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# Specificity of Rabbit Pulmonary Cytochrome P-450 Isozymes in the Activation of Several Aromatic Amines and Aflatoxin $B_1$

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SUMMARY

The involvement of pulmonary or hepatic cytochrome P-450 monooxygenase enzymes in the activation of the promutagens 2-aminoanthracene, 2-aminofluorene, 2-acetylaminofluorene, and aflatoxin $B_1$ has been investigated using *Salmonella* strain TA98. These agents were more readily metabolized to mutagenic products by the 16,000 × *g* supernatant fraction from lung than from liver despite the low pulmonary cytochrome P-450 content, and more revertants per nanomole of total cytochrome P-450 were consistently obtained with pulmonary than with hepatic microsomal preparations. In reconstituted monooxygenase systems containing the major pulmonary cytochrome P-450 isozymes (P-$450_I$ or P-$450_{II}$), P-$450_{II}$ was highly effective in the activation of 2-aminoanthracene and 2-aminofluorene and also active with 2-acetylaminofluorene, whereas these substrates were not activated by P-$450_I$. This difference was confirmed by the results of antibody inhibition studies carried out with pulmonary microsomal preparations. The higher activity of pulmonary preparations relative to hepatic preparations can be accounted for by the relatively high proportion of P-$450_{II}$ in the lung (approximately 50% total cytochrome P-450 content) as compared with the liver (less than 5%). However, antibody to P-$450_{II}$ did inhibit the hepatic microsomal activities by 50–70%, indicating that P-$450_{II}$ may be important in the activation of these agents in both tissues even though it is a minor component in the liver. Aflatoxin $B_1$ was activated only by P-$450_I$ in reconstituted monooxygenase systems, although the antibody inhibition studies indicated activation by both P-$450_I$ and P-$450_{II}$ in pulmonary microsomal preparations.

## INTRODUCTION

In the process of their excretion from the body, many exogenous compounds undergo oxidative metabolism to highly reactive, electrophilic intermediates or products which are mutagenic, carcinogenic, or otherwise toxic (1–4). This process of activation is catalyzed by the cytochrome P-450-dependent monooxygenase systems (3–5), which are most highly concentrated in liver but have also been detected in other tissues, including skin, kidney, intestine, gonads, adrenals, placenta, and lung (5).

Several cytochrome P-450 isozymes with different substrate specificities have been described (6–10). Therefore, the presence or absence of particular P-450 isozymes may contribute to tissue-specific toxicity. An assessment of the potential of extrahepatic tissues to mediate the activation of exogenous chemicals can only be made if we are able to identify the P-450 isozymes in these tissues and to determine their substrate specificities.

Two cytochrome P-450 isozymes, P-$450_I$ and P-$450_{II}$, have been purified from rabbit lung and characterized extensively (11–18). These enzymes, which are present in rabbit pulmonary preparations in approximately equal amounts (11, 16, 17), have distinct substrate specificities (11–13) but have not been investigated with respect to the activation of promutagens.

In the present study, the activation of 2AA,[4] 2AF, 2AAF, and $AFB_1$ by rabbit pulmonary and hepatic preparations has been investigated using the *Salmonella* test of Ames *et al.* (19). In experiments with either antibodies to specific cytochrome P-450 isozymes or monooxygenase systems reconstituted from purified enzymes, the observed activities have been related to the relative concentrations and activities of these isozymes.

## EXPERIMENTAL PROCEDURES

*Preparation of 16,000 × g supernatant fractions ($S_{16}$).* Groups of 15 rabbits (New Zealand White or Dutch Belt

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[4] The abbreviations used are: 2AA, 2-aminoanthracene; 2AF, 2-aminofluorene; 2AAF, 2-acetylaminofluorene; $AFB_1$, aflatoxin $B_1$; DMSO, dimethyl sulfoxide; IgG, immunoglobulin G; *N*-hydroxy-AAF, *N*-hydroxyacetylaminofluorene; *N*-hydroxy-AF, *N*-hydroxyaminofluorene.

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males, 2–3 kg) were killed with $CO_2$ and the lungs and livers were excised and washed in cold 0.15 M KCl (adjusted to pH 7.4 with $Na_2HPO_4$). Pulmonary and hepatic homogenates were prepared as previously described (16). The homogenates were centrifuged for 20 min at 16,000 × *g* and the supernatant fractions were decanted and stored at −80°. Pulmonary $S_{16}$ fractions free of bacterial contamination could not be obtained directly. Activation systems containing $S_{16}$ were therefore sterilized by filtration through 0.45-μm pore size Acrodisc (Gelman) syringe filter assemblies. Prefiltration through glass fiber, 5.0, 1.2, 0.8, and 0.45-μm pore size filters using a pressure filtration cell (Gelman) was required. For consistency, hepatic preparations were filtered according to the same protocol.

