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Immunological Comparison of Hepatic and Extrahepatic Cytochromes P-450

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SUMMARY

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Antibodies were raised to two apparently homogeneous cytochromes P-450 prepared from liver microsomes of phenobarbital- and 3-methylcholanthrene-treated rats; microsomal fractions were prepared from the livers, lungs, kidneys, testes, spleens, hearts, small intestines, colons, stomachs, and brains of untreated and phenobarbital- and 3-methylcholanthrene-treated rats. Cross reactivity was observed in each tissue with at least one of the two antibodies as judged by immunodiffusion analysis. Inhibition of benzo(a)pyrene hydroxylase or 7-ethoxycoumarin *O*-deethylase activity was observed with microsomes prepared from each tissue with the exception of spleen and colon; inhibition curves were dependent upon the substrate and antibody used as well as the pretreatment of the animals from which the microsomes were prepared. As a general rule, microsomes prepared from 3-methylcholanthrene-treated rats were inhibited most readily by antibody raised to cytochrome P-450 isolated from liver microsomes prepared from 3-methylcholanthrene-treated rats, and the same general rule held for phenobarbital induction; however, several exceptions were noted. On the basis of these results, the microsomal P-450s of extrahepatic tissues are, in general, immunologically similar to the major hepatic microsomal P-450s in the rat.

INTRODUCTION

Microsomal mixed-function oxidases metabolize a wide variety of endogenous and exogenous substrates, including steroids, fatty acids, drugs, carcinogens, insecticides, and alkanes (1–3). The major system involved in such metabolism consists of the flavoprotein NADPH-cytochrome P-450 reductase and the terminal oxidase P-450[1]; the latter protein appears to control the specificity of the system (4). While the majority of work with P-450 has been focused on hepatic systems, evidence has been presented that P-450s and mixed-function oxidase activities are present in a variety of other tissues, including lung (5, 6), kidney (6, 7), testis (6, 8), colon (9), brain (6, 10, 11), skin (12), bone marrow (13), eye (14), ovary (15), small intestine (16, 17), muscle (18), spleen (18), stomach (18, 19), and the thyroid (20), adrenal (20, 21), and pituitary (22) glands. These extrahepatic systems

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[1] Abbreviations used are: P-450, microsomal cytochrome P-450; PB, phenobarbital; 3MC, 3-methylcholanthrene; IgG, immunoglobulin G; PB-IgG, IgG prepared from antisera raised to P-450 prepared from liver microsomes of PB-treated rats; 3MC-IgG, IgG prepared from antisera raised to P-450 prepared from liver microsomes of 3MC-treated rats.

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may be important in the metabolism of compounds having these tissues as their targets. Of these extrahepatic P-450s, only lung P-450 has been purified to an extent that permits physical characterization (5). Except in the case of the lung, little is known about the similarity of these P-450s to the better characterized liver enzymes.

Recently three rat liver P-450s have been purified to apparent homogeneity in this laboratory, as judged by electrophoresis in six different systems, specific contents of P-450 per mg protein, sedimentation velocity and equilibrium measurements, *N*-terminal analysis, immunological techniques, and steady-state 7-ethoxycoumarin deethylase kinetics (23, 24). Antibodies were prepared to two of these P-450s (24) and used to examine the similarity of the hepatic and extrahepatic P-450s for the purpose of determining the usefulness of hepatic P-450s as models for the various extrahepatic P-450s.

MATERIALS AND METHODS

Preparation of microsomes. Male rats of Sprague-Dawley origin (Harlan, Cumberland, Ind.; 100–125 g each) were induced with PB or 3MC or used without treatment (23). Tissues were excised and microsomal fractions were prepared as described (25)—all buffers contained 20 μM butylated hydroxytoluene, 0.1 mM phenylmethylsulfonylfluoride (23), 1 mM dithiothreitol, and 1 mM EDTA. The microsomal pellets were washed twice with pyrophosphate buffer (25) to reduce levels of contaminating hemoglobin.

Immunological techniques. Hepatic P-450s from PB- and 3MC-treated rats ("B" fractions) were purified as previously described (23, 24); specific contents were 18 and 17 nmoles P-450 per mg protein, respectively. Antibodies were raised to the P-450s (24, 26) and IgG fractions were isolated (24, 27).

Double diffusion analysis utilized the general procedure of Thomas *et al.* (28–30). The agarose plates contained 0.2% (w/v) Emulgen 913 and all microsomal preparations were treated with 0.5% Emulgen 913 prior to addition to the sample wells.

