MOLECULAR PHARMACOLOGY, 24:156–162

The Relationship between Increases in the Hepatic Content of Cytochrome P-450, Form 5, and in the Metabolism of Aromatic Amines to Mutagenic Products following Treatment of Rabbits with Phenobarbital

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Received December 21, 1982; Accepted March 16, 1983

SUMMARY

Treatment of rabbits with phenobarbital is followed by increases in the hepatic microsomal concentration of cytochrome P-450, form 5, and in the hepatic microsomal metabolism of aromatic amines to mutagenic products. Inhibition by antibodies to form 5 of the activation of 2-aminoanthracene and 2-aminofluorene demonstrates that these increases are directly related. The extent of the apparent induction of form 5 by phenobarbital is determined from single radial immunodiffusion, immunostaining of form 5 on nitrocellulose sheets containing microsomal proteins transferred from polyacrylamide gels, and the amount of antibody required for 50% of maximal inhibition of activity. The results indicate that phenobarbital increases the hepatic microsomal concentration of cytochrome P-450, form 5, to the same extent that it increases form 5-mediated metabolism of aromatic amines to mutagenic products: 10- to 12-fold. In contrast to the effects of phenobarbital, treatment of rabbits with β-naphthoflavone decreases the hepatic microsomal concentration of cytochrome P-450, form 5, to less than detectable levels and has little effect on the metabolism of aromatic amines to mutagenic products. Our findings, along with the known effects of phenobarbital on cytochrome P-450, form 2, and the known catalytic activity of cytochrome P-450, form 4, lead to the following conclusions: (a) treatment of rabbits with phenobarbital results in increases in the hepatic microsomal concentrations of at least two structurally, immunochemically, and catalytically distinct isozymes of cytochrome P-450, forms 2 and 5; (b) the metabolism of aromatic amines to mutagenic products in rabbit hepatic microsomal preparations depends on the relative concentrations of at least two isozymes of cytochrome P-450, forms 4 and 5, that change in response to different inducers.

INTRODUCTION

Cytochrome P-450-mediated metabolism of many exogenous and endogenous compounds can be increased substantially by the administration of any of a vast number of chemicals to a multitude of animal species. As a group, these chemicals are classified as "inducers" because they lead to an increase in the synthesis of various enzymes. Although the effects of some of these chemicals, like 3-methylcholanthrene, are generally confined to the cytochrome P-450 system, others, like phenobarbital, increase the synthesis of a number of other enzymes as well.

The inductive effect of phenobarbital on rabbit hepatic cytochrome P-450-dependent metabolism has been associated primarily with an increase in the concentration of the cytochrome P-450 isozyme, LM2 (form 2) (1). The substrate specificity of form 2 is consistent with this association (1, 2), as is the inhibitory effect of antibodies to form 2 on hepatic microsomal activities that increase following the administration of phenobarbital (3).

Cytochrome P-450, form 2, is also present in rabbit lung, where it makes up a minimum of 30% of the total cytochrome P-450 (4). This isozyme from rabbit lung was formerly called "P-450$_I$" but has been identified as form 2 on the basis of immunochemical (3), catalytic (3), and structural (5) criteria. However, the pulmonary content of form 2 is not altered by phenobarbital. The same lack of a pulmonary response to inducers is evident with cytochrome P-450$_{II}$, another isozyme from rabbit lung that has been purified and characterized (6), but little

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0026-895X/83/040156-07$02.00/0

has been reported about the properties of this isozyme in rabbit liver. Immunochemical evidence (7, 8) indicates that cytochrome P-450$_{II}$, which also makes up a minimum of 30% of the pulmonary P-450 (4), is present in rabbit liver at a relatively low concentration (8). P-450$_{II}$, when electrophoresed on polyacrylamide gels in the presence of sodium docecyl sulfate, migrates between the cytochrome P-450 isozymes, form 4 and form 6, and corresponds in monomeric molecular weight with a protein in the rabbit hepatic microsomal fraction observed originally by Haugen *et al.* (9) in the area subsequently designated as "zone 5" by Johnson (10). Therefore, in accordance with the prevailing nomenclature for cytochrome P-450 from rabbits (9, 10), we shall now refer to P-450$_{II}$ as form 5.[4]

Although there are several substrates, e.g., 4-ipomeanol (11) and *p*-xylene (12), whose metabolism is catalyzed by both form 2 and form 5, their substrate specificities are quite different. The metabolism of benzphetamine, aminopyrene, ethylmorphine, 7-ethoxycoumarin, and *p*-nitroanisole is catalyzed by form 2 but not by form 5 (3), whereas the metabolism of several aromatic amines to mutagenic products is catalyzed only by form 5 (7). The exceptionally high activity of form 5 with aromatic amines, combined with the use of an inhibitory antibody preparation, have enabled us to investigate the effect of inducers on the hepatic content and activity of this isozyme.