*Preparation of microsomal fractions.* Fresh pulmonary and hepatic $S_{16}$ fractions were prepared and sterilized according to the above protocol except that the final 0.45-μm filtration was carried out with Naglene vacuum filter units (Sybron). (All subsequent steps were carried out using aseptic technique.) The filtered $S_{16}$ fractions were centrifuged for 40 min at 160,000 × *g*, the 160,000 × *g* supernatant was decanted, the surface of the pellet was rinsed, and the pellet was resuspended in 0.25 M sucrose (adjusted to pH 7.4 with $Na_2HPO_4$) to approximately 20 mg of protein per milliliter and stored at −80°.

*Purification of monooxygenase components.* The hepatic cytochrome P-450, P-450LM$_4$,[5] was isolated from rabbits treated with 3-methylcholanthrene (20 mg/kg in corn oil i.p. 2 days before sacrifice) according to the method of Philpot and Arinc (20). The two major rabbit pulmonary cytochrome P-450 isozymes (P-450$_I$ and P-450$_{II}$) were also isolated by previously reported procedures (14, 15). The purities of P-450LM$_4$, P-450$_I$, and P-450$_{II}$ were 15.6, 20.6, and 5.6 nmoles/mg of protein, respectively.

Cytochrome P-450 reductase (reductase) was purified to 40,000–50,000 units/mg of protein by a modification (16) of the method of Yasukochi and Masters (21). The cytochrome P-450 and reductase were diluted to 9 nmoles/ml and 50,000 units/ml, respectively, in 100 mM phosphate buffer (pH 7.4), filtered through a 0.45-μm pore size filter and stored at −80°.

*Preparation of antibodies against P-450$_I$ and P-450$_{II}$.* Antibodies to pulmonary cytochrome P-450$_I$ (anti-P-450$_I$) and P-450$_{II}$ (anti-P-450$_{II}$) were elicited from goats using electrophoretically homogeneous P-450$_I$ and P-450$_{II}$ as previously described (16).

*Analytical procedures.* Cytochrome P-450 concentrations were determined by the method of Omura and Sato (22) as modified by Estabrook *et al.* (23). Protein concentrations were determined by the method of Lowry *et al.* (24).

*Reagents and biochemicals.* Glucose 6-phosphate, NADP$^+$, glucose 6-phosphate dehydrogenase, and L-histidine-HCl were obtained from Sigma Chemical Company (St. Louis, Mo.); 2AF and 2AAF were obtained from Aldrich Chemical Company (Milwaukee, Wisc.), 2AA from ICN Pharmaceuticals Inc. (Plainview, N. Y.), and $AFB_1$ from Calbiochem Ltd. (San Diego, Calif.). Solutions of promutagens were made in DMSO (Fisher Scientific Company, Pittsburgh, Pa.) immediately prior to use.

*Salmonella mutagenesis assay.* Plates (Falcon No. 1028 MutaAssay dishes) contained 25 ml of Vogel-Bonner Medium E (25) supplemented with 0.5% glucose in 1.5% Difco purified agar. Top agar was prepared by adding 10.0 ml of a 0.5 mM L-histidine-0.5 mM (+)-biotin solution, 2.5 ml of a 20% glucose solution, and 2.0 ml of a 50-times concentration of Vogel-Bonner Medium E salts to 100 ml of 0.6% agar.

*Salmonella typhimurium* strain TA98 was obtained from Dr. Bruce N. Ames (Berkeley, Calif.) and stored at −80° as recommended (19). Cultures were grown in Oxoid nutrient broth No. 2 (Oxoid Ltd., Basingstoke, Hants., England) at 37° overnight with limited shaking and further grown with fast shaking to an absorbance at 650 nm of 1.0 (0.8–1.2 × $10^9$ colony-forming units per milliliter) and then placed on ice.

Mutagenicity assays other than those using purified monooxygenase enzymes were performed by plating the following in triplicate: cells (0.1 ml), substrate (in 50 μl of DMSO), and 0.5 ml of the appropriate activation system containing $S_{16}$ or microsomal fractions in top agar following standard procedures (19). The agar was allowed to solidify for up to 30 min and the plates were incubated at 37° for 48 hr after which the histidine-revertant colonies were counted manually. Counts are expressed as the mean and standard deviation of triplicate plates, unless otherwise indicated.

The activation systems contained (per milliliter) $MgCl_2$ (8 μmoles), KCl (33 μmoles), sodium phosphate buffer (pH 7.4) (100 μmoles), NADP$^+$ (4 μmoles), glucose 6-phosphate (5 μmoles) and up to 0.5 ml (10–20 mg of protein) of $S_{16}$ or up to 0.2 ml (2–4 mg of protein) of microsomal fractions. When microsomes were used, 0.5 or 1.0 unit of glucose 6-phosphate dehydrogenase (Sigma Type XV) was added.

