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CHARACTERISTICS OF RAT HEPATOCYTES SORTED BY FLUORESCENCE-ACTIVATED FLOW CYTOMETRY

EFFECTS OF MIXED FUNCTION OXIDASE INDUCERS

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Abstract—Fluorescence-activated flow cytometry has been used to separate rat liver parenchymal cells into five equal computer-generated regions on the basis of their mixed function activities towards diethoxyfluorescein. Enzymic activities in the sorted fractions showed 2.3–2.9-fold enrichment of 3-cyano-7-ethoxycoumarin *O*-deethylase, lactate dehydrogenase and pyruvate kinase activities in the cells of region 5. Cytochrome P-450 PBla and glutamine synthase activities were enriched 1.8- and 7-fold respectively in region 2 hepatocytes. Pretreatment of rats with phenobarbitone or 3-methylcholanthrene did not change the pattern of enrichment with respect to monooxygenase or lactate dehydrogenase activities. However, cytochrome P-450 PB3a and MC1a respectively were concentrated in the cells from region 5. In contrast, ethoxyquin caused enrichment of cytochrome P-450 in the hepatocytes in region 2. This fraction also contained the highest percentage of γ-glutamyltranspeptidase positive cells.

It is now established that hepatocytes do not represent a homogeneous population. Gradations in the concentrations of many enzymes across the liver lobule have been described (reviewed in Ref. 1). From the point of view of the response of the liver to hepatotoxins or hepatocarcinogens which require metabolic activation, differences in the distribution of mixed function oxidase activities may be particularly important.

Localization of cytochrome P-450 in the liver lobule by microspectrophotometry shows a higher overall concentration in the central areas [2]. Immunohistochemical studies suggest the zone of enrichment is dependent both on the isoenzyme and the strain of rat [3–5]. Functionally, higher rates of *O*-deethylation of 7-ethoxycoumarin occur in the centrilobular areas of the liver [6]. Treatment of rats with compounds such as phenobarbitone, causes large increases in the concentration of specific cytochrome P-450 isoenzymes, while to a degree, maintain a zonal distribution of these proteins across the liver lobule [3–5, 7].

In isolated hepatocyte preparations, 2–5-fold differences in mixed function oxidase activities are found in fractions separated on the basis of the cells' physical characteristics such as density gradient or centrifugal elutriation [8, 9]. More recently, fluorescence activated flow cytometry has been used to characterise cells separated by density gradient techniques [10], or to sort cells directly on the basis of their mixed function oxidase activities [11]. Using this latter technique, the aim of this study was to characterise the sorted hepatocytes from control rats or those that have been pretreated with the mixed function oxidase inducers, phenobarbitone, 3-methylcholanthrene or ethoxyquin.

MATERIALS AND METHODS

Chemicals. Collagenase and NADPH were from Boehringer Mannheim (F.R.G.). Ethoxyquin (6-ethoxy-2,2,4-trimethyl-1,2-dihydropyridine) came from Köch Light Ltd. (Suffolk, U.K.). Diethoxyfluorescein and 3-cyano-7-hydroxycoumarin were purchased from Molecular Probes (OR, U.S.A.). 3-Cyano-7-ethoxycoumarin was prepared as described previously [12]. L-[$U^{14}C$]glutamic acid (specific radioactivity 10.5 GBq/mole) was from Amersham International (Amersham, Bucks, U.K.).

Animals and pretreatment procedure. Male Fischer F344/N rats (140–160 g) were used. Sodium phenobarbitone, (0.1% w/v in the drinking water) was given for 7 days. Ethoxyquin (0.5% v/w in powdered MRC 41B diet) was fed *ad libitum* for 7 days. 3-Methylcholanthrene was dosed 20 mg/kg intraperitoneally once a day for 3 days.

Hepatocyte preparation and flow cytometry. Hepatocytes were prepared by collagenase perfusion as described by Paine and Legg [13]. Preparations with cell viabilities (as determined by trypan blue exclusion, with the aid of a haemocytometer) of >80%, were used except in the case of ethoxyquin pretreated rats where cell viabilities of >70% were used. Hepatocytes (1×10^7 cells) were incubated for 4 min at 37° in phosphate buffered saline (10 ml) containing diethoxyfluorescein (0.05 mM). After centrifugation (50 *g* for 2 min) and resuspension in phosphate buffered saline (10 ml), fluorescence activated flow cytometry was carried out using a Cytofluorograf 50 H instrument. Excitation wavelength (argon ion laser) was 488 nm. Emission bandpass filter was 530 nm [11]. Hepatocytes from one incubation were sorted into two regions at a time. Sequential sorts were used to obtain all five regions. Cells (5×10^4) were sorted into multi-well plates containing 0.3 ml phosphate buffered saline. With the present instrumentation, the number of cells sorted into each region was very consistent but only

