Characterization, localization and regulation of a novel phenobarbital-inducible form of cytochrome P_{450}, compared with three further P_{450}-isoenzymes, NADPH P_{450}-reductase, glutathione transferases and microsomal epoxide hydrolase

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Summary

Two cytochromes P_{450} (PB_1 and PB_2) have been isolated from the livers of rats treated with phenobarbital. PB_2 (mol. wt. 53500) is novel and is the first example of a phenobarbital-inducible enzyme with a Soret peak at 447 nm. Using an enzyme-linked immunosorbent assay, some immunochemical and structural similarities were observed between these cytochromes. PB_1 and PB_2 were induced by phenobarbital, Aroclor 1254, trans-stilbene oxide and to a lesser extent by isosafrole. Immunohistochemical localization of these proteins in the liver of untreated rats showed PB_1 to be localized in a large area and PB_2 in a narrow range of cells around the central vein. This demonstrates the heterogeneity of hepatocytes even within the centrilobular area and indicates that the synthesis of these two proteins is regulated differently although both are induced by the same agent, phenobarbital.

Two 3-methylcholanthrene inducible cytochromes MC_1 (mol. wt. 54500) and MC_2 (mol. wt. 57000) were present at very low levels, MC_2 mostly in the periportal region but also diffusely distributed throughout the lobule including some centrilobular cells, MC_1 concentrated in the centrilobular region.

The localization of two major groups of glutathione transferases (GST's) was also different. 'C' type proteins (Yb Yb') and microsomal epoxide hydrolase (EH) , were concentrated around the central vein, whereas the 'B' type proteins (Ya Yc) and cytochrome P_{450} reductase were distributed in a larger area of this region.

Thus, the localization was different for some members of the same enzyme family, whilst similarities in the localization existed across the border of the families:

- PB_2, MC_1, EH and GST 'C' type proteins were concentrated in a narrow area around the central vein;
- PB_1 and GST 'B' type proteins occupied a large centrilobular area;
- MC_2 levels were very low, predominantly periportal but also diffusely throughout the lobule.

Treatment of the animals with inducers increased the staining intensity and in several cases extended the areas of cells containing these proteins over the adjacent zone without fundamentally altering their distributions. However, treatment with ß-naphthoflavone led to a shift of MC_1 to the periportal area. This suggests that the expression of these proteins in certain cells is not an irreversible quality of differentiation but depends on the degree of suppression and derepression of regulatory components. The differences in the localization between the predominantly detoxifying enzymes EH and GST's and the cytochromes P_{450} which are frequently involved in the activation of carcinogens in all likelihood represent an important factor in the susceptibility of certain regions to chemical carcinogens, although it must be kept in mind that the method allows detection of immunoreactive protein but not that of enzyme activity.

* **Abbreviations:** P_{450}, cytochrome P_{450}; GST, glutathione transferase; EH, epoxide hydrolase; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

Introduction

The regulation of drug metabolizing enzymes within hepatocytes and the cells of other tissues is probably an extremely important factor in the initiation of chemical-induced carcinogenesis. Firstly, because the relative proportions of the cytochrome P_{450} (P_{450})* isoenzymes, frequently involved in the activation of procarcinogens, and the glutathione transferases (GST's), primarily involved in the deactivation of reactive metabolites, will determine the extent of reaction of these metabolites with DNA. Secondly, these enzymes are present as families of proteins and within each family large differences have been observed in the specificity of the isozymes in the activation of procarcinogens in the case of the P_{450}'s (1-3) and in the deactivation of the ultimate carcinogens in the case of the GST's (4, 5). It is therefore not only the absolute concentration of the P_{450}'s and GST's that is important, but also which isozymes are actually present and in which cells they are localized.