In all cases, pulmonary and hepatic preparations used were obtained from the same animals and each agent was tested concurrently with preparations from both tissues. Activation systems containing $S_{16}$ fractions were sterilized as described above. Activation systems containing the microsomal fraction were prepared from sterile components. When antibodies were included, the stock solutions of the antibodies were diluted to 5–7.5 mg of IgG per milliliter with 100 mM phosphate buffer (pH 7.4) before sterilization through 0.45 μm filters. Microsomal activation systems containing antibodies were held at 4° for at least 5 min before use to ensure that the antibody-antigen reaction occurred.

Experiments using purified monooxygenase enzymes were carried out as follows: Didodecanoyl-L-α-lecithin (25 mg/ml in chloroform, final content 500 μg/nmole of cytochrome P-450) was added to a glass vial, evaporated to dryness at 45–50°, and then cooled. Cytochrome P-450 (P-450LM$_4$, P-450$_I$, or P-450$_{II}$; stock solution 9 nmoles/ml) and cytochrome P-450 reductase (6250 units/nmole of cytochrome P-450) were added, mixed, and incubated at 37° for 5 min. These mixtures were diluted as required

[5] P-450LM$_4$ (Form 4, P-448) is the major hepatic cytochrome P-450 isolated from 3-methylcholanthrene or β-naphthoflavone treated rabbits (7, 8, 20) and is also present in untreated rabbits (7, 20).

in 100 mM phosphate buffer (pH 7.4), and aliquots added to 1-dram vials followed by the addition of the buffer and salt components described above for a final volume of 1.6 ml. Substrate (up to 40 μl in DMSO) and bacterial culture (0.32 ml) were added and the reaction was initiated by addition of the NADPH-generating mix. The vials were then incubated at 37° with shaking for 15 min, and cooled to 0°. Aliquots (0.6 ml) were plated in triplicate, the plates were incubated, and the revertants were counted as described above.

## RESULTS

Unlike hepatic preparations, sterile pulmonary preparations could not be obtained without filtration. Because of the fibrous nature of pulmonary tissue, a step-wise filtration procedure, as described under Experimental Procedures, was developed to obtain suitably sterile pulmonary samples.

Rabbit pulmonary and hepatic $S_{16}$ fractions were active in the formation of mutagenic products from 2AA, 2AAF, 2AF, and $AFB_1$ (Fig. 1a–d). The data are reported as the mean number of revertants per plate versus the amount of $S_{16}$ protein per plate at a single substrate concentration. The substrate concentrations used were those at the midpoints of the linear portions of substrate concentration curves obtained with the intermediate protein levels (data not shown). In the absence of substrate the numbers of spontaneous revertants ranged from 20 to 40 per plate.

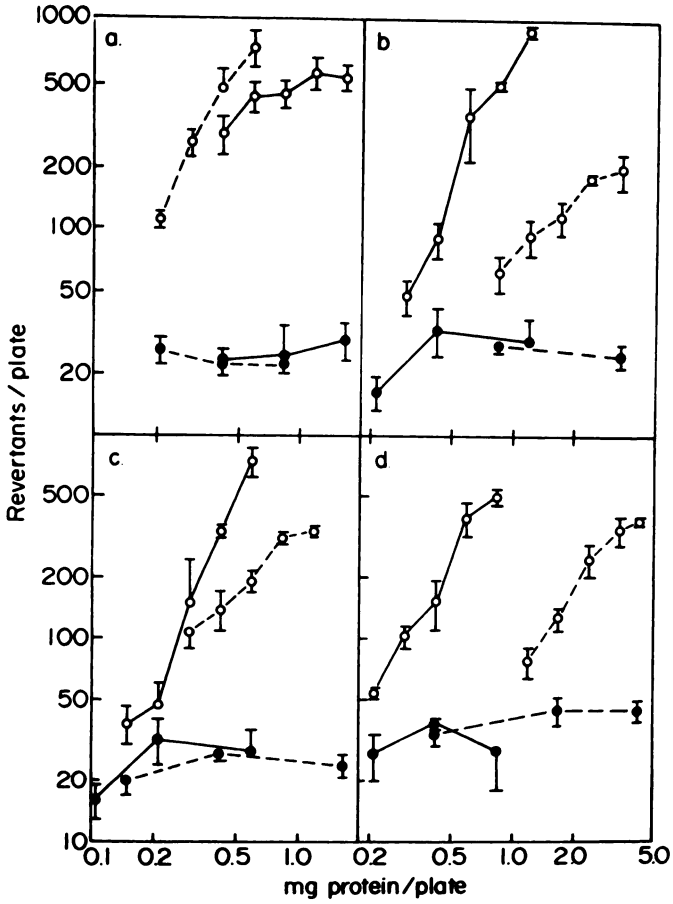


FIG. 1. *Formation of mutagenic products by hepatic and pulmonary $S_{16}$ fractions*

Experimental details are given under Experimental Procedures. ——, Hepatic; – – –, pulmonary preparations. *Open symbols* indicate activation with $S_{16}$; *closed symbols* indicate spontaneous number of revertants with $S_{16}$.

a. 2AF, 1.1 nmoles/plate.
b. 2AAF, 4.5 nmoles/plate.
c. 2AA, 0.23 nmole/plate.
d. $AFB_1$, 0.03 nmole/plate.

