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CHARACTERIZATION OF RAT LIVER NUCLEI ISOLATED IN ANHYDROUS MEDIA AND THEIR FRACTIONATION USING NON-AQUEOUS GRADIENTS

DONALD F. KENNEDY and ANTHONY P. MATHIAS

Department of Biochemistry, University College London, London WC1E 6BT, England

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Abstract—The properties of nuclei, which had been isolated from freeze-dried rat liver in anhydrous glycerol, and purified by a variety of steps, were investigated. The nuclei retained high levels of a number of nuclear enzymes including NAD pyrophosphorylase, and DNA and RNA polymerases. The nuclei isolated by these methods contained considerably higher amounts of soluble and non-histone proteins than did those isolated in aqueous solutions of sucrose. Some specific differences in the profiles of the proteins in these two categories were noted between nuclei prepared by these two methods. Rat liver nuclei isolated in non-aqueous solvents may be fractionated either in gradients prepared by dissolving metrizamide in glycerol–dimethylsulfoxide or in gradients composed of propane-1,3-diol and 3 chloro-1,2-propanediol. The fractionation provides a partial resolution of diploid stromal from diploid parenchymal nuclei and of diploid nuclei from the tetraploid nuclei.

Many pharmacological investigations ultimately require an analysis at the level of the cell nucleus. An understanding of nuclear function and its responsiveness to drugs may be achieved in a number of ways, including the investigation of isolated nuclei. In an ideal isolation, the nuclei would be free from non-nuclear contamination, of an unaltered appearance, unchanged in chemical composition, obtained in high yield, and would retain their full biological activity. Nuclear isolation is most commonly carried out in aqueous media by various modifications of the technique of Chauveau *et al.* [1], in which the tissue is homogenized in solutions of sucrose containing divalent cations, followed by centrifugation of the homogenate through sucrose solutions of various densities. The disadvantages of this, and indeed of any procedure for aqueous isolation, are the losses of small ions, metabolites, coenzymes and soluble proteins and the possible redistribution of cellular components [2–5]. An alternative is the use of non-aqueous solvents, as, for example, in the method of Kirsch *et al.* [6]. With this method, powdered lyophilized tissue is disrupted mechanically in anhydrous glycerol at 4° and centrifuged to provide intact, lyophilized nuclei. The rationale behind a non-aqueous isolation of nuclei is the possibility that water-soluble proteins would be lost during an aqueous isolation [7, 8] and that cytoplasmic constituents including proteins would be taken up by the nucleus. Obviously a procedure which prevents losses and cross-contamination would be of benefit for nuclear analysis of almost any kind. Translocation may be prevented by rapid freezing of the tissue, followed by lyophilization, before homogenization in a non-aqueous solvent.

Liver cells can be classified into two types, stromal and parenchymal. The parenchymal nuclei of rat liver exhibit polyploidization which increases in extent with age [9]. The nuclear types are separable, by virtue of their content of DNA and size difference, by density gradient centrifugation [10, 11]. The study of each nuclear type separately is preferable to the study of a mixed nuclear population.

The most commonly used solute for density gradient centrifugation, in aqueous media, is sucrose. This solute has three disadvantages: (1) the ability to penetrate cell membranes; (2) a high viscosity in solution; and (3) a limited density range. One compound which overcomes these disadvantages is *N*-(2,4,6-tri-iodo, 3-*N*-methyl acetylamino, 5-acetylamino benzoyl) glucosamine, which has been given the trivial name of metrizamide [12]. It is an inert substance, of value in whole body radiological investigations. Until the present time this solute has been used in density gradients only in aqueous solution experiments. We have shown that it can be used in solution in non-aqueous solvents to form gradients for centrifugation. Also we have initiated the use of propane-1,3-diol as a solvent, which has the advantage of a lower viscosity than glycerol. Density gradients can be formed by mixing the diol with 3-chloro-1,2-propanediol.

MATERIALS AND METHODS

Preparation of isolated rat liver nuclei. Male Sprague–Dawley rats, usually weighing 250 g, were killed by cervical dislocation, and their livers were immediately excised and immersed in isopentane chilled to −160° in liquid nitrogen. The frozen liver tissue was freeze-dried at −50°. The dry tissue was finely powdered under a nitrogen atmosphere, using a pestle and mortar chilled in solid CO_2 suspended in acetone, and subsequently stored at 40° over P_2O_5 as dessicant. Analar glycerol, which was used in all the experiments described in this paper, was dried before use by gentle heating, with stirring, to 160° followed by cooling and storage over P_2O_5. The dried liver powder was resuspended in anhydrous glycerol, in the ratio of 0.5 g powder to 30 ml glycerol, in a dry atmosphere, at 4°, for approximately 18 hr to allow even suspension of the

liver tissue. Homogenization was achieved with the Polytron PT10, at a setting of 3.0 for 5 min. The homogenate was forced in a syringe through a double layer of Nyebolt Boulting cloth (10–132 gauge) and centrifuged at 120,000 g_{av} for 1 hr at 4° using a Beckman SW 50.1 rotor in the Beckman L2-65B ultracentrifuge. The pellet either was used as a source of nuclei or was purified further by resuspension by gentle stirring with a glass rod in a dry atmosphere in glycerol–3-chloro-1,2-propanediol (9:1, v/v) (Fluorochem Ltd., Glossop, Derbyshire, U.K.; purified by vacuum distillation) or glycerol–metrizamide (Nyegaard & Co. A. S., Oslo, Norway) and then repeating the above centrifugation. The aqueous method used was that of Widnell and Tata [13].