Our results show that treatment of rabbits with phenobarbital dramatically increases the content of form 5 in the liver. We have verified this finding with a number of immunochemical techniques and conclude that phenobarbital significantly increases the hepatic concentrations of the two isozymes of cytochrome P-450 that predominate in rabbit lung. These increases have a marked effect on the ability of the liver to metabolize aromatic amines to mutagenic products as well as to metabolize other chemicals that are substrates for form 5 and/or form 2.

METHODS

Treatment of rabbits and preparation of microsomal fractions. Adult, male New Zealand white rabbits (Dutchland Farms) were untreated, treated with phenobarbital in the drinking water (1 mg/ml) for 3 days, treated with phenobarbital i.p. (50 mg/kg) daily for 3 days or treated with β-naphthoflavone i.p. (80 mg/kg) daily for 3 days. Rabbits were killed 1 day after the final treatment, and hepatic microsomal fractions were prepared from sterilized 16,000 × *g* supernatant fractions as described previously (7).

Assay for mutagenic activity. The metabolism of 2-aminoanthracene and 2-aminofluorene to mutagenic products was detected with the *Salmonella* mutagenesis assay developed by Ames *et al.* (13). The assay was somewhat modified for our purposes, and details of the cultures of *Salmonella typhimurium* (strain TA98), incubations, and counting procedures used have been published (7). All activities were determined from triplicate plates that contained 2-aminoanthracene (2.5 nmoles) or 2-aminofluorene (10 nmoles) and microsomal fractions equivalent to 20–200 pmoles of cytochrome P-450.

Other microsomal assays. The rate of *O*-demethylation of *p*-nitroanisole was determined by following the formation of *p*-nitrophenol at 417 nm. Incubations contained *p*-nitroanisole (200 μM), NADPH (100 μM), and microsomal protein (1–2 mg/ml) in phosphate buffer (100 mM, pH 7.7). The assays were carried out at 37°. Cytochrome P-450 content was measured by the method of Omura and Sato (14), and protein estimations were done according to the method of Lowry *et al.* (15). All spectral determinations were made with an Aminco DW2-A spectrophotometer (American Instrument Company, Silver Spring, Md.).

Immunochemical methods. Antibodies to cytochrome P-450, forms 2 and 5, were elicited in goats from purified pulmonary antigens (3). Inhibiting of microsomal activities by these antibodies and their specificities for forms 2 and 5 have been described in detail (3, 7). Inhibition experiments were carried out with antibody concentrations of 10 mg of IgG[5] per nanomole of total cytochrome P-450. Quantitation of forms 2 and 5 was done by the single radial immunodiffusion method of Mancini (16) as modified for our purposes (8). All determinations were made at three protein concentrations, and values were obtained from standard curves. Electrophoresis on polyacrylamide gels was carried out according to the method of Laemmli (17). Methods for the transfer of proteins from polyacrylamide gels to nitrocellulose sheets and subsequent immunostaining were based on the work of Towbin *et al.* (18). An antibody peroxidase-antiperoxidase method was used for the detection of antigens on nitrocellulose (19).

Materials. Chemicals and supplies for the mutagenicity assays have been described (7). NADPH, Type I, was purchased from Sigma Chemical Company (St. Louis, Mo.) and *p*-nitroanisole from Eastman (Rochester, N. Y.). Goat peroxidase-antiperoxidase and rabbit anti-goat IgG were obtained from Cappel (Cochranville, Pa.). All other chemicals and supplies were standard stock items.