When the hepatic and pulmonary $S_{16}$ fractions were compared on the basis of the amount of $S_{16}$ protein required to obtain a given number of revertants, the hepatic fraction was more active with 2AA (up to 2 times), 2AAF (2.5–5 times) and $AFB_1$ (5 times), whereas the pulmonary fraction was more active with 2AF (up to 2 times). However, on the basis of cytochrome P-450 content (the $S_{16}$ fraction from liver in these preparations contained approximately 10 times more cytochrome P-450 per milligram of protein than the $S_{16}$ from lung), the activity of the pulmonary fraction was equal to or greater than that of the hepatic fraction.

These comparisons are approximate owing to both the differences in the slopes of the response curves obtained with the fractions from the two tissues (Fig. 1a–d) and the inability to measure directly cytochrome P-450 concentrations in the pulmonary $S_{16}$ fraction. The activation by pulmonary and hepatic preparations was therefore further investigated using microsomal fractions, thus allowing direct comparisons on the basis of cytochrome P-450 content.

As with the $S_{16}$ fractions, all of the substrates were metabolized to mutagenic products by both pulmonary and hepatic microsomal fractions (Fig. 2a–d). The data shown in these figures are expressed as the mean number of revertants per plate versus nanomoles of cytochrome P-450 present. The numbers of revertants obtained in the absence of cofactor are given for the highest substrate concentrations used. In all cases the numbers of revertants were dependent on the substrate concentration. The results are in agreement with those obtained with the $S_{16}$ fractions (Fig. 1a–d). For a given substrate concentration, a comparison of the amounts of cytochrome P-450 required for equivalent activity shows that the pulmonary microsomal fraction was 3–4, 20–25, and 25–30 times more active than the hepatic fraction in the formation of mutagenic products from 2AAF, 2AA, and 2AF, respectively (Fig. 2d, a, and b). With $AFB_1$, similar activities were obtained with both microsomal fractions, the pulmonary fraction being slightly more active (Fig. 2c). Both 2AA and 2AF were extremely potent mutagens in systems containing the pulmonary microsomal fraction; significant numbers of revertants were detected with less than 2.5 pmoles of cytochrome P-450 per plate.

In order to ascertain which of the two pulmonary P-450 isozymes was responsible for the mutagenic activity in incubations containing pulmonary microsomal fraction, their activities were determined in monooxygenase systems reconstituted from purified enzymes (Table 1). For comparative purposes, the hepatic isozyme induced by treatment of rabbits with 3-methylcholanthrene (P-450$LM_4$) was also studied. [Of four rabbit hepatic cytochrome P-450 isozymes examined for their ability to activate 2AA, Norman *et al.* (26) obtained positive results

