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# Hydroxylation of *p*-Nitrophenol by Rabbit Ethanol-Inducible Cytochrome P-450 Isozyme 3a

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**SUMMARY**

The hydroxylation of *p*-nitrophenol to 4-nitrocatechol was investigated using rabbit hepatic microsomes and six purified isozymes of cytochrome P-450. The microsomal activity was maximal at pH 6.8 and at 100 μM *p*-nitrophenol. At higher substrate concentrations inhibition was observed. At pH 6.8 and 100 μM *p*-nitrophenol, isozyme 3a exhibited the highest activity of the purified isozymes: 3.4-fold more active than isozyme 6, and 8-fold more active than isozymes 2 and 4. The isozyme 3a-catalyzed hydroxylation reaction was stimulated 2.4-fold by the addition of a 4:1 ratio of cytochrome $b_5$/P-450. At optimal concentrations of cytochrome $b_5$, isozyme 3a was 8- to 9-fold more active than isozymes 2 and 6 and 20-fold more active than isozyme 4. Under the same conditions, isozyme 3a-catalyzed butanol oxidation was inhibited 40%. Antibodies to isozyme 3a inhibited greater than 95% of the *p*-nitrophenol hydroxylase activity of microsomes from untreated or from ethanol- or acetone-treated rabbits. The microsomal hydroxylase activity was linearly correlated with the microsomal concentration of isozyme 3a (correlation coefficient of 0.94) and had an intercept near zero. The results from reconstitution, antibody inhibition, and correlation experiments indicate that isozyme 3a is the principal catalyst of rabbit microsomal *p*-nitrophenol hydroxylation. The ability of the ethanol-inducible isozyme to catalyze catechol formation may be important in the ethanol-enhanced toxicity of aromatic compounds such as benzene.

Hepatic microsomes catalyze the metabolism of a myriad of endogenous and exogenous compounds (1, 2). The metabolic profile of different microsomal preparations is due, in part, to the relative concentrations of distinct isozymes of cytochrome P-450. Various factors influence the level of individual isozymes, including age, sex, nutritional state, and exposure to xenobiotics (3, 4). Although cytochrome P-450-dependent metabolism can be identified, the assignment of the roles of specific isozymes in microsomal catalysis is a more formidable task. The development of specific antibodies that inhibit catalytic activity provides an extremely powerful tool for such investigations (4).

Chronic ethanol treatment increases the metabolism of many compounds, including halogenated alkanes and ethers (5, 6), primary and secondary alcohols (7, 8), cyclic (9) and acyclic nitrosamines (10), and benzene (11) and substituted benzenes (12). It has been suggested that increases in metabolism after ethanol treatment are the result of the induction of an ethanol-inducible isozyme of cytochrome P-450. Antibodies to rabbit isozyme 3a, the ethanol-inducible isozyme (13), were used to define the role of this isozyme in microsomal incubations (14, 15). The activity of isozyme 3a accounted for the increased microsomal activity toward alcohols and aniline after ethanol treatment (15). Although the enzyme represented only about 5% of the total cytochrome P-450 in microsomes from untreated rabbits (14), it catalyzed about 30% of the alcohol oxidation and aniline hydroxylation (15). Rabbit microsomal acetone hydroxylation and *N*-nitrosodimethylamine demethylation (at low *N*-nitrosodimethylamine concentrations) were inhibited 90–97% by anti-3a antibody, an indication that isozyme 3a is the principal, if not the sole, catalyst for these two substrates (16, 17).

Recently, Reinke and colleagues (18, 19) reported that, in rats, the hydroxylation of *p*-nitrophenol to 4-nitrocatechol is extensively induced by ethanol when measured in microsomes or perfused livers. In the present report, purified isozyme 3a and antibodies to the enzyme were used to demonstrate that isozyme 3a is the predominant catalyst of *p*-nitrophenol hydroxylation in rabbit hepatic microsomes. Therefore, the hydroxylation reaction is relatively specific for monitoring the levels of isozyme 3a. The results suggest that the ethanol-inducible isozyme may have a role in the formation of potentially toxic catechols (20).