RESULTS

Treatment of rabbits with phenobarbital increased the ability of the hepatic microsomal fraction to metabolize 2-aminoanthracene and 2-aminofluorene to mutagenic products (Fig. 1). With our assay conditions, the activities were directly dependent on the amount of microsomal sample as shown for two preparations from rabbits treated with phenobarbital (Fig. 1). The same dependency for preparations from untreated rabbits has been described in detail elsewhere (7). The increase in the hepatic metabolism of 2-aminoanthracene to mutagenic products, which followed treatment of rabbits with phenobarbital (either in the drinking water or administered i.p.), was found to correlate with increases in the hepatic microsomal content of the cytochrome P-450 isozyme, form 5 (Table 1). This correlation was initially suspected when we observed that inhibition by antibodies of the mutagenic activity mediated by form 5 increased from about 35% in microsomes from untreated rabbits to about 85% in microsomes from rabbits treated with phenobarbital (Table 1). Similar effects of phenobarbital in the drinking water on the hepatic microsomal metabolism of 2-aminofluorene to mutagenic products were also observed (data not shown).

The relationship between antibody concentration and inhibition of the mutagenic activity of 2-aminoanthracene is shown in Fig. 2. For these experiments, in which microsomal preparations from four animals were pooled, maximal inhibition was 56% for the control sample and 84% for the treated sample. The concentrations of antibody (milligrams of IgG per nanomole of total cytochrome P-450) that produced the same percentage of

[4] The use of the terms "form 2" and "form 5" for isozymes of cytochrome P-450 found in rabbit liver and lung is not meant to imply that the proteins from both tissues are necessarily identical with respect to either primary structure or microheterogeneous populations.

[5] The abbreviations used are: IgG, immunoglobulin G; SDS, sodium dodecyl sulfate.

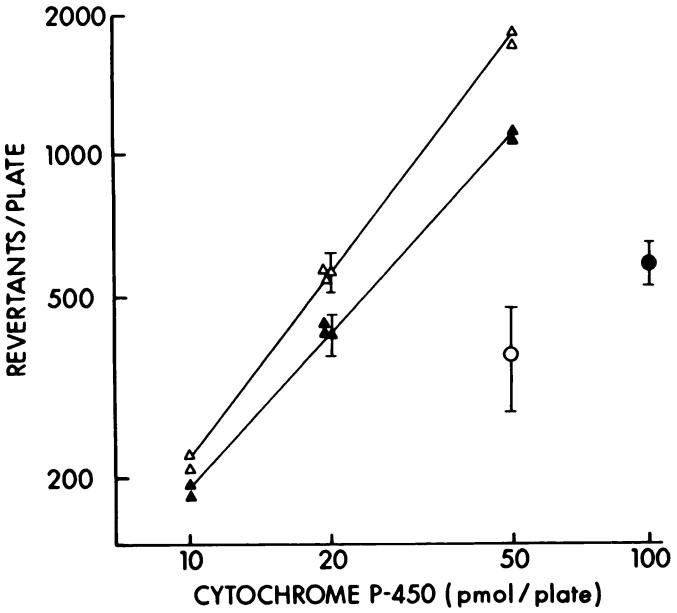

FIG. 1. *Mutagenic activities of 2-aminoanthracene and 2-aminofluorene metabolized by hepatic microsomal preparations from untreated rabbits and rabbits treated with phenobarbital*

For rabbits treated with phenobarbital (drinking water) results (△, 2-aminofluorene; ▲, 2-aminoanthracene) are given as the mean and standard deviation of the number of histidine revertants per plate for six preparations assayed at 20 pmoles of total cytochrome P-450 per plate and as individual values for two preparations assayed at 10, 20, and 50 pmoles total of cytochrome P-450 per plate. For preparations from untreated rabbits, the results (○, 2-aminofluorene; ●, 2-aminoanthracene) are given as the mean and standard deviation ($n = 4$) for preparations assayed at 50 (2-aminoanthracene) or 100 (2-aminofluorene) pmoles of total cytochrome P-450 per plate.

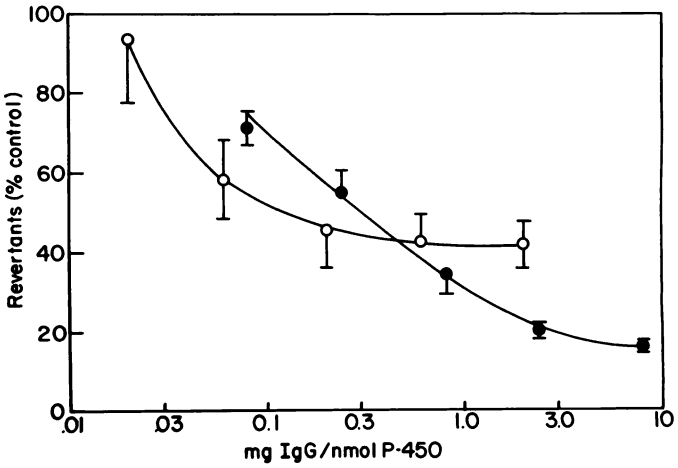

FIG. 2. *Inhibition of the mutagenic activity of 2-aminoanthracene by antibodies to cytochrome P-450, form 5*