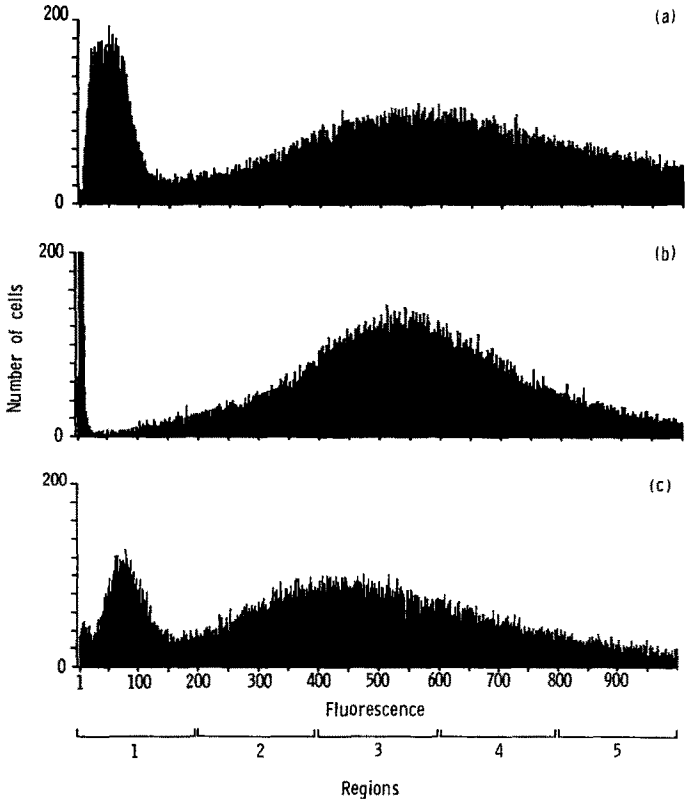

Fig. 1. Fluorescence activated sorting of hepatocytes from pretreated rats following incubation with diethoxyfluorescein. Hepatocytes (1×10^7) were incubated in PBS (10 ml) at 37° in air for 4 min with diethoxyfluorescein (0.05 mM). Following centrifugation and resuspension, cells were analysed using fluorescence activated flow cytometry: excitation wavelength 488 nm, emission filter >530 nm, laser power 500 mW. Traces represent (fluorescence photomultiplier gain control setting in parenthesis) (a) hepatocytes from phenobarbitone pretreated rats (2.9); (b) 3-methylcholanthrene (1.90); (c) ethoxyquin (3.45).

nominally 5×10^4 as judged using a haemocytometer. Results for enzyme content in the sorted regions were therefore expressed in relative terms rather than absolute activities. Cell suspensions were used either directly (mixed function oxidase activities) or centrifuged (200 *g* for 2 min), and the pellets frozen in liquid nitrogen and assayed the next day.

Enzyme assays. In freshly sorted cells, mixed function oxidase activities were determined using a continuous fluorimetric procedure based on the *O*-deethylation of 3-cyano-7-ethoxycoumarin [12]. In unsorted hepatocyte suspensions from control or ethoxyquin pretreated rats, the conversion of diethoxyfluorescein to ethoxyfluorescein was quantitated by HPLC as described previously [11]. For the remaining enzymes, cell pellets were resuspended in phosphate buffered saline (0.2 ml) containing reduced glutathione (1 mM) and the suspensions were subjected to four cycles of freezing and thawing. Pyruvate kinase (EC 2.7.1.40), glutamate dehydrogenase (EC 1.4.1.3), glutamine synthase (EC 6.3.1.2) and ferrochelatase (EC 4.9.9.11) were assayed by standard methods [14–17]. Alanine aminotransferase (EC 2.6.1.2) and lactate dehydrogenase (EC 1.1.1.17) were determined in the supernatant fraction following centrifugation (100,000 *g* for 1 hr at 4°) [18, 19]. Nuclear ploidy was determined in sorted cells as described previously [11].

