The Fractionation of Nuclei from Mammalian Cells by Zonal Centrifugation

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1. Purified liver nuclei from adult rats separate into two main zones when centrifuged in the slow-speed zonal rotor. One zone contains diploid nuclei, the other tetraploid. 2. The effect of age on the pattern of rat liver ploidy was examined. Tetraploid nuclei are virtually absent from young animals. They increase in proportion steadily with age. Partial hepatectomy disturbs the pattern of ploidy. 3. The zonal centrifuge permits the separation of diploid, tetraploid, octaploid and hexadecaploid nuclei from mouse liver. 4. Rat liver nuclei are isopycnic with sucrose solutions of density 1.35 at 5°.

The liver is a complex organ. The main functional units are the parenchymal cells. These are arranged in plates or cords around a network of venous capillaries (called sinusoids) supported by reticular fibrils (Harkness, 1958). The bile canaliculi pass between the parenchymal cells and link up to form the tree of bile ducts. The cellular elements may be divided into two categories: those within the lobules and those in the interlobular spaces (Daoust, 1958). The first group comprises the parenchymal cells and littoral cells. The term 'littoral' describes the cells lining the blood sinusoids. They include the Kupffer cells, which are part of the reticulo-endothelial system, and a mixture of so-called undifferentiated cells (Abercrombie & Harkness, 1951). In the interlobular spaces are bile-duct cells, connective-tissue cells and cells of blood-vessel walls. In normal adult rat liver parenchymal cells form approx. 60% of the cell population, though they constitute 90% of the mass (Fukuda & Sibatani, 1953). The littoral cells account for 33% of the total cells. It has long been known that the parenchyma of rodents is a polysomatic tissue (Jacobj, 1925; Beams & King, 1942; Swift, 1950; Epstein & Gatens, 1967). The non-parenchymal cells are diploid. Rat liver is one of the most commonly used materials for biochemical research. The heterogeneity in type of cell and degree of ploidy is a major complication in the interpretation of biochemical experiments involving isolated nuclei. We have sought methods for the separation of cell nuclei into different classes according to their size by using the zonal centrifuge developed by Anderson and his colleagues (Anderson, 1966*a,b*). Falzone, Barrows & Yiengst (1962) studied the settling of rat liver nuclei in a density-gradient column under gravity. The sedimentation of nuclei in low centrifugal fields has been described by

Albrecht (1968). An investigation under isopycnic conditions has been reported by Fisher, Holbrook & Irvin (1963).

MATERIALS AND METHODS

Animals. Norwegian Hooded rats fed *ad libitum* on Purina chow were used. The mice were albinos of the N.I.H. strain.

Isolation of nuclei. The nuclei were prepared from rat and mouse tissues by a modification of the method of Widnell & Tata (1964). Preliminary experiments indicated that it was essential to adjust the pH of all solutions containing sucrose to 7.4 if crenellation and clumping of the nuclei was to be avoided. Sufficient saturated NaHCO₃ solution was added to raise the pH to 7.4. The final concentration was approx. 1 mM. The medium for homogenization was 0.32 M-sucrose, 3 mM with respect to MgCl₂, adjusted to pH 7.4. The nuclei were purified by centrifugation through 2.4 M-sucrose, 1 mM with respect to MgCl₂, pH 7.4. At least three animals were used in all experiments. The tissues from individual animals were pooled. The yield of nuclei based on the recovery of DNA was about 60% in most experiments. Most of the losses arose from incomplete breakage of cells.

Zonal centrifugation of nuclei. All runs were carried out at 5°. A gradient of sucrose (1000 ml.) ranging from 20 to 50% (w/w), 1 mM with respect to MgCl₂, pH 7.4, and linear with respect to volume, was introduced into the MSE A-XII rotor (Measuring and Scientific Equipment Co. Ltd., London, S.W. 1) while it was running at 600 rev./min. in an MSE Mistral centrifuge. The pumping speed was 50 ml./min. (Hiloflow pump; F. A. Hughes and Co. Ltd., Epsom, Surrey). This was followed by an underlay of 55% (w/w) sucrose. The sample of nuclei examined in the zonal centrifuge was equivalent to approx. 6 g. wet wt. of tissue in the experiments with liver. The sample (10–20 ml. in 15% sucrose–1 mM-MgCl₂, pH 7.4) was introduced through the core line, followed by an overlay of 10% (w/w) sucrose until the leading edge of the sample was 6 cm. from the axis of rotation. A stroboscopic lamp (3DP; E.M.I. Electronics Ltd., Hayes, Middx.) was used to observe the