Nuclear morphology. The quality of the nuclear preparation was judged by examination of the nuclear pellet, resuspended in glycerol, with the Wild M20 phase contrast microscope. Photographs were taken with a Polaroid camera attachment. Further structural evaluation was carried out by electron microscopy, and two methods of fixation were employed, hereafter referred to as methods 1 and 2 respectively. In the first, samples of the pellets were incubated in anhydrous absolute ethanol at 4° for 30 min, rehydrated in aqueous ethanol solutions of 90, 70, 50 and 25% (v/v) and finally distilled water and incubated in 5% glutaraldehyde, 0.2 M sodium cacodylate buffer (pH 7.0) at 4° for 90 min. The nuclei were rinsed and incubated in 2% osmium tetraoxide, 0.2 M sodium cacodylate buffer (pH 7.0) at 4° for 2.5 hr. Samples were subsequently dehydrated, embedded, cut and viewed. In the second method, nuclei were fixed with half-strength Karnovsky solution [14], a formaldehyde–glutaraldehyde fixative of high osmolarity, at pH 7.2, plus 1% cetyl pyridinium chloride for 60 min. They were washed in sodium cacodylate buffer (pH 7.2) for 5 min, with two changes, and incubated in 1% osmium tetroxide, sodium cacodylate buffer (pH 7.2) for 60 min. Dehydration in ethanol solutions of 25, 30, 50, 70 and 90% (v/v) was then carried out followed by staining with 1% uranyl acetate in 100% ethanol. The preparation was rinsed in dried absolute ethanol and embedded in Spurr's resin and subsequently cut and viewed.

Biochemical determinations. Proteins were determined by the method of Lowry *et al.* [15] with crystallized bovine serum albumin as the standard. DNA was estimated by the method of Burton [16] using calf thymus DNA as the standard. For the measurement of RNA, washing with 0.2 N perchloric acid, as used in the DNA assay, was performed. The pellet was incubated in 0.3 N potassium hydroxide at 37–40° for 1 hr; the mixture was cooled and then centrifuged. The extinction of the supernatant fraction was read, with suitable dilution, at 260 nm. Estimations were based on a concentration of 32 μg RNA/ml, giving an optical density of 1. Nuclear numbers were estimated using the Coulter Counter model F with an aperture diameter of 100 μm and settings of 0.5, 32 and 5.0 for attenuation, aperture and threshold respectively. Concentrations of metal ions was carried out on nuclei resuspended in aqueous media, using the Perkin–Elmer model 305 atomic absorption spectrophotometer. Glucose-6-phosphatase activity was measured by the method of Hers and de Duve [17] at pH 6.3. After a 10-min incubation at 37°, the release of inorganic phosphate from 0.05 M glucose-6-phosphate was estimated by the colorimetric method of Fiske and Subbarow [18]. The enzymatic activity was expressed as μmoles phosphorus liberated/min/mg of DNA. NAD pyrophosphorylase activity was estimated by the method detailed in the Boehringer–Mannheim Biochemica Catalogue (15152 ENAF). The enzymatic activity was expressed as μmoles product/hr/mg of DNA. RNA polymerase activity, of both A and B enzymes, was determined by measuring the incorporation of [^{3}H]UTP into acid-precipitable RNA in the assay used by Austoker *et al.* [19]. DNA polymerase activity was measured by estimation of the incorporation of [^{3}H]TTP into acid-precipitable DNA in the manner of Holmes [20].

Extraction of nuclear proteins. All operations prior to dialysis were conducted at 4° and samples were immediately frozen in liquid nitrogen. All solutions used contained 0.1 mM phenylmethylsulfonyl fluoride as a protease inhibitor. The purified nuclear pellets were suspended in 2 ml of 0.14 M NaCl and centrifuged at 5000 *g* for 5 min. This was repeated twice and the combined supernatant fractions were taken as the 0.14 M NaCl-soluble proteins. The non-histone proteins were then extracted from the nuclear residue by suspension in 2 ml of 8 M urea (freshly deionized by the use of A. R. Amberlite monobase resin), containing 1.4 mM 2-mercaptoethanol, and homogenization using the Polytron PT10 at a setting of 1.5 for 10–15 sec. This extraction was repeated and the combined supernatant fractions were considered as the non-histone chromosomal proteins. Histones were extracted from the remaining pellet by resuspension in 1 ml of 0.25 N HCl and centrifugation at 3000 *g* for 5 min. The process was repeated and the supernatant fractions were combined to provide the histone fraction. The sediment was finally extracted by boiling in 1 ml of 5% (sodium dodecyl sulphate (SDS) containing 0.01 M sodium phosphate, pH 7.4, for 5 min. After protein estimations had been carried out, all the samples were diluted to a concentration of 1 mg/ml with dialysis buffer and 1 ml of this sample was dialyzed against 500 ml of 0.01 M sodium phosphate, pH 7.4, 0.1% (w/v) SDS, 10% (w/v) glycerol and 0.14 M 2-mercaptoethanol, for 20 hr with three changes of buffer. The samples were then boiled for 3 min, centrifuged at 8000 *g* for 3 min, and aliquots taken for gel electrophoresis.