7-Ethoxycoumarin *O*-deethylase activity was assayed as described (24, 31) using 5–100 pmoles of microsomal P-450, depending upon the specific activity with each tissue. Incubations were carried out for 10 min at 37° with a substrate concentration of 0.3 mM. Benzo(a)pyrene hydroxylation was assayed with same incubation time and P-450 concentrations using the fluorimetric procedure of Dehnen *et al.* (32). IgG fractions were preincubated with microsomes for 15 min at 23° prior to addition of substrate and NADPH-generating system.

Other assays. Because of high levels of hemoglobin in some of the preparations, P-450 was estimated from reduced-CO vs. oxidized-CO difference spectra ($\Delta\epsilon_{450}$ = 104 mM^{-1} cm^{-1}) (33) using a Cary 219 spectrophotometer in the automatic baseline correction mode at 20° ± 1°. Protein assays utilized the methods of Lowry *et al.* (34).

Materials. 7-Ethoxycoumarin was prepared as previously described (5); 7-hydroxycoumarin was purchased from Aldrich and recrystallized twice from hot water (mp 220–222°, uncorr). Other materials were obtained from commercial sources.

RESULTS

Induction of P-450 and mixed-function oxidase activities. P-450 was detected spectrally in each of the tissues examined except small intestine and colon (Table 1). Detection is limited by the presence of hemoglobin and methemoglobin rather than instrumental factors; these other hemoproteins did not permit the estimation of cytochrome P-420 (i.e., inactive P-450) in these preparations. The heart contained a rather high level of P-450 compared to other extrahepatic tissues[2]. P-450 levels were clearly elevated by PB and 3MC in the liver, lung, and testis.

7-Ethoxycoumarin *O*-deethylase activity was induced by 3MC in the liver, lung, kidney, and brain (Table 2). Benzo(a)-pyrene hydroxylase activity was induced by PB in the liver and testis and was induced by 3MC in the liver, lung, kidney, heart, stomach, and brain.

[2] The recovery of heart protein in the microsomal fraction was only 2%, compared with a typical 10–15% value for liver. The liver contains roughly 1000 times as much P-450 as does the heart.

TABLE 1

Induction of P-450 in various rat tissues

Pretreatments of rats are indicated with the column headings.

Tissue	P-450[a]		
	Untreated	PB	3MC
	nmoles per mg protein		
Liver	0.96 ± 0.05	2.02 ± 0.14	1.66 ± 0.35
Lung	0.009 ± 0.003	0.027 ± 0.005	0.034 ± 0.009
Kidney	0.064 ± 0.003	0.076 ± 0.008	0.076 ± 0.001
Testis	0.019 ± 0.004	0.066 ± 0.010	0.062 ± 0.007
Spleen	<0.005	0.016 ± 0.002	0.014 ± 0.010
Heart	0.12 ± 0.01	0.18 ± 0.004	0.12 ± 0.06
Small intestine	<0.004	<0.002	<0.008
Colon	<0.008	<0.006	<0.007
Stomach	0.030 ± 0.011	0.015 ± 0.002	0.025 ± 0.006
Brain	0.017 ± 0.007	0.028 ± 0.001	0.024 ± 0.007

[a] Mean of two determinations ± SD

TABLE 2

Induction of 7-ethoxycoumarin de-ethylase and benzo(a)pyrene hydroxylase activities in various rat tissues

All values are means of three determinations ± SD. Pretreatments of rats are indicated with the column headings. The activities are expressed on the basis of P-450.

Tissue	7-Ethoxycoumarin *O*-deethylase			Benzo(a)pyrene hydroxylase		
	Untreated	PB	3MC	Untreated	PB	3MC
	nmoles 7-hydroxycoumarin/nmol P-450/min			*nmoles 3-hydroxybenzo(a)pyrene/nmole P-450/min*		
Liver	1.12 ± 0.43	0.76 ± 0.11	8.55 ± 0.99*	0.22 ± 0.01	0.52 ± 0.04*	1.62 ± 0.30*
Lung	0.60 ± 0.14	1.04 ± 0.08	8.85 ± 0.52*	0.35 ± 0.04	0.048 ± 0.024	1.98 ± 0.14*
Kidney	0.98 ± 0.13	0.35 ± 0.11	12.9 ± 4.5*	0.082 ± 0.005	0.070 ± 0.017	1.86 ± 0.22*
Testis	0.65 ± 0.30	N.S.[a]	N.S.	0.19 ± 0.01	0.44 ± 0.03*	0.14 ± 0.03
Spleen	N.S.	N.S.	N.S.	≥0.060 ± 0.003[b]	N.S.	0.11 ± 0.03
Heart	0.13 ± 0.04	N.S.	N.S.	0.024 ± 0.009	0.026 ± 0.001	0.060 ± 0.010*
Small intestine	N.S.	N.S.	N.S.	≥0.070 ± 0.014[b]	≥0.10 ± 0.01[b]	≥0.070 ± 0.010[b]
Colon	N.S.	0.35 ± 0.14*	N.S.	N.S.	N.S.	N.S.
Stomach	N.S.	N.S.	N.S.	0.011 ± 0.004	0.013 ± 0.001	0.043 ± 0.015*
Brain	0.10 ± 0.02	N.S.	1.90 ± 0.74*	0.024 ± 0.002	0.028 ± 0.002	0.036 ± 0.004*

