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Metabolic-Intermediate Complex Formation Reveals Major Changes in Rat Hepatic Cytochrome P-450 Subpopulations in Addition to Those Forms Previously Purified after Phenobarbital, β-Naphthoflavone, and Isosafrole Induction

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SUMMARY

By coupling a simple separatory procedure with the ability of cytochrome P-450 subpopulations to sequester themselves as metabolic-intermediate (MI) complexes, previously undocumented alterations of cytochrome P-450 subpopulations by inducing agents have been detected. Phenobarbital induces at least two forms of cytochrome P-450 differing in their chromatographic properties and molecular weight, with neither form possessing the ability to generate MI complexes from isosafrole. β-Naphthoflavone induces at least two forms having different chromatographic properties, molecular weights and abilities to generate MI complexes; these two forms differ from both of the forms induced by phenobarbital. Isosafrole induces at least three forms, all of which generate MI complexes but which differ from each other in chromatographic properties and molecular weight. Thus, in addition to the well-characterized forms of cytochrome P-450, phenobarbital, β-naphthoflavone, and isosafrole also cause changes in heretofore uncharacterized forms, and it is these "other" forms which are responsible for the changes in isosafrole MI complex formation seen after β-naphthoflavone and phenobarbital treatment.

INTRODUCTION

Cytochrome P-450 catalyzes the oxidation of a wide variety of drugs as well as endogenous substrates such as steroids (1, 2). The multiplicity of the hepatic microsomal drug-metabolizing system was first postulated by Axelrod (3) and was subsequently proven by many workers who have purified and characterized several forms of cytochrome P-450, usually after the induction of that form in response to treatment with phenobarbital, 3-methylcholanthrene, pregnenolone 16α-carbonitrile, isosafrole, or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (4–8).

A form of cytochrome P-450 induced by phenobarbital and a form induced by 3-methylcholanthrene have been extensively studied since they constitute a large percentage of the cytochromes of induced animals. Using an immunoquantitation method, which relies upon an antibody raised to a purified cytochrome having no cross-reactivity with any form (previously purified or as yet uncharacterized), Thomas *et al.* (9) reported that the phenobarbital-inducible form accounts for 35% of the total microsomal cytochrome P-450 in phenobarbital-pretreated adult rats, whereas the 3-methylcholanthrene-inducible form accounts for 68% of the total microsomal cytochrome P-450 in 3-methylcholanthrene-pretreated rats. Harada and Omura (10) reported that microsomes from phenobarbital or 3-methylcholanthrene-treated rats contain approximately 60% and 90% of the total microsomal cytochrome P-450 as the phenobarbital or 3-methylcholanthrene-inducible forms, respectively. The reason for the 20–25% greater contribution of the major form to the total cytochrome in these latter studies is not known, but may relate to antibody specificity as mentioned above. Although these two cytochromes account for a large proportion of the cytochromes present in an induced animal in both studies, they account for less than 3% (9) or 20% (10) of the cytochromes present in an untreated adult animal. A "constitutive" form of cytochrome P-450 which has been purified and characterized accounts for less than 3% in either an induced or noninduced animal (9). Very little is known about the remainder of the cytochromes in uninduced rat liver or about the noncharacterized forms of the cytochromes in an induced animal.

The chromatographic method of Warner and Neims (11), which resolves microsomal cytochrome P-450 into four fractions, offers the advantage of examining more of the total cytochromes present, owing to an over-all re-

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covery of greater than 75%. However, as noted by Lu and West (12), chromatography of microsomal protein after animal treatments may yield corresponding fractions that do not contain the same forms of cytochrome P-450. The induction of a form of cytochrome P-450 might go undetected if it elutes under the same conditions as the cytochrome(s) that exist constitutively and cannot be differentiated on the basis of its molecular weight.