The activities of the P_{450} System, the GST's and microsomal epoxide hydrolase (EH), another important enzyme in the regulation of toxic metabolites (6), can all be induced by treatment of animals with foreign compounds (7). Induction is also known to alter the relative proportions of the P_{450} and the GST isozymes (8-11). It has recently become apparent that more than one P_{450} form can be induced by a single inducing agent and therefore the induced enzymes may well be regulated in a similar manner (8, 10). However, the extent of this similarity has not been determined. In this paper we report the isolation and characterization of a novel phenobarbital-inducible form of P_{450} as well as that of the phenobarbital-inducible form already characterized extensively in other laboratories (13). In order to study the regulation of these proteins, and that of two 3-methylcholanthrene-inducible isozymes, their response to a variety of inducing agents and also their localization within the liver lobule has been determined. The relationship between the localization of the P_{450} isozymes relative to the GST's and EH has been compared and these results are discussed in relation to the general regulation of drug metabolising enzymes and its implications with regard to chemical-induced carcinogenesis.

Materials and methods

Purification and characterization of proteins

Male Sprague-Dawley rats (150-200 g from the Süddeutsche Versuchstierfarm, Tuttlingen, FRG) were used. Animals received either phenobarbital (80 mg/kg in 0.9% NaCl) or 3-methylcholanthrene (20 mg/kg, dissolved in corn oil) i.p. for three consecutive days before use. P_{450} isozymes PB_1 and PB_2 were purified from liver microsomes from phenobarbital-treated rats. The method used was a modified version of that already applied to the purification of rabbit cytochromes (14, 15). Microsomes were solubilized with sodium cholate in 12.5 mM phosphate buffer, pH 7.4, containing 0.lmM EDTA and 0.1 mM dithiothreitol (buffer A) as described previously (14). Glycerol was then added (20% v/v) and the sample applied to a column of DEAE cellulose (DE 52, Whatman) equilibrated with buffer A. Further details of this purification procedure are shown in Figure 1. The yield of both cytochromes was low being < 1% of the original microsomal cytochrome content.