## Materials and Methods

**Preparation of microsomes and purification of enzymes.** New Zealand White male rabbits (2.0–2.5 kg) were either untreated; given 10% (v/v) ethanol for 14 days, 1% (v/v) acetone for 7 days, or 0.062% (w/v) isoniazid for 10 days in place of the drinking water; or given one of the following intraperitoneally and then killed 24 hr after the treatment: trichloroethylene (11 mmol/kg) by a single injection, pyrazole (200 mg/kg) by a single injection. All animals were fasted for 12–14 hr before death. Hepatic microsomes were prepared immediately

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after the animals were killed, washed with pyrophosphate buffer, and stored at −70° until used (15). The isozymes of cytochrome P-450 were purified by published procedures (13, 21, 22) and had specific contents (nmol of P-450/mg of protein) as follows: isozyme 2, 18.0; isozyme 3a, 16–20.7; isozyme 3b, 18.9; isozyme 3c, 15.0; isozyme 4, 19.2; and isozyme 6, 16.2. Rabbit cytochrome $b_5$, prepared according to the method of Strittmatter *et al.* (23), had a specific content of 59 nmol of cytochrome $b_5$/mg of protein. NADPH-cytochrome P-450 reductase, purified according to the method of French and Coon (24), catalyzed the reduction of 45–60 μmol of cytochrome *c*/min/mg of protein. Protein was determined by the method of Lowry *et al.* (25), cytochrome $b_5$ by the method of Raw and Mahler (26), and P-450 as previously described (21). The preparation and characterization of antibody to isozyme 3a have been described (14, 15). The concentration of isozyme 3a in microsomal preparations was determined by immunoblots (14).

**Enzyme assays.** The hydroxylation of *p*-nitrophenol to 4-nitrocatechol (1,2-dihydroxy-4-nitrobenzene) was determined as described by Reinke and colleagues (18, 19). Reaction mixtures (1.0 ml) were stopped by the addition of 0.5 ml of 0.6 N perchloric acid and centrifuged. The 4-nitrocatechol formed was determined spectrally in 1.0 ml of the supernatant at 546 nm after the addition of 0.10 ml of 10 N NaOH. An extinction coefficient of 9.53 $mM^{-1}$ $cm^{-1}$ was determined for 4-nitrocatechol for the conditions used. The absorption spectra of the reaction product were identical to those of standard 4-nitrocatechol. The identity of the product was further confirmed by thin layer chromatography. A reaction mixture was acidified and extracted with ethyl acetate after the addition of 0.1 M ascorbic acid. The extract was dried under nitrogen, redissolved in 10 μl of methanol, and applied to a silica gel G thin layer plate. The plate was developed with benzene/acetic acid (5:1) or benzene/methanol/acetic acid (45:8:4) giving $R_f$ values for 4-nitrocatechol of 0.24 and 0.54, respectively. The product of the reaction mixture had an $R_f$ identical to that of authenic 4-nitrocatechol in both solvent systems.

Butyraldehyde was determined by gas chromatography as described previously (15). The components of the individual reaction mixtures are given in the legends of the figures and tables. For reactions in which cytochrome $b_5$ was included, the cytochrome $b_5$ was added to the reaction mixture before the addition of the reconstituted P-450 system as described by Gorsky and Coon (27). Rates are the average of duplicate or triplicate incubations corrected for zero time blanks which were quenched prior to the addition of NADPH. All reaction mixtures were run at 30°after a 3-min preincubation at the same temperature for times that represented the initial rate of product formation. Each experiment was repeated at least once and the results presented are representative data of the experiment. The results of replicate experiments varied by less than 10%.

**Materials.** Desferrioxamine was kindly provided by Ciba-Geigy. *p*-Nitrophenol was obtained from Sigma Chemical Co., an 4-nitrocatechol was obtained from Aldrich Chemical Co. The source of other materials has been described (13–15, 21).

## Results

Fig. 1 shows the time course for the microsomal-dependent hydroxylation of *p*-nitrophenol to 4-nitrocatechol. The reaction was linear for 6 min with 0.1 mg of protein and decreased to 2 min or less with increasing concentrations of microsomal protein. In experiments not shown, it was found that the addition of 1 mM ascorbic acid increased the linearity of the reaction without affecting the initial rate of product formation or any of the following characteristics. The formation of product was linear with microsomal protein to 0.5 mg and exhibited a pH optimum of 6.8. Maximal rates of hydroxylation were obtained at a substrate concentration of 100 μM. At higher substrate concentrations, inhibition was observed. This effect was not dependent on the pH or the protein concentration. The inhibition was not the result of product inhibition since identical initial rates were obtained in the presence or absence of 11 nmol of 4-nitrocatechol, the maximal amount of product formed in initial rate experiments (results not shown).