Electrophoresis of proteins. Gels were composed of 10% acrylamide, 0.2% *N,N'*-methylene bisacrylamide, purified according to the methods of Loening [21], dissolved in 0.1 M sodium phosphate, pH 7.4, containing 0.1% SDS, and were poured in to Pyrex glass tubes, 12 cm long and 6 mm internal diameter. Polymerization was accomplished by the addition of 0.03 ml of *N,N,N',N'*-tetramethyl-ethylenediamine (TEMED) and 0.3 ml of 10% ammonium persulfate (w/v) to 40 ml of the acrylamide solution. Gels were poured and over-layered with buffer to a height of 2–3 mm. Polymerization was allowed to proceed for 60 min, and the gels were then drained of the buffer layer. Stacking gels of a 3.5% acrylamide solution were then poured on top of the 10% gels, to a height of 0.5 cm, layered with buffer as before, and polymerized for 60 min before being topped up with buffer, covered and left overnight.

Samples of the various protein fractions were applied to gels, each sample containing approximately 200 μg protein in 200 μl of 0.1 M sodium phosphate, pH 7.4,

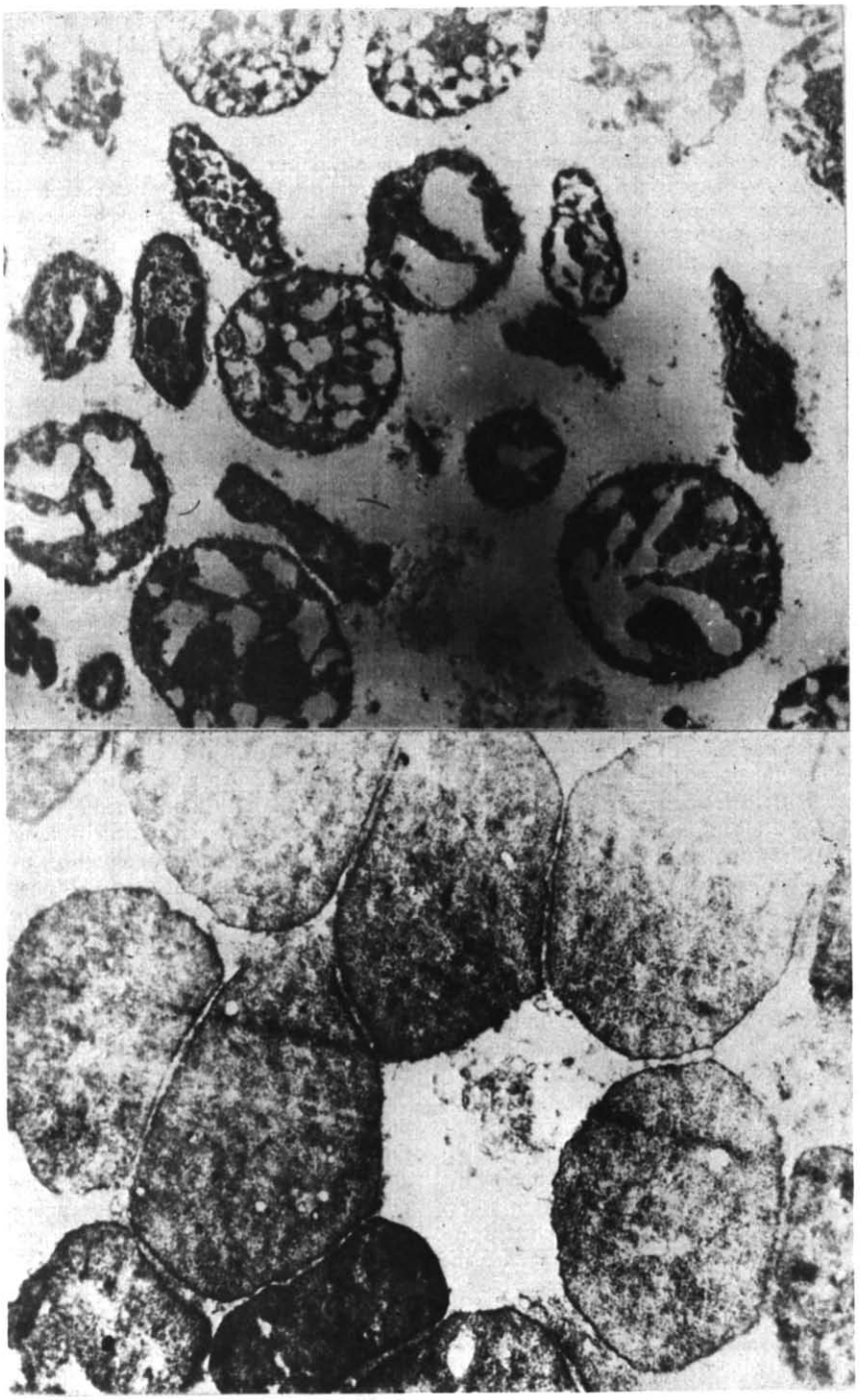

Fig. 1. Electron micrographs of nuclei isolated from freeze-dried rat livers using anhydrous glycerol. Top, nuclei stained by method 1. Bottom, nuclei stained by method 2. See text for details.

