Biochimica et Biophysica Acta, 628 (1980) 480—488

BBA 29190

THE FRACTIONATION OF SUSPENSIONS OF ISOLATED HEPATOCYTES BY RATE ZONAL CENTRIFUGATION

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(Received August 7th, 1979)

Key words: Hepatocyte fractionation; Zonal centrifugation; DNA synthesis; Metrizamide; (Rat liver)

Summary

Parenchymal cells, isolated from rat liver by a simple non-enzymic technique, were fractioned according to ploidy class by rate zonal centrifugation on sucrose density gradients. This method of fractionation applied to liver cells prelabelled in vivo with tritiated thymidine separated different size classes of cells synthesising DNA.

Introduction

Mammalian liver is composed of a variety of cell types, including Kupffer, endothelial, and bile duct cells, but interest is usually focussed on the parenchymal cells, which constitute the major cell type and endow the organ with most of its characteristic biochemical properties [1]. The parenchymal cell population itself is far from uniform: except in the neonate it is distributed between different ploidy classes [2] and a segregation into distinct functional types may also occur [3]. In addition, cells of all these classes may traverse the cell cycle at different frequencies and rates [4]. Procedures to enable the separation of the various classes of parenchymal cells would assist studies of normal and pathological liver and allow investigations of problems, such as the formation of polyploid cells and the development of hepatomas, to proceed from a histochemical to a biochemical level.

Numerous techniques now exist for preparing suspensions of isolated cells from rat liver [5] and several attempts to fractionate such suspensions into

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Abbreviation: BBOT, 2,5-bis(5-*t*-butylbenzoxazol-2-yl)thiophen.

individual cell types have been recorded (e.g. Refs. 6—8). This paper describes the application of rate zonal centrifugation in zonal rotors, with its advantages of rapidity and high capacity, to liver cell suspensions isolated from young rats at the stage of rapid liver polyploidisation, and reports on the incorporation of radioactively labelled precursor into the DNA of fractionated liver cell populations.

Materials and Methods

Animals. The rats were from an inbred line of C strain Wistars fed ad libitum on Purina chow diet. In the radioactive labelling experiments, female rats weighing about 90 g (roughly 40 days old) were each injected intraperitoneally with 100—150 μCi tritiated thymidine (23.3 Ci/mmol, The Radiochemical Centre, Amersham, Bucks, U.K.) in 0.2 ml isotonic saline either 2 or 24 h before killing.

Liver dissociation. Liver suspensions were prepared using a modification [9] of the method of Rappaport and Howze [10], which employs the chelating agent, sodium tetraphenylboron (BDH Ltd., Poole, Dorset). After dissociation the isolated cells were collected by centrifugation ($20 \times g$, 4 min at 4°C), resuspended in approximately 40 ml buffer 1 (0.146 M NaCl; 0.0053 M KCl; 0.0007 M Na_2HPO_4; 0.0055 M dextrose; 0.0188 M Tris-HCl, pH 7.4, measured at 25°C) per dissociated liver, and again harvested by centrifugation. The cells were washed once more in this way before final resuspension in buffer 1.

Cell diameters. These were measured using a Wild (Heerbrugg, Switzerland) M 20 phase-contrast microscope fitted with a Leitz (Wetzlar, F.R.G.) measuring eyepiece.

Rate zonal centrifugation. A gradient ranging from 20 to 36.6% (w/w) sucrose (AnalaR grade) in 1 mM Tris-HCl (pH 7.4) of capacity 1 l and linear with respect to volume was pumped into the A-XII zonal rotor of an MSE Mistral centrifuge at 600 rev./min and 4°C. This was followed by an underlay of 55% (w/w) sucrose. The sample containing $8—10 \cdot 10^6$ cells, suspended in 10 ml of buffer 1, was applied to the top of the gradient as an inverse sample gradient, formed by a device consisting of two interconnected syringes, one containing the sample, and the other 10 ml of buffer 1. This procedure minimised possible hydrodynamic instabilities [11]. The sample band was displaced from the centre of the rotor with an overlay of Tris-HCl, pH 7.4 (90 ml). After centrifugation at 600 rev./min for approximately 20 min, during which the migration of the cells in the centrifugal field was followed by eye or with the aid of a stroboscopic device [11], the gradient was unloaded, also at 600 rev./min, by displacement towards the rotor centre by underlay, at a rate of 50 ml/min. The light-scattering profile of the rotor effluent was recorded at 600 nm and 40-ml fractions were collected manually.