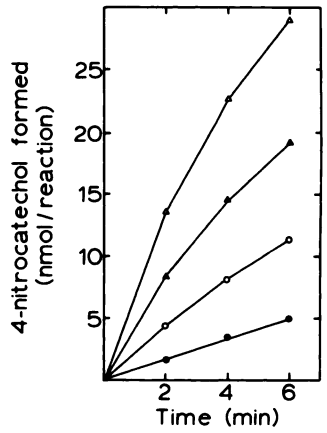


**Fig. 1.** Hydroxylation of *p*-nitrophenol by rabbit hepatic microsomes. The reaction mixtures contained 100 mM potassium phosphate buffer, pH 6.8, 100 μM *p*-nitrophenol, hepatic microsomes from acetone-treated rabbits at various concentrations, and 1 mM NADPH in a final volume of 1.0 ml. The reactions were initiated with NADPH after preincubation for 3 min at 30° and were terminated with 0.5 ml of 0.6 N perchloric acid after various incubation times at 30°. The amount of 4-nitrocatechol was determined as described in Materials and Methods. The microsomal protein concentrations were 0.1 mg (●), 0.25 mg (○), 0.50 mg (▲), and 1.0 mg (△).

Microsomes prepared from ethanol- or acetone-treated rabbits showed a significant increase in the NADPH-dependent hydroxylation of *p*-nitrophenol. Microsomes from untreated rabbits catalyzed the formation of 1.02 ± 0.12 nmol of 4-nitrocatechol/min/mg of protein ($n$ = 3). Ethanol treatment increased the rate about 4-fold to 3.98 ± 0.20 nmol of 4-nitrocatechol/min/mg of protein ($n$ = 3), whereas acetone treatment resulted in about a 7-fold increase, 7.13 ± 0.32 nmol of 4-nitrocatechol/min/mg of protein ($n$ = 3). Both compounds induce isozyme 3a in rabbit hepatic microsomes (14).

The inducibility of *p*-nitrophenol hydroxylation by ethanol and acetone suggested that the reaction was catalyzed by isozyme 3a. As shown in Fig. 2, antibody to the purified isozyme inhibited the formation of 4-nitrocatechol by microsomes from acetone-treated rabbits. At optimal concentrations of the antibody, the reaction was inhibited about 95%. Similar inhibition was observed when microsomes from untreated or ethanol-treated rabbits were used in the incubation mixtures, and when the incubations were run at pH 7.6.

Initial experiments with a reconstituted system of purified isozyme 3a, dilauroylglyceryl-3-phosphorylcholine, and NADPH-cytochrome P-450 reductase at pH 7.6 gave a turnover number for 4-nitrocatechol formation that was lower than that obtained with hepatic microsomes (Table 1). The low turnover number for purified isozyme 3a suggested that component(s) present in the microsomes were absent in the reconstituted system. Morgan *et al* (28) reported that cytochrome $b_5$ inhibited isozyme 3a-catalyzed ethanol oxidation. However, as shown in Table 1, the addition of cytochrome $b_5$ stimulated the isozyme 3a-catalyzed hydroxylation of *p*-nitrophenol. At pH 7.6, the rate was stimulated 4.5-fold and 5.6-fold by cytochrome $b_5$ at of cytochrome $b_5$/isozyme 3a molar ratios of 2:1 and 4:1, respectively. Maximal stimulation of the hydroxylase activity by cytochrome $b_5$ was observed at $b_5$/isozyme 3a ratios between

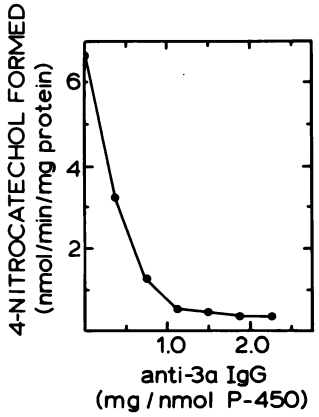


**Fig. 2.** Inhibition of microsomal *p*-nitrophenol hydroxylation by anti-3a IgG. The reaction mixtures contained 100 mM potassium phosphate buffer, pH 6.8, 100 μM *p*-nitrophenol, 0.50 mg of microsomal protein from acetone-treated rabbits, various concentrations of anti-3a IgG, and preimmune sheep IgG so that the total IgG concentration was constant at 3.0 mg and 1 mM NADPH in a final volume of 1.0 ml. The reactions were preincubated for 3 min at 30° and were initiated by the addition of NADPH. After 2 min, the reactions were quenched with 0.5 ml of 0.6 N perchloric acid and 4-nitrocatechol was determined as described in Materials and Methods.

