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SUBCELLULAR FRACTIONATION OF LIVER ORGANELLES FROM PHENOBARBITAL-TREATED RATS BY COUNTER-CURRENT PARTITION AND SUCROSE GRADIENT CENTRIFUGATION

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Abstract—Counter-current partition, sucrose density gradient centrifugation and enzymic analysis were used to explore the changes in rat liver organelles induced by phenobarbital There was a small increase in partition coefficient and marker enzyme activity of the endoplasmic reticulum The modal density of the marker enzyme showed a significant decrease reflecting the proliferation of the smooth endoplasmic reticulum The mitochondria showed a bimodal distribution with a small reduction of marker enzyme activity In contrast, lysosomes and peroxisomes were relatively unaffected by phenobarbital treatment Phenobarbital caused a small but statistically insignificant increase in γ-glutamyl transferase activity density gradient centrifugation studies indicated that the increased activity was predominantly in the biliary canalicular elements In contrast, cytosolic γ-glutamyl hydrolase activity was strikingly reduced by phenobarbital treatment

Phenobarbital is a commonly used anticonvulsant which increases microsomal protein [1] and the activities of hepatic microsomal drug metabolising enzymes [2] It induces cytochrome P450 and, in most cases, NADPH-cytochrome reductase [3, 4] Phenobarbital has also been shown to increase liver size by stimulating mitosis and cell division [5] The most striking changes induced by phenobarbital include proliferation of smooth endoplasmic reticulum with accompanying dispersion of rough endoplasmic reticulum cisternae, the production of a mitochondria lacking cristae and the accumulation of myelin figures These abnormalities at first affect only centrilubular cells but progressively involve cells further out into the periphery of the lobule [5].

Tissue homogenates are usually fractionated by centrifugation techniques, where differences in size and density are used to effect separations. Much useful information can be obtained comparing normal and diseased tissue by this approach [6] Counter-current partition, which separates particles on the basis of their surface properties [7, 8], has recently been applied to the fractionation of organelles of rat liver [9]. More recently counter-current partition with the toroidal coil centrifuge was used to fractionate a rat liver homogenate with enhanced separation of plasma membrane from lysosomes and endoplasmic reticulum [10]. We have used counter-current partition and sucrose density gradient centrifugation to explore changes in the enzyme activities and properties of rat liver organelle induced by phenobarbital

MATERIALS AND METHODS

Injections. Non-fasted male rats (150–200 g) Sprague–Dawley strain, were injected i.p. with phenobarbital (80 mg/kg; Macarthy's Ltd., Essex, U.K), once daily for four days. Control animals were injected with 0.15 M NaCl. About 18 hr after the final injection the animals were killed.

Preparation of rat liver homogenates for sucrose gradient centrifugation Rats were stunned and killed by cervical dislocation. The livers were removed immediately and a portion (2 g) of perilobular tissue minced with a razor blade and disrupted in a medium-sized manual Dounce homogeniser (Kontes Glass Co , Vineland, NJ) in 10 ml ice-cold 0.25 M sucrose containing 1 mM EDTA pH 7.4, and 20 mM ethanol. Homogenization was by 10 strokes of the type A pestle followed by 10 strokes of the type B pestle Fibrous material was removed by filtration through a 50 μM nylon mesh

Density gradient centrifugation Liver homogenate (5 ml) was layered onto 28 ml sucrose density gradient, extending linearly with respect to volume, from a density of 1.05 g/ml to one of 1.28 g/ml and resting on a 6 ml sucrose cushion of density 1.32 g/ml in a Beaufay automatic rotor [11]. All density gradient solutions contained 1 mM EDTA, pH 7.4 and 20 mM ethanol. The rotor was accelerated to 35,000 rev/min and run for 35 min with an integrated angular velocity of 3.3×10^{10} rad^2 sec^{-1} The rotor was then slowed to 8000 rev/min for automatic unloading and collection of 16 fractions into tared tubes.

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Table 1 Marker enzyme activities in rat liver homogenates from control and phenobarbital-treated animals