Table 1. Content of protein, DNA and RNA of nuclei isolated in anhydrous solvents

Nuclei purified in	Protein*	DNA*	RNA*	RNA/DNA	Yield†
Glycerol‡	85.4 ± 3.3	8.5 ± 0.6	2.2 ± 0.2	0.26	48 ± 2
3-Chloro-1,2-propanediol§	79.1 ± 3.7	8.8 ± 0.7	2.2 ± 0.4	0.25	45 ± 2
Metrizamide‖	82	10			40

* Data expressed as pg/nucleus ± S.E.M.
† Yield expressed as percentage of DNA in the original homogenate.
‡ Second centrifugation through glycerol alone; eight experiments.
§ Second centrifugation through glycerol–3-chloro-1,2-propanediol (85:15, v/v); eight experiments.
‖ Second centrifugation through glycerol–DMSO (1:1, v/v), with 19% metrizamide by weight; data from one experiment.

0.1% SDS plus 10% (w/v) glycerol and 15% of 0.2% (w/v) bromphenol blue as a tracer dye. Electrophoresis was carried out at 9–10 milliamps/gel for 7.5 hr. Gels were removed and stained in a solution of 0.2% (w/v) Coomassie Blue in H_2O–methanol–acetic acid (6:3:1, v/v) for 12 hr. After destaining in the above solvent mixture, the gels were scanned, using a Joyce–Loebel Transtab type D8 Mark 2 scanner connected to a Bryan's chart recorder, model 27000.

Centrifugation in non-aqueous density gradients. The nuclear pellet was resuspended in either a 1:1 (v/v) mixture of glycerol and dimethylsulfoxide (DMSO), a 1:1 (v/v) mixture of glycerol and dimethylsulfoxide containing 5% metrizamide by weight, or propane-1,3-diol (B.D.H. Ltd., Enfield, Middlesex, U.K.). Samples were layered on the appropriate gradient and centrifuged using a Beckman SW 40 Ti rotor, with 14-ml capacity polyallol tubes, on the Beckman LZ-65B ultracentrifuge at 4°. The shape of the gradient was checked by the addition of a dye, usually methylene blue, to the heavy medium of the gradient and subsequent monitoring in a flow cell at 570 nm. After centrifugation, the gradients were fractionated in aliquots of approximately 1.5 ml from bottom to top, using a MSE gradient recovery system and a peristaltic pump to withdraw the tube contents. The number of nuclei per fraction and the proportions of the nuclear types therein were estimated with the Improved Neubauer haemocytometer under phase contrast microscopy.

RESULTS

Phase contrast microscopy of nuclei, isolated by the method of Kirsch *et al.* [6] by centrifugation solely through glycerol, showed a continuous nuclear membrane, essentially free of cytoplasmic tabs. When the nuclei were resuspended in glycerol and centrifuged through a 9:1 (v/v) mixture of glycerol and 3-chloro-1,2-propanediol, there appeared to be slight clumping of the chromatin. Nucleoli were clearly visible. To gain detailed information regarding the morphology of nuclei isolated in non-aqueous solvents, it is necessary to examine them by electron microscopy. It must be acknowledged that the transfer of the nuclei to an aqueous environment during the staining procedure introduces the possibility of morphological changes. Method 1 (Fig. 1) revealed nuclei containing large clear areas, particularly in the bigger nuclei, while method 2 (Fig. 1) revealed nuclei of virtually uniform granularity with the nucleoli barely discernible. The nuclei are bounded by a membrane and appear free of cytoplasmic tabs. The chemical characteristics of nuclei isolated in glycerol alone are summarized in Tables 1 and 2. An additional centrifugation through glycerol, 3-chloro-1,2-propanediol or solutions of glycerol in metrizamide made little difference in the data (Table 1). The activities of a number of enzymes were examined, including glucose-6-phosphatase as a microsomal marker and NAD pyrophosphorylase as a nuclear marker. The results are given in Table 3.

The purity of the nuclei, manifested by microscopy, is confirmed by the very low levels of the microsomal enzyme, glucose-6-phosphatase, that are found in the nuclear fraction. The bulk of the NAD pyrophosphorylase, regarded as a nuclear enzyme, appears to reside in the nuclei. Two enzymes were examined as a measure of nuclear biochemical viability, namely the RNA and DNA polymerases. Comparisons of nuclei isolated in aqueous sucrose and in glycerol showed that RNA polymerase A activity was high and similar in both preparations but that RNA polymerase B activity was

Table 2. Content of cations of nuclei isolated in glycerol*

Ion	Concn
Na^+	173 (165)
K^+	561 (410)
Ca^{2+}	99 (9)
Mg^{2+}	63 (54)

*Concentrations are expressed as μmoles/g dry weight. Published results [22] are given in parentheses. For the determination of dry weight, a sample of the nuclei, resuspended in double-distilled water, was taken to dryness in an oven at 110°.