With the use of compounds capable of forming MI[1] complexes with cytochrome P-450 (13, 14), an estimation of the various subpopulations of cytochrome P-450 in microsomes has been attempted by Werringloer *et al.* (15). By monitoring MI complex formation in the various subfractionations of the microsomal cytochrome P-450 achieved through the simple separatory procedure of Warner and Neims (11), a greater sensitivity in the detection of the different forms of cytochrome P-450 is accomplished. This work reports on the alterations in the subpopulations of cytochrome P-450 due to the induction of forms of cytochrome P-450 which differ in character from those forms previously purified and characterized, and in some cases the increases upon induction exceed those of the well-characterized forms.

EXPERIMENTAL PROCEDURES

Male Sprague-Dawley rats (200–300 g, Simonsen Labs) were used for all experiments. Animals either were not induced or were induced with phenobarbital (80 mg/kg i.p. daily for 4 days) or with β-naphthoflavone (80 mg/kg in corn oil i.p. daily for 3 days). Twelve hours before sacrifice, animals in all three groups were administered isosafrole (160 mg/kg in corn oil i.p.) This time interval allowed maximal MI complex formation from the isosafrole to occur without any inductive effect (increase in cytochrome P-450 concentration) from the isosafrole itself. To determine whether alteration in extent of MI complex formation could occur from changes in the pharmacokinetics of isosafrole upon induction, the extent of MI complex formation in uninduced, phenobarbital-induced, and β-naphthoflavone-induced groups was investigated at much higher doses of isosafrole: 320, 640, and 960 mg/kg. At the 12-hr interval there was no increase in extent of MI complex formation over that seen at the 160-mg/kg dose. Another group of rats was treated with isosafrole (160 mg/kg in corn oil i.p. daily) for 3 days during which time both induction of cytochrome P-450 and formation of MI complex occurred.

Microsomes were prepared by the method of Franklin and Estabrook (16) and suspended in 10 mM sodium phosphate buffer (pH 7.4) to a concentration of 0.33 ml of buffer per gram of liver (original wet weight). Aliquots were removed for protein (17) and cytochrome P-450 (18) determinations and for the determination of the amount of cytochrome present as an isosafrole MI complex. The MI complex present was determined with an Aminco DW-2 spectrophotometer set in the dual-wavelength mode, by monitoring the absorbance change at 456 nm relative to 490 nm upon reduction of the microsomes with sodium dithionite. It was quantitated using an extinction coefficient of 75 $mM^{-1}cm^{-1}$ (19). The amount of uncomplexed cytochrome P-450 was then determined from the additional absorbance produced at 450 nm relative to 490 nm upon gassing with carbon monoxide.

Microsomes were solubilized and chromatographed on a DEAE-cellulose (DE-52) ion-exchange column by a modified method of Warner and Neims (11). Microsomes were solubilized for 1 hr by the addition of a solubilizing buffer [0.66 ml/g of liver (original wet weight)] containing 10 mM sodium phosphate, 0.15 mM EDTA, 0.75% (w/v) sodium cholate, 30% (v/v) glycerol, and 0.3% (v/v) Emulgen 911, and adjusted to pH 7.4. The solubilized microsomes, containing 300 nmoles of cytochrome P-450, were then applied to the column (30 × 2.5 cm) and developed with 100 ml of buffered detergent (10 mM sodium phosphate, 0.1 mM EDTA, 0.5% (w/v) sodium cholate, 20% (v/v) glycerol, and 0.2% (v/v) Emulgen 911), followed by a 500-ml linear gradient (0–250 mM) of NaCl in buffered detergent.

Eluted fractions were analyzed for MI complex and uncomplexed cytochrome P-450. In addition, the apparent molecular weights of the eluted proteins were determined by polyacrylamide gel electrophoresis in the presence of 0.1% SDS as described by Laemmli (20). For this determination, samples were dialyzed overnight against buffer (pH 6.8) containing 60 mM Tris-chloride and 1% SDS and then concentrated over crystalline sucrose. A volume equivalent to approximately 10 μg of protein (to which was added 1% SDS, 15% glycerol, 0.1% bromophenol blue, and 5% β-mercaptoethanol) was heated at 100° for 3 min and applied to a 1-cm stacking gel of 3% acrylamide followed by an 8-cm running gel of 7.5% acrylamide. Gels were electrophoresed at 40 mamp through the stacking gel and 70 mamp through the running gel. Gels were stained overnight with 0.05% Coomassie Brilliant Blue in methanol-acetic acid-water (2:1:7) according to the method of Fairbanks *et al.* (21).

