [^{14}C]thymidine and [^{3}H]amino acid mix, the chromosomes have a buoyant density of ρ=1.24 g/cm^3 and the DNA and protein is located in a common peak.

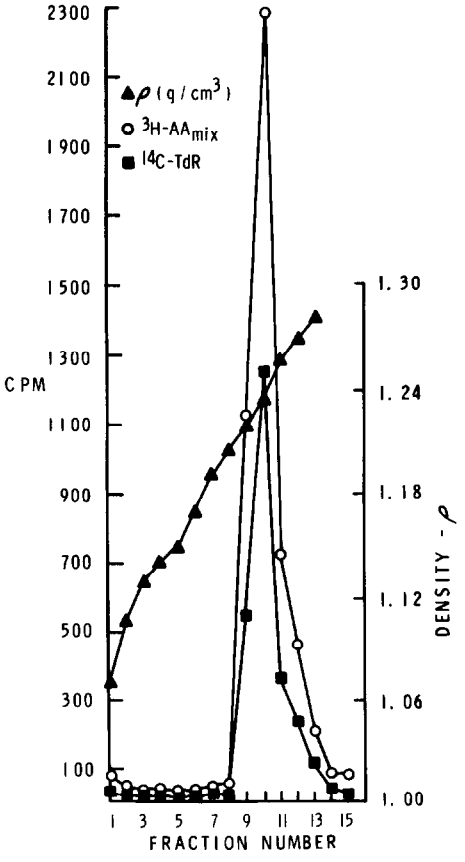

Fig.2. Isopycnic banding of metaphase chromosomes doubly labeled with [^{14}C]thymidine and [^{3}H]amino acid mix. Tubes containing 5 ml of a preformed 0.35 M to 0.75 M metrizamide-buffer gradient were prepared and centrifuged as described in the text.

It is important to demonstrate that the proteins which are associated with a particular chromosome are faithful to that chromosome and do not rearrange and transfer from chromosome to chromosome either normally or in the presence of metrizamide. To show this, the following experiments were designed. CHO cultures were grown in the presence of either [^{3}H]thymidine, [^{3}H]amino acid mix, or no label. Chromosomes from each were kept separate and independently isolated and purified. Four separate mixing experiments were then performed. In two of the experiments [^{3}H]thymidine labeled chromosomes were mixed with unlabeled chromosomes. In the other two experiments [^{3}H]amino acid labeled chromosomes were mixed with unlabeled chromosomes. In one experiment in each of the above two sets metrizamide was present, in the other experiment in each set it was omitted. The rationale for the series of experiments is as follows: it is assumed that DNA present in a particular chromosome remains in that chromosome so long as the chromosome maintains its morphologic integrity. Therefore, when [^{3}H]thymidine-labeled chromosomes are mixed with unlabeled chromosomes only the chromosomes which were originally labeled will ever be labeled and thus show up as such by autoradiography. When these labeled and unlabeled chromosomes are mixed on a volume/volume basis the percent of labeled chromosomes present in the final mixture should be proportional to the original mixing volumes (i.e. a linear function). In these experiments the knowledge of exact numbers of labeled and unlabeled chromosomes per unit volume is not necessary. The number of labeled and unlabeled chromosomes in a unit volume will determine the slope of the line, but the only important point is that the graph of the line must be linear. It is shown in fig.3 that when [^{3}H]thymidine labeled chromosomes are mixed with unlabeled chromosomes both in the presence and absence of metrizamide there is a linear function when the percent of labeled chromosomes on a slide is plotted against the mixing proportions (v/v) labeled: unlabeled. The only way that a linear relationship may be obtained as if there is no exchange of label (i.e. chromosomal components) among chromosomes.

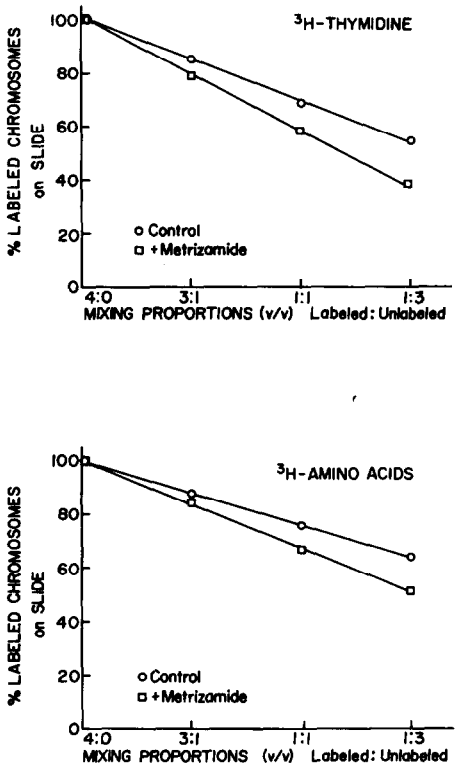

Fig.3. Experiments demonstrating the fidelity of DNA and proteins in chromosomes. Four separate mixing experiments were conducted as described in the text. In two of the experiments [^{3}H]thymidine labeled chromosomes were mixed with unlabeled chromosomes. In the other two experiments, [^{3}H]amino acid labeled chromosomes were mixed with unlabeled chromosomes. In one experiment in each of the above two sets metrizamide was present, in the other experiment in each set it was omitted. For each time point in each experiment the chromosomes were mixed and centrifuged onto a microscope slide with a Cytocentrifuge (Shandon). The slides were then prepared for autoradiography and allowed to develop for 1 month. At each of the mixing proportions in each experiment 1000 chromosomes were analyzed to obtain the percent labeled chromosomes on the slide. Clumps of chromosomes and uninterpretable grain patterns were omitted from analysis. In these experiments the chromosomes were either very heavily labeled or unlabeled. There were no chromosomes which had only a few grains associated with them. It is estimated that if 1.0% of the DNA or proteins transferred from one chromosome to another, they would be easily seen by this technique. The knowledge of exact numbers of labeled and unlabeled chromosomes per unit volume will determine the slope of the line, but is not necessary for interpretation of the results. The only way that a linear relationship may be obtained is if there is no exchange of label (i.e. chromosomal components) among chromosomes.