positions of zones against a scale marked on the underside of the rotor. This was illuminated from below by a lamp mounted under a translucent Perspex screen, fitted into the bottom of the bowl of the centrifuge. The stroboscopic lamp permitted an exact determination of rotor speed. Most runs were of 1 hr. duration. The contents of the rotor were displaced by pumping in more underlay. Displacement lasts for 26 min. The effluent was passed through a flow cell of 1 cm. path length in a Gilford 2000 extinction meter and monitored at 600 m μ . The cell compartment was cooled to 7–8°. The effluent was collected either manually in large fractions selected according to extinction, or in 100 13 ml. fractions in a Chromofrac fraction cutter (Baird and Tatlock Ltd., Chadwell Heath, Essex) modified to give a 15 sec. interval between changes. The tubes used for collecting fractions were cooled in ice-cold water.

Determination of nuclear volumes. Nuclei were recovered from the effluent from the zonal rotor at the end of a run by combining the appropriate fractions and diluting with an equal volume of 0.32 M-sucrose, 3 mM with respect to MgCl₂, pH 7.4. The nuclei were sedimented by centrifugation at 700 g for 5 min. at 4° and resuspended in a small volume of 0.32 M-sucrose. A drop of the suspension was placed in a Thoma haemocytometer chamber. The nuclear diameters were measured under oil immersion by using a phase-contrast microscope (Wild M20; Heerbrugg, Switzerland) fitted with a Baker eye-piece micrometer. One small division of the eye-piece corresponded to 0.088 μ at an overall magnification of $\times 1000$. Both major and minor axes were measured of prolate-spheroidal nuclei. Their volumes were calculated from the formula:

$$\text{Volume} = \frac{\pi ab^2}{6}$$

where a is the length of the major axis and b the length of the minor axis. For most fractions a minimum of 200 nuclei were measured. Calculations of the volumes of both spherical and ellipsoidal nuclei were done with an Olivetti Programma 101 programmed to accept the eye-piece readings.

Nuclear counts. The nuclei in fractions obtained directly from the zonal rotor were counted in a Coulter counter after suitable dilution (1 in 100–300) with 0.32 M-sucrose 0.16 M with respect to NaCl. Nuclei recovered from zonal fractions were counted in a haemocytometer chamber or in a Coulter counter.

Chemical analyses. DNA was measured by the method of Burton (1956) as described by Widnell & Tata (1964).

Assay of RNA polymerase. The activity of the DNA-dependent RNA polymerase of the nuclei was assayed in the presence of (NH₄)₂SO₄ and Mn²⁺ as described by Widnell & Tata (1966). The regions of the gradient corresponding to the diploid and tetraploid zone were collected in eight fractions. The nuclei were recovered and assayed immediately with [5-³H]UTP (ammonium salt, 1860 μ C/ μ mole; The Radiochemical Centre, Amersham, Bucks.). The labelled RNA was isolated and counted by the method of MacGregor & Mahler (1967).

RESULTS

The chart obtained when nuclei prepared from adult Norwegian Hooded rats weighing 300 g. were examined is shown in Fig. 1. The data are replotted in Fig. 2 on specially drawn graph paper that has a linear scale of distance from the axis of rotation. The slope of the extinction scale takes into account

Fig. 1. Chart of the light-scattering profile obtained on emptying the rotor after running a sample of nuclei from adult rat liver at 600 rev./min. for 60 min. at 5°. The direction of sedimentation is from left to right. The chart speed was 0.5 in./min. The horizontal bar is equivalent to 1 in. of chart paper. The first peak on the left is at 6 cm. from the axis of rotation. The ordinate gives extinction at 600 m μ . Full-scale deflexion is equivalent to E 0.2. The abscissa is linear with respect to volume of contents of rotor. The small peak on the extreme right is caused by the interface between the underlay and the end of the gradient.

Fig. 2. Data from Fig. 1 replotted with a linear scale of distance from the axis of rotation. The height of the extinction scale at any given radius is adjusted to maintain the actual area under peaks proportional to the amount of material present.