Organelles (Marker enzymes)	Activity			
	Control	Phenobarbital treated	Control	Phenobarbital treated
	(mU/mg Protein)		(mU/mg DNA)	
Plasma membrane				
5'-Nucleotidase	30.0 ± 4 8(18)	17 1 ± 2 7(9)	174 ± 19(5)	109 ± 14(5)[c]
γ-Glutamyl transferase	0 060 ± 0 036(5)	0 102 ± 0 058(5)	0 372 ± 0 239(5)	0 657 ± 0 400(5)
Alkaline phosphatase	1 18 ± 0.14(5)	1 12 ± 0 14(5)	7 24 ± 0 89(5)	7 13 ± 0 99(5)
Alkaline phosphodiesterase	0 033 ± 0 005(5)	0 033 ± 0 002(5)	0 204 ± 0 038(5)	0 206 ± 0 013(5)
Lysosomes				
N-Acetyl-β-glucosaminidase	1 24 ± 0 36(10)	1 25 ± 0 23(10)	9 10 ± 0 88(5)	8 82 ± 0 25(5)
Acid phosphatase	22.7 ± 2 7(7)	22 7 ± 2 3(10)	118 ± 13(5)	111 ± 14(5)
β-Glucuronidase	7.04 ± 0 89(7)	6 11 ± 0 54(11)	46 3 ± 6 1(5)	38 8 ± 1 5(5)[c]
Mitochondria				
Malate dehydrogenase	3250 ± 670(9)	3110 ± 320(11)	21600 ± 1800(5)	20800 ± 1600(5)
Glutamate dehydrogenase	7 22 ± 0 73(7)	6 63 ± 1.28(6)	44 7 ± 3 6(5)	38 3 ± 4 1(5)[c]
Endoplasmic reticulum				
Neutral α-glucosidase	0 017 ± 0 001(5)	0 018 ± 0 001(5)	0 104 ± 0 003(5)	0 116 ± 0 005(5)[b]
Peroxisomes				
Catalase	289 ± 80(10)	283 ± 68(11)	1730 ± 390(5)	1680 ± 270(5)
Cytosol				
Lactate dehydrogenase	2010 ± 472(10)	1960 ± 270(10)	13600 ± 2100(5)	13400 ± 1400(5)
γ-Glutamyl hydrolase	0 950 ± 0 160(5)	0.286 ± 0 067(5)	5 85 ± 1 09(5)	1 69 ± 0 32(5)[a]

1 mille unit of activity corresponds to the processing of 1 nmole substrate/min Results show mean ± S E with number of animals shown between parentheses

Statistical analysis by Students t test (a) $P < 0\ 001$, (b) $P < 0\ 02$, (c) $P < 0\ 05$

Counter-current partition experiments Counter-current partition experiments were performed as described previously [9]. Briefly, rat liver homogenates were prepared in poly(ethylene glycol)-rich upper phase solution from a mixture containing 5.8% (w/w) dextran T-500, 4 0% (w/w) poly(ethylene glycol) 6000, 0.25 M sucrose, 10 mM sodium phosphate–phosphoric acid buffer, pH 7.4, 0.2 mM Na_2 EDTA, pH 7.4 and 1.1 mM ethanol. Upper phase (0.65 ml) containing homogenate was loaded onto 0.65 ml dextran-rich lower phase in the sample well of the counter-current device [9], and complete polymer mixture was added to the other 17 wells The phase mixtures, liver homogenate and counter-current device were all maintained at 4°. Seventeen transfers were effected. Before each transfer, the two phases were mixed by twenty inversions of the entire device and the phases separated by centrifugation at 250 *g* for 5 min. Sucrose (0.25 M, 0.40 ml) was then added to each well and, after mixing to form a homogeneous suspension, the content of each well was removed and stored at −20° for subsequent analysis

Analytical methods. The distribution of organelles in each fraction from the sucrose gradient and partition experiments was determined by assaying marker enzymes as described previously [9, 12, 13]. γ-Glutamyl transferase (biliary tract-plasma membrane) was measured as described by Smith *et al.* [14]. Protein in CCD fractions [15] and density fractions [16] was assayed with bovine serum albumin as standard, and DNA [17] was measured with calf thymus DNA (Sigma type 1) as standard.

Sources of chemicals Dextran T500 (batch 3447) was obtained from Pharmacia (Uppsala, Sweden) Poly(ethylene glycol) 6000 (PEG, batch 29577) was obtained from BDH Ltd. (Poole, Dorset) All other reagents were standard AR grade

RESULTS

The effect of phenobarbital on enzyme activities

There is a significant increase ($P < 0\ 01$) in the liver weight of phenobarbital treated animals (5.98 ± 0.39 g/100 g, N = 5) compared to controls (4.48 ± 0.13 g/100 g, N = 5), but the changes in protein and DNA/g wet weight were not significant The similar protein/DNA ratio in both groups [controls, 30.6, mg/mg; N = 5, phenobarbital treated, 31 6, mg/mg; N = 5] indicate that enzyme changes largely reflect activity per cell.