* Significantly greater than value obtained with microsomes from untreated rats ($p < 0.05$).

[a] Not significant ($p < 0.10$)

[b] This value is based upon the concentration of P-450 as being the limit of detection.

FIG. 1. *Double diffusion analysis using microsomes from 3MC-treated rats and 3MC-IgG (A), PB-treated rats and PB-IgG (B), and 3MC-treated rats and pre-immune IgG (C).*

Panel A: The center wells contained 3MC-IgG (25 mg/ml). Wells *a* and *g* contained buffer only. The other peripheral wells contained microsomes from the following tissues of 3MC-treated rats: b) liver, c) lung, d) kidney, e) testis, f) spleen, h) heart, i) small intestine, j) colon, k) stomach, and l) brain. The P-450 concentrations are as listed in Table 3. Staining and destaining were as described (28).

Panel B: The center wells contained PB-IgG (25 mg/ml) and the peripheral wells are arranged as in Fig. 1A using microsomes prepared from PB-treated rats.

Panel C: The center wells contained pre-immune IgG (31 mg/ml) and the peripheral wells contained microsomes from 3MC-treated rats in the arrangement used in Panel A.

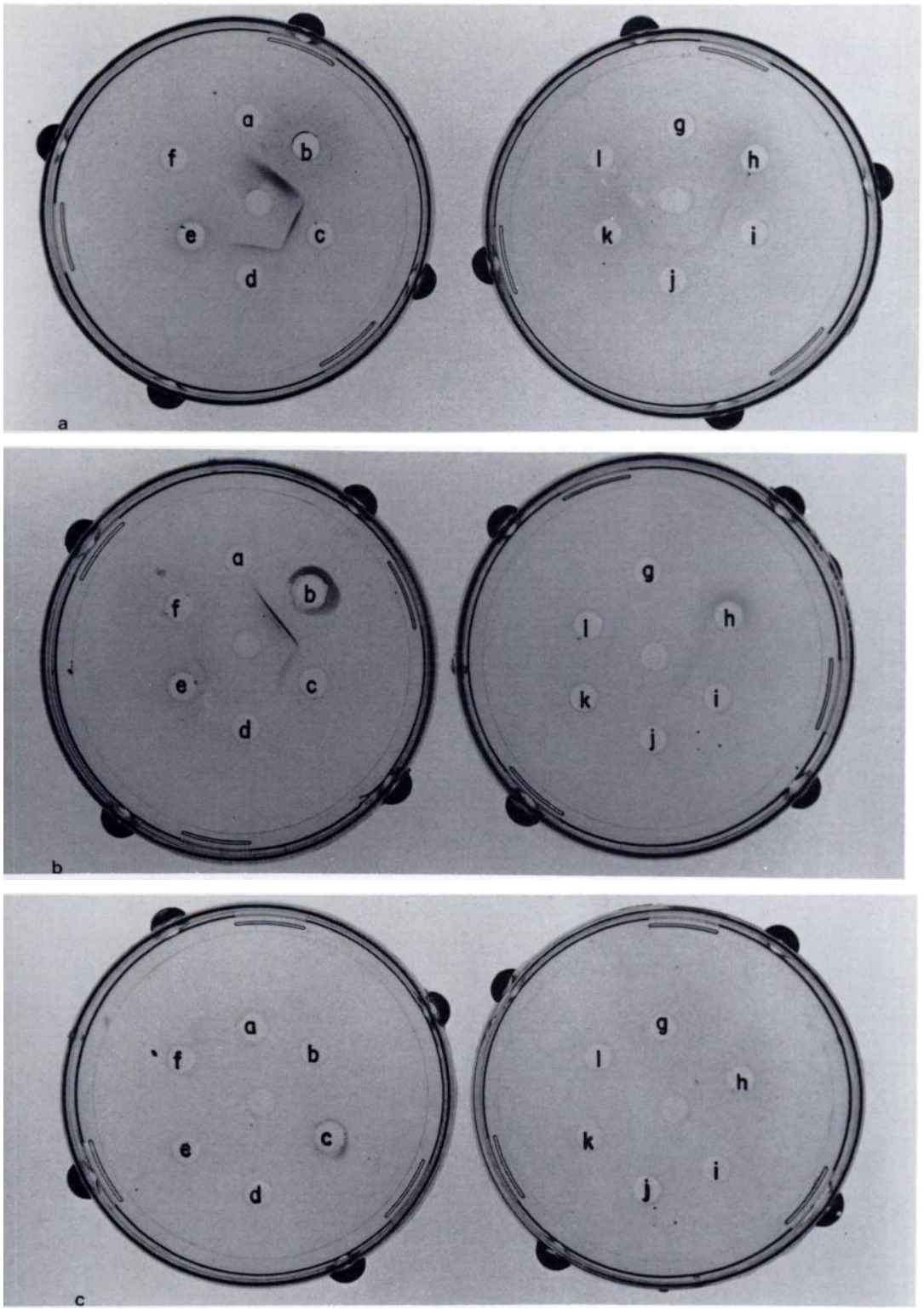


FIG. 1

TABLE 3

Summary of double-diffusion analyses

The destained plates were scored using a graded system ranging from "−" (no band observed) to "+++" (strongest band intensity).

Source of microsomes	P-450	Extent of precipitin band formation	
		3MC-IgG	PB-IgG
	μM		
3MC liver	31	++++	++