Sucrose concentrations in fractions were measured with an Abbé refractometer (Bellingham and Stanley, Ltd., London N15) and cells were harvested by centrifugation ($1500 \times g$ for 10 min at 4°C). Cell pellets were resuspended in 1.0 ml buffer 1 and portions used for the preparation of stained smears and particle volume determination as well as for scintillation counting of incor-

porated radioactivity. Cell recoveries from the gradients were in the range 80—95%.

Differential cell counts. Smears of resuspended cell fractions were air-dried, fixed 15—30 min in 10% formal/saline, and washed in saline. The smears were Feulgen stained for DNA and mounted in Xam (G. Gurr, Ltd., London SW6). The slides were examined in the Wild M 20 microscope fitted with a Whipple eyepiece grid (Graticules, Ltd., Garrick Street, London SW1) and at least 400 cells per gradient fraction were scored for ploidy type. For microspectrophotometric determinations of nuclear ploidy, a Barr and Stroud integrating microdensitometer (1 Pall Mall East, London, SW1Y 5AU) was used by courtesy of Dr. U. Mittwock, Dept. of Genetics and Biometry, University College London.

Particle volume determination. The numbers and volume distributions of the resuspended cells were estimated using a Coulter Counter Model F (Coulter Electronics, Ltd., Dunstable, Beds., U.K.) fitted with a 100 μM aperture type and previously calibrated with ragweed pollen of known volume (obtained from Coulter Electronics, Ltd.). Cell counts were made at a succession of equal threshold (i.e. volume) increments between a minimum threshold setting which excluded debris and gave the total cell concentration, and a maximum setting at which only cell aggregates, a very small proportion of the particle population, were counted. When processed manually the data were plotted as a counts vs. threshold curve (approximately sigmoidal in shape) and the threshold value giving half the total cell count was determined. On conversion to μm^3 this yielded the median cell volume of the cell population. In the case of resuspended cells from the zonal gradient fractions, the data were processed using an IBM 360: curves were fitted to particle counts vs. threshold data with a polynomial regression analysis program from the collection of the University College Computer Centre.

In subsequent steps introduced into the program the mean and modal threshold values for the particle population as well as the standard deviation about the mean were computed and all these items was printed in tabular form for any series of zonal gradient fractions.

Radioactivity determinations. 0.5 ml samples of resuspended gradient fractions were washed successively with 20 ml of ice-cold 3% (v/v) perchloric acid and 80% (v/v) C_2H_5OH. The pellets were digested in 0.25 ml hyamine hydroxide (Nuclear Enterprises, Ltd., Edinburgh, U.K.) overnight at 37°C then transferred after addition of 2 ml CH_3OH into 15 ml scintillation fluid (4 g, 2,5-bis(5-*t*-butylbenzoxazol-2-yl)thiophen (BBOT) in 1 l toluene) and counted in a liquid scintillation spectrometer (Beckman or Intertechnique) at approximately 20% efficiency.

Results and Discussion

The cell suspensions used in these experiments consisted very largely of single parenchymal cells. Doublets or aggregates of higher order were infrequent (less than 5%). Contamination with erythrocytes and nuclei or other subcellular debris was very slight, although some small nucleated cells probably non-parenchymal, were detectable. In gross morphology the parenchymal cells

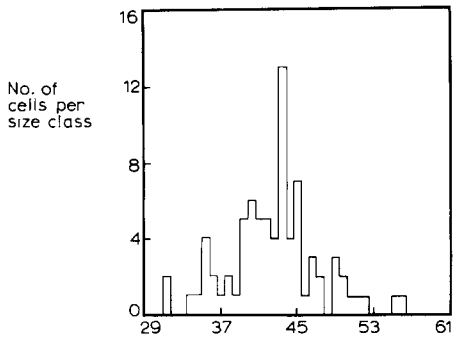

Fig. 1. Size distribution of summed maximum and minimum diameters (in μm) of 80 randomly selected liver cells isolated from a 200 g female rat.

were irregular, presenting roughly circular or elliptical profiles.