TABLE 1

**Effect of pH and cytochrome $b_5$ on isozyme 3a-catalyzed *p*-nitrophenol hydroxylation**

Reaction mixtures contained 100 mM potassium phosphate buffer at the indicated pH, 100 μM *p*-nitrophenol, 1.0 mM ascorbic acid, 0.5 mg of hepatic microsomes from acetone-treated rabbits, or a reconstituted enzyme system composed of 0.1 μM P-450 isozyme 3a, 0.3 μM NADPH-cytochrome P-450 reductase, and 30 μg/ml of dilauroylglyceryl-3-phosphorylcholine, 0.2 or 0.4 μM cytochrome $b_5$ as indicated, and 1 mM NADPH in a final volume of 1.0 ml. After a 3-min preincubation at 30°, the reactions were initiated with NADPH and terminated with 0.5 ml of 0.6 N prechloric acid after a 3-min (microsomal system) or 10-min (reconstituted system) incubation. The 4-nitrocatechol was determined as described in Materials and Methods.

| Enzyme system | *p*-Nitrophenol hydroxylase activity | | |
|---|---|---|---|
| | pH 6.8 | pH 7.2 | pH 7.6 |
| | *nmol of 4-nitrocatechol/min/nmol of P-450* | | |
| Microsomes | 2.50 | 2.25 | 1.90 |
| Reconstituted system | 4.02 | 2.02 | 1.00 |
| Reconstituted system + 0.2 μM cytochrome $b_5$ | 9.11 | 6.94 | 4.57 |
| Reconstituted system + 0.4 μM cytochrome $b_5$ | 9.78 | 7.68 | 5.63 |

4:1 and 5:1 and was independent of the P-450-reductase/isozyme 3a ratio, which was varied from 1:1 to 3:1. The magnitude of the cytochrome $b_5$ effect was dependent on the pH. The stimulation by cytochrome $b_5$ decreased from 5.6-fold at pH 7.6 to 2.4-fold at pH 6.8, the optimal pH for the microsomal reaction. In parallel incubations, cytochrome $b_5$ inhibited isozyme 3a-catalyzed butanol oxidation by 20% at pH 7.6 and 40% at pH 6.8 (data not shown).

In the presence and absence of cytochrome $b_5$, the *p*-nitrophenol hydroxylase activity increased as the pH of the reaction mixtures was decreased. Since *p*-nitrophenol was a $pK_a$ of 7.15, the effect of pH could result from a change in the concentration of the protonated form of the substrate. As shown in Fig. 3, the reconstituted system was inhibited by concentrations of *p*-nitrophenol greater than 100 μM at pH 6.8, 7.2, and 7.6. At 500 μM *p*-nitrophenol, the rates were about 30% lower than those obtained at the optimal concentration of 100 μM (data not shown). The double reciprocal plots for the saturation curves