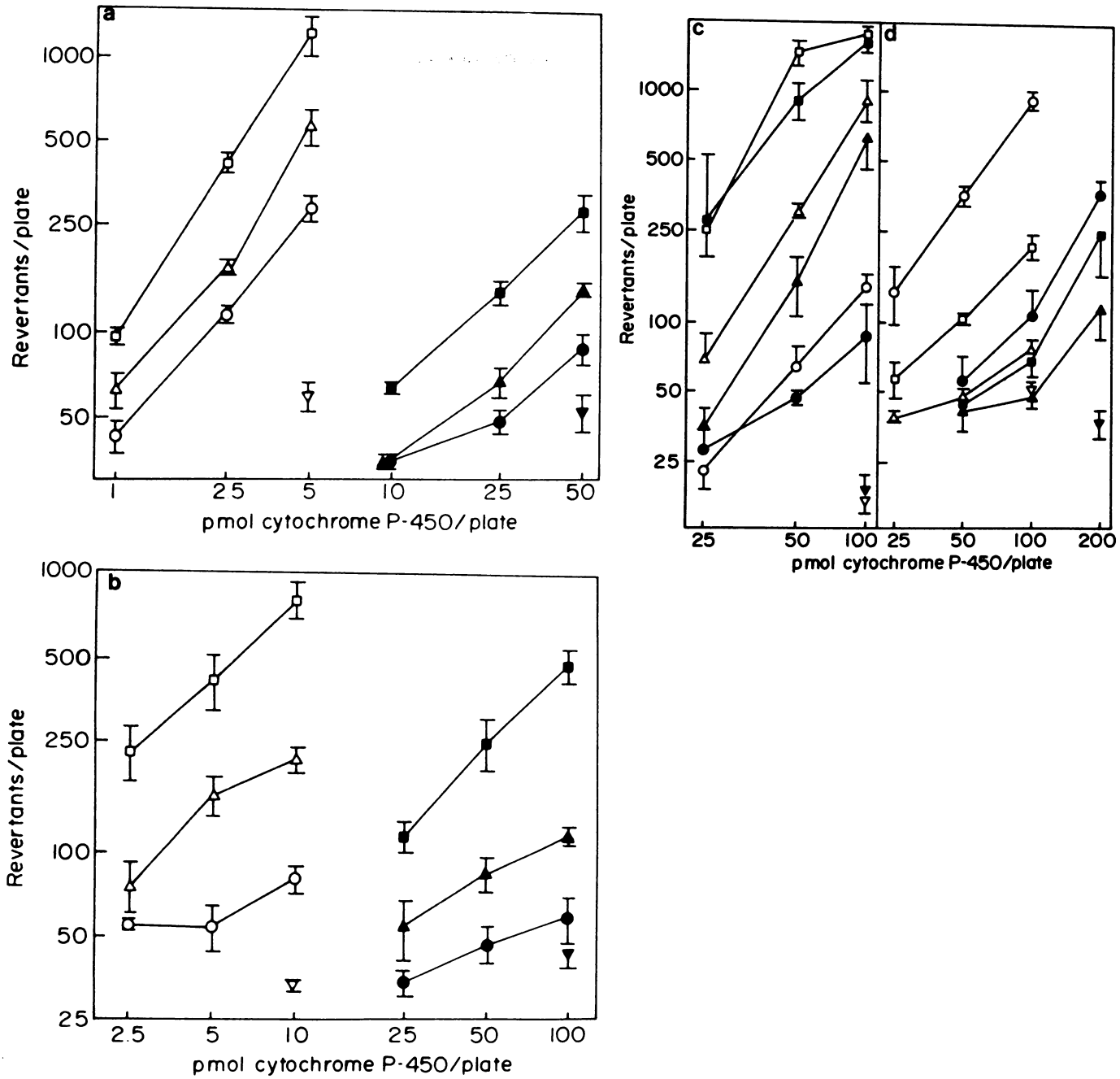


FIG. 2. *Formation of mutagenic products by hepatic and pulmonary microsomal fractions*

Experimental details are given under Experimental Procedures. *Open symbols* indicate pulmonary fractions; *closed symbols*, hepatic fractions. Mean sponteneous numbers of revertants were 31 ± 7 and 29 ± 7 for the pulmonary and hepatic fractions, respectively.

a. 2AA at 0.52 (○), 2.6 (△), and 10 (□) nmoles/plate, and 10 (▽) nmoles/plate without NADPH.

b. 2AF at 0.55 (○), 2.8 (△), and 11 (□) nmoles/plate, and 11 (▽) nmoles/plate without NADPH.

c. $AFB_1$ at 0.016 (○), 0.08 (△), and 0.32 (□) nmoles/plate, and 0.32 (▽) nmoles/plate without NADPH.

d. 2AAF at 2.2 (△), 9.0 (□), and 45 (○) nmoles/plate and 45 (▽) nmoles/plate without NADPH.

only with P-450LM$_4$.] The promutagens 2AA, 2AF, and 2AAF were substrates for both P-450$_{II}$ and P-450LM$_4$ but were inactive with P-450$_I$. P-450LM$_4$ showed the greater activity with 2AAF; however, P-450$_{II}$ gave 10–20 times more mutagenic activity with 2AA and 2AF than did P-450LM$_4$. With $AFB_1$, only incubations containing P-450$_I$ gave a significant increase in the number of revertants.

Confirmation of these results was obtained with pulmonary microsomal preparations and antibodies to the two pulmonary isozymes. The data obtained are summarized in Table 2, where both the number of revertants and the percentage inhibition are presented. A preimmune IgG was included as a control for the incubations that contained the antibody preparations. Anti-P-450$_{II}$ inhibited the ability of the pulmonary microsomal preparations to form mutagenic products from 2AA, 2AF, and

TABLE 1

*Mutagenic activation by purified monooxygenase systems*

The protocol used is described under Experimental Procedures. Substrate additions per milliliter of incubation mixture were 5.2, 14, 90, and 0.4 nmoles for 2AA, 2AF, 2AAF, and $AFB_1$, respectively. Cytochrome P-450 additions per milliliter of incubation mixture were 10 pmoles for 2AA and 2AF, and 100 pmoles for 2AAF and $AFB_1$. All values are mean numbers of revertants per plate ± standard deviation (three plates).

| | Phosphate buffer | $P450_I$ | $P450_{II}$ | P-450LM$_4$ |
|---|---|---|---|---|
| 2AA | 39 ± 8 | 63 ± 5 | 2608 ± 139 | 198 ± 11 |
| 2AF | 40 ± 10 | 78 ± 13 | 2247 ± 211 | 121 ± 6 |
| 2AAF | 20 ± 12 | 43 ± 7 | 97 ± 3 | 355 ± 43 |
| $AFB_1$ | 30 ± 16 | 291 ± 6 | 35 ± 9 | 32 ± 6 |

2AAF by greater than 85%; anti-$P-450_I$ had no effect. Both antibodies inhibited the activation of $AFB_1$ by about 50%.