It is now clear that the sodium tetraphenylboron-based liver dissociation technique introduced by Rappaport and Howze [10] causes some damage to both cell ultrastructure and biochemistry [13]. However, rat liver cell cultures have been established using cells dissociated by a similar tetraphenylboron-using technique [14] and, of particular relevance to fractionation studies, the overall dimensions of the cells appear to be little affected by the disaggregation procedure. Measurement of the maximum and minimum diameters of 80 cells isolated from the liver of a 200 g (approx. 8 weeks old) female gave an average of 21.2 μm (Fig. 1); this may be compared with an estimate by Lound [15] of 21.0 μm for the mean in vivo diameter of parenchymal cells in the liver of mature Columbia-Sherman rats, and with 22.8 μm reported [6] for the enzymatically dissociated liver cells of 200 g Sprague-Dawley rats. Similarly the median cell volumes of cell suspensions prepared from 39-day-old rats given in Table I are comparable with Lound's in vivo figure of 5400 μm^3 [15].

Typical yields were of the order of $5 \cdot 10^7$ cells/g wet weight of liver. This represents a recovery of about 40% of the parenchymal cells, assuming that 1 g of rat liver contains $2 \cdot 10^8$ cells, of which 60% are parenchymal [12]. This estimated recovery implies that much liver tissue has been destroyed or otherwise lost during cell dissociation. However, Table I demonstrates that there is an increase with age in the mean cell volume of the isolated cells and Table II shows that this phenomenon of cell enlargement is associated with the process of polyploidisation. The constitution of the isolated parenchymal cell suspensions therefore seems accurately to reflect the known sequence of events in

TABLE I

MEDIAN CELL VOLUMES OF LIVER CELL SUSPENSIONS ISOLATED FROM C STRAIN WISTAR RATS OF DIFFERENT AGE-WEIGHT CLASSES

Approximate age (days)	29	39	50
Weight class (g)	50—60	90—100	140—150
Number of cell suspensions evaluated *	6	8	12
Mean median cell volume (μm^3)	3555	5662	6674
S.D. (μm^3)	667	343	851

* Each cell suspension was prepared from the liver of one individual animal.

TABLE II

THE PERCENTAGE FREQUENCIES OF NUCLEI IN THREE CELL PLOIDY CLASSES IN PARENCHYMAL SUSPENSIONS ISOLATED FROM RATS OF THREE WEIGHT CLASSES

For each weight category a cell suspension from a single rat was evaluated, at least 140 nuclei being scored by microdensitometry of Feulgen-stained cells.

Weight (g)	Mononucleate diploid	Binucleate diploid	Mononucleate tetraploid
50	81.7	12.7	5.6
100	24.2	64.4	11.4
150	5.4	45.1	49.5

vivo with respect to polyploid cell formation, suggesting that selective destruction of particular classes of parenchymal cells has not occurred.

Zonal fractionations

In these experiments sucrose was chosen as a suitable gradient solute on the grounds of its high water solubility, low toxicity, and cheapness (gradient volumes of 1 l being used in rate separations). Though sucrose may present osmotic problems because of its low molecular weight, it had been previously found, in, for example, the case of pigeon erythrocytes that useful fractionations could be achieved on sucrose gradients in zonal rotors [16]. Liver cells in gradient fractions examined immediately after the various centrifuge separations reported here had retained their original morphologies, though there were indications of shrinkage at the higher sucrose concentrations.

Most previous attempts to fractionate liver cell suspensions by sedimentation methods have exploited isopycnic centrifugation, and usually only one zone of parenchymal cells has been found [6,17,18]. This was also the case in our experiments: liver cells banded isopycnically on linear gradients (0—50% (w/w) in 1 mM Tris-HCl, pH 7.4) as a single peak with a modal buoyant density averaging 1.18 (39.3% (w/w) sucrose). In the instance of cells isolated from the pooled livers of three 150 g females, and centrifuged to equilibrium (45 min at 8300 rev./min, 4°C) on a linear 33—48% (w/w) sucrose gradient (in 1 mM Tris-HCl, pH 7.4) in an MSE AHS zonal rotor, a single symmetrical cell band was obtained, extending through about 50% of the 600 ml gradient. Ploidy frequencies in the central eleven fractions (10 ml each) containing the bulk of the cells were determined by scoring 400 randomly selected cells per fraction. No indication of a fractionation according to ploidy was found, though a fractionation of functional types, as has been reported by Drochmans and colleagues, and others [6,19,20], could conceivably have occurred.