Table 1 shows the organelle marker enzyme activities in liver homogenates from control and phenobarbital-treated rats. The activity of 5'-nucleotidase, a plasma membrane marker, showed a significant reduction in phenobarbital-treated animals The other marker enzymes for the plasma membrane showed no significant change Phenobarbital treatment caused a small increase in the mean activity of γ-glutamyl transferase, the biliary tract plasma membrane marker enzyme, but this was not statistically significant. Neutral α-glucosidase expressed/mg protein was not increased significantly by drug treatment but there was a significant increase in activity per mg DNA. Of the cytosolic marker enzyme activities measured, γ-glutamyl hydrolase showed a marked

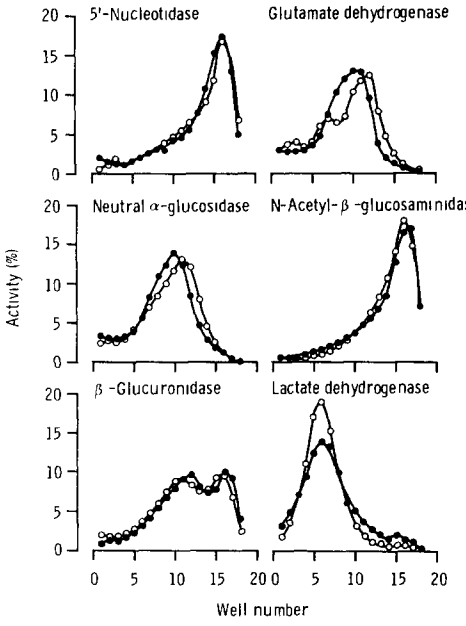

Fig 1 The distribution of organelles from rat liver homogenates of control (closed circles) and phenobarbital-treated (open circles) animals after partition between 5 8% (w/w) dextran and 4 0% (w/w) poly(ethylene glycol) Percent recovered enzyme activity is plotted against well number The results are the mean of 3 experiments for normal and 2 experiments for phenobarbital-treated animals

decrease in activity while lactate dehydrogenase remained unchanged. *N*-Acetyl-β-glucosaminidase and acid phosphatase showed no significant changes in total activity, but that of β-glucuronidase showed a small decrease when expressed per mg DNA. There

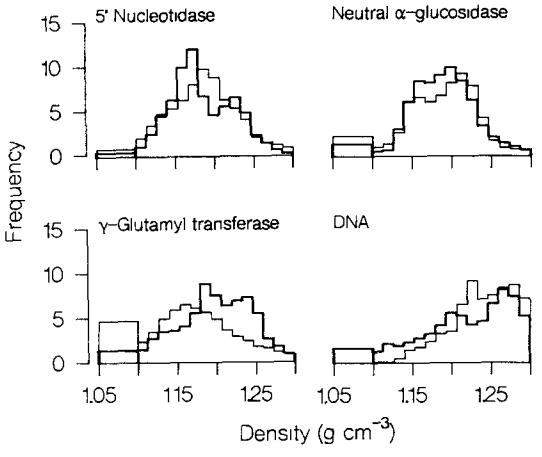

Fig 2 Isopycnic centrifugation of liver homogenates from control (thin line) and phenobarbital-treated (thick line) rats Graphs show frequency-density distribution of marker enzymes and DNA Frequency is defined as the fraction of total recovered activity present in the subcellular fraction divided by the density span covered The abscissa interval of densities 1 05–1 10 g cm^{-3} represents the activity remaining in the sample layer and is presumed to be soluble The results show the mean of 3 experiments for each treatment group

was no change in the total malate dehydrogenase activity but the specific enzyme marker for the mitochondria (glutamate dehydrogenase) showed a small decrease in activity per cell. The specific activity of catalase was similar in liver homogenates from both groups of animals

The effect of phenobarbital on organelle distribution

Figure 1 compares the distribution, after 17 transfer counter-current partition, of liver organelle marker enzymes from control and phenobarbital-treated animals. There was no significant change in the distribution or modal partition coefficient of 5'-nucleotidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, or lactate dehydrogenase. However, the partition coefficient of neutral α-glucosidase showed a small increase in the phenobarbital-treated animals Figure 1 clearly shows that the distribution of glutamate dehydrogenase, the mitochondrial marker enzyme, became bimodal in phenobarbital-treated animals.

Figure 2 compares the sucrose density gradient distribution of various organelle marker enzymes from drug-treated and control animals. 5'-Nucleotidase and γ-glutamyl transferase activities showed unimodal distributions in control animals, with peak densities of 1 19 and 1 16 g cm^{-3}, respectively The distribution of both enzyme activities were resolved into two components after phenobarbital treatment 5'-Nucleotidase had peaks of activity at densities 1.16 and 1 22 while γ-glutamyl transferase peaked at densities, 1 19 and 1 24 Neutral α-glucosidase showed a small increase in the low density component after phenobarbital treatment In control tissue (results not shown) lysosomes were located at density 1.21, as reflected by *N*-acetyl-β-glucosaminidase and acid phosphatase, and peroxisomes at density 1.24, as reflected by catalase distribution No significant change in the density distribution of these enzyme activities was seen after treating the animals with phenobarbital.