3MC lung	0.33	+++	++
3MC kidney	2.2	+++	+
3MC testis	0.86	+	+
3MC spleen	0.19	±	−
3MC heart	2.0	±	−
3MC small intestine	≤0.15	++	+
3MC colon	≤0.15	+	+
3MC stomach	0.31	+	+
3MC brain	0.44	±	−
PB liver	27	+++	++++
PB lung	0.79	+	++
PB kidney	2.8	+	+
PB testis	0.65	+	+
PB spleen	0.10	−	+
PB heart	1.5	−	±
PB small intestine	≤0.04	+	++
PB colon	≤0.04	−	+
PB stomach	0.08	−	+
PB brain	0.44	−	+
Untreated liver	31	+++	++
Untreated lung	0.18	++	+
Untreated kidney	0.55	+	+
Untreated testis	0.44	+	+
Untreated spleen	≤0.15	+	+
Untreated heart	0.65	++	+
Untreated small intestine	≤0.15	+	+
Untreated colon	0.20	+	+
Untreated stomach	0.30	+	+
Untreated brain	0.42	±	+

Immunodiffusion analyses. At least one microsomal preparation from each tissue showed some visible reaction with one of the liver P-450 antibodies in double-diffusion analyses (Table 3; Fig. 1). None of the microsomes reacted with pre-immune serum. The concentration of spectrally-detectable P-450 did not appear to limit the sensitivity of the qualitative assays, as several of the preparations having very low P-450 concentrations produced moderately strong precipitin lines (e.g., small intestine microsomes from PB-treated rats).

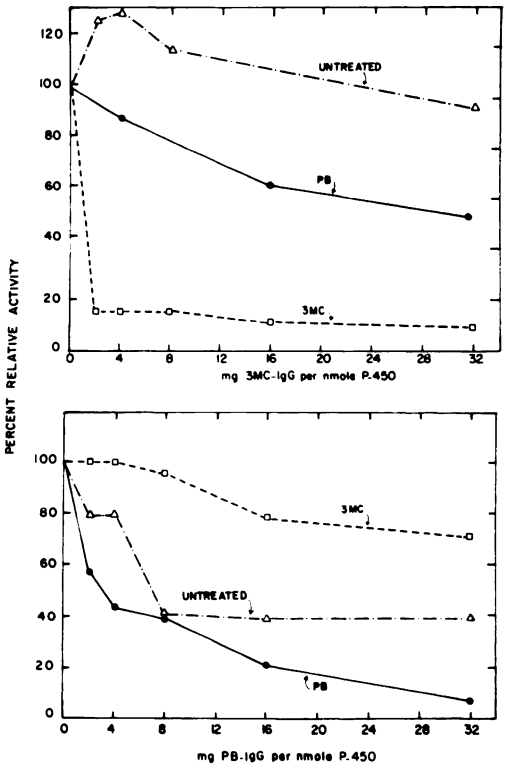


FIG. 2. *Inhibition of liver microsomal benzo(a)pyrene hydroxylase activity by antibodies prepared to liver microsomal P-450s*

Treatments of rats used for preparation of liver microsomes are labeled on the graphs.