Table 3. Enzymic activities of whole tissue and nuclei isolated in glycerol*

Enzyme	Nuclei	Whole tissue
Glucose-6-phosphatase†	0.14 ± 0.01	7.56 ± 0.24
NAD-pyrophosphorylase‡	2.43 ± 0.2	2.59 ± 0.04

*Mean values and S. E. M. were calculated for five experiments. The activities were measured on suspensions of the samples in double-distilled water. Ninety per cent of the activity in the original homogenate was recovered in the post-nuclear supernatant fraction after recovery of the nuclei.
† Activity expressed as μmoles phosphate/min/mg of DNA.
‡ Activity expressed as μmoles product/hr/mg of DNA.

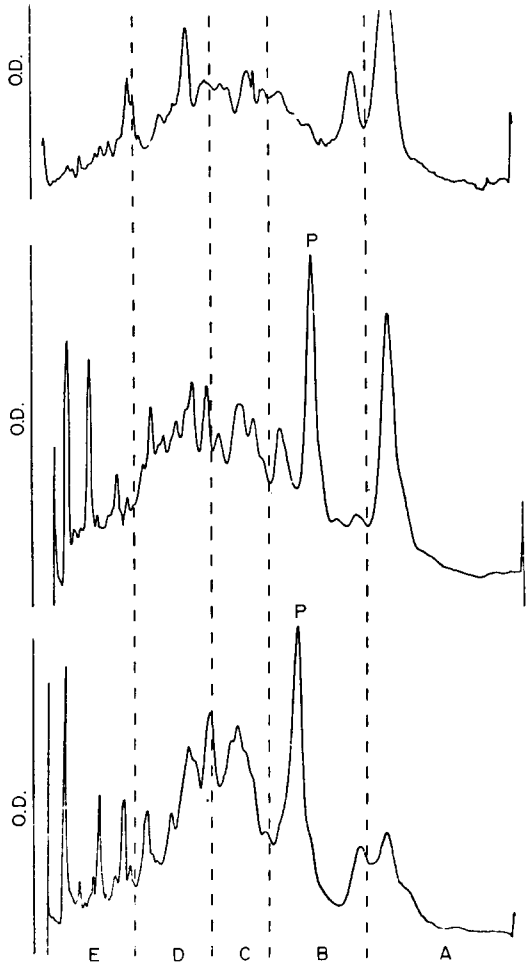

Fig. 2. Densitometer traces of the soluble proteins from rat liver nuclei separated by gel electrophoresis and stained with Coomassie Blue. The direction of migration is from right to left. Top, nuclei isolated in aqueous solvents (AN); centre, nuclei isolated in glycerol (NAG); and bottom, nuclei isolated in glycerol with an additional purification using 3-chloro-1,2-propanediol (NACk).

somewhat depressed in non-aqueous nuclei. The glycerol nuclei contained DNA polymerase activity about eight times that of the aqueous nuclei. This agrees with results published by Foster and Gurney [23].

The results in Table 1 indicate that one of the main differences between nuclei prepared in aqueous solvents and those isolated under anhydrous conditions is the substantially higher amounts of protein in the non-aqueous nuclei. Whereas the protein:DNA ratio is about 4:1 in aqueous nuclei, the value in nuclei prepared in glycerol is about 10:1. To investigate this in greater detail several types of nuclei were compared, as follows: (1) nuclei isolated by the aqueous procedure of Chauveau *et al.* [1], called the aqueous nuclear sample (AN); (2) nuclei isolated by the glycerol-based technique described and referred to as the non-aqueous glycerol sample (NAG); and (3) nuclei isolated as in (2) above but with an additional centrifugation through an 85:15 (v/v) mixture of glycerol and 3-chloro-1,2-propanediol. This is known as the NACk sample. The

Table 4. Content of protein of nuclei prepared in aqueous or organic solvents*

Fraction	AN	NAG	NACk
Soluble	1.56 ± 0.3	8.57 ± 1.43	7.8 ± 0.4
NHC†	1.86 ± 0.14	3.12 ± 0.48	3.2 ± 0.9
Histones	0.96 ± 0.07	0.97 ± 0.14	1.1 ± 0.28
Residual	0.33 ± 0.06	0.68 ± 0.19	0.77 ± 0.2

*Results are expressed as mg/10^8 nuclei and are mean values (±S. E. M.) from five experiments. Abbreviations: AN, nuclei prepared in aqueous sucrose; NAG, nuclei isolated in glycerol; and NACk, nuclei isolated in glycerol and 3-chloro-1,2-propanediol.
†NHC, non-histone chromsomal proteins.