DISCUSSION

These studies used enzymic analysis, counter-current partition and sucrose gradient centrifugation to explore the changes in hepatic organelles induced by phenobarbital 5'-Nucleotidase is located predominantly in the plasma membrane [18] The decrease in enzyme activity is probably due to cell hyperplasia in the phenobarbital-treated rats [5]. The specific activity of 5'-nucleotidase in the rapidly dividing hepatocytes of the newborn and in regenerating rat liver has also been shown to be lower than that in the normal resting liver [19, 20] 5'-Nucleotidase activity is also lower than normal in some hepatomas [21] and in leukaemic lymphocytes [22] compared to controls. Although counter-current partition did not show any phenobarbital-induced changes in the surface properties of the plasma membranes, sucrose gradient centrifugation showed that a denser component to this organelle had been produced. The changes in the density distribution of the plasma membrane may have resulted from the formation of

additional protein-rich intercellular junctional complexes and desmosomes [23] which accompanies phenobarbital-induced hepatocyte proliferation [24]

γ-Glutamyl transferase has been shown to be primarily associated with the plasma membrane [25] of biliary tract cells [26] The reported phenobarbital-induced increase in hepatic γ-glutamyl transferase activity in rat [27, 28], rabbit [27, 29], and in mouse neoplasm [30], was not found to be statistically significant in the present study This may reflect differences in assay procedure and the large variation in activities between individual animals. Phenobarbital is known to cause the increased metabolism and excretion of endogenous substances [2], and the increase in γ-glutamyl transferase activity is probably related to this phenomena. The subcellular fractionation studies indicate a marked increase in the dense component of γ-glutamyl transferase and this may reflect a selective increase in the biliary tract cells membranes [31]

The bulk of the activity of neutral α-glucosidase is found in the endoplasmic reticulum [32]. This enzyme showed a small but significant increase in activity in livers from phenobarbital-treated animals. Microsomal glycosidases, which act at neutral pH, remove glucose from the oligosaccharide components of newly assembled proteins [33] It is therefore possible that the increase in neutral α-glucosidase, induced by phenobarbital treatment, could be a reflection of the increased processing of newly synthesized glycoproteins. The changes in the modal density and partition of neutral α-glucosidase may be a reflection of the increased proportion of smooth endoplasmic reticulum [34] which usually follows phenobarbital treatment Electron microscopic studies have previously indicated a proliferation of smooth endoplasmic reticulum membranes in the liver of phenobarbital-treated animals [5].

The specific role of γ-glutamyl hydrolase, a soluble enzyme [25], has not been determined Ding *et al* [26] reported that the highly purified γ-glutamyl transferase from the plasma membrane of rat biliary tract cells has only 7% hydrolytic activity Therefore, the striking decrease in γ-glutamyl hydrolase must be due to a specific reduction of the soluble enzyme. This observation further distinguishes γ-glutamyl hydrolase and transferase activities

In the present study phenobarbital did not cause significant lysosomal changes It has, however, been reported that the activity of some lysosomal enzyme activities (hyaluronidase, *N*-acetyl-β-glucosaminidase, acid phosphatase and cathepsin D) were reduced in rapidly dividing cells [35] This observation was thought to be due to the depletion of lysosomal enzymes during the reorganisation of hepatocyte structure for cell division Evidence of increase in some hepatic lysosomal enzyme activities, after phenobarbital treatment, has also been presented [36, 37] However, Mulder [38] has shown that the activities of four lysosomal enzymes were reduced by phenobarbital treatment. Rivera-Calimlin and co-workers [39] have presented biochemical and electron microscopic evidence that oral administration of phenobarbital can increase intestinal lysosomal enzyme activity The cause of these differing results are not clear but could be related to the animal species used, tissue examined, route of drug administration or the duration of treatment

Phenobarbital treatment results in the production of numerous small mitochondria [40] Wanson *et al* [24] later localised these organelles to the centrolobular hepatocytes. The decrease in glutamate dehydrogenase activity seen in the present study is probably related to the observation that phenobarbital induces dramatic changes in the structure of mitochondria [5] Counter-current partition shows that a new population of mitochondria was produced by phenobarbital treatment Electron microscopic examination and functional studies of the two populations of mitochondria could be of particular interest.

There was no alteration in the properties of peroxisomes from phenobarbital treated animals However, it is possible that the increase in low density fraction of nuclei is due to the production of new binucleated cells as observed by Staubli and co-workers [40]. These studies illustrate important effects of phenobarbitone on the liver cell organelles and further indicate that the application of two techniques based on different physical properties show distinct facets of these changes

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