Inhibition of mixed-function oxidase activities. With most tissues benzo(a)pyrene hydroxylase was preferentially inhibited in 3MC rat microsomes by liver 3MC-IgG and in PB rat microsomes by liver PB-IgG (Figs. 2–6). However, 3MC-IgG was more effective than PB-IgG in inhibiting the activity in testis and heart microsomes prepared from PB-treated rats (Figs. 5, 6). The hydroxylase activity of PB rat lung microsomes was not inhibited by either antibody (Fig. 3). Activity in the microsomes of 3MC treated rat stomach, small intestine, and brain (Fig. 7) was inhibited by IgGs; the low levels of benzo(a)pyrene hydroxylase in these tissues prepared from untreated and PB-treated rats did not permit a complete examination of antibody specificity. No inhibition was observed using either antibody with spleen microsomes from 3MC-treated

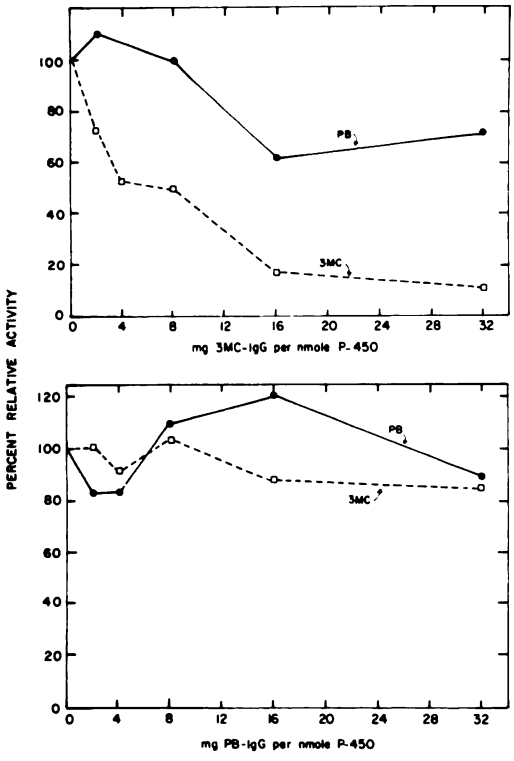


FIG. 3. *Inhibition of lung microsomal benzo-(a)pyrene hydroxylase by antibodies prepared to liver microsomal P-450s*

Treatments of rats used for preparation of lung microsomes are labeled on the graphs.

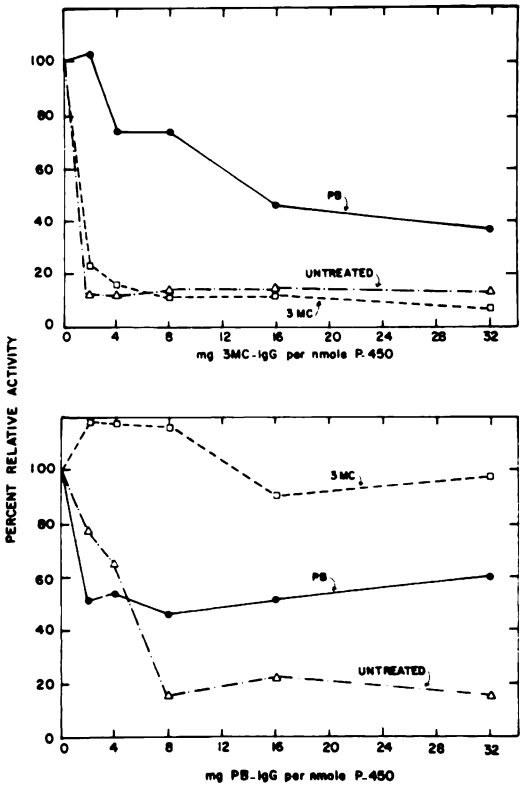


FIG. 4. *Inhibition of kidney microsomal benzo-(a) pyrene hydroxylase by antibodies prepared to liver microsomal P-450s*

Treatments of rats used for preparations of kidney microsomes are labeled on the graphs.

rats (not shown).