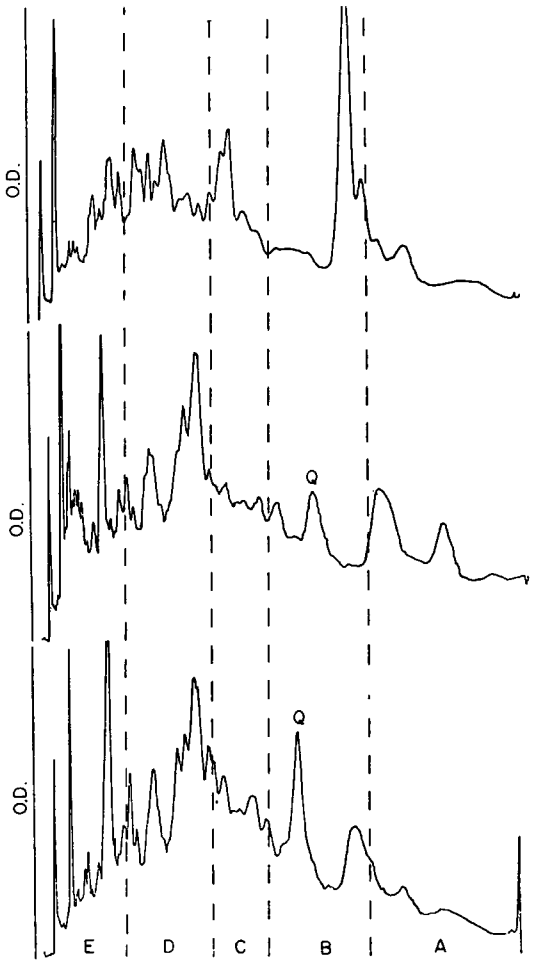

Fig. 3. Densitometer traces of the non-histone chromosomal proteins from rat liver nuclei separated by gel electrophoresis and stained with Coomassie Blue. The direction of migration is from right to left. Top, nuclei isolated in aqueous solvents (AN); centre, nuclei isolated in glycerol (NAG); and bottom, nuclei isolated in glycerol with an additional purification using 3-chloro-1,2-propanediol (NACk).

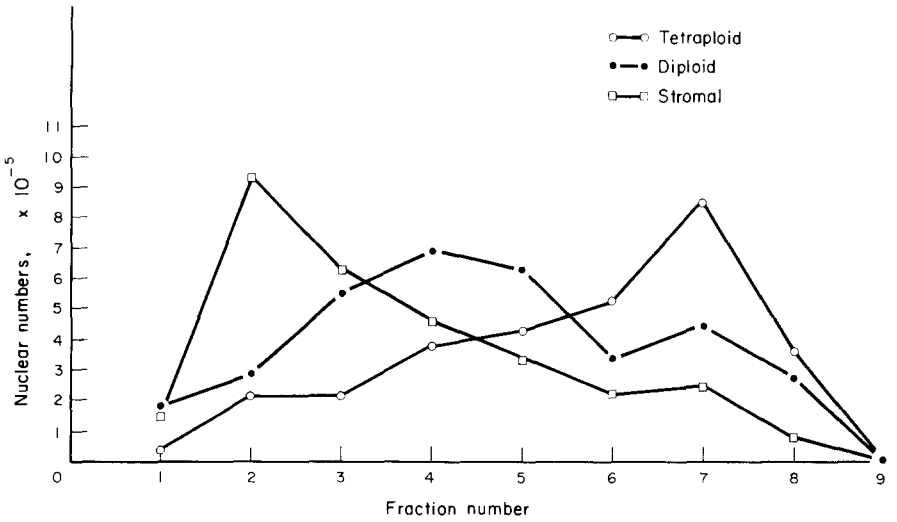

Fig. 4. Distribution of different types of rat liver nuclei after centrifugation on a linear gradient (0–20%, w/v) of metrizamide in glycerol–dimethylsulfoxide (1:1, v/v) for 2 hr at 4° and 2500 rev/min. The top of the gradient is at the left. The sample containing 9.6 × 10^6 nuclei was loaded in 0.4 ml glycerol–DMSO.

amount of each class of protein extracted from each of these preparations is given in Table 4. It will be noted that isolation in glycerol produces nuclei with about double the amount of non-histone proteins and more than five times as much soluble protein, whereas the amounts of histones are virtually the same. The various categories of protein were electrophoresed as described in Materials and Methods. The gel profiles of the histones were identical for all three classes of nuclei. Although there was rather more residual protein in the non-aqueous nuclei, the profiles did not differ to any appreciable extent. It is with the soluble and non-histone chromosomal proteins that the largest differences appear. Scans of the soluble proteins are represented in Fig. 2 and the non-histone chromosomal proteins in Fig. 3. From the gel profiles of the soluble protein fraction, it can be seen that both NAG and NACk samples display a greater number of peaks in the high molecular weight range and one major peak (labeled P), with a molecular weight of about 29,000 which is missing from the AN sample. For the non-histone chromosomal proteins, the NAG and NACk preparations contain more high molecular weight peaks and one particular peak (labeled Q) of about 30,000 daltons which is not seen with the AN sample. The latter contains two peaks of about 19,500 and 22,000 which are virtually absent in the other preparations. When run in parallel slots on the slab gels, it is apparent that P and Q are not the same protein. The proteins of nuclei isolated under anhydrous conditions in organic solvents obviously merit much more detailed investigation.

Glycerol, although possessing many advantages as a medium for the suspension of nuclei, suffers from a high viscosity. This may be reduced by the addition of another inert solvent, dimethylsulfoxide. Many sub-

