DENSITY GRADIENT ISOLATION OF RAT LIVER NUCLEI WITH HIGH DNA CONTENT

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ABSTRACT

Rat liver nuclei, after preliminary isolation in 2.2 molar sucrose solution, were separated into density classes by centrifugation at 95,000 g for 45 to 85 minutes in a sucrose density gradient (density range, 1.28 to 1.33). Nuclei from normal liver separated into three bands with average DNA phosphorus content per nucleus of 0.67, 0.84, and 0.93 picogram for top, middle, and bottom bands, respectively. Nuclei from regenerating liver (26 hours after one-third hepatectomy) yielded three bands and a pellet fraction with average DNA phosphorus content per nucleus of 0.76, 1.02, 1.38, and 1.51 picograms (top to bottom of tube). This method appears capable of yielding nuclei which have increased their DNA content prior to mitosis, and this procedure should be valuable in studies of biochemical changes which occur in nuclei preparing for mitosis.

In a preliminary communication (7) we reported the isolation of rat liver nuclei with high deoxyribonucleic acid (DNA) content by density gradient centrifugation of nuclei from regenerating liver. In the interim, Falzone and coworkers (6) have reported a successful fractionation of normal rat liver nuclei into diploid and tetraploid classes by a density gradient technique which differs considerably from that used in this laboratory. The present paper is a more detailed report of our work on this subject.

Normally, individual cells within a tissue are dividing in a rather random manner. However, considerable synchronization of cell division can be obtained in liver during short intervals following partial hepatectomy. At a given time, one should find more cells preparing to divide and thus a greater proportion of nuclei with increased DNA content in regenerating tissue than in normal tissue. Furthermore, since DNA has a higher density than other nuclear constituents, such as protein or lipid (with the exception of ribonucleic

acid (RNA), which has higher density but lower concentration than DNA), one might expect that those cells or nuclei with increased DNA content would have higher densities than those with lesser DNA content, when other factors remain constant.

A physical technique useful for the separation of particles of various densities is available in density gradient centrifugation. With this technique nuclei of normal and regenerating liver of the rat can be separated into various density classes which differ in DNA content.

MATERIALS AND METHODS

Male Wistar strain rats, approximately 5 to 7 months old and weighing 275 to 400 grams, were used in these experiments. For each experiment, three rats were selected and subjected to one-third hepatectomy by removal of the left lateral lobe. After 26 hours, the animals were sacrificed, and the liver was homogenized in 0.25 molar sucrose containing 0.0018 molar calcium chloride (9) and centrifuged at 600 g for 15 minutes in a refrigerated centrifuge. The crude nuclear fraction was suspended in 2.2 molar sucrose

and purified by centrifugation for 1 hour at 44,000 g in the number 30 rotor of a Spinco model L ultracentrifuge by a procedure similar to that of Chauveau et al. (3). Nuclei from normal liver (from rats not previously subjected to partial hepatectomy) were also prepared by the same procedure. The purified nuclei were suspended in 1 ml of 4.4 molal sucrose for application of density gradient centrifugation.

Density gradients were prepared by layering solutions of sucrose of the concentrations shown in Table I into plastic tubes for the SW-39-L swinging bucket rotor of the Spinco model L ultracentrifuge. After allowing the tubes to stand at 4°C for 24 hours, a suspension of purified rat liver nuclei in 4.4 molal sucrose (prepared as above) was added as a top layer to each tube and centrifuged at 95,000 g (or 30,000 RPM) for 45 to 85 minutes.

TABLE I

Composition of Sucrose Solutions Used in Preparing Density Gradients by Layering Technique*

Position of layer in tube	Volume of layer (ml)	Sucrose concentration		Density (gm/ml)
		gm/1000 gm H_2O	Molal	
Top	1	1500	4.40	1.286
Middle	2	1800	5.28	1.310
Bottom	1	2000	5.86	1.328

* 1 ml of nuclear suspension (in 4.40 molal sucrose solution) was added to each tube as a top layer immediately before centrifugation.