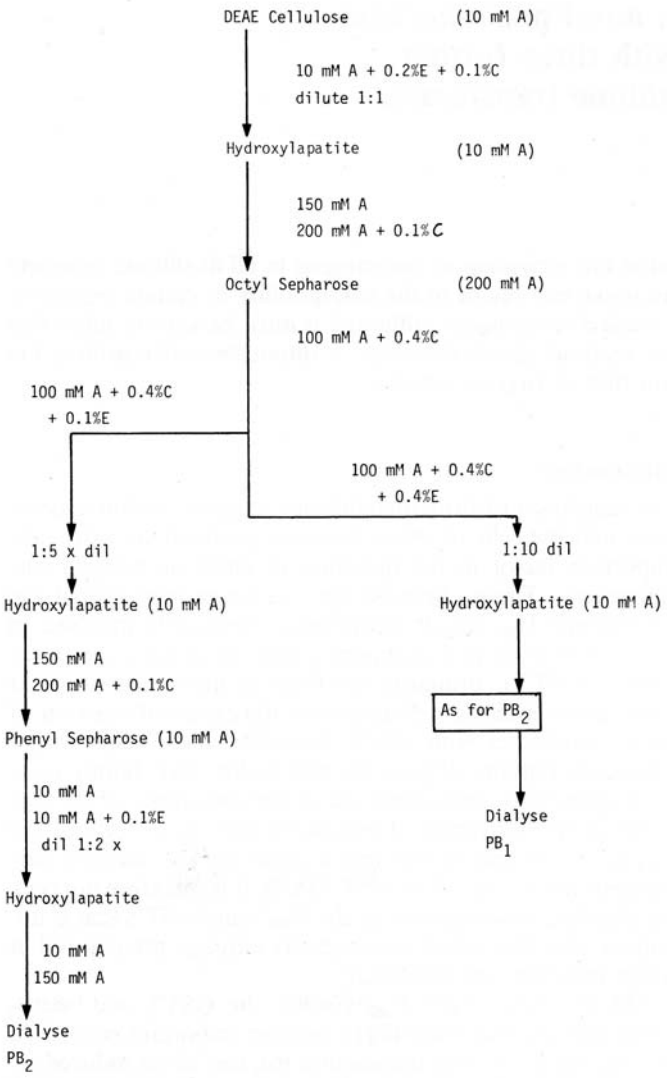

Fig. 1. Schematic representation of the purification of P_{450}'s (PB_1 and PB_2) from the livers of phenobarbital-treated rats. Equilibration buffer: (concentration in mM), E: Emulgen 911, C: sodium cholate, A: potassium phosphate buffer, pH 7.7 containing 20% glycerol (v/v), 0.1 mM EDTA and 0.1 mM dithiothreitol.

Cytochrome P_{450} reductase was eluted from the DEAE cellulose column with a KCl gradient (0-500 mM in buffer A containing 0.1 % cholate and 0.2% Emulgen 911) and was further purified by the method of Yasukochi and Masters (16). Final preparations of these and other proteins were stored at -70°C. P_{450} PB_1 had a specific content of 14.7 nmol/mg protein and P_{450} PB_2 a specific content of 15.6 nmol/mg protein. In addition to these two proteins two further cytochromes were prepared from the livers of 3-methylcholanthrene-treated rats (MC_1 and MC_2) as reported previously (10). These proteins had specific contents of 16.5 and 16.4 nmol/mg protein for MC_1 and MC_2, respectively.

Rat liver microsomal EH and the cytosolic GST's B and C (nomenclature according to Reference 20) were purified as reported previously (17, 18). The EH had a specific activity of 543 units/mg protein. One unit was defined as the amount of enzyme required to hydrolyse 1 nmol of styrene oxide per min at 37°C (19). The GST's B and C had specific contents of 18.58 and 10.50 units/mg protein, respectively, 1 unit being the amount of enzyme required to conjugate 1 µmol of chlor-2,4-dinitrobenzene per min at 25°C (20). Antisera to the cytochromes, GST B and C and EH were prepared either in rabbits using the procedure described previously (10), or were raised in goats. Animals were injected s.c. at several sites with 300 µg of antigen in Freunds complete adjuvant (1 ml diluted 1:1 with water), followed by two further injections of 200 µg in incomplete adjuvant (diluted 1:1) at two weekly intervals. A further injection was carried out if required. Blood samples were obtained two weeks following the final injection. Antisera and their IgG fractions were prepared by conventional procedures (21). Enzyme-linked immunosorbent assays (ELISA) were carried out by the method of Zimmer *et al.* (22). SDS-electrophoresis and P_{450} concentration were determined as described previously (15). Spectra were recorded using a Perkin Elmer Model 356 spectrophotometer. Protein determinations were by the method of Lowry *et al.* (23).

Immunohistochemical studies

Treatment of animals. Female Wistar rats (150-200 g from Zentralinstitut für Versuchstierkunde, Hannover, FRG) were used. Animals were kept on a standard diet (Altromin pellets, Altromin, Lage, FRG) and water *ad libitum.* Certain groups of animals were treated with either phenobarbital (80 mg/kg), 3-methylcholanthrene (50 mg/kg), isosafrole (150 mg/kg), ß-naphthoflavone (40 mg/kg), pregnenolone-16α-carbonitrile (50 mg/kg) or Aroclor 1254 (50 mg/kg). Phenobarbital was dissolved in 0.9% NaCl, the other compounds in olive oil. All inducers were administered by stomach tube once daily for 4 days.

Immunohistology. The livers of the animals were carefully removed and the large median lobe was excised. Liver slices of ~0.5 cm thickness were fixed in 99% ethanol-1% acetic acid for 12-15 h at 0-4°C and embedded in paraffin (24). 5-7 µm thick sections were mounted on acetone-cleaned slides, deparaffinated in xylene and passed from absolute ethanol into 0.05 M Phosphate buffered saline (0.15 M NaCl) pH 7.2 (PBS).