EXPLANATION OF PLATE 1

Photographs of unstained nuclei from rats weighing 160g. under phase contrast, obtained with a Zeiss Ultraphot fitted with a $\times 100$ Planapo Chromat oil immersion objective. (a) Unfractionated nuclei; (b) diploid zone (rotor effluent 195-245 ml.); (c) diploid zone (rotor effluent 245-315 ml.); (d) tetraploid zone (360-510 ml.).

Fig. 3. Histograms of the nuclear volumes of the nuclei from the two main zones shown in Figs. 1 and 2: zone 1 (effluent volume 210–350 ml.) and zone 2 (360–490 ml.). The horizontal bar on the ordinate represents ten nuclei.

Table 1. Values of the content of DNA and the volume of adult rat liver nuclei separated into zones by centrifugation

	Zone 1	Zone 2
Total nuclei	189 × 10 ⁶	117 × 10 ⁶
DNA per nucleus (μg.)	7.5 ± 0.1	14.7 ± 0.5
Corrected* DNA per nucleus (μg.)	7.5 ± 0.1	15.7 ± 0.5
Nuclear volume (μ ³)	200	410

* The correction allows for contamination of zone 2 by diploid nuclei.

the variation of zone volume with distance from the axis of rotation. This method of presenting the information obtained from the spectrophotometric traces gives a clearer and more realistic indication of the migration and separation of zones. The peak at 6 cm. marks the position of the sample band. A small amount of material, apparently mostly membranous, remains at this position. Part of the change in extinction represents the passage through the flow cell of the interface between the overlay and the gradient. The end of the gradient is also marked by a small peak. Two main zones of nuclei are seen. The fractions corresponding to each zone were combined. The nuclei were recovered and the nuclear volumes measured. The volumes are plotted as histograms in Fig. 3. The amount of DNA per nucleus was determined. The results for 300 g. rats are given in Table 1. These data demonstrate that the two well-separated zones contain predominantly diploid and tetraploid nuclei respect-

Fig. 4. Zonal profile of nuclei from adult rat kidney. All conditions were as for Fig. 1.

ively. Prolonging the duration of the centrifugation, though it increased the separation of the apices of each zone, resulted in broader peaks without appreciable decrease in the contamination of the tetraploid zone. The nuclei from the diploid and tetraploid zones appear in satisfactory condition when examined by phase-contrast microscopy (Plate 1). Zonal centrifugation removes virtually all the cytoplasmic contaminants present in the original nuclear pellet. For the purposes of photography the diploid zone was collected in two halves. The slower-moving fraction contains a higher proportion of nuclei that are appreciably smaller than those in the faster fraction and also considerable numbers of prolate spheroids. Presumably these arise from stromal cells.

The nuclei from rat kidneys are known to be diploid (Fraser & Davidson, 1953). Ten animals yielded 22.6 g. of kidney tissue. In the zonal centrifuge the nuclei formed one main zone (Fig. 4) in the same position as zone 1 from the liver. The nuclei were similar in size to the liver diploid nuclei. Very few nuclei of the tetraploid size were present. This experiment confirms that the liver nuclei in zone 1 (Figs. 1 and 2) are diploid in character.

The clear separation of diploid from tetraploid nuclei permitted an assay of the two classes of nuclei for their DNA-dependent RNA polymerase activity *in vitro*. The rats used for this experiment each weighed 300 g. The region of the two main zones was divided into eight fractions. The specific activity per 10⁹ nuclei was markedly higher in the tetraploid region. The total incorporation per 10⁹ nuclei in the diploid zone was 35 μμmoles, and in the tetraploid zone 393 μμmoles. This observation underlines the need for caution in interpreting experiments in which the effects of hormones or

Fig. 5. Zonal profile of rat liver nuclei from animals ranging from 4.5g. to 100g. weight. Experimental conditions were as in Figs. 1-3. The histograms of the nuclear volumes of the fractions indicated by the numbered horizontal bars are shown alongside the zonal traces.

Fig. 6. Zonal profile of rat liver nuclei from animals ranging from 150g. to 350g. weight. Experimental conditions were as in Figs. 1-3. The histograms of the nuclear volumes of the fractions indicated by the numbered horizontal bars are shown alongside the zonal traces.

physiological stress on the RNA polymerase activity are studied.