After centrifugation, three fractions or bands were obtained with nuclei of normal rat liver, but in the case of regenerating liver a pellet fraction also occurred. The bands were found in the following locations, as measured from the meniscus of the centrifuge tube: top band, 9 to 11 mm; middle band, 14 to 30 mm; bottom band, 32 to 37 mm; and pellet (in regenerating liver), 43 to 45 mm. A J-shaped hypodermic needle, attached to a syringe mounted on a smoothly adjustable rack and pinion, was used to remove the bands of nuclei. A determination of the number of nuclei in each band was obtained by direct counting of a sample in a hemocytometer after dilution to a measured volume. Each fraction was assayed for DNA by Burton's modification (1) of the diphenylamine reaction, for RNA by the orcinol reaction (11), and for protein nitrogen by Kjeldahl digestion and direct Nesslerization after preparation of samples by the procedure of Schneider (15).

RESULTS

Previous studies in this laboratory indicated that, in the first mitotic cycle following one-third

hepatectomy in rats, maximum incorporation of adenine into DNA occurred at 26 hours, and the greatest mitotic activity at 28 to 29.5 hours, following the partial hepatectomy (5, 10). In the present experiments the interval 26 hours after partial hepatectomy was selected in order to obtain nuclei which were actively synthesizing DNA but had not attained their greatest mitotic activity.

Data on the relative distribution of nuclei in the various fractions are presented in Table II. The two upper bands of nuclei from normal liver

TABLE II

Distribution of Nuclei in Various Bands

Band	Percentage of total nuclei	
	Normal liver	26-hr. regenerating liver
Top	4.2	1.4
Middle	8.5	13.0
Bottom	87.0	78.0
Pellet	(No pellet obtained)	7.5

TABLE III

DNA Phosphorus of Nuclei of Normal and Regenerating Liver

Band	DNA phosphorus (picograms per nucleus)	
	Normal	26-hr. regenerating
Top	0.67 ± 0.17	0.76 ± 0.06
Middle	0.84 ± 0.18	1.02 ± 0.13
Bottom	0.93 ± 0.14	1.38 ± 0.20
Pellet	(No pellet obtained)	1.51 ± 0.34

and from regenerating liver represent only small proportions of the total nuclei. The bottom band contains by far the greatest proportion of nuclei. The pellet fraction, found only in the case of regenerating liver, contains 7.5 per cent of the total nuclei.

A comparison of the DNA phosphorus per nucleus in each of the bands from normal and from regenerating liver is found in Table III. The lower bands, consisting of the densest nuclei, have the greatest content of DNA phosphorus per nucleus. Also, values for regenerating liver are higher than the corresponding values for normal liver. The

pellet fraction from regenerating liver has a higher content of DNA phosphorus per nucleus than the other bands. The average value of 1.51 picograms of DNA phosphorus per nucleus in the pellet fraction from regenerating liver is 62 per cent greater than the average value of 0.93 picogram per nucleus in the bottom fraction of normal liver of the young adult rat.

Table IV presents data for RNA phosphorus and protein nitrogen per nucleus for each of the fractions obtained from regenerating liver. From these data no correlation between the position of the band within the gradient solution (and hence the density of the nuclei) and either RNA phosphorus or protein nitrogen seems evident, in contrast to the correlation which was found with DNA content.

TABLE IV

RNA Phosphorus and Protein Nitrogen of Nuclei of Regenerating Liver

Band	RNA phosphorus (picograms per nucleus)	Protein nitrogen (picograms per nucleus)
Top	0.59 ± 0.20	4.7 ± 1.0
Middle	0.22 ± 0.08	3.1 ± 0.5
Bottom	0.15 ± 0.04	4.1 ± 0.6
Pellet	0.35 ± 0.09	2.7 ± 0.5

Photomicrographs of isolated nuclei from regenerating liver from the bottom band and the pellet fraction are presented in Figs. 1 and 2, respectively.

DISCUSSION

The possible effect of lipid content on nuclear density has not been investigated. The lipid content of rat liver nuclei varies somewhat (method of preparation, strain of rat, etc.) but is approximately 10 per cent or less of the dry weight of isolated nuclei (4). In view of the relatively small amount of lipid actually present in the nucleus, it seems that its effect on nuclear density should be quite small. Thus the available evidence seems to support our original assumption that the density of the nucleus reflects its DNA content.