Endogenous peroxidases were inhibited by treatment of sections with 1 % hydrogen peroxide in PBS for 1 h (24). Slides were washed for 5 min in PBS followed by PBS supplemented with 1% bovine serum albumin plus 0.35 M NaCl (BSA/PBS) (25), then incubated with rabbit antibodies against the different enzymes for 24 h at 4°C followed by treatment with horseradish peroxidase-labelled goat anti-rabbit IgG antibodies (0.1 mg/ml) for 20 min at room temperature. Unreacted antibodies were washed off by three successive washings for 5 min each in BSA/PBS. Peroxidase activity was revealed by incubation in 3,3'-diaminobenzidine and H_2O_2 (26). After washing in PBS, sections were treated with 0.1% OsO_4 in PBS for 1 min, dehydrated and mounted under coverglass. Control incubations were performed either by substitution of the first antiserum with non-immune rabbit or goat serum or by omission of

anti-rabbit IgG antibodies. Alternatively, the median lobe was frozen immediately after removal and serial sections of 10 μm were prepared at -15°C on a cryostat microtome. The sections were mounted on albumin-coated slides, air-dried, washed with PBS for 5 min and then fixed with a *p*-benzoquinone solution (0.5% *p*-benzoquinone, 0.02 M $CaCl_2$, 0.2 M sodium cacodylate, pH 7.4) for 5 min. The subsequent treatment was as described above.

Materials. Horseradish peroxidase labelled IgG fractions and other immunochemicals were obtained from Medac (Hamburg, FRG). Isosafrole was a gift from Dr. T. Orton, ICI Pharmaceuticals Ltd., Alderly Park, UK. H styrene oxide was synthesized as described previously (27). All other chemicals were of the highest grade available and were from commercial sources.

Results

On chromatography of liver microsomal samples from phenobarbital-treated rats two P_{450} preparations were obtained. These two samples were of high purity (Figure 2a) and had clearly distinguishable mol. wt. (Figure 2a) of 52 000 and 53 500 for PB_1 and PB_2, respectively, (Figure 2b). The absolute spectra of these two cytochromes are shown in Figure 3. The spectra of the ferric cytochromes and ferrous cytochromes (not shown for PB_1) were indistinguishable with Soret peaks at 418 and 413 nm, respectively. However, a significant difference in the position of the ferrous P_{450} carbon monoxide complex was measured with maxima at 450 and 447 nm for PB_1 and PB_2. A comparison of the properties of these cytochromes with the other isozymes used in this investigation is made in Table I. All of the proteins used could be clearly distinguished from each other on the basis of their mol. wt. and substrate specificities. PB_1 was only active in the metabolism of 7-ethoxycoumarin, PB_2 did not metabolize either 7-ethoxycoumarin or 7-ethoxyresorufin at a detectable rate.