Effect of age on liver polyploidy. We have observed that the relative proportion of the diploid and tetraploid peaks varies with the age of the animals. The effect of age on polyploidy in rodent liver has been studied by caryometry (Nadal & Zajdela, 1966) and microphotometrically by using the Feulgen reaction (Alfert, 1958). In Figs. 5 and 6 are plotted the zonal patterns and, alongside, the corresponding histograms of the sizes of the nuclei from livers of animals ranging from 1 day to 12 months old. The numbers of rats and the total weights of the livers are given in Table 2.

Newborn rats have very few tetraploid nuclei. The tetraploid zone becomes distinguishable between the fourth and fifth week after birth and increases progressively until, in old rats (350 g.), it

forms the major band. The region of the gradient between 12 and 15 cm. radius in the run with these old rats contains few nuclei, but an appreciable proportion (25%) are octaploids. A distinct trend towards increased size is noticeable with age in both diploid and tetraploid nuclei. The diploid nuclei in young animals have a volume of approx. $120\mu^3$, which increases to $200\mu^3$ in older animals. This may reflect a change in the relative proportions of littoral and parenchymal cells in the diploid region (Falzone *et al.* 1962). The volume of tetraploid nuclei shifts from $350\mu^3$ in 100 g. rats to $450\mu^3$ in 350 g. rats.

Effect of partial hepatectomy. The median and left lateral lobes of the liver were removed from a number of rats each weighing 200 g. (Higgins & Anderson, 1931). The animals were allowed a period of regeneration of 24 hr. before being killed. The zonal profile of the nuclei from the regenerating tissue is shown in Fig. 7. The sizes of the nuclei found in the various zones are given in Fig. 8. Comparison of these values with those for 200 g. rats (Fig. 6) shows that in regenerating rat liver the diploid peak has split into two components (zones 1 and 2, Fig. 8) and there is an increase in the relative proportion of the tetraploid region (zone 3). Increased polyploidy after partial hepatectomy has been observed previously (Nadal & Zajdela, 1966; James, Schopman & Delfgaauw, 1966). An additional small peak (zone 4) is found beyond zone 3. Zone 1 contains approx. 45% of prolate spheroids. The nuclei are small, with a volume of approx. $80\mu^3$. Zone 2 has nuclei ranging in volume from 40 to $300\mu^3$. The non-spherical nuclei (24%) are mostly small. The spherical nuclei are similar in size to those from normal animals of the same weight. Zone 3 contains tetraploid nuclei, whereas in zone 4 some octaploid nuclei are found.

Table 2. Numbers of animals used in investigations of the effect of age on polyploidy in liver

Average wt. (g.)	No. of rats	Sex	Total wt. of liver (g.)	Age
4.5	12		1.3	24 hr.
25	9	F	9.8	3 weeks
50	9	2M, 7F	18.1	4 weeks
100	9	6M, 3F	48.3	5 weeks
150	5	M	18.8	7 weeks
200	3	M	25.7	13 weeks
300	3	M	24.1	6 months
350	2	M	19.0	12 months

Fig. 7. Zonal profile of rat liver nuclei prepared from 200 g. animals that had undergone partial hepatectomy 24 hr. before being killed.

Fig. 8. Histograms of nuclei taken from the zones indicated by the numbered horizontal bars in Fig. 7. The prolate spheroids are indicated by hatching.

Polyplody in mouse liver. Mouse liver is known to differ from that of rat in the presence of a considerable proportion of octoploid nuclei in both mono- and bi-nucleate cells (Epstein & Gatens, 1967). We have therefore examined the liver nuclei of albino mice of the N.I.H. strain. Twelve animals, of average weight 27g., gave 13.6g. of liver. Information from zonal experiments is shown in Figs. 9–11. In contrast with nuclei from adult rat liver, three peaks are seen. The first corresponds to diploid nuclei and is partially resolved into two components. The slower-moving component (zone 1A) contains nuclei, probably derived from non-parenchymal cells, of volume approx. $100\mu^3$ with about 20% of prolate spheroids. Zone 1B has nuclei of volume $150\mu^3$, all of which were spherical. These are parenchymal diploid nuclei. The tetra-

ploid region (zone 2, nuclei of volume approx. $320\mu^3$) is followed by a third zone containing nuclei with volumes approx. $700\mu^3$ (octaploids). Beyond this are found some nuclei with sizes expected of hexadecaploid nuclei.

