ZONAL CENTRIFUGATION STUDIES ON LABELLING PATTERNS OF MITOCHONDRIAL AND NUCLEAR DNA IN RAT LIVER AND HEPATOMA

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1. Introduction

The zonal centrifuge is well established as a tool for studying the heterogenity of liver organelles including mitochondria [1, 2]. We have applied it to the examination of the distribution of DNA amongst the heavier particles in liver and hepatoma homogenates. Mitochondrial DNA (mit DNA) takes up precursors more rapidly than nuclear DNA (nuc DNA) in mammalian tissues [3–7]. We have resolved crude nuclear-mitochondrial pellets on sucrose gradients by zonal centrifugation and have located DNA and RNA in the fractions containing succinate dehydrogenase activity. The parameter to which we have paid particular attention is the specific activity ratio mit DNA : nuc DNA. This ratio, which showed considerable scatter, was much greater than unity in normal liver, whereas in tumour tissue it was near unity. Results thus expressed enable liver and hepatomas to be tentatively compared even if differences exist in precursor pool labelling. Specific activity values indicated heterogeneity within the nuclear region. With hepatomas an additional DNA peak was located in the lysosomal region.

2. Materials and methods

2.1. *Tissues*

The rats were of a hooded strain as bred at the Chester Beatty Research Institute. Normal rat liver was isolated from 1–4 month old males. The hepatomas were subcutaneous transplants of primary liver tumour induced by intermittent feeding with ethionine. The generation time was of the order of 21 days.

After an overnight fast the rats were killed by cervical dislocation at about 10 a.m. The liver or tumour mass was immediately removed and placed in 10 vol of ice-cold homogenising medium (0.25 M sucrose containing 5 mM Tris, pH 7.2). Subsequent procedures were carried out at 0°–4°. Homogenisation was carried out in a Potter-Elvehjem homogeniser with a Teflon pestle rotating at 2000 revs/min (clearance 0.2 mm) until dispersion was achieved, which required 2 strokes for liver and 4–8 strokes for hepatomas.

2.2. *Radioactive labelling*

Labelled precursors were obtained from the Radiochemical Centre, Amersham. In double-labelling experiments 300 μC of [CH_3-^{3}H]-thymidine was injected intraperitoneally 18 hr, and 10 μC of [6-^{14}C]-orotate 45 min before death. For single-label experiments, 10 μC (normal liver) or 40 μC (tumour) of [2-^{14}C]-thymidine was injected, usually 18 hr before death.

2.3. *Centrifugation*

Nuclear-mitochondrial pellets were prepared by centrifuging sieved Potter-Elvehjem homogenates for 10 min at 7,600 *g*. The pellet was resuspended in homogenising medium using a hand-operated Teflon/glass homogeniser, and 20 ml of the suspension was

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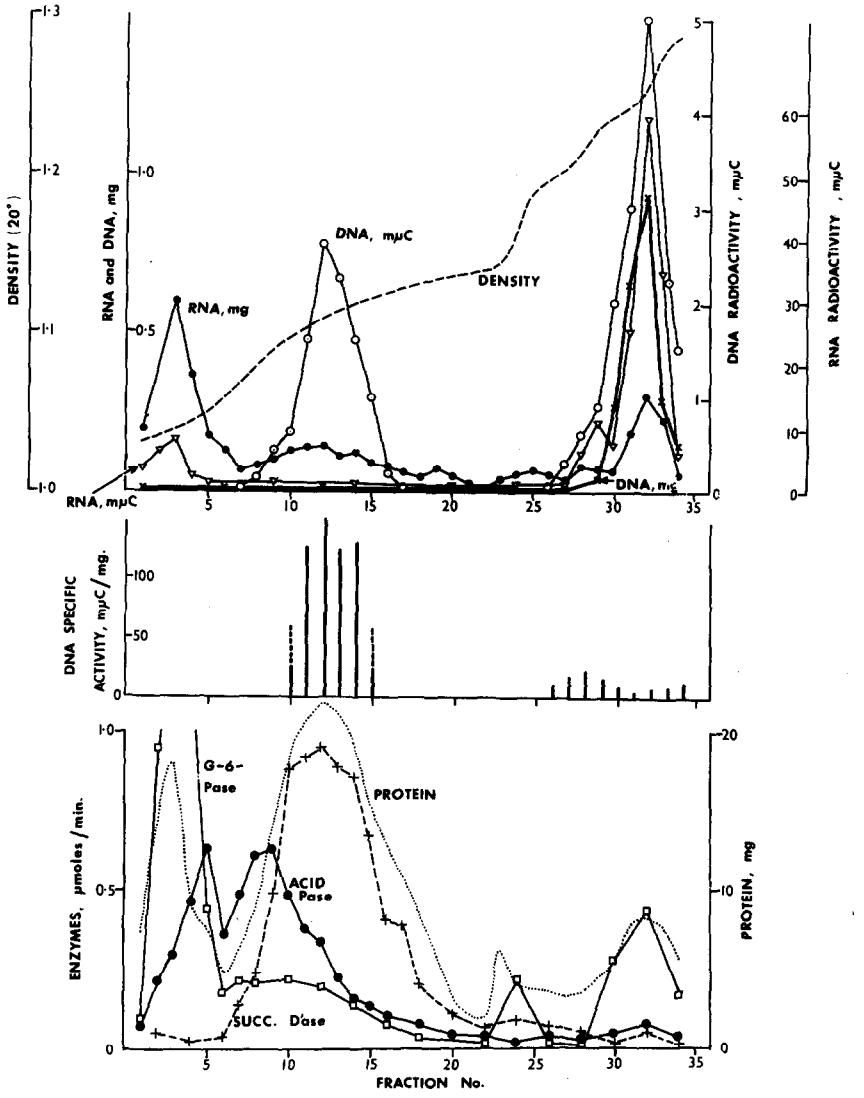