RESULTS

Solubilization of the microsomes occurred with very little loss of cytochrome P-450 (<10%). The solubilized cytochrome P-450 eluted from the DEAE-cellulose column as four fractions with the total recovery of cytochrome P-450 applied to the column, ranging from 72 to 77%. The proportion of cytochrome P-450 eluting in each of the four fractions varied with the inducing agent used. The original composition of the microsomes with respect to the subpopulations of cytochrome P-450 eluting in each fraction could then be determined from the product of the proportion of cytochrome P-450 occurring in each fraction and the concentration of cytochrome P-450 per milligram of protein found in the microsomes (Table 1). Uninduced microsomes have a cytochrome P-450 concentration of 0.67 nmole/mg of protein and 13%, 56%, 22%, and 7% of the applied cytochrome P-450 elutes in Fractions I, II, III, and IV, respectively. Thus, the amounts of cytochrome P-450 capable of eluting in Fractions I–IV were 0.09, 0.38, 0.16, and 0.05 nmole/mg of original microsomal protein, respectively (Table 1). Phenobarbital induction doubled the microsomal cytochrome P-450 concentration. After induction the cytochromes were of the type that eluted predominantly in

[1] The abbreviations used are: MI complex, metabolic-intermediate complex; SDS, sodium dodecyl sulfate.