Since ploidy classes are known to differ in size (Tables I and II and Ref. 21) it might be expected that rate sedimentation would effectively resolve them. Figs. 2 and 3 summarise the results of two rate zonal fractions of parenchymal cells prelabelled in their DNA with tritiated thymidine 2 or 24 h before isolation. With respect to cell type separation they are typical of our results for rate zonal fractionations of liver cell suspensions from rats of this age (40 days). Panels 2a and 3a illustrate the turbidity profiles of the gradients. Though essentially unimodal, the cell distribution is not symmetrical, indicating hetero-

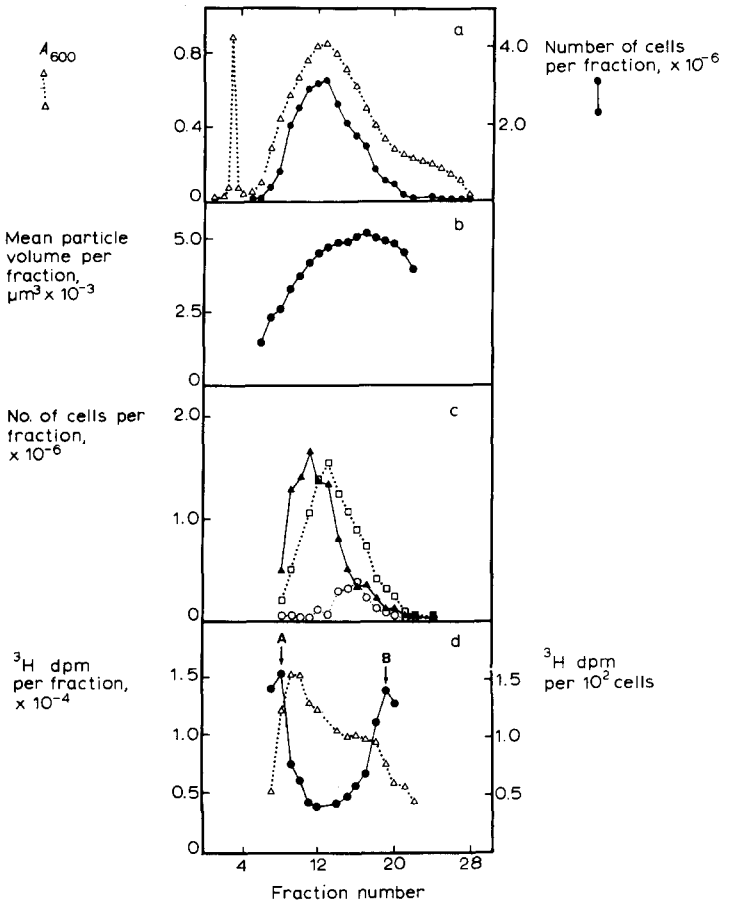

Fig. 2. Rate zonal fractionation of liver cells from a 90 g female rat which has received 100 μCi of [^{3}H]-thymidine 2 h before killing. Abscissa: gradient fraction number. Ordinates: (a) △·····△, turbidity in A_{600} units; ●———●, numbers of cells per fraction (X10^{-6}). (b) median volumes, in μm^3 (X10^{-3}), of particles in gradient fractions. (c) numbers of different parenchymal cell types in gradient fractions (X10^{-6}). ▲———▲, mononucleate diploids; □·····□, binucleate diploids; ○·····○, mononucleate tetraploids. (d) △·····△, ^{3}H dpm per fraction (X10^{-4}); ●———●, ^{3}H dpm per 10^2 cells. A and B indicate peaks of specific activity.

geneity of particle sedimentation rate within the cell population. Indeed, panels 2c and 3c show that rate zonal centrifugation achieves a partial separation of cells according to ploidy, though there is a significant overlap of the mononucleate diploid and binucleate diploid-mononucleate tetraploid populations. This could result from a clumping of dissimilar cell types although the tetraphenylboron isolation technique generates suspensions with a very high proportion of single cells which have little tendency to adhere, contrasting in the latter property with enzymically dissociated liver cells.