Therefore when [^{3}H]amino acid chromosomes were mixed with unlabeled chromosomes both in the presence and absence of metrizamide and a linear function is obtained as shown in fig.3, it demonstrates conclusively that the proteins which are associated with a particular chromosome remain associated with that chromosome.

There are a number of ways by which the density of chromosomes may be specifically altered. This type of experiment could play a very important role in the fractionation of a genome so the following experiment was done to demonstrate that chromosome populations with altered buoyant densities may be separated on metrizamide gradients. CHO chromosomes labeled with [^{14}C]thymidine were treated with 0.25 N HCl for 1 h at 4°C to remove histones. They were then washed once and mixed with [^{3}H]thymidine labeled CHO chromosomes in buffer containing 0.75 M metrizamide, 5 mM NDA, and isopycnically buoyed as described earlier. Since proteins band isopycnically in metrizamide at a greater density than DNA, removal of protein would yield a less dense chromosome. The results shown in fig.4 indicate that the chromosomes which had some histone removed have a lower density than the control and that it is possible to separate the two populations by their buoyant density in metrizamide. It should be noted that the densities of both chromosome populations are higher than 1.24

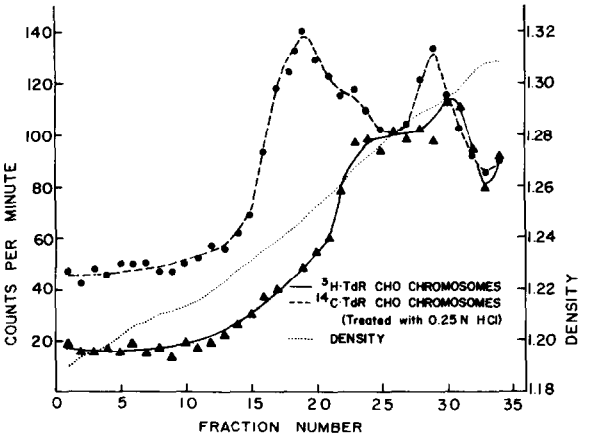

Fig.4. Isopycnic banding of metaphase chromosomes showing a density difference induced by removal of histone proteins from the chromosomes. Centrifuge conditions are described in the text.

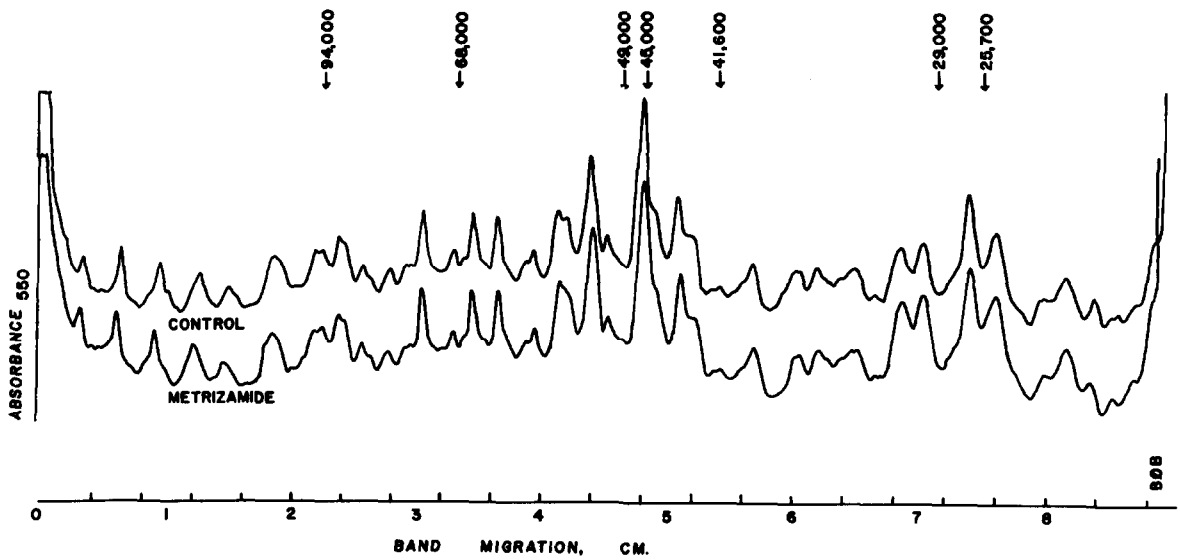

Fig.5. Comparative gel scans of control isolated chromosomes and chromosomes exposed to metrizamide.

g/cm^3 as shown in fig.2. This difference is apparently due to the presence of NDA in the gradient.

Chromosomal proteins from HeLa were analyzed by SDS slab gel electrophoresis (fig.5) as described in the General methods. As compared to the control chromosomes sample, chromosomes after exposure to metrizamide had identical polypeptide populations.

4. Discussion

The introduction of iodinated density gradient media for isopycnic centrifugation of metaphase chromosomes represents a significant advance over previous technology. In this report we have shown that metrizamide is a useful and dependable medium for banding chromosomes according to their buoyant density. The morphology of the metaphase chromosome after exposure to metrizamide is excellent and the total protein complement and the DNA: protein ratio is not altered. The major deterrent to its general use is its expense.

Acknowledgements

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