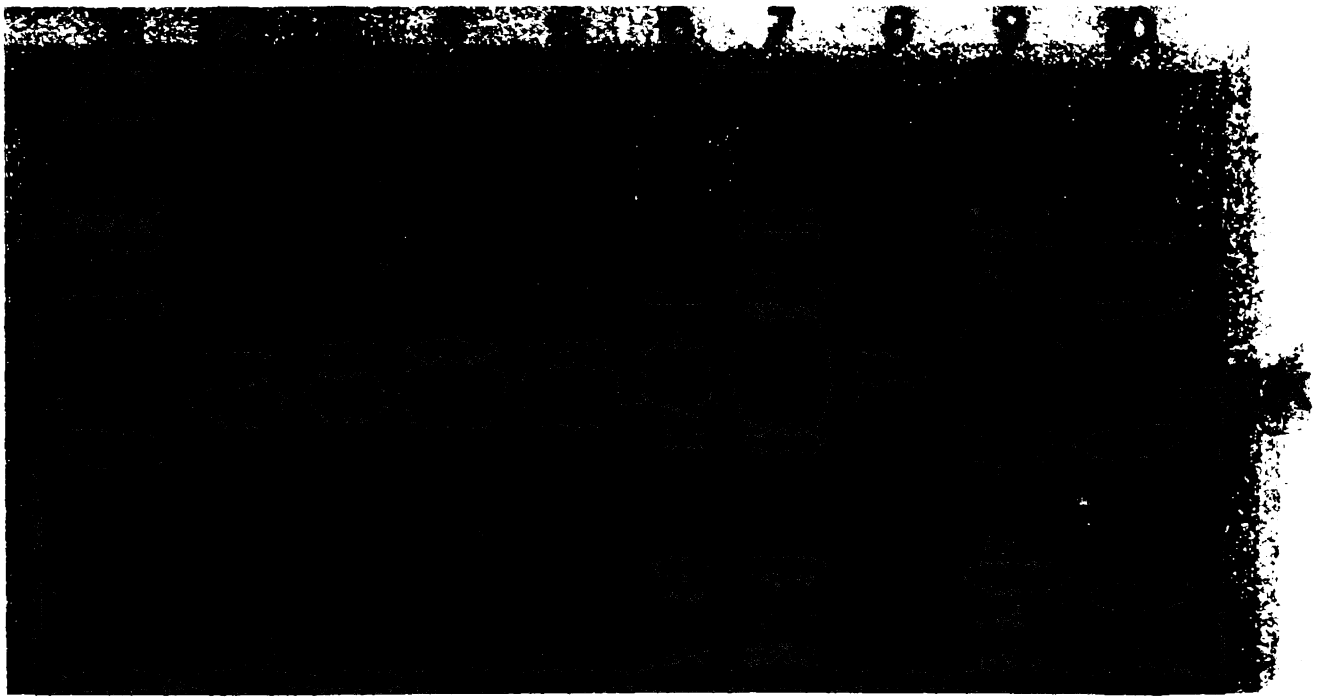

FIG. 1. *Polyacrylamide gel electrophoresis of microsomal proteins eluting in Fractions II and III from a DEAE-cellulose column*

Approximately 10 μg of protein (in the presence of SDS, glycerol, bromophenol blue, and mercaptoethanol) were boiled and electrophoresed through a 1-cm stacking gel of 3% acrylamide and an 8-cm running gel of 7.5% acrylamide. Gels were stained overnight with 0.05% Coomassie Brilliant Blue according to the method of Fairbanks *et al.* (21).

Wells *1* and *10* contain molecular weight standards of carbonic anhydrase, ovalbumin, bovine serum albumin, phosphorylase B, and β-galactosidase which range in molecular weights from 31,000 to 116,250. Wells *2–5* contain proteins eluting in Fraction II from uninduced, phenobarbital-induced, β-naphthoflavone-induced, and isosafrole-induced rats, respectively, and Wells *6–9* contain proteins eluting in Fraction III from the same animals.

TABLE 1

Effect of inducing agents on the elution profile of cytochrome P-450 from solubilized microsomes upon DEAE-cellulose column chromatography

Microsomes from rats either noninduced or induced with phenobarbital, β-naphthoflavone, or isosafrole were solubilized and chromatographed on a DEAE-cellulose column using a linear gradient of 0–250 mM NaCl in buffered detergent. The cytochrome P-450 eluted as four fractions in amounts varying according to the inducing agent used.

Animal treatment[a]	Microsomal cytochrome P-450 ± SE	Microsomal cytochrome P-450 (±SE) eluting in				% Recovery[c] ± SE
		Fraction I[b]	Fraction II[b]	Fraction III[b]	Fraction IV[b]	
	nmoles/mg					
None (4)	0.67 ± 0.02	0.09 ± 0.03	0.38 ± 0.06	0.16 ± 0.01	0.05 ± 0.01	76 ± 5
Phenobarbital (5)	1.41 ± 0.07	0.16 ± 0.04	0.72 ± 0.05[d]	0.43 ± 0.03[d]	0.06 ± 0.01	74 ± 6
β-Naphthoflavone (4)	1.56 ± 0.07	0.03 ± 0.01	1.07 ± 0.06[d]	0.39 ± 0.03[d,e]	0.08 ± 0.02	72 ± 6
Isosafrole (5)	1.50 ± 0.13	0.14 ± 0.05	1.07 ± 0.05[d]	0.21 ± 0.02	0.08 ± 0.02	77 ± 3

[a] See Experimental Procedures for regimen. Numbers in parentheses indicate number of preparations examined.

[b] Derived from the following equation: [% of cytochrome eluting in each fraction] × [microsomal cytochrome P-450 concentration (nmoles/mg protein)].

[c] Of 300 nmoles of cytochrome P-450 applied to the DEAE-cellulose column.

[d] Significantly different with respect to noninduced animals ($p < 0.005$).

[e] This cytochrome P-450 fraction shows a ferrous carbon monoxide spectral maximum at 448 nm. All others were 450 ± 0.5 nm.

Fractions II and III (0.72 and 0.43 nmole/mg, respectively). This compares with 0.38 and 0.16 nmole/mg eluting in Fractions II and III in uninduced microsomes. There was also a slight increase in the amount eluting in Fraction I after phenobarbital induction. β-Naphthoflavone induction, like phenobarbital, doubled the microsomal P-450 concentration, and again the induced forms were of the type that eluted in Fractions II and III (1.08 and 0.39 nmoles/mg, respectively). Isosafrole, which also doubled the microsomal cytochrome P-450 concentration, differed from both phenobarbital and β-naphthoflavone in that the induced cytochrome P-450 eluted predominantly in Fraction II, with very little eluting in Fraction III. However, similar to phenobarbital induction a minor amount of the induced cytochrome eluted in Fraction I.