Repetition of some of the experiments using 7-ethoxycoumarin *O*-deethylase activity (Figs. 8–10) yielded similar plots, although differences were observed in the case of lung microsomes (Fig. 9). PB- and 3MC-IgGs were about equally effective in inhibiting activity in 3MC-treated rat lung microsomes; PB-IgG was more effective than 3MC-IgG in the inhibition of 7-ethoxycoumarin *O*-deethylase activity in lung microsomes prepared from PB-treated rats. With both substrates, inhibition of activity in microsomes prepared from untreated rats was rather variable; in several cases little inhibition was observed (Figs. 2, 8–10), while inhibition was greater with untreated rat kidney microsomes than with either preparation of induced kidney microsomes in the case of liver PB-IgG (Fig. 4). (Preimmune IgG did not inhibit activities, and neither PB-IgG nor 3MC-IgG inhibited microsomal NADPH-cytochrome *c* reductase activity.)

DISCUSSION

The results indicate that hepatic and extrahepatic P-450s are, in general, rather similar as judged by immunological techniques. The conclusion that immunoprecipitin lines (Fig. 1) are due to reaction of IgGs with P-450 is strengthened by the inhibition of P-450 activities by the IgGs (Figs. 2–10).

The results presented should be considered qualitative rather than quantitative because of several possible complications. First of all, while the antigens used here satisfied a variety of criteria for homogeneity, we cannot completely rule out the possibility of the existence of multiple forms of P-450 that might have gone un-

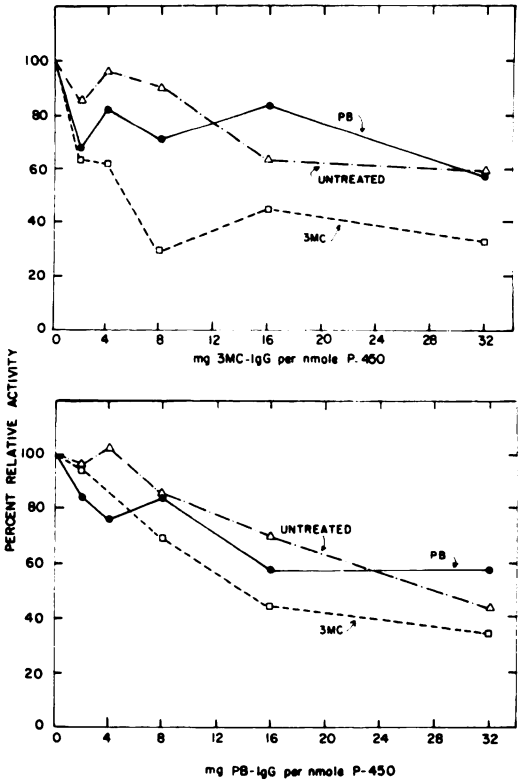


FIG. 5. *Inhibition of testis microsomal benzo(a)pyrene hydroxylase by antibodies prepared to liver microsomal P-450s*

Treatments of rats used for preparation of testis microsomes are labeled on the graphs.

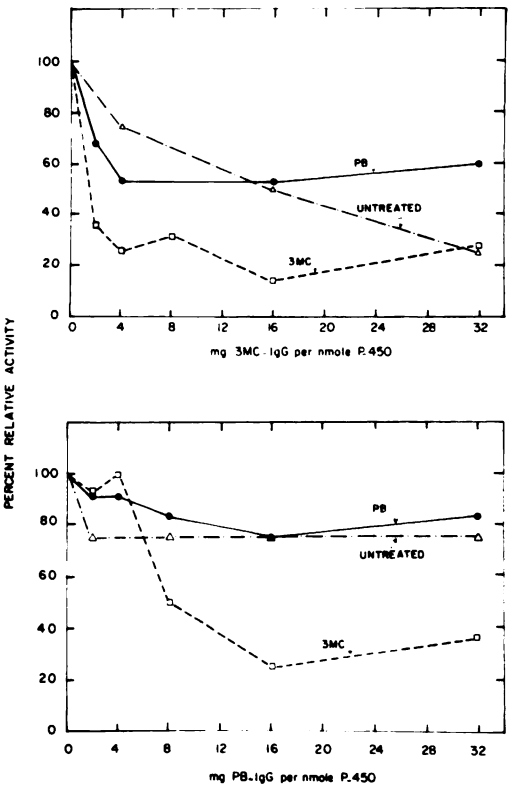


FIG. 6. *Inhibition of heart microsomal benzo(a)pyrene hydroxylase by antibodies prepared to liver microsomal P-450s*

Treatments of rats used for preparation of heart microsomes are labeled on the graphs.