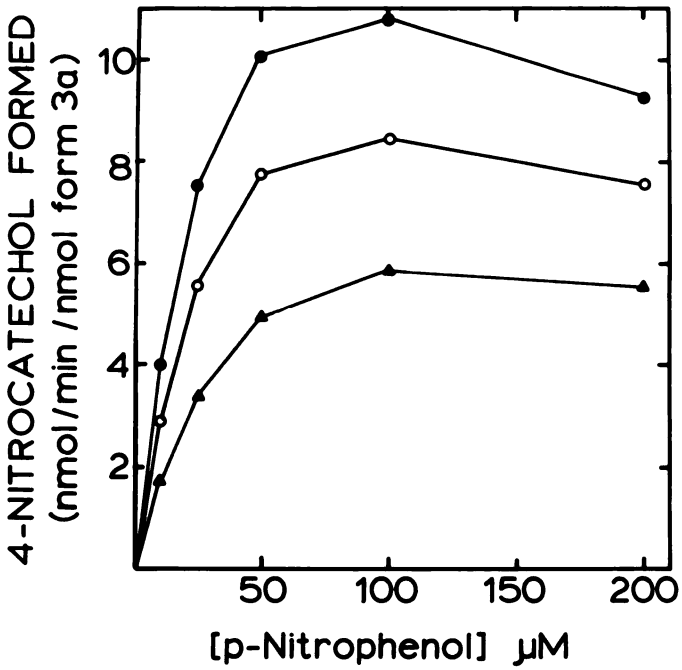


**Fig. 3.** Effect of *p*-nitrophenol concentration on isozyme 3a-catalyzed formation of 4-nitrocatechol. The reaction mixtures contained 100 mM potassium phosphate buffer at the indicated pH, various concentrations of *p*-nitrophenol, a reconstituted system as described in Table 1, 1 mM ascorbic acid, 0.4 μM cytochrome $b_5$, and 1 mM NADPH. Reactions were terminated by the addition of 0.5 ml of 0.6 N perchloric acid after a 10-min incubation at 30°. The 4-nitrocatechol formed was determined as described in Materials and Methods. The pH values of the reaction mixtures were 6.8 (●), 7.2 (○), or 7.6 (▲).

in Fig. 3 were not linear. As a result, it was not possible to accurately evaluate the effect of pH on the apparent $K_m$ for *p*-nitrophenol; the pH did not significantly change the apparent $K_m$ when the parameter was determined by extrapolation of a straight line asymptote estimated from the lowest substrate concentrations. To further evaluate the effect of substrate ionization, the pH profile for *p*-nitrophenol hydroxylation was compared to the pH profile for *n*-butanol oxidation, a substrate that is not ionized over the pH range used. As shown in Fig. 4, the pH profiles for both substrates are nearly identical. The butanol oxidation activity at pH 6.8 was not inhibited by either catalase or desferrioxamine. Thus, the change of pH affects the enzyme-catalyzed reaction, but not by generating hydroxyl radicals from $H_2O_2$ and a reduced iron-chelate (29). In addition, increasing the ionic strength at pH 6.8 by the addition of up to 200 mM KCl had no significant effect on the activity of isozyme 3a. The similarity of the pH profiles for the two substrates suggests that the protonation of the enzyme may be responsible for the increase in activity observed at lower pH values.

Stimulation of the reconstituted system by cytochrome $b_5$ suggested that if cytochrome $b_5$ is participating in the microsomal reaction, then the induction of microsomal *p*-nitrophenol hydroxylase activity could result from changes in the cytochrome $b_5$ concentration and not from changes in the isozyme 3a concentration. To test this hypothesis, hepatic microsomes were prepared from untreated rabbits or from rabbits treated with ethanol, acetone, pyrazole, or trichloroethylene, inducers of isozyme 3a (14). The concentrations of cytochrome $b_5$ and isozyme 3a and the *p*-nitrophenol hydroxylase activity were determined for each microsomal preparation. Fig. 5 shows that