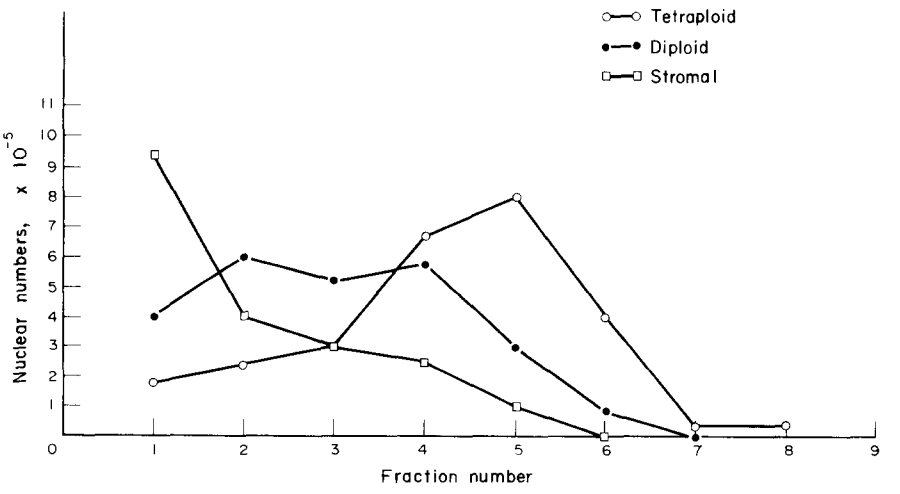

Fig. 5. Distribution of different types of rat liver nuclei after centrifugation in a concave gradient of metrizamide in glycerol–dimethylsulfoxide (1:1, v/v) ranging from 5 to 20% (w/v) at 3200 rev/min for 30 min at 4°. Sample, 7.7 × 10^6 nuclei suspended in 0.4 ml.

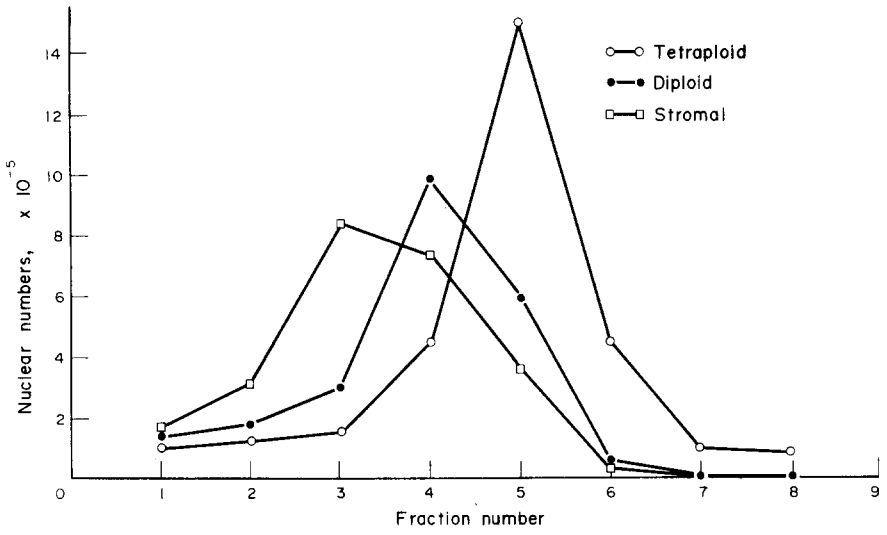

Fig. 6. Distribution of different types of rat liver nuclei after centrifugation in a concave gradient of 3-chloro-1,2-propanediol in propane-1,3-diol ranging from 0 to 50% (v/v) at 3000 rev/min for 40 min at 5°. Sample, 7×10^6 nuclei in 0.4 ml propanediol.

stances, both solid and liquid, were examined for non-aqueous fractionation of nuclei. Trifluorethanol, although promising in many ways, had to be abandoned because it caused clumping of nuclei. Metrizamide proved to be one of the most useful.

Isolated nuclei, resuspended in a 1:1 (v/v) mixture of glycerol and dimethylsulfoxide, were layered over a linear gradient of 0–20% (w/v) metrizamide in the same medium. A sample of 0.4 ml composed of 10^7 nuclei, which contained roughly one-third tetraploid parenchymal nuclei, one-third diploid parenchymal nuclei and one-third diploid stromal nuclei, was layered over the gradient, which was centrifuged at 2500 rev/min for 2 hr. Fractions were collected and nuclear numbers and type determined. The profile obtained is illustrated in Fig. 4. A partial resolution has been achieved. Fraction 2 contains two-thirds diploid stromal nuclei. In fraction 4 nearly half the nuclei are diploid parenchymal, and in fraction 7 more than 90% are tetraploid nuclei. In an attempt to improve the separation, a concave gradient ranging from 5 to 20% (w/v) metrizamide in a 1:1 (v/v) mixture of glycerol and dimethylsulfoxide was employed. Centrifugation was at 3200 rev/min for 30 min in a Beckman SW 40 Ti rotor at 4°. Fractions were collected and examined as before and the resultant profile is illustrated in Fig. 5. Fraction 1 contains two-thirds diploid stromal nuclei. Fraction 3 consists of 45 per cent diploid parenchymal nuclei and fraction 5 contains 67 per cent tetraploid nuclei. Nearly 80 per cent of the nuclei in fraction 6 are tetraploid. The identification of the nuclei in these and other experiments involving fractionation depends on both microscopy and measurements of the content of DNA/nucleus. The nuclei in the tetraploid region of the