Fig. 1. Zonal pattern for normal liver samples (10 g) from 6-week rats given [^{3}H]-thymidine and [^{14}C]-orotate 18 hr and 45 min respectively before death. Values usually represent the amount per tube. Values for DNA specific activity (per mg DNA) are shown by spikes, the broken portions signifying uncertainty due to difficulty in determining trace amounts of DNA; with [^{14}C]-thymidine in single-labelling experiments the patterns were similar.

taken for zonal centrifugation with an A-XII zonal rotor (M.S.E. Ltd.). Several gradient shapes were prepared [8] and tried. The most satisfactory for the requisite separation, as shown in fig. 2, was produced by sequentially adding to 300 ml of 0.3 M sucrose, in a closed mixing vessel, firstly 500 ml of 0.85 M sucrose and secondly 500 ml of 1.50 M sucrose. All the solutions contained 5 mM Tris, pH 7.2. Finally the rotor was filled with 2 M sucrose as underlay (cushion). After loading the gradient and sample an overlay of 100 ml of 0.08 M sucrose was added, and the rotor accelerated from 400 to 4,000 revs/min for normal liver or 4,500 revs/min for hepatomas. During acceleration, further overlay

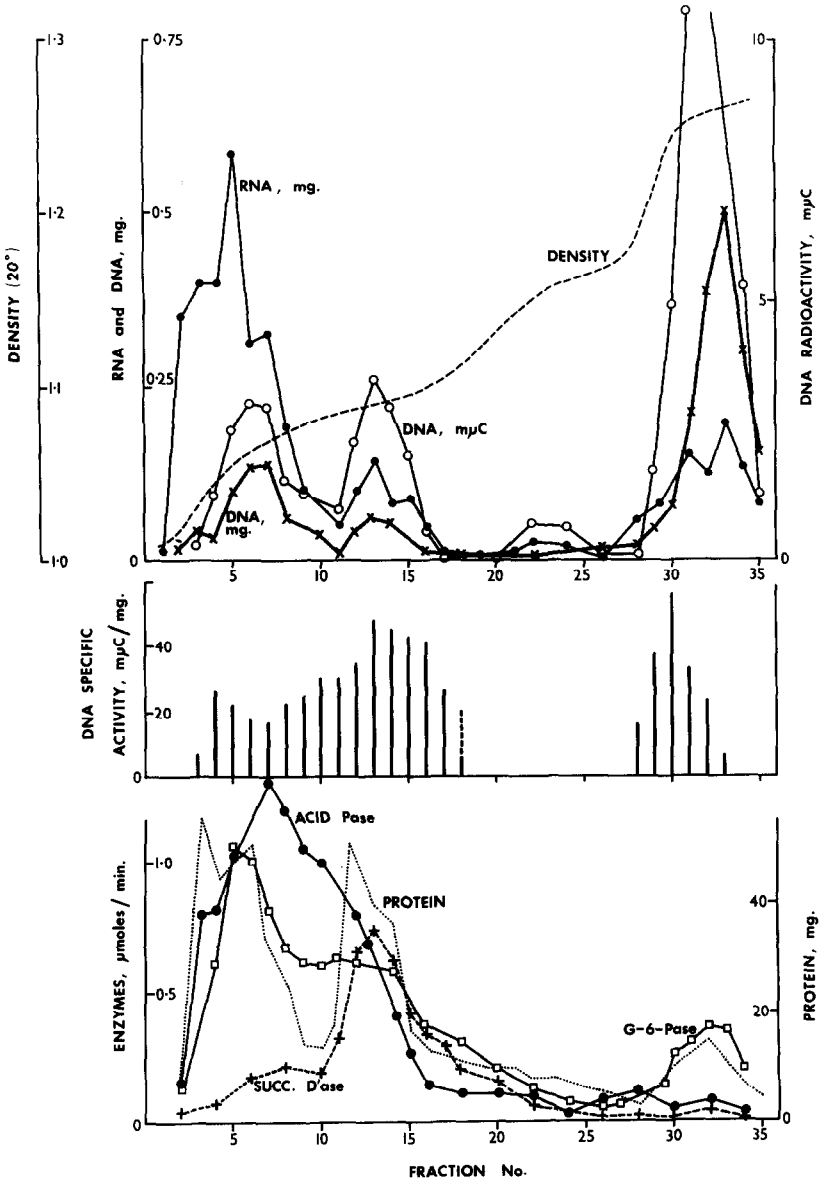

Fig. 2. Zonal pattern for hepatoma samples (18 g) from rats given [^{14}C]-thymidine 18 hr before death.