Hepatic microsomes from untreated rabbits (○, 50 pmoles of total cytochrome P-450 per plate) and rabbit treated with phenobarbital i.p. (●, 20 pmoles of total cytochrome P-450 per plate) were incubated with varying amounts of antibody to cytochrome P-450, form 5, and 2-aminoanthracene (2.5 nmoles/plate). The results are given as the mean and standard deviation ($n = 4$) of the number of histidine revertants per plate (expressed as percentage of control) versus antibody concentration (milligrams of IgG protein per nanomole of total cytochrome P-450). Appropriate amounts of preimmune IgG were included so that the total IgG was the same for all assays. The uninhibited activities were 8.7 ± 1.9 and 61.5 ± 2.5 revertants/mg × 10^3 for the samples from untreated and phenobarbital-treated rabbits, respectively.

maximal inhibition were greater for the microsomes from rabbits treated with phenobarbital, a result which indicated that phenobarbital had increased the hepatic content of form 5. This conclusion was substantiated by quantitative analysis of form 5 by single radial immunodiffusion. Phenobarbital increased the microsomal content of form 5 approximately 10-fold per milligram of microsomal protein (from 0.03 to 0.30 nmole) and 6-fold as a percentage of the total cytochrome P-450 content (Table 1). The increase in form 5 content correlated well with the 11- to 12-fold increase in the mutagenic activity that was inhibited by the antibodies to form 5 (Table 1).

Electrophoresis of hepatic microsomal proteins (20 μg) on polyacrylamide gels (10%) in the presence of SDS and staining with Coomassie blue showed that the amount of protein with the monomeric molecular weight of form 5, 58,000 M_r, was increased by treatment of rabbits with phenobarbital (Fig. 3A). The presence of form 5 in this protein band, the increase in form 5 caused by phenobarbital, and the specificity of the immunoreactivity of the antibody preparation were verified by immunostain-

TABLE 1

Mutagenic activity of 2-aminoanthracene and the content of cytochrome P-450, form 5, in hepatic microsomal preparations from untreated rabbits and rabbits treated with phenobarbital

Treatment	Mutagenic activity of 2-aminoanthracene[a]			Cytochrome P-450, form 5[b]	
	Total activity	Effect of antibody to form 5		Content	Proportion
		Activity inhibited	Inhibition		
	revertants/mg × 10^3	*revertants/mg × 10^3*	*%*	*nmoles/mg*	*% total P-450*
Control ($n = 3$)	14.2 ± 5.8	4.9 ± 1.9	36 ± 8	0.03 ± 0.01	2 ± 1
Phenobarbital (drinking water, $n = 4$)	66.4 ± 12.2	57.7 ± 10.4	87 ± 4	0.28 ± 0.03	12 ± 1
Phenobarbital (i.p., $n = 3$)	63.4 ± 31.5	54.4 ± 27.4	85 ± 2	0.31 ± 0.11	11 ± 2
Ratio: phenobarbital (water)/control	4.7	11.8	2.4	9.3	6.0
Ratio: phenobarbital (i.p.)/control	4.5	11.1	2.4	10.3	5.5

[a] Mutagenic activity was determined by the test devised by Ames *et al.* (13) under the conditions described under Methods.

[b] The content of cytochrome P-450, form 5, was determined by single radial immunodiffusion as described under Methods.

[c] The mean concentrations of total cytochrome P-450 were 1.5, 2.3, and 2.8 nmoles/mg of protein for control, phenobarbital (drinking water), and phenobarbital (i.p.), respectively.