Immunoblotting of cytochrome P-450 and cytochrome P-450 reductase. The small numbers of sorted cells available precluded sub-cellular fractionation. Pelleted hepatocytes (3×10^4 cells) were solubilised in urea/SDS sample buffer in the usual way and run on 7.5% polyacrylamide–SDS gels as described elsewhere [19]. Proteins were blotted onto nitrocellulose and probed with the appropriate antisera. Goat anti-rabbit IgG second antibody conjugated with horseradish peroxidase was used. Immunoreactive bands were visualised by incubating sheets with 4-chloro-1-naphthol and the intensity of the bands quantitated using an LKB soft laser scanning densitometer [3]. The ratio of cytochrome P-450 concentrations between the different regions was determined, rather than absolute quantitation. There was a linear relationship between the hepatocyte number and the staining intensity over the range 1×10^4 to 1×10^5 cells. Polyclonal antisera against cytochrome P-450 reductase and the isoenzymes of cytochrome P-450 PB1a (P-450 IIC6), PB3a (P-450 IIB1) and MC1a (P-450 1A2) were used

[3, 4]. Antibodies to other constitutive cytochrome P-450 isoenzymes were not available. PB1a is an antibody to a constitutive form, marginally induced by phenobarbitone, PB3a is a major phenobarbitone and MC1a is a major 3-methylcholanthrene inducible form of cytochrome P-450. Two bands were recognised by the PB3a antibody on Western blots of cell lysates from rats pretreated with either phenobarbitone (cf. Fig. 2) or ethoxyquin, confirming earlier reports [4, 20]. The areas of both these bands were summated to give the cytochrome P-450 content in these experiments. Total cytochrome P-450 was determined in cell lysates from the CO-reduced versus reduced difference spectra [21].

Cell histochemistry. Sorted hepatocytes (1×10^4) were deposited onto microscope slide using a Cytospin centrifuge and briefly air dried. The cells were fixed in ice-cold acetone and subsequently stained for γ-glutamyltranspeptidase activity [22] and with haematoxylin for nuclear visualisation. On each slide 500 cells or nuclei were counted.

RESULTS AND DISCUSSION

Flow cytometry of hepatocytes following incubation with diethoxyfluorescein. Figure 1 shows the fluorescence analysis of hepatocytes by flow cytometry, following 4 min incubation with diethoxyfluorescein, using cells from animals pretreated with the mixed function oxidase inducers phenobarbitone, 3-methylcholanthrene, or ethoxyquin. These patterns are similar to those of controls [10]. However, the fluorescence photomultiplier gain control had to be adjusted to compensate for the different rates of metabolism. In unsorted hepatocytes, relative to controls, 3-methylcholanthrene pretreatment induces the metabolism of diethoxyfluorescein, while phenobarbitone has little effect [11]. In all instances, the hepatocytes were sorted into five equal computer generated regions with the non-viable cells in region 1 and those having the highest rates of diethoxyfluorescein metabolism in region 5.

Enzymatic activities in sorted sub-populations: control rats. Relative activities in the sorted regions of a number of enzymes which have a zonal distribution across the liver lobule are shown in Table 1. As non-viable hepatocytes comprise a large proportion of region 1 [11] and activities of soluble (cytosolic) enzymes such as lactate dehydrogenase might be expected to be abnormally low compared with membrane bound components, e.g. glutamate dehydrogenase (cf. Table 1, region 1), results are expressed relative to the cells of region 2. Table 1 shows marker enzymes that are concentrated in the centrilobular areas of the liver, e.g. pyruvate kinase [23] and glutamate dehydrogenase [1, 24] show the greatest enrichment in the cells from region 5. However, this fraction also has low activities of another centrilobular enzyme glutamine synthase [25] and enrichment of the periportal markers lactate dehydrogenase and alanine aminotransferase [1, 23]. In the present experiments, ferrochelatase has a similar distribution in the sorted hepatocytes to the mixed function oxidases. The activities of ferrochelatase might be expected to reflect the overall requirement of the cell for haem.

Table 1. Activities and relative content of enzymes in sorted hepatocyte sub-populations from control rats following incubation with diethoxyfluorescein and fluorescence activated cell sorting