The activation of the aromatic amines was inhibited by anti-$P-450_{II}$ in a concentration-dependent manner (shown for 2AA in Fig. 3). Maximal inhibition with anti-$P-450_{II}$ was obtained at 10–20 mg of IgG per nanomole of cytochrome P-450; no inhibition was obtained with anti-$P-450_I$ at concentrations up to 20 mg of IgG per nanomole of cytochrome P-450 (Fig. 3). (It should be noted that anti-$P-450_I$, at a concentration of less than 10 mg of IgG per nanomole of cytochrome P-450, does inhibit the metabolism of a number of substrates in pulmonary microsomal preparations; see ref. 16.)

The effects of anti-$P-450_I$ and anti-$P-450_{II}$ on the activation of 2AA, 2AF, 2AAF, and $AFB_1$ by hepatic microsomal preparations are shown in Table 3. Neither antibody had a measurable effect on the activation of $AFB_1$, but anti-$P-450_{II}$ did inhibit the mutagenic activition of 2AA, 2AF, and 2AAF by 55, 69, and 48%, respectively. As demonstrated with 2AA (Fig. 3), less than 0.2 mg of IgG (anti-$P450_{II}$) per nanomole of cytochrome P-450 was required to obtain 50% maximal inhibition. Similar inhibition of the pulmonary activation of 2AA required approximately 2 mg of IgG per nanomole of cytochrome P-450. Anti-$P-450_I$ was ineffective in these systems at concentrations of up to 5 mg of IgG per nanomole of cytochrome P-450 (Table 3; Fig. 3).

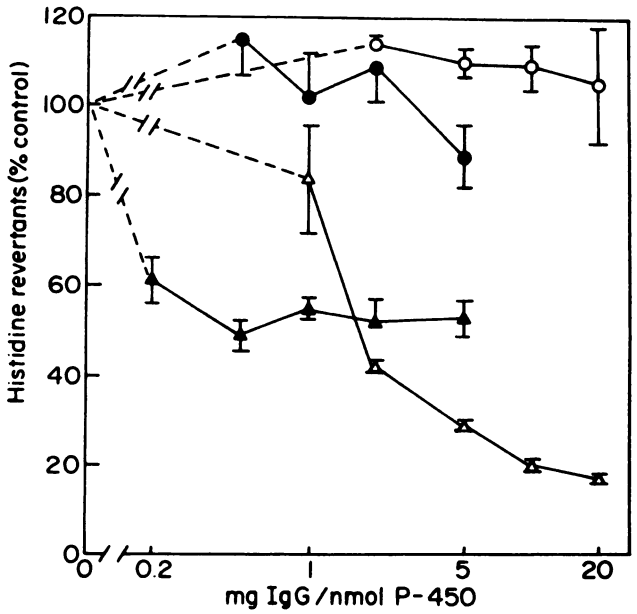


FIG. 3. *Effect of antibodies to the two major pulmonary cytochrome P-450 isozymes on the mutagenicity of 2AA in microsomal fractions*

The protocol used was as described under Experimental Procedures. *Open symbols* indicate pulmonary fractions; *closed symbols* indicate hepatic microsomal fractions. Substrate (10 nmoles) was added to each plate with 5 pmoles of pulmonary or 50 pmoles of hepatic of cytochrome P-450 and up to 20 μg of anti-$P450_I$ or anti-$P450_{II}$/pmole of pulmonary cytochrome P-450 and 5 μg/pmole of hepatic cytochrome P-450. Appropriate amounts of control preimmune IgG were added so that the total amounts of IgG per plate were the same. ○, anti-$P-450_I$; △, anti-$P-450_{II}$.