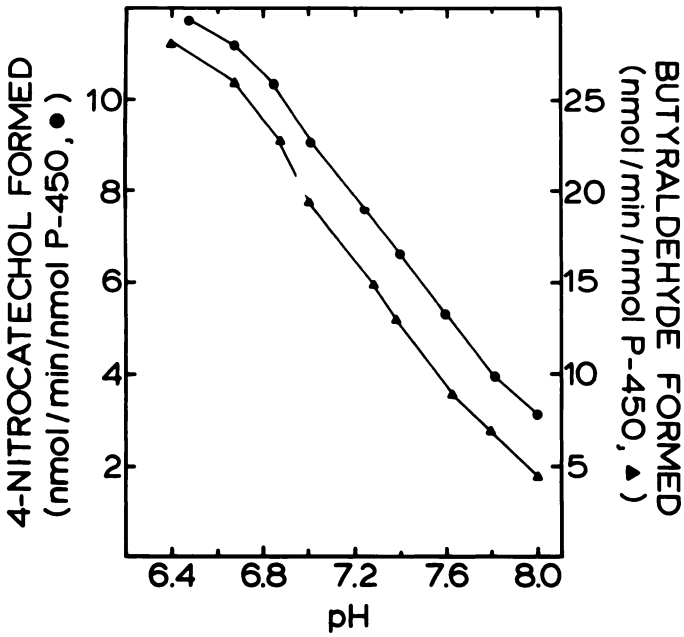


**Fig. 4.** Effect of pH on isozyme 3a-catalyzed butanol oxidation and *p*-nitrophenol hydroxylation. Reaction mixtures contained 100 mM potassium phosphate buffer at the indicated pH, 100 μM *p*-nitrophenol or 30 mM *n*-butanol, a reconstituted enzyme system as described in Table 1, 0.4 μM cytochrome $b_5$, and 1 mM ascorbic acid for *p*-nitrophenol hydroxylation, and 1 mM NADPH in a final volume of 1.0 ml. Reactions were terminated after a 10-min incubation at 30° and products were measured as described in Materials and Methods.

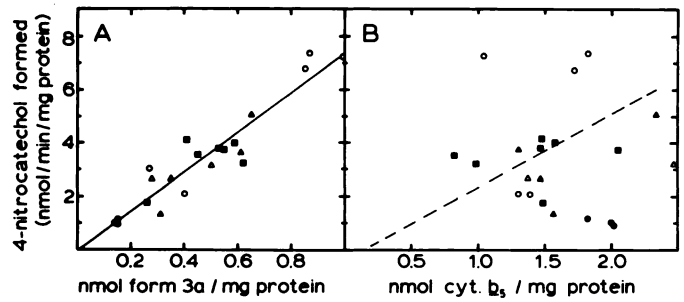


**Fig. 5.** Correlation analysis between the microsomal concentration of isozyme 3a (A) or cytochrome $b_5$ (B) and microsomal *p*-nitrophenol hydroxylase activity. The *p*-nitrophenol hydroxylase activity for each microsomal preparation was determined in reaction mixtures of 1.0 ml containing 0.5 mg of microsomal protein, 100 mM potassium phosphate, pH 6.8, 100 μM *p*-nitrophenol, and 1.0 mM NADPH. The reactions were terminated after a 2-min incubation at 30°. The concentration of isozyme 3a was determined immunochemically by immunoblots (14) and the concentration of cytochrome $b_5$ was measured spectrally (26). Microsomes from untreated (●) or from acetone- (○), pyrazole- (▲), trichloroethylene- (△), or ethanol-treated (■) rabbits were used. The lines of best fit were determined by linear regression analysis. In A, the correlation coefficient was 0.94 with a slope of 7.35 nmol of 4-nitrocatechol/min/nmol of isozyme 3a and an intercept of −0.024 nmol of 4-nitrocatechol/min/mg of protein. In B, the correlation coefficient was 0.09 with a slope of 4.09 nmol of 4-nitrocatechol/min/nmol of cytochrome $b_5$, and an intercept of −0.408 nmol of 4-nitrocatechol/min/mg of protein.

there was a good linear correlation between the concentration of isozyme 3a and the *p*-nitrophenol hydroxylase activity. The correlation coefficient obtained was 0.94, with a slope of 7.35 nmol of 4-nitrocatechol formed/min/nmol of isozyme 3a and an intercept of −0.02 nmol of 4-nitrocatechol formed/min/mg of protein. In contrast, there was no significant correlation between the hydroxylase activity and the concentration of cytochrome $b_5$ (correlation coefficient = 0.09). There was also no correlation between the hydroxylase activity and the ratio of cytochrome $b_5$ to isozyme 3a (results not shown).

The nearly complete inhibition of the microsomal *p*-nitrophenol hydroxylase activity by anti-3a IgG and the linear correlation between the concentration of isozyme 3a and the hydroxylase activity with a near-zero intercept suggested that isozyme 3a was the predominant catalyst in microsomal suspensions. Table 2 summarizes the hydroxylase activity of six purified rabbit isozymes in the presence and absence of cytochrome $b_5$. Isozyme 3a was the most active of the six isozymes examined in the presence and absence of cytochrome $b_5$. Forms 2, 4, and 6 also catalyzed the hydroxylation reaction, but the rates were 87% (forms 2 and 4) and 70% (form 6) lower than the form 3a rate. Forms 4 and 6 were unaffected by the addition of cytochrome $b_5$, whereas form 2 was stimulated 2.3-fold. In the presence of cytochrome $b_5$, the rates of product formation for forms 2, 4, and 6 were 89%, 95%, and 87% lower, respectively, than the form 3a rate.