Enzyme	Activity in unsorted cells (nmol/min/10^6 cells)	Relative content in sorted regions					Zonation in lobule
		1	2	3	4	5	
Alanine aminotransferase	1502 ± 196	0.47 ± 0.05	1	1.71 ± 0.16	1.88 ± 0.15	1.95 ± 0.28	Portal (1)
Lactate dehydrogenase	2480 ± 340	0.17 ± 0.03	1	1.65 ± 0.15	2.15 ± 0.49	2.37 ± 0.40	Portal (1, 23)
3-Cyano-7-ethoxy coumarin-*O*-deethylase	0.05 ± 0.01	0.32 ± 0.05	1	1.16 ± 0.03	1.46 ± 0.19	2.66 ± 0.50	Central ?
Cytochrome P-450 PB1a	0.36 ± 0.02*	0.31 ± 0.02	1	0.76 ± 0.13	0.56 ± 0.10	0.56 ± 0.11	Central (3)
Cytochrome P-450 reductase	ND	0.18 ± 0.04	1	0.85 ± 0.03	0.63 ± 0.08	0.57 ± 0.16	Central (3)
Glutamine synthase	4.75 ± 1.8	0.50 ± 0.03	1	0.52 ± 0.06	0.24 ± 0.04	0.14 ± 0.04	Central (24)
Glutamate dehydrogenase	4036 ± 232	0.75 ± 0.08	1	1.28 ± 0.19	1.51 ± 0.12	1.64 ± 0.14	Central (1, 23)
Pyruvate kinase	100 ± 11	0.60 ± 0.01	1	2.01 ± 0.12	2.03 ± 0.22	2.92 ± 0.15	Central (22)
Ferrochelatase	0.50 ± 0.07	0.58 ± 0.04	1	0.78 ± 0.05	1.07 ± 0.09	1.28 ± 0.23	—

* Total cytochrome P-450 estimated spectrophotometrically (nmol/10^6 cells). Results represent the mean ± SE for four experiments. ND = not determined.

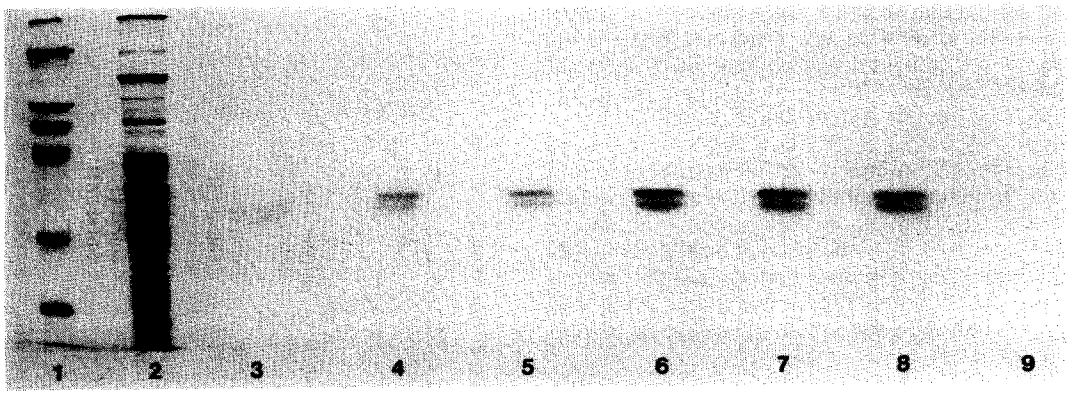

Fig. 2. Separation of hepatocyte proteins by SDS-gel electrophoresis and immunoblotting for cytochrome P-450 PB3a. Hepatocyte (3×10^4) lysates from a PB pretreated rat were subjected to electrophoresis using a 7.5% SDS-gel. Lanes 1 and 2 were stained with Coumassie blue for protein. Lane 1, molecular weight markers (myosin, β-galactosidase, phosphorylase b, bovine serum albumin, ovalbumin and carbonic anhydrase); lane 2 unsorted hepatocytes. The remaining lanes represent immunoblots probed with antibodies to cytochrome P-450 PB3a. Lanes 3 to 7, regions 1 to 5 respectively; lane 8 unsorted cells; lane 9 unsorted cells from an untreated control rat.

The magnitude of the enrichment of marker enzymes in the sorted regions is similar to that reported after the separation of cells using digitonin, centrifugal elutriation or density gradient techniques [8, 9]. However, using flow cytometry, sorted fractions do not appear to be related to acinar zones within the liver lobule.

Results suggest monooxygenase activities are highest towards diethoxyfluorescein and 3-cyano-7-ethoxycoumarin in the sorted cells from region 5. It cannot be excluded that Phase II conjugating enzyme systems may modify the cellular fluorescence detected using these substrates. Both sulphation and glucuronidation activities are concentrated in the pericentral areas of the liver lobule [26]. However, hepatocytes from region 5 show the highest levels of unscheduled DNA synthesis following exposure to a number of model carcinogens such as aflatoxin B, 2-acetylaminofluorene or retrorsine [11]. It was unexpected that the concentration of both the constitutive cytochrome P-450 PB1a and of cytochrome P-450 reductase was greatest in the sorted cells from region 2, suggesting these components may not be rate determining in the activation of these carcinogens.