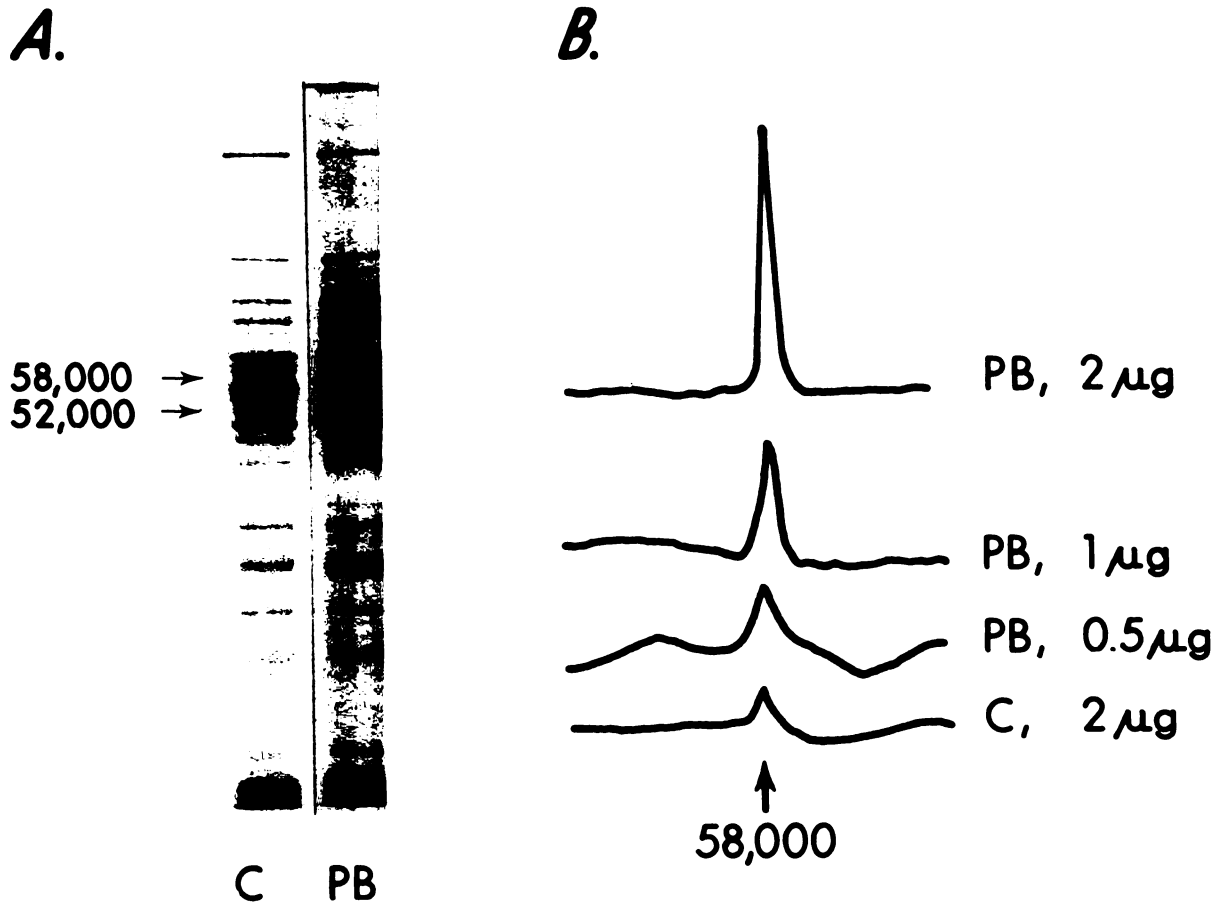

FIG. 3. *Hepatic microsomal electrophoretic patterns and densitometric scans*

A. Electrophoresis of rabbit hepatic microsomal proteins on polyacrylamide gels in the presence of SDS. Microsomal samples (20 μg of protein) from an untreated rabbit (*C*) and a rabbit treated with phenobarbital (*PB*) were treated with SDS, β-mercaptoethanol, and heat, and then electrophoresed on polyacrylamide gels (10%). Standard procedures were used for sample treatment, electrophoresis, and staining with Coomassie blue as noted under Methods. The bands corresponding to the cytochrome P-450 isozymes, forms 2 and 5, are marked *52,000* and *58,000*, respectively.

B. Densitometric scans of diaminobenzidine reaction product on nitrocellulose paper containing microsomal proteins transferred from polyacrylamide gels and immunostained for cytochrome P-450, form 5. Microsomal samples from a rabbit treated with phenobarbital (*PB*; 0.5, 1.0, and 2.0 μg of protein) and from an untreated rabbit (*C*; 2 μg of protein) were electrophoresed on polyacrylamide gels and then transferred electrophoretically to nitrocellulose paper. Specific immunostaining for cytochrome P-450, form 5, was then carried out using the peroxidase-antiperoxidase method. The scans were obtained with a Zeineh soft-laser scanning densitometer (LKB). The position of the band observed on the nitrocellulose paper coincided exactly with the band corresponding to 58,000 M_r observed on the polyacrylamide gels (A).

ing of microsomal proteins (0.5–2 μg) transferred from polyacrylamide gels to nitrocellulose sheets (Fig. 3B). With microsomal proteins from either untreated rabbits or those treated with phenobarbital, staining was confined to a protein with a monomeric molecular weight of 58,000. The amount of antigen (form 5) was clearly greater in the preparations from rabbits treated with phenobarbital (Fig. 3B).