TABLE 2

*Inhibition of mutagenic activation in pulmonary microsomal fractions by antibodies to purified forms of cytochrome P-450*

The protocol used is described under Experimental Procedures. Antibody or IgG additions were 20 μg/pmole of cytochrome P-450. Substrate additions were 10, 17, 45, and 0.32 nmoles/plate for 2AA, 2AF, 2AAF, and $AFB_1$, respectively. Microsome additions per plate were 5 pmoles of cytochrome P-450 for 2AA and 2AF and 50 pmoles for 2AAF and $AFB_1$. The mean number of spontaneous revertants obtained was 23 ± 3. All values are mean numbers of revertants per plate ± standard deviation. The mean number of revertants as a percentage of the preimmune serum control is given in parentheses.

| | 2AA | 2AF | 2AAF | $AFB_1$ |
|---|---|---|---|---|
| Microsomes only | 962 ± 155 (91) | 224 ± 13 (93) | 378 ± 127 (134) | 933 ± 70 (89) |
| Preimmune serum | 1056 ± 117 (100) | 238 ± 77 (100) | 289 ± 149 (100) | 1040 ± 111 (100) |
| Anti-$P-450_I$ | 862 ± 101 (81) | 218 ± 32 (91) | 317 ± 39 (111) | 514 ± 100 (48) |
| Anti-$P-450_{II}$ | 163 ± 9 (13) | 55 ± 2 (14) | 63 ± 18 (15) | 542 ± 65 (51) |

TABLE 3

*Inhibition of mutagenic activation in hepatic microsomal fractions by antibodies to the pulmonary cytochrome P-450 isozymes*

The protocol used is described under Experimental Procedures. IgG, 5 μg/pmole of cytochrome P-450, was used. Substrate additions were 10, 17, 90, and 0.32 nmoles/plate for 2AA, 2AF, 2AAF, and $AFB_1$, respectively. Microsome additions per plate were 50 pmoles for 2AA and $AFB_1$, 100 pmoles for 2AF, and 150 pmoles for 2AAF. All values are mean numbers of revertants ± standard error computed from the results of two separate experiments using a function taking into account both inter- and intra-experiment variation. The number of spontaneous revertants was 27 ± 2. The numbers of revertants as a percentage of the preimmune serum control, corrected for minus cofactor, are given in parentheses.

| | 2AA | 2AF | 2AAF | $AFB_1$ |
|---|---|---|---|---|
| Microsomes only | 353 ± 27 (92) | 681 ± 35 (92) | 338 ± 34 (122) | 324 ± 71 (113) |
| Preimmune serum | 377 ± 51 (100) | 737 ± 57 (100) | 282 ± 37 (100) | 291 ± 58 (100) |
| Anti-$P-450_I$ | 375 ± 19 (99) | 768 ± 42 (104) | 383 ± 63 (140) | 286 ± 125 (98) |
| Anti-$P-450_{II}$ | 196 ± 29 (45) | 254 ± 55 (31) | 157 ± 25 (51) | 256 ± 112 (86) |

## DISCUSSION

Differences among the substrate specificities of cytochrome P-450 isozymes are both extensive and complex. Such differences are exemplified by the catalytic properties of P-450$_{I}$ and P-450$_{II}$, the major rabbit pulmonary isozymes of cytochrome P-450. For example, significant differences in the metabolite profiles obtained with these cytochromes have been observed with the pulmonary carcinogen benzo[*a*]pyrene (12) and the pulmonary toxins *p*-xylene and 4-ipomeanol,[6] although these substrates are metabolized at similar rates. In addition, the metabolism of other compounds tested is catalyzed to a detectable extent only by P-450$_{I}$. This has been demonstrated in microsomal preparations through the use of antibodies and in purified systems for benzphetamine and 7-ethoxycoumarin (11, 16) and norbenzphetamine (13). We have now been able to determine that P-450$_{II}$ catalyzes some reactions for which P-450$_{I}$ exhibits little or no activity.

We have shown in purified systems that the aromatic amines, 2AA, 2AF, and 2AAF, are metabolized to mutagenic products by P-450$_{II}$ but not by P-450$_{I}$. This finding does not preclude the possibility that P-450$_{I}$ can metabolize these compounds to nonmutagenic products. However, Johnson *et al.* (27) were unable to detect any metabolism of 2AAF by "form 2," a cytochrome P-450 isozyme from rabbit liver that cannot be distinguished from P-450$_{I}$ (14–16). This difference is also apparent in microsomal preparations; the activation of these compounds to mutagenic products is greatly inhibited by anti-P-450$_{II}$ but is unaffected by anti-P-450$_{I}$.

Cytochrome P-450$_{II}$ is particularly active in the metabolism of 2AA and 2AF to mutagenic products—approximately 25 times more active than the hepatic isozyme, P-450LM$_4$ (form 4). P-450LM$_4$ has been shown by Norman *et al.* (26) to be 5–10 times more effective in the production of mutagenic metabolites from 2AA than three other rabbit hepatic cytochrome P-450 isozymes (forms 2, 3, and 6) tested. Our results with P-450LM$_4$ and 2AA are comparable to those of Norman *et al.* (26) when the differences in the concentrations of cytochrome used are considered.