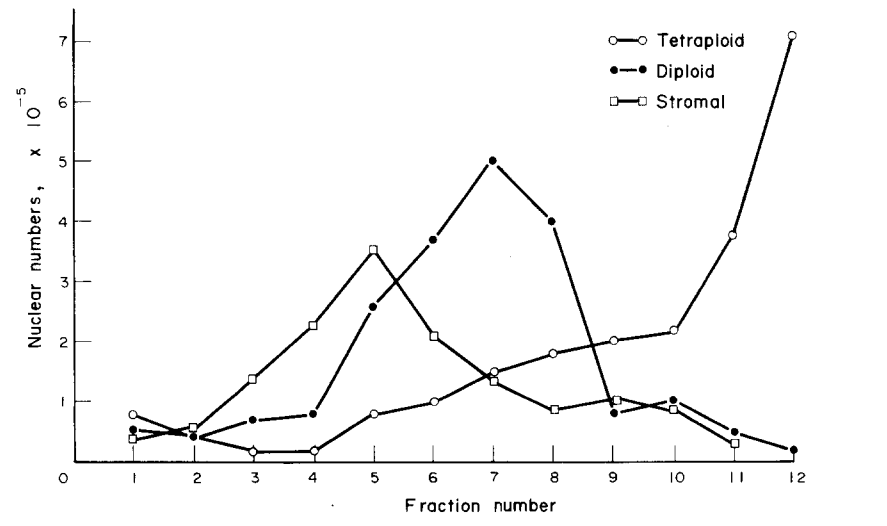

Fig. 7. Distribution of different types of rat liver nuclei after centrifugation in a concave gradient of 3-chloro-1,2-propanediol in propane-1,3-diol ranging from 0 to 50% (v/v) at 3200 rev/min for 70 min at 5°. Sample, 6×10^6 nuclei in 0.14 ml propanediol.

gradient contained approximately twice the amount of DNA as those in the diploid fraction. Exposure to metrizamide gradients caused only a small loss of activity of DNA and RNA polymerases.

An alternative to metrizamide was investigated. Gradients constructed with propane-1,3-diol and 3-chloro-1,2-propanediol were examined. Propane-1,3-diol has the advantage that it is considerably less viscous than glycerol. Centrifugation was carried out for 40 min (Fig. 6) and 70 min (Fig. 7) in a Beckman SW 40 Ti rotor maintained at 4°. The increased centrifugation time enhances nuclear separation, and fractions 4 and 5 are two-thirds diploid stromal. Fraction 7 is two-thirds diploid parenchymal. Ninety-five per cent of the nuclei in fraction 12 are tetraploid.

DISCUSSION

In the present paper, we have described a modification to the method of Kirsch *et al.* [6] of isolating nuclei from freeze-dried stored rat livers. The degree of preservation of nuclear structure and function was evaluated by ultra-structural studies and biochemical determinations, and the suspension of isolated nuclei was further fractionated on non-aqueous gradients of various shapes and compositions.

Light microscopy of nuclei isolated in glycerol showed nuclei with reasonable contours, pronounced nucleoli and a spherical shape, generally. Unfortunately, electron microscopy was of less value in the further morphological characterization of nuclei. It is recognized that the pH, ionic strength, specific ionic composition, dielectric constant, temperature, length of fixation and method of application of the fixative solution are critical factors in determining the quality of tissue fixation. In addition, a fixative may have a damaging effect on the structure of an organelle [24]. Thus, bearing in mind the evidence of phase contrast micrographs, it seems that the clear areas observed in Fig. 2 are artifacts.

The isolated nuclei contained more protein than those isolated in conventional sucrose media. There are two possible explanations for this. First, there may be contamination by cytoplasm. Second, the non-aqueous nuclei may retain certain soluble molecules including proteins. The micrographs and the studies of marker enzymes show that the first explanation is untenable. Thus, the high protein must reflect the prevention of loss or movement of macromolecules during the isolation procedure.

The inorganic ion estimations agree with published results, with the exception of the value obtained for the Ca^{2+} concentration. Siebert and Humphrey [22] have shown that the concentrations, in micro-equivalents/g dry weight, of Ca^{2+} are roughly equivalent in cytoplasm and nuclei. Therefore, it appears unlikely that the high Ca^{2+} concentration that we have found is due to cytoplasmic contamination. The presence of RNA and DNA polymerase activities indicates that the nuclei have retained their biochemical integrity.

In the various protein fractions extracted from the non-aqueous nuclei, there are greater amounts of high molecular weight material than there are in the aqueous nuclear samples. This may reflect a tendency for losses of all types of protein from aqueous nuclei. Perhaps the most interesting point to emerge from the protein scans is the presence of a very distinct protein peak, labeled P and Q in the soluble and non-histone chromosomal fractions, respectively, in the non-aqueous nuclear samples and which is not present in the corresponding aqueous nuclear sample (Figs. 3 and 4).

The separation of the three main types of liver nuclei was satisfactory in metrizamide gradients but was most effectively achieved on a concave gradient composed of propane-1,3-diol and 3-chloro-1,2-propanediol. This gradient type was not an arbitrary choice but a progression from discontinuous density gradients through linear and concave gradients and embraced a study of many different organic solvents and solutes.

The separation that we have achieved could provide a means of investigation of the biochemical properties of the different nuclear types, in which the nuclei have been in a non-aqueous environment from the excision of the liver to the actual investigation of the nuclear type itself, and offers a prospect for detailed investigation of drugs that act at the level of the nucleus. It would circumvent the problem of loss of the drug or its metabolites from the nucleus, provided that these were not soluble in organic solvents.

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