(50–70 ml) was taken up by the rotor. After 75 min, the rotor was decelerated to 400 revs/min and the gradient displaced with 2 M sucrose. The outflow was monitored at 650 mμ with a Unicam SP 800 recording spectrophotometer, then collected in 40 ml fractions at 0°.

2.4. *Analyses*

Procedures for the estimation of enzyme activity and protein content, and the correction of these values for sucrose interference have been previously described [9]. Glucose-6-phosphatase was used as a marker for endoplasmic reticulum fragments, acid β-glycerophosphatase (acid phosphatase) for lysosomes, and succinate dehydrogenase (succinate-INT

Table 1
Content of DNA and RNA in mitochondrial peak, and mitochondrial to nuclear specific activity ratios.

Tissue	DNA μg/mg protein*	DNA mμC/mg DNA, mit : nuc	RNA** μg/mg protein*	RNA** mμC/mg RNA, mit : nuc
Normal rat liver				
4–6 weeks of age	0.81, 1.06	18, 39	10.8, 14.8	0.06, 0.14
12–16 weeks of age	0.68 ± 0.08 (7)	18 ± 3.5 (5); 56*** 70***	15.2 ± 1.1 (3)	0.09 ± 0.01 (3)
Hepatoma	1.95 ± 0.31 (3)	0.96 ± 0.16 (3)	[Orotate not used]	

Values are given as mean ± standard error (with no. of observations); each specific activity value represents an average for the peak.

* The total amount of mitochondrial protein per g of wet tissue was typically 34 mg with liver and 15 mg with hepatoma.

** Uncorrected for microsomal contamination (shoulder of glucose-6-phosphatase activity in the mitochondrial (succinate dehydrogenase) peak).

*** 10 hr *in vivo* label; the other DNA specific activity values are for an 18 hr period.

reductase) for mitochondria. The biochemical characterisations thus made were corrobated by electron microscopy. To 30 ml of each zonal fraction in the presence of a protein carrier, perchloric acid was added to 5%. The nucleoprotein pellets were rapidly and thoroughly washed with ice-cold 3% perchloric acid and then extracted by a modified Schneider procedure [10]. DNA was estimated by a diphenylamine procedure [11], RNA by a cupric chloride-orcinol method [12], and radioactivity by scintillation counting with internal standardisation.

3. Results and discussion

Representative distribution patterns are shown in figs 1 and 2. Table 1 summarises values for DNA and RNA. Under the conditions of centrifugation used, neither mitochondria nor nuclei attain isopycnic positions after 75 min. Normal liver mitochondria were located in the region of density 1.12, whereas tumour mitochondria were at a density of about 1.09.

The presence of glucose-6-phosphatase activity in the mitochondrial peak suggests that part of the RNA is microsomal. The specific activity of the "mitochondrial" RNA was considerably lower than that in the nuclear region (table 1) and of the same order as that in the microsomal region. A single experiment on foetal liver gave a rather broad mitochondrial peak with RNA specific activity greater than that of the nuclear region.

In agreement with isopycnic experiments [7, 13], labelling of DNA was fairly uniform in the mitochondrial population although not in the nuclear population. The specific activity maximum for DNA in the nuclear region did not coincide with the fraction richest in DNA. Fig. 1 shows that the specific activity of the DNA at the succinate dehydrogenase peak (148 mμC/mg DNA) is 28 times or 7 times greater than that of the nuclear DNA, depending on whether comparison is made with the nuclear fraction which contains the most DNA, or with the fraction which contains the DNA of highest specific activity and which is presumably enriched with those diploid nuclei that are engaged in DNA synthesis [14].

Whereas normal liver showed much lower specific activity values for nuc DNA than for mit DNA, the values were of the same order in the hepatomas and also in samples of foetal and regenerating liver (single samples; not tabulated). Our results, together with previous findings for DNA in tumours and regenerating liver [15, 16], bear out the view that a fall in the mitochondrial to nuclear DNA specific activity ratio reflects cell proliferation [5].

Besides showing the usefulness of the zonal centrifuge for examining cytoplasmic DNA, the present rate-zonal study, in conjunction with the isopycnic studies cited, confirms that this DNA is mainly mitochondrial and virtually eliminates the possibility that such DNA is due to microbial contamination. However, in some hepatoma runs a trace of

high specific activity was encountered between the mitochondrial and nuclear peaks (fractions 20–25 in fig. 2) associated with high bacterial contamination as shown by plating and by light microscopy.

A feature of the tumour pattern (fig. 2) is a DNA peak coincident with maximum acid phosphatase activity. This suggests that considerable intracellular digestion of DNA has occurred, leading to the location of diphenylamine-positive material in phago- and auto-lysosomes [17, 18]. The data recently reported [19] for a "microsome-associated DNA" in mouse liver could conceivably refer to a lysosomal DNA. A further feature of the tumour pattern is the notably high level of DNA per unit weight of protein in the succinic dehydrogenase peak.

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