The partial inhibition by anti-P-450$_{II}$ of the metabolism of the aromatic amines to mutagenic products by hepatic microsomal preparations suggests that cytochrome P-450$_{II}$ is present in rabbit liver as well as lung. We have estimated from purification data (11), ligand complex formation (13), and antibody radial diffusion experiments (17) that approximately one-half of the rabbit pulmonary cytochrome P-450 is P-450$_{II}$. From the hepatic and pulmonary activities inhibited by anti-P-450$_{II}$, it can be estimated that only 1–2% of the hepatic cytochrome is P-450$_{II}$. Alternatively, a comparison of the amounts of antibody (per nanomole of total P-450) required for 50% maximal inhibition in pulmonary and hepatic microsomal incubations indicates that less than 5% of the hepatic cytochrome P-450 is P-450$_{II}$. Although the accuracy of these calculations cannot be determined at this time, it is reasonable to conclude that the markedly greater ability of pulmonary as compared with hepatic microsomal preparations to metabolize 2AA and 2AF to mutagenic products is a function of the relative proportions of the highly active cytochrome P-450$_{II}$ in the two tissues.

The potential difference between the abilities of lung and liver to produce high intracellular concentrations of mutagenic products, particularly from 2AA and 2AF, is probably much greater than is indicated by the results obtained with microsomal preparations. Pulmonary cytochrome P-450 in the rabbit is not evenly distributed throughout the lung, and appears to be highly localized in certain cell types, particularly the nonciliated bronchiolar epithelial (Clara) cell (17, 18). This is the case for P-450$_{I}$ and P-450$_{II}$, both of which have also been identified in the alveolar type II cell but appear to be absent from the alveolar macrophage (17). In contrast, the distribution of cytochromes P-450$_{I}$ and P-450$_{II}$ in the rabbit liver appears to be much more diffuse (17, 18).

The reasons for the pronounced differences in the activation of 2AAF and 2AF in both purified and microsomal systems are difficult to assess. The active product from 2AAF in the purified system is likely *N*-hydroxy-AAF, a weak mutagen compared with *N*-hydroxy-AF (28, 29), and the activities of 2AF and 2AAF with P-450$_{II}$ appear to reflect this difference. However, with P-450LM$_4$, greater activity was obtained with 2AAF than with 2AF. Either 2AAF is a much better substrate for P-450LM$_4$ than is 2AF or *N*-hydroxy-AAF is further metabolized to a more potent mutagen in this system. It is unlikely that *N*-hydroxy-AF is formed from *N*-hydroxy-AAF in this system, as no deacetylase activity can be detected in the P-450LM$_4$ or reductase preparations. However, the mutagenicity of *N*-hydroxy-AAF can be enhanced by horseradish peroxidase plus hydrogen peroxide,[7] possibly via a nitroxyl free radical, and a similar mechanism for the P-450LM$_4$ system cannot be excluded. In microsomal systems, 2AAF or *N*-hydroxy-AAF can be deacetylated to 2AF or *N*-hydroxy-AF, respectively (28, 29). Some of the differences observed in the microsomal systems may be a function of the deacetylase. Preliminary data indicate that the level of this enzyme in lung is substantially lower than in liver, but whether or not it is rate-limiting is still being investigated.

The activation of the aromatic amines can be contrasted with that of AFB$_1$, which is activated by P-450$_{I}$ in both purified and microsomal systems. The role of P-450$_{II}$ in this reaction is not certain; anti-P-450$_{II}$ inhibited the pulmonary microsomal activity by about 50%, but purified P-450$_{II}$ was inactive with AFB$_1$. One possible explanation for this is a P-450$_{II}$-dependent pathway for the activation of AFB$_1$ that requires the participation of an enzyme (e.g., cytochrome $b_5$) not included in the purified system. The activation of AFB$_1$ in hepatic and pulmonary microsomal systems (per nanomole of P-450) was similar and no significant inhibition of AFB$_1$ activation in hepatic microsomal systems was obtained with antibody to either P-450$_{I}$ or P-450$_{II}$. The lack of inhibition in the hepatic microsomal system is not unreasonable because, as the pulmonary and hepatic preparations are equally efficient (per nanomole of cytochrome P-450) in

[6] R. M. Philpot, C. R. Wolf, B. R. Smith, J. R. Bend, S. N. Sthatham, and M. R. Boyd, unpublished observations.

[7] P. J. Wirth and S. S. Thorgeirsson, personal communication.

the activation of $AFB_1$, the contribution of $P\text{-}450_I$ and $P\text{-}450_{II}$ to the hepatic activation of $AFB_1$ should reflect their low concentrations relative to the total hepatic cytochrome P-450 content.

In conclusion, we have identified a cytochrome P-450 isozyme, $P\text{-}450_{II}$, in rabbits that is highly active in the metabolism of aromatic amines to mutagenic products as determined by the mutagenesis assay devised by Ames *et al.* (19). At least 90% of the metabolism of these compounds to mutagenic products in pulmonary microsomal preparations, and approximately 50–70% in hepatic preparations, is catalyzed by this isozyme even though it accounts for only a small percentage of the total hepatic cytochrome.

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