detected; thus the term "apparently homogeneous" is used to describe the P-450s used to raise the antibodies. The levels of cytochrome P-420 (presumably formed from P-450) and apo-P-450 could not be estimated in the extrahepatic tissues and could influence the immunodiffusion and antibody inhibition experiments. Moreover, the intensity of the immunodiffusion precipitin lines does not seem directly related to P-450 concentration. Membrane organization may effect the accessibility of P-450s to antibody in the various preparations used in the inhibition experiments. Thomas *et al.* (28) have previously demonstrated that such inhibition curves are, for reasons not entirely understood, dependent upon the substrate used as well as the sources of antibodies and tissues. Some P-450s may not be solubilized in the double-diffusion studies. Attempts were made to obtain subunit molecular weights of P-450s immunoprecipitated from the various microsomal preparations using the general procedure of Welton *et al.* (33); however, suitable detergent conditions could not be established in which microsomal P-450s could be efficiently solubilized without solubilization of immunoprecipitates. Finally, the possibility exists that not all P-450s necessarily react with either of these antibodies, as suggested by the lack of antibody inhibition of some tissue hydroxylases (e.g., Figs. 3, 10).

The conclusion of immunological similarity in these tissues is a general statement; under certain conditions each tissue contains P-450s that cross-react with the liver antibodies. In making more specific conclu-

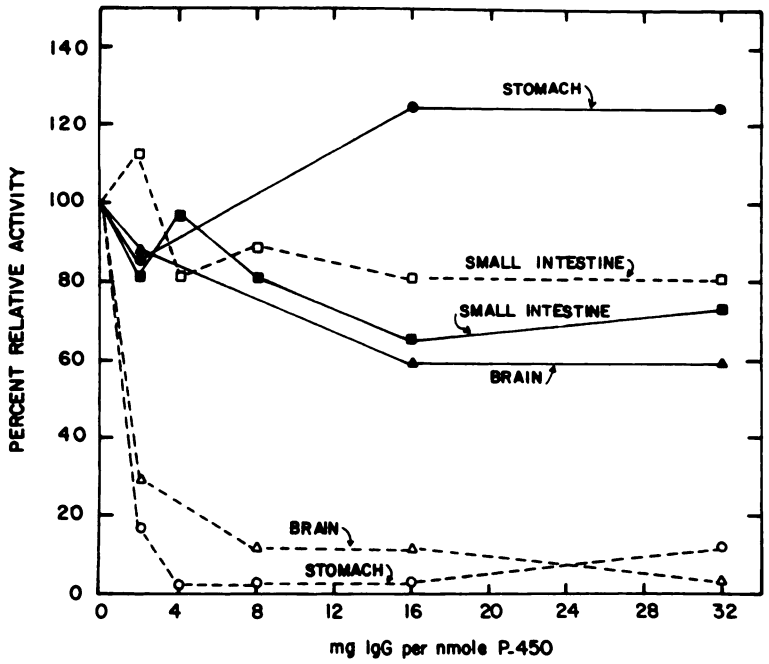
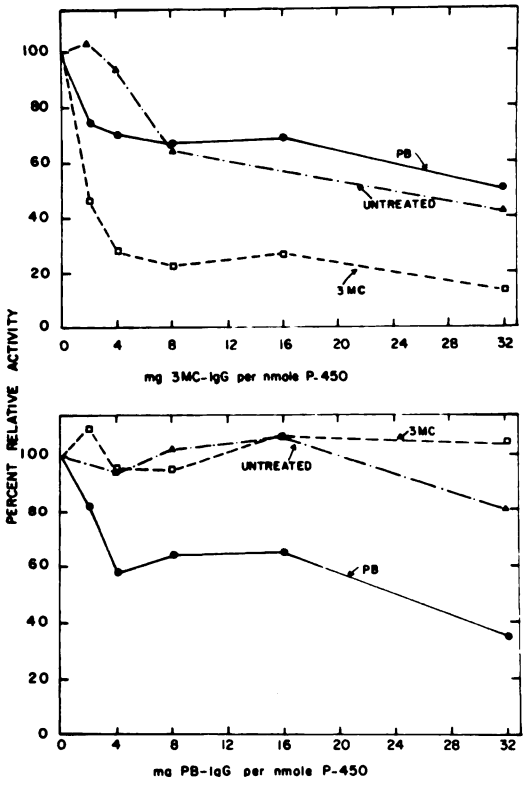


FIG. 7. *Inhibition of benzo(a)pyrene hydroxylase activity in stomach, brain, and small intestine microsomes prepared from 3MC-treated rats*

The indicated microsomal preparations were mixed with PB-IgG (——) or 3MC-IgG (----) prior to incubation.