Although RNA has a higher density than DNA, RNA generally comprises only a small proportion of the total nuclear nucleic acids. The figure for RNA phosphorus per nucleus in the top band

from regenerating liver is excessively high in relation to the RNA values found for the other bands. If all the RNA of this band were intranuclear RNA, and if the nuclear density is assumed to be a combined function of DNA and RNA content, then one would calculate that nuclei of the top band would be denser than nuclei in the middle band. This paradox can most easily be resolved by assuming that the high value for the RNA phosphorus per nucleus in the top band may be due to some degree of contamination of the nuclei in this band with microsomal RNA, which would not add to the density of the nuclei in the top band. The greater variability of the data for RNA and protein content of nuclei in the top band also suggests that this fraction may be contaminated in some experiments.

From an examination of photomicrographs of the fractionated nuclei, differences are noted in the relative sizes of the nuclei. The top bands in both normal and regenerating liver contain nuclei that are smaller than those in the lower fractions. In addition, the nuclei in the pellet fraction, obtained in experiments on regenerating liver, contain four or more nucleoli in many cases (Fig. 2). It seems probable that nuclei in the bottom and pellet fractions are derived from parenchymal cells, whereas those in the upper and middle bands originate principally from other (stromal) cells. Further work is desirable on the cellular origin of the nuclei in the various bands.

Falzone and coworkers (6) have noted similar differences in nuclear size and DNA content in their work on normal liver of non-pregnant, adult female rats. It seems probable, as these workers state, that the nuclei of lower density represent those nuclei in the diploid state while the denser ones represent those in the tetraploid state. In experiments on normal liver, the bottom band is probably composed to a large extent of the tetraploid nuclei characteristic of adult rats, in which octaploid nuclei are rare (12, 16). At 1 to 2 days after partial hepatectomy, however, there is an increase in the greater-than-tetraploid nuclei as the liver prepares for the first waves of mitosis (14, 16). Correspondingly, nuclei which are approaching the octaploid condition evidently are concentrated in the pellet fraction, which is obtained in the present experiments only from regenerating liver.

The density gradient sedimentation technique, without centrifugation, used by Falzone *et al.* (6)

differs considerably from the technique used in this study, but it seems likely that both methods will be useful in the fractionation of nuclei. In the technique of Falzone *et al.*, the following points are to be noted: The banding of nuclei is dependent on the *velocity* of sedimentation of the nuclei; and, because of the lower densities of the gradient, the nuclei sediment without centrifugation. If nuclei are allowed to remain in the lower density gradient, all nuclei will eventually settle to the bottom of the gradient. The technique used in the current study, however, utilizes centrifugation of the nuclei through a denser gradient in which the nuclei reach an *equilibrium* with the gradient during the centrifugation. In this denser gradient, nuclei from normal control liver do not settle to the bottom of the gradient if allowed to stand or if subjected to prolonged centrifugation. Three bands of nuclei were obtained with normal liver in the present experiments, whereas Falzone *et al.* obtained two bands (two other bands were reported to be attributable to cytoplasmic contamination and to clumping of nuclei).

Falzone *et al.* (6) found that the density of the nuclei (calculated from Stokes' law) varied directly with the density of the ambient medium but was always slightly higher than the latter. Presumably, sucrose in the medium equilibrates with intranuclear fluid. It seems probable that in the present experiments, in which higher density gradients are used, nuclei become isopyknic with the medium when the density of the latter is equal to the average density of the nuclear components, principally DNA, RNA, protein, and lipid.

The average values of DNA phosphorus per nucleus which we have calculated for *unfractionated* nuclei, *viz.* 0.91 and 1.31 picograms for nuclei of normal and regenerating liver, respectively, are in reasonable agreement with the range of values which can be calculated (assuming that the phosphorus content of DNA is 9.0 per cent (2)) from the data of other investigators for normal (6, 8, 9, 14, 17) and regenerating liver (13, 14). The average value of 1.51 picograms DNA phosphorus per nucleus found in the pellet fraction of regenerating liver (Table III), in comparison with the corresponding value of 0.93 for the bottom band of normal liver nuclei, suggests that our density gradient fractionation procedure may provide a method for the isolation of nuclei which have attained, or are approaching, the condition of doubling the DNA content prior to cell division. This method should be valuable in studies of biochemical changes which occur in nuclei preparing for mitosis. With this objective, experiments are in progress in this laboratory to determine relative rates of incorporation of labeled precursors into DNA of nuclei of regenerating liver fractionated by density gradient centrifugation.