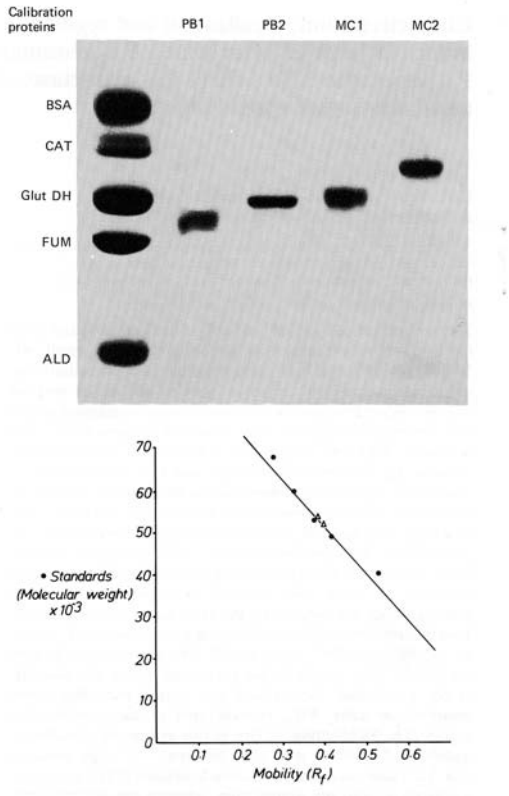

Fig. 2. (a) SDS electrophoresis P_{450} PB_1 and PB_2. 5 μg of each protein was used. **(b)** Mol wt. determination of PB_1 and PB_2. The standards used were bovine serum albulin (67 000), catalse (60 000), glutamic dehydrogenase (53 000), fumarase (49 000) and alsolase (40 000).

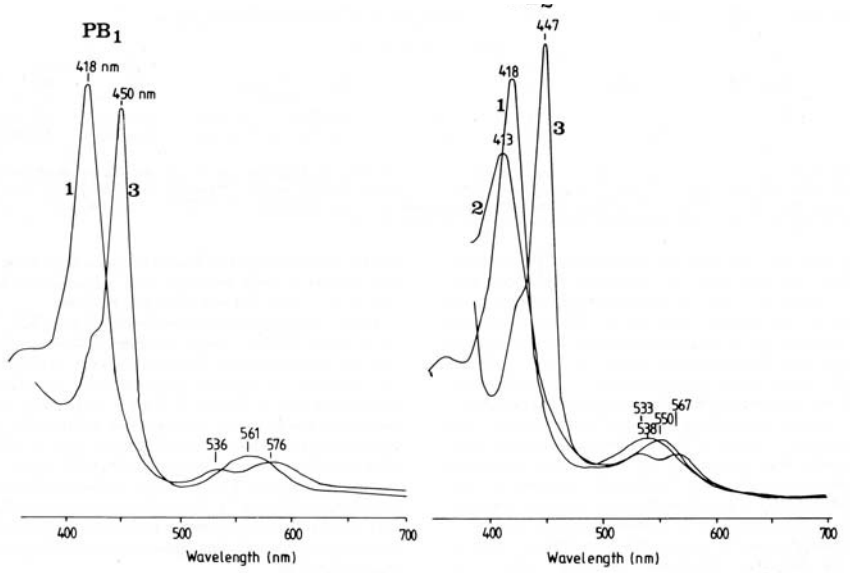

Fig. 3. Absolute spectra of P_{450} PB_1 and PB_2. 1 = Ferric cyt. P_{450} 2 = Ferrous cyt. P_{450} 3 = Ferrous cyt. P_{450}–carbon monoxide complex.

Table I. Summary of the data for the P-450 forms used in this study

P-450 form	Literature nomenclatures	CO-complex (nm)	Mol. wt.	Substrate specificity (nmol/min/nmol P-450)	
				7-EC	7-ERF
PB_1	P-450b P-450 PB B	450	52 000	3.4	ND
PB_2	–	447	53 500	ND	ND
MC_1	P-450d P-450 ISO G	447	54 500	ND	9.0
MC_2	P-450c	447	57 000	10.1	70.3

7-EC, 7-ethoxycoumarin; 7-ERF, 7-ethoxyresorufin; ND, not detectable.
Nomenclatures are from references 28 and 29.