The relationship between the effects of phenobarbital on the metabolism of 2-aminoanthracene to mutagenic products and the microsomal content of form 5 for pooled microsomal preparations is shown in Table 2. Induction of form 5 by phenobarbital was determined from single radial immunodiffusion, from immunonstaining of samples on nitocellulose, and from the amount of antibody required for 50% maximal inhibition of form 5-mediated activity. The results in all cases were similar and showed that form 5 had been induced approximately 11-fold. This determination was in good agreement with the ratio between the antibody-inhibited activities of the two microsomal preparations (Table 2).

The induction of cytochrome P-450, form 2, by phenobarbital (4) was confirmed in the same animals used for the study of form 5. Electrophoresis of microsomal proteins in the presence of SDS and staining with Coomassie blue clearly showed that the amount of protein corresponding in monomeric molecular weight to form 2, 52,000 M_r, was increased by phenobarbital (Fig. 3A). Results of single radial immunodiffusion experiments indicated that phenobarbital administered i.p. increased the content of form 2 from 0.16 to 1.22 nmoles/mg of protein (Table 3). The increase in form 2 by phenobarbital included in the drinking water was about 25% less. The induction of form 2 by phenobarbital was accompanied by an increase in the inhibition of *p*-nitroanisole *O*-demethylation by antibodies to form 2 from 48% to greater than 90%. The absolute amount of activity inhibited was also significantly increased (Table 3). The

TABLE 2

Correlation of the content of cytochrome P-450, form 5, with inhibition of the mutagenic activity of 2-aminoanthracene by the antibody to cytochrome P-450, form 5

Results are given for samples pooled from four rabbits.

Treatment	Total activity[a]	Effect of antibody to form 5		Estimations of form 5 content		
		Activity inhibited	Inhibition	Single radial immunodiffusion[b]	Immunostaining[c] (peak area)	Antibody inhibition I_{50}[d]
	revertants/mg $\times 10^3$	*revertants/mg* $\times 10^3$	%	*nmoles/mg protein*	$mm^2/\mu g$ *protein*	*mg IgG/mg protein*
Control	8.7 ± 1.9	4.9	56	0.025 ± 0.006	3	0.045
Phenobarbital (i.p.)	61.5 ± 2.5	51.7	84	0.269 ± 0.014	37.7 ± 3.2	0.478
Ratio: phenobarbital/control	7.1	10.6	1.5	10.8	12.6	10.6

[a] The mutagenic activity was determined by the test devised by Ames *et al.* (13) under the conditions described under Methods.

[b] Single radial immunodiffusion was carried out by the procedures under Methods. The standard deviation was derived from three determinations made at different protein concentrations.

[c] Immunostaining for form 5 was done on nitrocellulose paper as described. The standard deviation for the phenobarbital value was calculated from three determinations at different protein concentrations. The control sample was assayed only at 2.0 μg of protein.

[d] The value reported is the amount of antibody required to produce 50% of the maximal inhibition observed.

specificity of the antibodies to form 2 was also confirmed. Microsomal samples from four untreated and four phenobarbital-treated rabbits all produced a single band after transfer from polyacrylamide gels to nitrocellulose sheets and immunostaining. This band corresponded to the monomeric molecular weight of form 2 (data not shown). The ratios of the peak areas of densitometric tracings of the bands on the nitrocellulose to single radial immunodiffusion determinations were 138.5 ± 19.7 and 135.4 ± 24.0 mm^2/pmole of form 2 for samples from untreated and phenobarbital-treated rabbits, respectively.

In contrast to phenobarbital, β-naphthoflavone produced only a 2-fold increase in the metabolism of 2-aminoanthracene to mutagenic products. No increase was observed in the activation of 2-aminofluorene (Table 4). On the basis of total cytochrome P-450 content, no change in the activation of 2-aminoanthracene or 2-aminofluorene was produced by treatment of rabbits with β-naphthoflavone (Table 4). Cytochrome P-450, form 5, could not be detected by single radial immunodiffuson in hepatic microsomes from rabbits treated with β-naphthoflavone (Table 4).