Effect of recentrifuging. In our experiments with rat liver the tetraploid zone contained between 10 and 20% of diploid nuclei. We have examined the effect of re-running the separated diploid and tetraploid zones. The rats weighed 225g. each. The nuclei from each zone were resuspended in

Fig. 9. Chart of zonal centrifugation of liver nuclei from adult mice. Conditions were as for Fig. 1.

Fig. 10. Replot of data in Fig. 9.

Fig. 11. Histograms of the volumes of liver nuclei recovered from the fractions indicated by the numbered bars in Fig. 10.

Fig. 12. Effect of recentrifuging nuclear zones. The re-run of the rat liver nuclei from zone 1 is shown by vertical hatching. The re-run of nuclei from zone 2 is shown by horizontal hatching.

20ml. of 15% (w/w) sucrose and re-run separately. The results are shown in Fig. 12. Zone 1 on re-running gives a major zone that contains diploid nuclei, about 10% of which are prolate spheroids, and no detectable tetraploid nuclei, and in addition a very small tetraploid zone. The diploid nuclei obtained on re-running zone 2 are somewhat larger than those in the re-run of the original zone 1. The tetraploid nuclei from the recentrifugation of zone 2 are still contaminated by a small percentage of diploid nuclei.

Sedimentation properties of liver nuclei. An advantage of the A rotor is that it permits continuous direct observation of the position of the

particle zones and enables an investigation to be made of their sedimentation behaviour. Fig. 13 shows the position of the tetraploid and diploid zones as a function of time at a rotor speed of 600 rev./min. The g value at this speed at a given distance, r , from the axis of rotation may be calculated by multiplying r by 4.03. The good reproducibility is apparent. The rate of sedimentation at twice the rev./min., and therefore a fourfold increase in gravitational field, is shown in Fig. 14. The radial velocity of both kinds of nuclei has been calculated at a number of points in the gradient. It increases in the predicted manner. This provides an internal check of the system.

The original choice of gradient and conditions of centrifugation was dictated by theoretical consideration of nuclear size and density and the need to preserve nuclear structure. An additional limitation is that imposed by the considerable time required to displace the gradient at the end of the run. The gradient must be sufficiently concentrated to prevent excessive sedimentation during the pumping-out period and sufficiently steep to ensure stability at low speeds. A number of gradients and conditions of sedimentation other than the standard ones have been tried. These include a gradient of 5–17% (w/w) sucrose at 600 rev./min. for 40 min. and 50–69% (w/w) sucrose at 2500 rev./min. for 6.5 hr. Though these conditions are not suitable for preparative purposes, the results derived from them augment the information available from the standard runs on the sedimentation behaviour of nuclei. On the assumption that the nuclear radii are unaffected by sucrose concentration and have values of 3.6μ and 4.67μ for the diploid and tetraploid nuclei, it is possible to compute from Stoke's law the density of the nuclei at given points in the gradient from the rate of sedimentation at those points (Fig. 15). The density of the nuclei varies with the density of the medium, presumably because of their permeability to sucrose solutions. By extrapolation it can be seen that the

Fig. 13. Distance of nuclei from axis of rotation as a function of time at 600 rev./min. Diploid nuclei, open symbols. Tetraploid nuclei, closed symbols. The different symbols are data taken from separate runs.

Fig. 14. Plot of sedimentation of the diploid nuclei ($\square$) and tetraploid nuclei ($\blacksquare$) at 1200 rev./min.

Fig. 15. Variation of nuclear density with the density of the medium. Symbols are as in Fig. 14. ρ_P , Density of nuclei; ρ_m , density of sucrose solution. The temperature was 5° .

nuclei would be isopycnic in sucrose solutions of density 1.35. The sucrose-impermeable space occupies 10.2% of the nuclear volume for diploid and tetraploid nuclei.

DISCUSSION

It is apparent that the zonal rotor affords a rapid and efficient way of differentiating nuclei according to their degree of ploidy and of recovering them in reasonable quantities. Provided that the initial isolation of the nuclei does not involve selective losses of a particular class, it permits a representative picture to be gained of the proportion of nuclei of different sizes. Sufficient material for direct biochemical analyses is available. The technique should prove invaluable in many biochemical and biological investigations, including the mode of action of carcinogens that act directly on nuclei, in elucidating the control mechanisms that operate during regeneration and in studies of the causes of polyploidy.

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