The effect of various inducing agents on the relative concentrations of PB_1 and PB_2 determined using the ELISA is shown in Table II. Reactivity between each antibody and its homologous antigen was strong, whilst weak but significant cross reactivity was also observed between anti PB_1 and PB_2 and between anti PB_2 and PB_1 Reactivity with both antibodies was observed in control microsomes although a much higher level of PB_1 appeared to be present. The concentration of these proteins was increased substantially by treatment of the animals with phenobarbital, Aroclor 1254 or trans-stilbene oxide. Interestingly, the novel phenobarbital-inducible form PB_2 was induced by phenobarbital (and also by Aroclor 1254)

to a greater extent than PB_1 whilst trans-stilbene oxide led to a similar increase of the two forms. Isosafrole treatment caused a slight increase in PB_1 and a larger increase in PB_2 concentration. Neither 3-methylcholanthrene or clofibrate induced PB_1, in fact these reagents appeared to repress the levels of this protein. It is of interest that in contrast to PB_1 the novel phenobarbital-inducible form PB_2 was also increased by 3-methylcholanthrene treatment, albeit considerably less than by phenobarbital.

Table II. ELISA for the detection of PB_1 and PB_2 in liver microsomal samples from rats treated with various inducing agents

	Reactivity (absorption units)								
	P-450 PB_1	P-450 PB_2	Control	PB	Aro	TSO	MC	Iso	Clo
Anti PB_1	0.31	0.07	0.13	0.60 (4.6)	0.38 (2.9)	0.61 (4.6)	0.03 (0.2)	0.20 (1.54)	ND
Anti PB_2	0.07	0.22	0.03	0.35 (11.7)	0.26 (8.6)	0.12 (4.0)	0.08 (2.6)	0.12 (4.0)	ND

P-450 stock solutions were 5 μg/ml. 10 μl was taken per assay. Microsomal samples (0.4 mg/ml; 10 μl/assay) were solubilized with sodium cholate (1.6 mg/ml). Other details are given in reference 22. Animals were treated with phenobarbital (PB), Aroclor 1254 (Aro), trans-stilbene oxide (TSO), 3-methylcholanthrene (MC), isosafrole (Iso), clofibrate (Clo). P-450 concentrations were: control 0.53, PB 1.09, TGSO 0.81, Clo 0.90, 3-MC 1.12, Iso 1.26 and Aro 2.39 nmol/mg protein. (n.n), n fold increase over control; ND, not detectable.

Immunohistochemical demonstration of these two proteins as well as that of MC_1 and MC_2, the GST's B and C and microsomal EH is shown in Figure 4 (*cf. to original publication*). Samples in this Figure were obtained from animals treated with isosafrole. Control animals gave practically the same distribution (for comparison see Figure 6; *cf. to original publication*), however the staining intensity was uniformly lower so that the localizaton showed up more distinctly after treatment with isosafrole. PB_1 was localized diffusely in the centrilobular region. Interestingly PB_2 was distributed in a different manner to PB_1 and was much more concentrated in a narrow area around the central vein appearing to be highly concentrated in particular cells (Figure 5; *cf. to original publication*). Relative to these two cytochromes the MC-inducible enzymes were present only in very low concentrations in the untreated liver. MC_2 occurred mostly in the periportal area but was also diffusely distributed throughout the whole lobule including some centrilobular cells whereas MC_1 was preferentially localized in the centrilobular region. The localization of glutathione S-transferase B was diffusely centrilobular, similar to P_{450} PB_1, that of GST C and EH were more concentrated around the central vein, similar to P_{450} PB_2. P_{450} reductase was present in broad areas of the centrilobular and midzonal regions.

The effect of pretreatment of rats with various agents on the enzyme distribution is summarized in Figure 6. In most instances PB_1 and PB_2 were induced by the same compounds, however the extent of induction of these isoenzymes varied. The same was the case for MC_1 and MC_2. These data are in good agreement with the findings of the ELISA (Table II, reference 10). Some inducers, however, e.g. Aroclor 1254 and isosafrole, affected both P_{450} groups. The intensified immunostaining following drug treatment was usually not associated with an altered distribution of the cytochromes (Figure 6) the enzymes being localized at higher levels in similar areas as controls. In certain cases (e.g. Aroclor 1254) the immunostaining extended into adjacent lobular zones which in controls did not show any reaction.