DISCUSSION

In mice (20), rats (21, 22), or rabbits (23), inducers of the class typified by 3-methylcholanthrene increase the hepatic concentration of at least two isozymes of cytochrome P-450 whose structural, immunochemical, and catalytic properties are markedly different (20–24). Similar findings have not been as clearly demonstrated with other inducers, although results of single radial immunodiffusion experiments indicate that phenobarbital induces the synthesis of urelated isozymes of cytochrome P-450 in rats. The findings of Thomas *et al.* (25) indicate that phenobarbital increases (2- to 3-fold) the hepatic microsomal content of "P-450a" in adult female rats and immature rats of either gender. The content of cytochrome "P-450b," the major isozyme of P-450 induced in the rat by phenobarbital, was increased over 50-fold in the same animals as well as in adult male rats (25). Similar findings by Heuman *et al.* (26) indicated that phenobarbital also increases the hepatic microsomal content of "$P\text{-}450_{PCN}$," an isozyme that is distinct from "P-450b" and is primarily associated with the inductive effects of pregnenolone-16α-carbonitrile. In addition, it

TABLE 3

Metabolism of p-nitroanisole and the content of cytochrome P-450, form 2, in hepatic microsomal preparations from untreated rabbits and rabbits treated with phenobarbital

Treatment	*p*-Nitroanisole O-demethylation[a]			Cytochrome P-450, form 2[b]	
	Total activity	Effect of antibody to form 2		Content	Proportion
		Activity inhibited	Inhibition		
	nmoles product/mg	*nmoles product/mg*	%	*nmoles/mg*	*% total P-450*
Control ($n = 3$)	2.02 ± 0.50	0.97 ± 0.08	48 ± 11	0.16 ± 0.05	9 ± 5
Phenobarbital (Drinking water, $n = 4$)	3.78 ± 1.31	3.64 ± 1.26	95 ± 2	0.99 ± 0.26	44 ± 11
Phenobarbital (i.p., $n = 3$)	4.60 ± 1.16	4.23 ± 1.20	92 ± 5	1.22 ± 0.35	45 ± 4
Ratio: phenobarbital (water)/control	1.8	3.8	2.0	6.2	4.6
Ratio: phenobarbital (i.p.)/control	2.3	4.4	1.9	7.6	4.7

[a] The rate of metabolism of *p*-nitroanisole was determined as described under Methods. The results are expressed as the mean ± the standard deviation.

[b] The content of cytochrome P-450, form 2, was determined by single radial immunodiffusion as described under Methods. The results are expressed as the mean ± the standard deviation. Individual determinations were from samples run at three different protein concentrations.

TABLE 4

Mutagenic activities of 2-aminoanthracene and 2-aminofluorene and the content of cytochrome P-450, form 5, in microsomal preparations from untreated rabbits and rabbits treated with β-naphthoflavone

Treatment	Mutagenic activity (revertants × 10^3)[a]				Cytochrome P-450[b]		
	2-Aminoanthracene		2-Aminofluorene		Total	Form 5	
	/mg protein	/nmole P-450	/mg protein	/nmole P-450		Content	Proportion
					nmoles/mg	*nmoles/mg*	*% total P-450*
Control (n = 3)	13.8 ±7.3	7.6 ± 2.2	10.3 ± 3.0	6.4 ± 0.5	1.72 ± 0.4	0.04 ± 0.01	2 ± 1
β-Naphthoflavone (n = 4)	25.3 ± 13.9	10.1 ± 6.7	11.2 ± 4.0	4.4 ± 2.4	2.83 ± 0.8	<0.01	<0.5

[a] Mutagenic activity determined as for data in Table 1.

[b] Total cytochrome P-450 was determined by the method of Omura and Sato (14). The content of cytochrome P-450, form 5, was determined by single radial immunodiffusion as described under Methods.

has been clearly established in the rat that phenobarbital does induce the synthesis of several microheterogeneous forms of cytochrome P-450 that have extensive immunochemical and structural similarities (27). These forms of cytochrome P-450 are coded for by different structural genes and are not products of post-translational modification (28).