Effects of pretreating rats with mixed function oxidase inducers. Two enzymes, lactate dehydrogenase and glutamate dehydrogenase were chosen as markers for periportal and centrilobular cells respectively. Table 2 shows with phenobarbitone, 3-methylcholanthrene and ethoxyquin, hepatocytes from region 5 had the highest relative activities. Cells from this region also had the highest activities of 3-cyano-7-ethoxycoumarin-*O*-deethylase, although the metabolism of 3-cyano-7-ethoxycoumarin is much more marked if induced by 3-methylcholanthrene pretreatment [11]. Some enrichment of diploid cells occurred in region 2, tetraploids in region 5, irrespective of the inducer employed. Similar distributions of the ploidy classes occur in sorted hepatocytes from control rats [11].

The small numbers of sorted cells prevented the preparation of microsomal fractions. However, as shown in Fig. 2, adequate resolution of the proteins from the solubilised hepatocytes could be achieved to permit immunoblotting and the detection of cytochrome P-450 isoenzymes. Following pretreatment with mixed function oxidase inducers, relative to controls, there is the expected 2–3-fold increase in overall cytochrome P-450 concentrations measured spectrophotometrically (Table 2). Because whole cell lysates were used, SDS gels could not be loaded with sufficient protein to achieve both an acceptable resolution of components and an accurate assessment of the concentration of constitutive cytochrome P-450 isoenzymic forms. In the case of phenobarbitone pretreated rats, in unsorted cells, there was a 20–30-fold increase in the PB3a isoenzyme relative to unsorted control hepatocytes (cf. Fig. 2). Similar results for the induction of isoenzymic forms of cytochrome P-450 by phenobarbitone or methylcholanthrene have been reported previously [3, 4]. With phenobarbitone or 3-methylcholanthrene, enrichment of isoenzymes PB3a and MC1a respectively both occur in the tetraploid-rich cells of region 5. Immunohistochemical studies show PB3a preferentially concentrated in the centrilobular zones while MC1a distribution is relatively even [3]. Ethoxyquin pretreatment, which like phenobarbitone causes the induction of cytochrome P-450 PB3a [20], results in the highest concentration of this isoenzyme in the diploid-rich cells of region 2. Unlike phenobarbitone, ethoxyquin preferentially induces cytochrome P-450 PB3a in the periportal areas of the liver lobule (M. M. Manson, unpublished results). In unsorted hepatocytes there is a marked reduction of the metabolism of diethoxyfluorescein from 0.38 ± 0.05 nmol/min per 10^6 hepatocytes in controls to 0.01 ± 0.001 nmol/min per 10^6 cells in ethoxyquin pretreated animals (mean ± SE, four experiments). Similar low levels of metabolism were seen using isolated microsomal preparations (I. N. H. White, unpublished results). The *O*-deethylation of 3-cyano-7-ethoxycoumarin is much less affected (Table 2), suggesting cytochrome P-450 PB3a is not involved.

Table 2. Effects of pretreating rats with mixed function oxidase inducers on the properties of hepatocytes following fluorescence activated cell sorting