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FIGURES 1 AND 2

Photomicrographs of nuclei in two fractions isolated by density gradient centrifugation of nuclei from regenerating rat liver. Nuclei stained with methylene blue. Fig. 1, bottom band; Fig. 2, pellet fraction. × 680.

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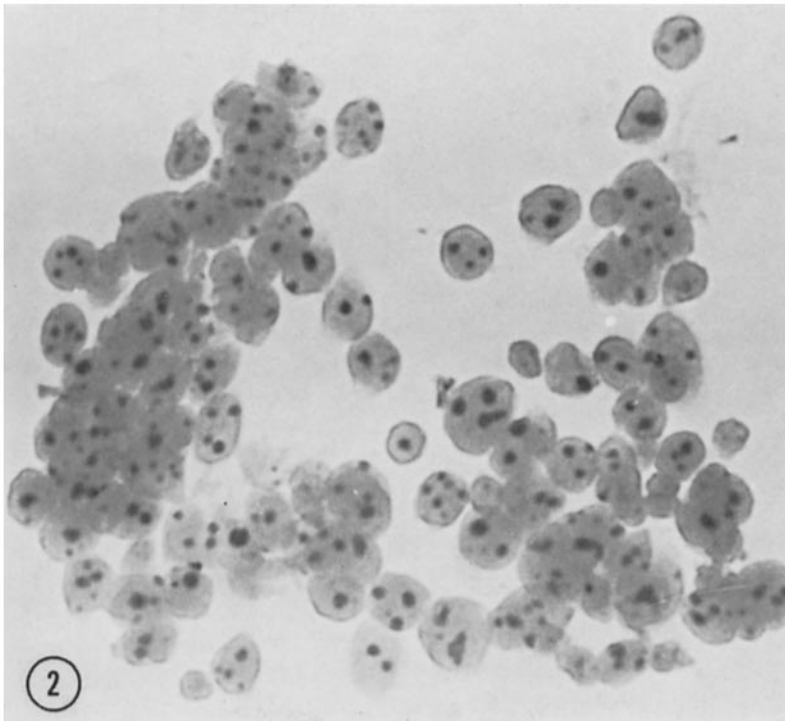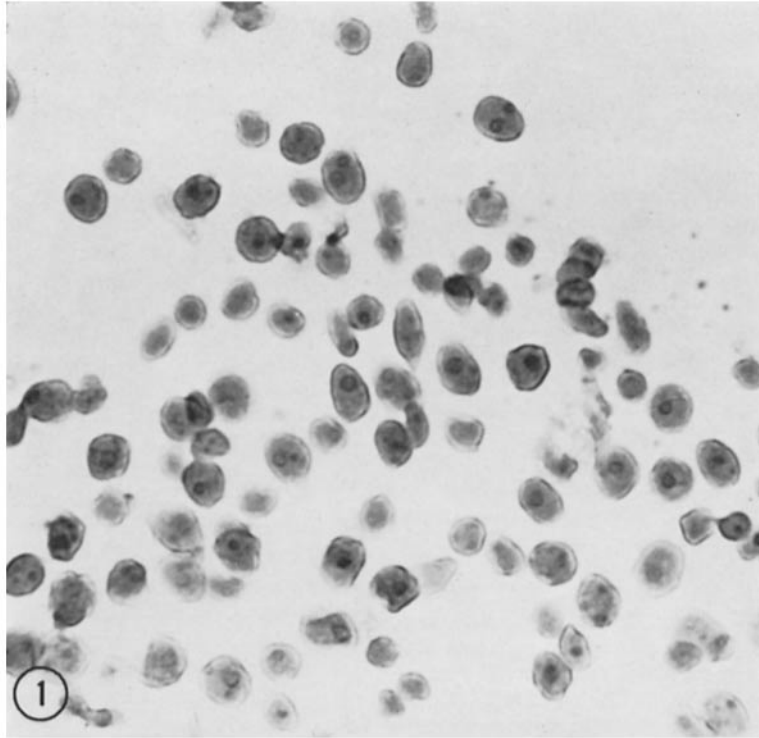
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