TABLE 2

Effect of inducing agents on the elution profile of cytochrome P-450 isosafrole metabolic-intermediate complexes after DEAE-cellulose column chromatography of solubilized microsomes

Microsomes were prepared from rats that were either noninduced or induced with phenobarbital or β-naphthoflavone and treated with isosafrole for 12 hr before sacrifice. Microsomes were also prepared from rats treated with isosafrole daily for 3 days. Microsomes were solubilized and chromatographed on a DEAE-cellulose column using a linear gradient of 0-250 mM NaCl in buffered detergent. The cytochrome P-450-MI complex was determined from the absorbance change at 456 nm relative to 490 nm upon reduction of the microsomes or the eluted fractions with sodium dithionite, and quantitated using an extinction coefficient of 75 mM^{-1} cm^{-1}. The amount of uncomplexed cytochrome P-450 was then determined from the additional absorbance produced at 450 nm relative to 490 nm upon gassing with carbon monoxide.

Animal pretreatment[a]	% Cytochrome P-450 existing as isosafrole-MI complex[b]		Microsomal cytochrome P-450 originally existing as an MI complex[c] which elutes in			
	In microsomes	After chromatography	Fraction I	Fraction II	Fraction III	Fraction IV
None (4)	18	14	0.016 (18)	0.075 (19)	0.002 (1)	0.000 (0)
Phenobarbital (5)	7	7	0.023 (15)	0.065 (9)	0.004 (1)	0.002 (4)
β-Naphthoflavone (4)	44	35	0.007 (23)	0.560 (52)	0.004 (1)	0.002 (2)
Isosafrole (5)	50	44	0.051 (38)	0.555 (53)	0.015 (7)	0.005 (6)

[a] See Experimental Procedures for regimen; numbers indicate number of preparations examined.

[b] Twelve hours after a single dose of isosafrole (160 mg/kg) except isosafrole-pretreated animals, which received no additional isosafrole.

[c] Calculated by the following equation: [microsomal cytochrome P-450 eluting in each fraction (from Table 1)] × [% of cytochrome P-450 in that fraction existing in the MI complexed state (values in parentheses in these columns)].

The ability of the "constitutive" and induced forms of cytochrome P-450 to form metabolic intermediate complexes from isosafrole *in vivo* was investigated as a possible means of identifying subpopulations existing within the fractions eluting from DEAE-cellulose (Table 2). The presence of cytochrome P-450 in a complexed state did not alter the DEAE-elution profile, nor did it alter the yield of total cytochrome eluting off of the column. However, there was some loss (up to 22%) of MI complex during the solubilization and chromatographic procedures. Twelve hours after an isosafrole injection, microsomes from an uninduced animal contained 18% of the cytochrome P-450 in the complexed state. The complexed cytochromes eluted predominantly in Fractions I and II. In animals previously induced with phenobarbital, only 7% of the microsomal cytochrome P-450 existed in the complexed state 12 hr after the isosafrole injection. The complexed cytochromes again eluted in the first two fractions and in relative amounts similar to those seen in uninduced animals (0.016 and 0.023 in Fraction I and 0.075 and 0.065 in Fraction II for uninduced and phenobarbital induced, respectively). Thus, despite 2- and 3-fold increases in the total amount of cytochrome P-450 (complexed plus uncomplexed) eluting in Fractions II and III (Table 1), there was no increase in the amount of complexed cytochrome eluting there (Table 2).

Twelve hours after a single injection of isosafrole in rats induced with β-naphthoflavone, 44% of the microsomal cytochrome P-450 was in a complexed state. Once again the complexed cytochromes eluted predominantly in Fractions I and II. The induced cytochrome eluting in Fraction III, like that in Fraction III after phenobarbital induction, was almost devoid of isosafrole MI complex. The induced cytochrome eluting in Fraction II after β-naphthoflavone induction differs from that seen after phenobarbital induction by the 10-fold increase in the amount capable of existing as an MI complex (0.56 as compared with 0.065 nmole/mg).