Fundamental changes in the localisation of MC_1 and less pronounced of MC_2 were observed following β-naphthoflavone administration. This was the only case, where both MC-inducible cytochromes clearly showed pronounced periportal localization (Figure 7; *cf. to original publication*). This is particularly interesting relative to the induction of these two proteins by 3-methyl-cholanthrene or Aroclor 1254 (Figure 6). On the other hand, after β-naphthoflavone administration, GST B and GST C as well as microsomal EH remained undetectable in the periportal region (Figure 7). These enzymes as well as P_{450} reductase, constantly showed

central and midzonal localization, independent of the inducing agent and the resulting pattern of P_{450} isoenzymes.

Discussion

A novel form of P_{450} (PB_2) has been isolated from liver microsomes of phenobarbital-treated rats. This form can be distinguished from a second enzyme induced by this compound (PB_1) on the basis of molecular weight, carbon monoxide spectrum and substrate specificity. A number of isozymes and polymorphic forms of P_{450} have been isolated from the livers of phenobarbital-treated rats (8, 13, 28, 29). However, the mol. wt. and the absorption maxima of the carbon monoxide complexes of these cytochromes are distinctly different from those of PB_2 which therefore appears to be an as yet unreported P_{450} form. In the rabbit liver there are also indications of the presence of at least two phenobarbital-inducible cytochromes [form 2 (30, 31) and form 5 (3, 32)]. Form 2 appears to be analogous to PB_1 In spite of being a minor form in control rabbit liver form 5 accounts for the majority of the hepatic activity in the activation of many aromatic amines (32). It would therefore be of interest to determine the possible analogy between PB_2 and form 5 by investigating the activity of PB_2 towards these substrates. Forms 2 and 5 are also major P_{450} forms in the rabbit lung (33). The 3-methylcholanthrene-inducible enzymes, MC_1 and MC_2, do appear to have counterparts in the rabbit liver (34). Immunochemical similarities between PB_1 and PB_2 indicate the presence of common structural domains in these proteins. We have made a similar observation for MC_1 and MC_2 (10, see also 29). The cross-reactivities of these proteins could influence both the quantitation as well as the immunohistochemical localization. At the antibody dilutions used this does not appear to have been a problem as the cytochromes were not localized in a similar fashion. In support of this certain inducing agents had a differential effect on PB_1 and PB_2 which would not have been the case should cross-reactivity have been an interfering factor. The differential regulation of all of these cytochromes during hepatocarcinogenesis substantiates these conclusions (35). It can, of course, never be excluded that as yet unidentified cross-reacting proteins contribute to the observed staining pattern.

P_{450} PB_2 is localised in a highly concentrated fashion in a small portion of cells in the centrilobular region. Highly specific localizations of P_{450} forms have been reported for extrahepatic tissues (36-39). This is the first observation of such a specific localization in hepatocytes. In contrast PB_1 is much more diffusely distributed. In recent publications by Baron *et al.* (40, 41) a cytochrome referred to as P_{450} PB B (probably PB_1) was also found to be predominantly localized in the centrilobular region.

In the liver of untreated rats P_{450} MC_1 and MC_2 were found to be present only in low concentrations throughout the whole liver lobule, MC_1 being preferentially localized around the central vein, whilst MC_2 showed a higher concentration in the periportal region. With the exception of this latter finding these data agree essentially with those of Baron *et al* (40, 41) who localized a cytochrome P_{450} MC B (probably equivalent to MC_2) relatively evenly within the liver lobule with a slightly higher concentration in the centrilobular region. In view of the very small intensity of the periportal staining found in our studies, this discrepancy may easily be explained with differences in animals and methods used. The localization of MC_1 has not been previously reported and it is interesting to note that it was found to be localized differently to MC_2. These studies demonstrate that there are significant differences between hepatocytes in their enzyme content and show that hepatocytes are not a homogeneous cell population with respect to drug metabolising enzymes not even within a given area.