Aggregates might form after application of the cells to the gradient, but microscopic examination of zonal fractions immediately after centrifugation has revealed clumps, usually just doublets, only in the last fractions, well after the main cell peak, and clumps were prominent only at the gradient-underlay interface. Also the majority even of cell doublets should sediment considerably

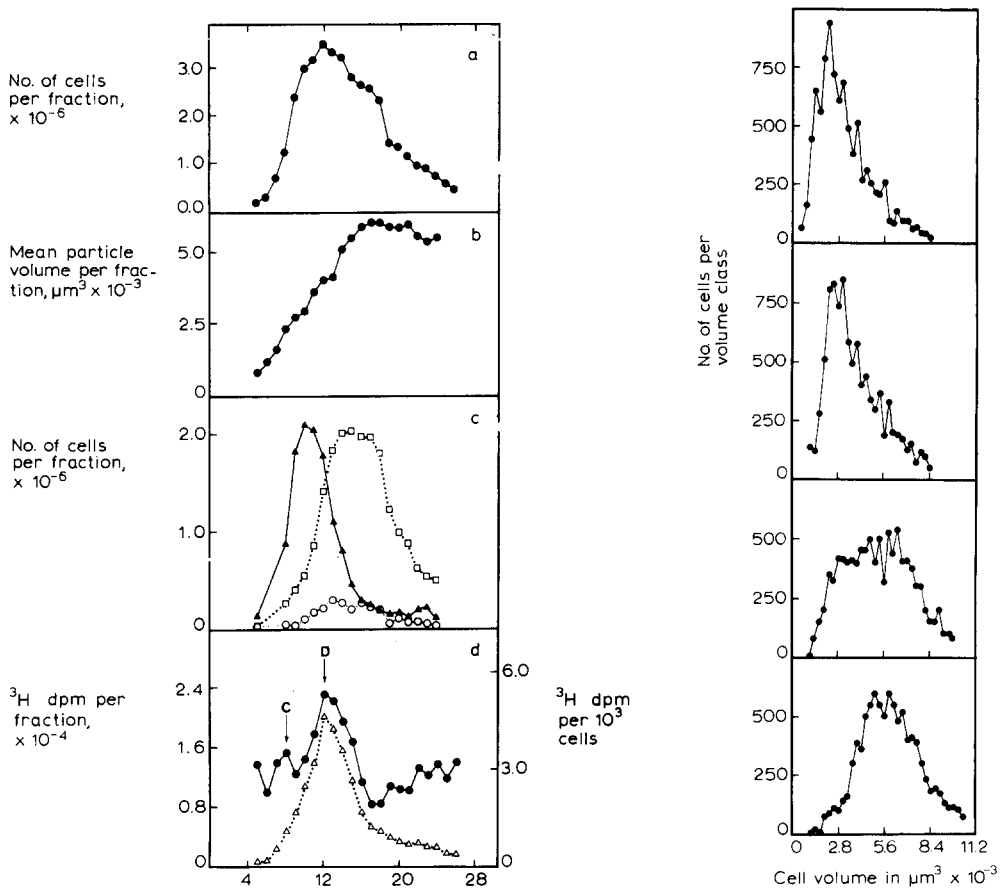

Fig. 3. Rate zonal fractionation of liver cells from a 90 g female rat which had received 50 μCi of [^{3}H]-thymidine 24 h before killing. Ordinates and abscissa are as in Fig. 2 except that no turbidity profile is given in panel a, and the specific activity profile in (d) is in ^{3}H dpm per 10^3 cells. C and D indicate peaks of specific activity.

Fig. 4. Volume of distribution of liver cell suspensions prepared from single female rats of different weights: (a) 50 g; (b) 70 g; (c) 100 g, and (d) 150 g.

faster than the single cells composing the main cell zone. Another contribution to impaired resolution could arise from cell shrinkage at higher sucrose concentrations; as panels 2b and 3b show there is a tendency for the mean cell volumes of fractions from the denser regions of the gradient to level out.