sions with regard to induction, etc., each tissue should be considered separately. For instance, Figures 2–10 indicate different inhibition curves for the hydroxylases in the various tissues, suggesting some basic differences in the P-450s or in their accessibility. Moreover, some differences are seen when the benzo(a)pyrene and 7-ethoxycoumarin hydroxylase activities are compared to each other in liver, lung, and kidney (Figs. 2–4, 8–10), suggesting variability between the abilities of the P-450s to metabolize these substrates. Immunologically similar P-450s can differ considerably in their physical properties (5, 24). The two P-450 preparations used to raise antibodies for this work show some cross-reactivity as judged by immunodiffusion analysis or immunoprecipitation, although the two proteins are clearly quite different as judged

FIG. 8. *Inhibition of liver microsomal 7-ethoxycoumarin O-deethylase activity by antibodies prepared to liver microsomal P-450s*

Treatments of rats used for preparation of liver microsomes are labeled on the graphs.

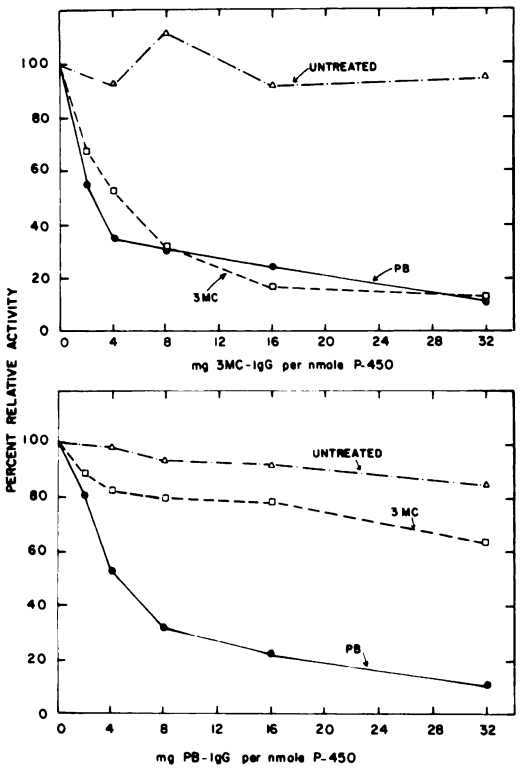


FIG. 9. *Inhibition of lung microsomal 7-ethoxycoumarin O-deethylase activity by antibodies prepared to liver microsomal P-450s*

Treatments of rats used for preparation of lung microsomes are labeled on the graphs.

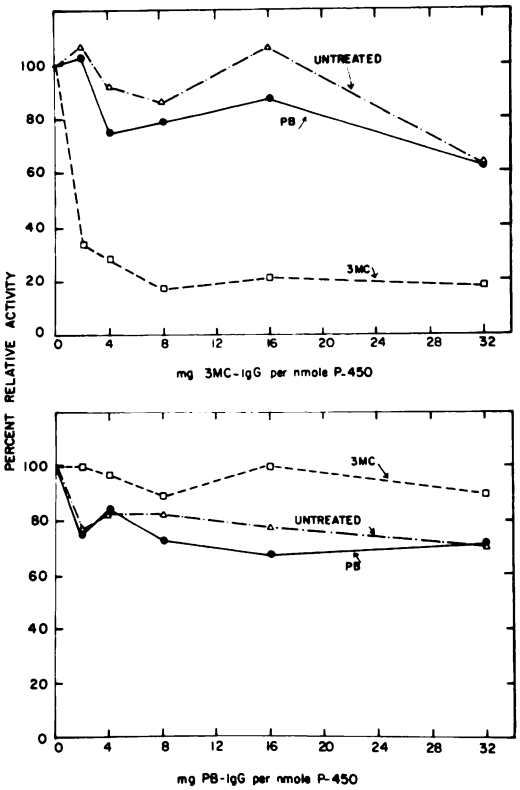


FIG. 10. *Inhibition of kidney microsomal 7-ethoxycoumarin O-deethylase activity by antibodies prepared to liver microsomal P-450s*

Treatment of rats used for preparation of kidney microsomes are labeled on the graphs.

by agarose and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, amino acid and *N*- and *C*-terminal analyses (24), substrate specificities (23, 24), and peptide maps (36). These studies presented here indicate that the various P-450s in hepatic and nonhepatic tissues contain similar immunological determinants. On this basis, the P-450s in the various tissues would appear to be at least somewhat similar, and the hepatic P-450s should be reasonably good models for the extrahepatic P-450s. The antibodies prepared to hepatic P-450s should prove to be useful in studying the characteristics of the extrahepatic P-450s and the reactions that they catalyze.

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