This conclusion is further substantiated by the finding that the GST's also show differences in the pattern of lobular distribution, GST B being localized diffusely in the central and midzonal area and GST C being localized in a narrower area around the central vein. The antibodies used in this study had been raised against GST B (Ya Yc) and GST C (Yb Yb'). These antibodies are not specific for these forms as anti GST B will also react with ligandin (subunits Ya Ya) and GST AA (subunits Yc Yc) and anti GST C (Yb Yb') will also react with GST A (Yb Yb) and GST X (Yb' Yb') (for nomenclature see 20, 42, 18). Investigations using antibodies to the specific subunits will be needed to identify the individual localizations of these six transferase forms. The localization of ligandin has already been reported to be in the centrilobular region (43). Redick *et al.* (44) have shown GST's B and C to be localized throughout the whole liver lobule with higher centrilobular concentrations. This is essentially in agreement with our findings, with the exception of the staining reported in the periportal region. In our study in normal liver low GST concentrations were found in the midzonal but animals were not principally altered following treatment with the majority of the inducers investigated. Predominantly the lobular region in untreated animals but extended into further zones after induction.

The enzyme distributions found in liver of untreated not in the periportal area. In agreement with previous findings (45) microsomal epoxide hydrolase was increased in centrilobular areas s already containing these proteins, strong induction leading to an expansion into adjacent lobular regions. These data indicate that hepatocytes which normally do not express certain enzymes still contain the regulatory system to respond to the enhanced functional demands evoked by inducing agents with adaptive enzyme synthesis. Depending on the type of inducer used, either PB_1 and PB_2 or MC_1 and MC_2, respectively, became in most cases increased simultaneously. Groups of P_{450}'s therefore appear to be regulated combined in a similar manner. However, the individual isoenzymes of each group may be stimulated to very different degrees, and thus PB_1 and PB_2 as well as MC_1 and MC_2 may be localized in part within different cells of the lobule. This indicates that besides a common regulatory principle there must exist separate mechanisms in the regulation of each of these proteins. This is substantiated by the fact, that in addition to quantitative alterations some of the inducing agents also cause qualitative differences in the enzyme localization. Following administration of β-naphthoflavone, a significant change in localization of MC_1 and MC_2 was observed, the isoenzymes becoming concentrated preferentially in the periportal region. These data indicate that β-naphthoflavone acts in a different manner to 3-methylcholanthrene, which induced these isoenzymes to similar levels in all three regions of the liver lobule. Treatment of animals with the potent inducer Aroclor 1254 resulted in a marked increase of all P_{450} isoenzymes several of them within the whole liver lobule.

Application of most of the inducers of the P_{450} isoenzymes investigated led to a concomitant increase of the predominantly detoxicating enzymes EH and GST's in the central and midzonal areas of the liver, but not in the periportal region. In those cases in which EH and GST's were increased in the same area of the liver lobule as the P_{450} forms, the localization of these enzymes is consistent with their role in the detoxication of toxic and carcinogenic metabolites produced by cytochrome P_{450}-mediated reactions. An uneven distribution of these enzymes, especially of the GST's relative to the cytochromes, however, as seen after treatment of animals with β-naphthoflavone, 3-methylcholanthrene or isosafrole is of toxicological importance, since it is known that these enzymes have different specificities towards different carcinogenic electrophiles (4, 5). As a consequence P_{450} MC_2 is present in cells of the periportal region which contain no or extremely low concentrations of the two GST's and EH. MC_2

is an enzyme with high activity in the conversion of polycyclic aromatic hydrocarbons (46), aromatic amines (2), and other procarcinogens to the ultimate carcinogenic species.

The inability of these cells to remove these metabolites may be a major contributing factor to the subsceptibility of this area of the liver to the cytotoxic and carcinogenic action of certain chemical agents, although it must be kept in mind that the method detects immunoreactive protein but not enzyme activity.

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