However, this incomplete separation of cell ploidy types could simply reflect genuine overlaps in the size distributions of these cell types, and this interpretation is supported by Coulter counter analyses of volume distributions of cell suspensions isolated from livers undergoing active polyploidisation. As Fig. 4 shows, the size distributions of liver cells isolated from rats between 50 g and 150 g are in all cases essentially continuous, yet, during this growth period there is a clear increase in mean cell volume associated with an upward shift in mean cell ploidy, and this is accompanied by the emergence of considerable heterogeneity of ploidy type (Tables I and II). Enzymatically dispersed cells also have continuous volume distributions, with effects on attempts at fractionation that

can be seen in the work of Tulp and colleagues [7] who allowed suspensions of collagenase-dissociated hepatocytes to sediment through Ficoll gradients at unit gravity. Although considerable separation of ploidy types was achieved, the fractionation was incomplete. By contrast, diploid and tetraploid parenchymal nuclei can be very clearly separated both by rate zonal sedimentation in sucrose gradients [22] and rate sedimentation at unit gravity in Ficoll gradients [7]. Cell mass may be much less sharply correlated with content of DNA than is the nuclear mass both because of the effects of variable position in the cell cycle and the possibility that cell mass is affected by the functional status of the cell.

Separation of labelled cells

The mean generation time of hepatocytes in growing rat liver has been estimated as 22 h, the S phase being 9 h [23]. 'Flash labelling' of cells with tritiated thymidine [24] 2 h before death should therefore identify S phase cells, whereas the labelling pattern after 24 h should reveal those cell types being formed immediately after a period of DNA synthesis. Although the incompleteness of the separation of the various ploidy classes complicates the interpretation of the specific radioactivity profiles, none of the fractions in the peaks of radioactivity represent a pure cell type, the gross and specific activity profiles at 2 h and 24 h (panels c and d of Figs. 2 and 3) are distinctly different.

The specific activity profiles of the cells prelabelled for 2 h (Fig. 2d) suggests that at least two cell size classes are particularly active in DNA synthesis, whereas at 24 h (Fig. 3d) the single major peak of specific activity corresponds with the mode of the total cell distribution. In peak A (panel 2d) of the 2 h profile it seems probable that mononucleate diploids, the most frequent cell class in the peak fraction (fraction 8), are mainly responsible for the incorporation. However, it would be anticipated that the volume of the S phase cells would be rather larger than that of the bulk of the cell population, so mononucleate diploids labelled with tritiated thymidine at 2 h might be expected to sediment at the leading edge of the cell type zone, rather than at the trailing edge, in fraction 8. Other cell types could also contribute to this radioactivity peak, such as one or more of the small non-parenchymal cells which tend to contaminate this region of the gradient.

From examination of the peak B of the 2 h profile (Fig. 2d), it can be seen that the specific activity mode corresponds to a gradient zone particularly rich in binucleate diploids and mononucleate tetraploids, both of which cell types are known to engage in DNA synthesis in rats of this age [25], and, in accordance with expectation, the peak is seen at the leading edge of the distributions of these two cell types.

The specific activity profile at 24 h (Fig. 3d) shows one major peak (D) with a mode in fraction 12, but there are indications of a subsidiary peak (C) centred on fraction 8, and similar in position with peak A of the 2 h profile. This would be consistent with the suggestion that small non-parenchymal cells are mainly responsible for incorporation in this section of the gradient because after completion of mitosis, daughter cells of a size resembling the parent cells are generated; in contrast, many mononucleate diploid parenchymal cells

would after S phase, remain in a size class double that of the parent cells, since mononucleate diploids tend to produce binucleate diploids by a process of karyokinesis without cytokinesis [2]. The modal region of the specific activity profile, fractions 12 and 13 (peak D), corresponds with the central regions of both the binucleate diploid and mononucleate tetraploid distributions. Formation of tetraploids from binucleate diploid precursors in a particular feature of this stage of liver development in rats [2]. Thus, it seems likely that much of this incorporated radioactivity is in the mononucleate tetraploids.

Interpretation of the radioactivity profiles must necessarily be cautious in view of the heterogeneity of cell types in the gradient fractions. Identifications of labelled cell types could be confirmed with autoradiography, but it is encouraging that different labelling patterns can be discriminated, and that these are consistent with what is known of the development of rat liver.

In conclusion, it has been demonstrated that the techniques of rate zonal centrifugation, hitherto used mainly for separations of subcellular organelles [21] or those cells naturally existing as suspensions such as reticulocytes [25] and mast cells [26], can also be applied to tissue cells dispersed by a non-enzymatic technique. It also appears that, for liver cells, distinct size classes of cells synthesising DNA can be recognised. This approach could have some value in tracing normal and pathological cellular events occurring in solid tissues.

Acknowledgements

Invaluable assistance with zonal centrifugation by Dr. David Ridge is gratefully recognised. This work was supported by a grant from the Science Research Council.

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