		Unsorted cells	Sorted regions 1	2	3	4	5
Pretreatment: Phenobarbitone							
Nuclear ploidy (%)	Diploid:	35 ± 1	34 ± 1	38 ± 1	28 ± 1	24 ± 1	21 ± 1
	Tetraploid:	60 ± 1	61 ± 1	58 ± 3	67 ± 1	71 ± 2	72 ± 2
Binucleate cells (%)		10 ± 2	5 ± 1	15 ± 3	9 ± 1	6 ± 1	4 ± 1
Cytochrome P-450 (PB3a)		1.54 ± 0.1*	0.5 ± 0.1	1	1.9 ± 0.4	2.4 ± 0.5	2.4 ± 0.3
3-cyano-7-ethoxycoumarin-*O*-deethylase		0.34 ± 0.05	0.2 ± 0.03	1	1.8 ± 0.1	2.1 ± 0.1	2.9 ± 0.1
Lactate dehydrogenase		2845 ± 304	0.1 ± 0.03	1	1.2 ± 0.1	1.3 ± 0.1	1.3 ± 0.1
Glutamate dehydrogenase		3245 ± 315	0.8 ± 0.06	1	1.2 ± 0.1	1.4 ± 0.1	1.2 ± 0.1
Pretreatment: Methylcholanthrene							
Nuclear ploidy (%)	Diploid:	50 ± 1	71 ± 3	63 ± 2	48 ± 3	38 ± 2	29 ± 2
	Tetraploid:	44 ± 1	24 ± 3	30 ± 1	47 ± 1	55 ± 3	63 ± 2
Binucleate cells (%)		25 ± 2	23 ± 2	30 ± 3	23 ± 1	18 ± 1	21 ± 1
Cytochrome P-450 (MC1a)		0.72 ± 0.09*	0.8 ± 0.2	1	1.4 ± 0.3	1.8 ± 0.2	2.3 ± 0.4
3-cyano-7-ethoxycoumarin-*O*-deethylase		1.58 ± 0.18	0.5 ± 0.03	1	1.8 ± 0.2	2.2 ± 0.3	2.7 ± 0.1
Lactate dehydrogenase		2828 ± 515	0.2 ± 0.1	1	1.4 ± 0.2	1.5 ± 0.2	1.6 ± 0.2
Glutamate dehydrogenase		3603 ± 851	0.7 ± 0.1	1	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1
Pretreatment: Ethoxyquin							
Nuclear ploidy (%)	Diploid:	39 ± 2	37 ± 1	50 ± 5	38 ± 4	33 ± 3	30 ± 3
	Tetraploid:	57 ± 1	57 ± 2	54 ± 4	55 ± 2	62 ± 1	63 ± 1
Binucleate cells (%)		10 ± 2	11 ± 2	12 ± 2	13 ± 2	26 ± 2	26 ± 2
Cytochrome P-450 (PB3a)		0.79 ± 0.07*	0.5 ± 0.1	1	0.6 ± 0.1	0.5 ± 0.1	0.4 ± 0.1
3-cyano-7-ethoxycoumarin-*O*-deethylase		0.20 ± 0.03	0.2 ± 0.1	1	1.7 ± 0.2	2.2 ± 0.1	2.7 ± 0.1
Lactate dehydrogenase		2855 ± 300	0.04 ± 0.01	1	1.2 ± 0.02	1.5 ± 0.1	1.6 ± 0.02
Glutamate dehydrogenase		3194 ± 303	0.7 ± 0.1	1	1.1 ± 0.03	1.5 ± 0.2	1.6 ± 0.1
γ-Glutamyl transpeptidase positive cells (%)		36.1 ± 6	33.5 ± 5	32.5 ± 5	28.5 ± 5	23.4 ± 4	7.0 ± 1

Boxed areas show cytochrome P-450 and enzyme content relative to those of region 2. Results represent the mean ± SE for four experiments.
* Total cytochrome P-450 estimated spectrophotometrically (nmol/10^6 cells).
Enzyme activities are expressed as nmol/min/10^6 cells.

Ethoxyquin was used in the present study since it also selectively induces γ-glutamyltranspeptidase in the periportal areas of the liver [22]. Staining sorted hepatocytes from ethoxyquin pretreated rats showed the percentage of γ-glutamyltranspeptidase positive-cells decreased from regions 1 to 5 (Table 2).

Binucleation in sorted hepatocytes. Table 2 shows that in unsorted hepatocytes, the percentage of binucleate cells following phenobarbitone or ethoxyquin pretreatment is not changed relative to the control value of 12 ± 2% (mean ± SE, four experiments). Exposure to 3-methylcholanthrene results in a significant increase in the percentage of binucleate cells in the unsorted fraction ($P < 0.01$). After phenobarbitone or 3-methylcholanthrene pretreatment (Table 2) the highest percentage of binucleate cells is in region 2, while after ethoxyquin, the highest percentage of binucleates is in region 5. It is suggested that the change in the distribution of binucleated cells and cytochrome P-450 PB3a after ethoxyquin pretreatment may be due to selective toxicity in the centrilobular area. Other antioxidants of this class, e.g. butylated hydroxytoluene are cytotoxic [27]. Overall cell viabilities were consistently lower in hepatocytes from ethoxyquin pretreated rats.

In conclusion, hepatocytes sorted on the basis of their monooxygenase activities towards diethoxyfluorescein show enrichment of a number of enzymic activities, binucleation and nuclear ploidy. By these criteria, sub-populations of cells are selected which clearly differ from those using digitonin or centrifugal techniques. Examination of hepatocytes sorted by flow cytometry may be of particular relevance in the study of the response of the liver to hepatotoxins or carcinogens which required metabolic activation.

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