In rabbits, phenobarbital is known to induce the synthesis of cytochrome P-450, form 2, but its effects have not been previously related to other isozymes of cytochrome P-450. However, Schwab *et al.* (29) have published evidence (see fig. 1 of ref. 29) that phenobarbital does increase the hepatic microsomal content of a protein with a monomeric molecular weight between those of the cytochrome P-450 isozymes, forms 4 and 6. We have now used several immunochemical methods to characterize this protein as cytochrome P-450, form 5, and to demonstrate that its concentration is significantly increased by the administration of phenobarbital. Although the techniques used rely on different properties of the antibody preparation (antigen-antibody precipitation for single radial immunodiffusion, antigen-antibody binding for immunostaining on nitrocellulose, and antibody inhibition of activity for the catalytic studies), they produced very similar results. The results allow for a correlation to be made between increases in protein of the monomeric molecular weight of form 5 and increases in activities known to be catalyzed by this isozyme. This demonstration of antibody specificity greatly reduces the possibility that the results obtained are compromised by cross-reactivity of the antibodies with other isozymes of cytochrome P-450. Therefore, we conclude that treatment of rabbits with phenobarbital is followed by the induction of at least two structurally (6), immunochemically (3), and catalytically (3, 4, 7, 11, 12) distinct isozymes of cytochrome P-450, forms 2 and 5.

Because of the high activity of cytochrome P-450, form 5, changes in the hepatic concentration of this isozyme have a significant effect on the metabolism of aromatic amines to mutagenic products in microsomal preparations. This effect, following treatment of rabbits with phenobarbital, is clear, but the case with β-naphthoflavone is not so obvious. β-Naphthoflavone and other polycyclic aromatic hydrocarbons increase the hepatic concentrations of cytochrome P-450, forms 4 and 6, in rabbits (10). Of the forms of cytochrome P-450 tested by Norman *et al.* (30) (forms 2, 3, 4, and 6), form 4 was by far the most active in the metabolism of 2-aminoanthracene to mutagenic products. However, treatment of rabbits with tetrachlorodibenzo-*p*-dioxin (31) or β-naphthoflavone (this study) increases the activation of 2-aminoanthracene by only 3-fold and less than 2-fold per milligram of protein, respectively. A similar result (1.5-fold) has been reported for the effect of 3-methylcholanthrene on the activation of 2-aminofluorene (32). Most of these reported increases in the metabolism of aromatic amines to mutagenic products, when calculated per nanomole of total cytochrome P-450, are actually less than control activities and they do not reflect the induction of form 4. However, a decrease in the concentration of form 5 upon treatment of rabbits with compounds that increase the concentration of form 4 could account for these findings. Evidence for this is the decrease in form 5 content following treatment of rabbits with β-naphthoflavone (Table 4).

Thus, the relative contribution of the cytochrome P-450 isozymes, forms 4 and 5, to the activation of aromatic amines appears to vary considerably with different microsomal preparations. In preparations from untreated rabbits, 50–70% of the activity is mediated by form 5 (7). [Because form 5 is 20–25 times more active than form 4, at least with 2-aminoanthracene and 2-aminofluorene (7), the concentration of form 4, if it mediates most or all of the remaining activity, should be 10–20 times greater than that of form 5 in these preparations. This conclusion is consistent with the report of Chiang *et al.* (33) that form 4 makes up 30% of the cytochrome P-450 in hepatic microsomal preparations from untreated rabbits.] Treatment of rabbits with inducers like 3-methylcholanthrene appears to shift the balance in favor of form 4 and, as shown in the present study, activity mediated by form 5 predominates in microsomal preparations from rabbits treated with phenobarbital.

Exogenous compounds that induce the synthesis of monooxygenase enzymes have proven to be useful in efforts to determine the relationship between microsomal activities and specific forms of cytochrome P-450. However, documentation of proposed relationships is difficult, particularly when the result obtained with inducers may be a consequence of multiple factors. It is clear that the concentrations of more than one isozyme can be increased, and the concentrations of others decreased, by

a single inducer. In addition, our finding that form 5 is induced in the rabbit by phenobarbital demonstrates that two catalytically related isozymes of cytochrome P-450, forms 4 and 5, respond to different inducers. Although the most apparent effects of inducers are well known, characterization of these less obvious effects, both inductive and repressive, is required for an accurate assessment of the extent to which cytochrome P-450-dependent monooxygenase systems are modified by exogenous compounds.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Barbara A. Domin for setting up the "Western blotting" assay. They thank Dr. E. Zieger, in whose laboratory the mutagenicity assays were carried out, for his encouragement during the course of this work, and Miss Susan Foster for her technical assistance.

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