After isosafrole induction, the amount of cytochrome P-450 in the microsomes existing as an isosafrole MI complex increased to 50%, similar to the extent seen after β-naphthoflavone induction. However, the complexed cytochromes differed in their distribution between the four fractions. After isosafrole induction they eluted in Fractions I, II, and III, whereas after β-naphthoflavone induction they eluted almost exclusively in Fraction II. Thus isosafrole is unique in its ability to induce cytochromes which elute in Fractions I and III and exist as isosafrole MI complexes.

The major protein staining bands (in the 45,000–55,000 range)[2] of the four fractions eluting from DEAE-cellulose after SDS-polyacrylamide gel electrophoresis were determined (Table 3). The major protein band in Fractions I and IV were of 48,000 and 55,000 molecular weight, respectively, and were not altered by the three inducing agents. The molecular weight of the major protein bands of Fractions II and III were dependent upon the inducing agent (Fig. 1). Before induction the major protein band in Fraction II had a molecular weight of 50,000 whereas after phenobarbital induction it was 51,000. After β-naphthoflavone or isosafrole induction, Fraction II contained three major protein bands of 49,000, 50,000 and 52,000 mol wt. In Fraction III two major protein bands of 51,000 and 54,000 mol wt were present in uninduced animals, but after phenobarbital and β-naphthoflavone induction, single major bands of 52,500 and 54,000 mol wt, respectively, were observed. Isosafrole induced several proteins in Fraction III, with molecular weights of 49,000, 50,000, 54,000 and 55,000.

DISCUSSION

The induction of cytochrome P-450 is a complex cellular event that is not well understood. It is usually

[2] The molecular weight of the same form of cytochrome P-450 may differ when determined in different laboratories, but the relative order of electrophoretic mobilities is quite reproducible (12). Because of the dissociation of the heme and the apocytochrome in the presence of SDS, it is also difficult to identify a protein as a cytochrome; therefore, induction of a protein is at best indirect evidence for induction of cytochrome P-450.

TABLE 3

Molecular weights of microsomal proteins in the fractions eluting from the DEAE-cellulose column

The molecular weights of the eluted proteins found in Fractions I through IV after various pretreatments were determined by polyacrylamide gel electrophoresis in the presence of 0.1% SDS as described by Laemmli (20). Only those major bands in the range of 45,000–55,000 mol wt are given.

DEAE eluate	Molecular weight of the major protein bands after treatment of animals with			
	Control	Phenobarbital	β-Naphthoflavone	Isosafrole
Fraction I	48,000	48,000	48,000	48,000
Fraction II	—	—	49,000	49,000
	—	49,500	—	—
	50,000	—	50,000	50,000
	—	51,000	—	—
	—	—	52,000	52,000
Fraction III	—	—	—	49,000
	—	—	—	50,000
	51,000	—	—	—
	—	52,500	—	—
	54,000	—	54,000	54,000
	—	—	—	55,000
Fraction IV	55,000	55,000	55,000	55,000

characterized by an increase in the cytochrome P-450 concentration (nanomoles per milligram of microsomal protein), often with the selective appearance of one or more specific forms of cytochrome P-450, resulting in an altered balance of the cytochrome subpopulations from that originally present. Induction of a form of cytochrome P-450, without a concurrent increase in the relative amount of a particular molecular weight species, and without a discernible increase in the cytochrome P-450 concentration in microsomes, might therefore go undetected.

The selective ability of subpopulations of cytochrome P-450 to form stable MI complexes during the oxidative metabolism of certain xenobiotics allows for the detection of induced subpopulations based upon their metabolic specificities rather than based upon molecular weight changes. Specificity in MI complex formation could be inferred from changes in the rate of formation after induction (22, 23) and has subsequently been shown for the extent of formation for both amines (24) and methylenedioxyphenyl derivatives (25). Utilizing the methylenedioxyphenyl derivative, isosafrole, Fennell *et al.* (25) showed a decrease in the extent of isosafrole MI complex formation in phenobarbital-pretreated animals and an increase in MI complex formation in 3-methylcholanthrene-pretreated animals as compared with non-induced animals. By coupling MI complex formation with a simple but reproducible separatory procedure which gives high yields (>70%), it was possible to monitor changes in the subpopulations of cytochrome P-450 which would not be detected by either MI complex formation in microsomes or column chromatography alone. Phenobarbital pretreatment, which induces a cytochrome P-450 with a molecular weight of 52,500 and elutes in Fraction III of this system (11), was also found in this study to induce another form(s) of cytochrome P-450 which eluted in Fraction II (Table 1). Like the induced form eluting in Fraction III, this cytochrome was unable to form MI complexes since there was no concomitant increase in this parameter (Table 2). Thus the decreased percentage of cytochrome P-450 capable of existing as an MI complex in the microsomes from phenobarbital-induced animals (7%) as compared with non-induced animals (18%) is the result of the inability of both of the induced cytochromes to form MI complexes. The change in the major protein band from 50,000 to 51,000 mol wt in Fraction II after phenobarbital induction, coupled with the reduction in the ability of cytochromes in this fraction to form MI complexes from 19 to 9% (Table 2), strongly suggests differential induction of certain forms. β-Naphthoflavone pretreatment, like phenobarbital, induces a form of cytochrome P-450 which is unable to form isosafrole MI complexes and which elutes in Fraction III. However, with β-naphthoflavone induction it has a molecular weight of 54,000, not 52,500. β-Naphthoflavone also induces cytochromes which elute in Fraction II, but in contrast to the phenobarbital-induced forms eluting in Fraction II, the β-naphthoflavone-induced cytochromes are capable of forming an MI complex with isosafrole.

In addition to its ability to sequester some forms of cytochrome P-450 as MI complexes, or perhaps as a consequence of that property, isosafrole also induces hepatic cytochrome P-450 (26). The induction results in the production of a unique form which has a molecular weight intermediate between those induced by phenobarbital and 3-methycholanthrene and which exhibits very limited mixed-function oxidase activity toward conventional laboratory substrates (7, 27). In addition to the unique form, isosafrole has been reported to induce other forms of cytochrome P-450 typical of phenobarbital and 3-methycholanthrene induction (7), although contrary studies exist (25). In the present paper, isosafrole was found to induce cytochromes which elute in three fractions: I, II, and III. In each fraction there was an increase in the amount of cytochrome P-450 capable of existing as an MI complex. Such an increase in MI complex formation is unique to isosafrole induction for Fraction III, similar to β-naphthoflavone induction for Fraction II, and much greater than was seen after phenobarbital induction for Fraction I. Thus, on the basis of their ability to form MI complexes, isosafrole appears to induce unique forms of cytochrome P-450 in Fractions I and III. In addition, isosafrole induces forms in Fraction II which may be identical with those induced by β-naphthoflavone, since this fraction contains proteins of the same molecular weight and cytochromes complexed to the same extent.

In conclusion, this work shows that, after phenobarbital and β-naphthoflavone induction, there are changes in cytochrome P-450 subpopulations in addition to those cytochromes which elute in Fraction III as characterized by Warner and Neims (11). With respect to MI complex formation, the greatest changes occur not with those cytochromes which elute in Fraction III with molecular weights of 52,500 and 54,000 [presumed to be cytochrome P-450$_b$ and P-450$_c$, (12), respectively] but with the cytochromes eluting in Fraction II. In addition to these cytochromes being present in greater amounts than the previously well-characterized forms, their importance in

drug metabolism should not be overlooked, since these are the forms responsible for the alterations in MI complex formation (and their concomitant inhibitory effects on oxidation reactions) seen after β-naphthoflavone or phenobarbital induction. With the advent of the concept of multiple forms of cytochrome P-450, one must be extremely careful not to overlook the fact that compounds might be causing subtle changes within the subpopulations of cytochrome P-450 not necessarily evidenced by novel molecular weight forms or by large increases in the amount of "constitutive" forms.

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