## Discussion

In 1975, Chrastil and Wilson (30) reported that rat hepatic microsomes catalyzed the NADPH-dependent hydroxylation of *p*-nitrophenol to 4-nitrocatechol. Reinke and co-workers (18, 19) confirmed the microsomal activity and demonstrated that ethanol treatment of rats induces *p*-nitrophenol hydroxylation 4- to 6-fold in microsomal incubations as well as in perfused liver. Similarly, rabbit hepatic microsomes efficiently catalyze the hydroxylation of *p*-nitrophenol to 4-nitrocatechol. Ethanol treatment induced the microsomal activity about 4-fold, and other compounds that induce isozyme 3a in rabbit were effective inducers of *p*-nitrophenol hydroxylase activity.

The present study has clearly established that rabbit hepatic isozyme 3a is the principal rabbit *p*-nitrophenol hydroxylase. The results of reconstitution experiments demonstrate that isozyme 3a has the highest activity of six purified rabbit isozymes. In the presence of cytochrome $b_5$, isozyme 3a is about 3-fold more active than isozyme 6, and about 10- and 20-fold more active than isozymes 2 and 4, respectively. Stimulation of the hydroxylase activity by cytochrome $b_5$ was unexpected since the hemeprotein inhibited the isozyme 3a-dependent oxidation of ethanol (28) and butanol. The stimulation of activity by cytochrome $b_5$ is not well understood although mechanisms have been proposed from studies using purified rat and rabbit isozymes with a variety of substrates (Refs. 27 and 31, and references therein). No attempt was made in the present study

TABLE 2

**p-Nitrophenol hydroxylase activity of purified isozymes of rabbit hepatic cytochrome P-450**

Reaction mixtures contained 100 mM potassium phosphate buffer, pH 6.8, 100 μM *p*-nitrophenol, 1.0 mM ascorbic acid, a reconstituted enzyme system containing 0.1 μM cytochrome P-450 as described in the legend to Table 1, 0.4 μM cytochrome $b_5$ when indicated, and 1 mM NADPH. Reactions were initiated with NADPH and quenched with 0.5 ml of 0.6 N perchloric acid after a 10-min incubation at 30°. The 4-nitrocatechol formed was assayed as described in Materials and Methods.

| Addition | Activity of isozyme | | | | | |
|---|---|---|---|---|---|---|
| | 2 | 3a | 3b | 3c | 4 | 6 |
| | *nmol of 4-nitrocatechol/min/nmol of P-450* | | | | | |
| None | 0.46 | 3.68 | <0.40[a] | <0.40[a] | 0.46 | 1.08 |
| 0.4 μM Cytochrome $b_5$ | 1.06 | 9.58 | <0.40[a] | <0.40[a] | 0.46 | 1.23 |

[a] Value represents the limit of detection in the assay used.

to determine the mechanism by which cytochrome $b_5$ stimulated isozyme 3a. The results represent yet another example in which the effect of cytochrome $b_5$ is dependent on the substrate.

Although the effect of cytochrome $b_5$ in the reconstituted system suggested that the induction of cytochrome $b_5$ could be responsible for an increase in microsomal *p*-nitrophenol hydroxylation, the lack of any correlation between the concentration of cytochrome $b_5$ and microsomal hydroxylase activity is not consistent with that hypothesis. The lack of a correlation could suggest that cytochrome $b_5$ does not participate in the microsomal reaction or that the measurement of total microsomal cytochrome $b_5$ is not an accurate reflection of the cytochrome $b_5$ that might interact with isozyme 3a. Additional experiments will be required to determine what role, if any, cytochrome $b_5$ has in the microsomal reaction. In contrast, an excellent correlation is obtained between the concentration of isozyme 3a and the hydroxylase activity. The slope of the line of best fit, 7.35 nmol of 4-nitrocatechol formed per min per nmol of 3a, is only 25% lower than the rate obtained for the purified enzyme in the presence of optimal concentrations of NADPH-cytochrome P-450 reductase and cytochrome $b_5$. The small difference in the two rates could be due to limiting concentrations of the reductase as proposed for isozyme 3a-catalyzed *n*-butanol oxidation (14). In addition, if cytochrome $b_5$ participates in the microsomal reaction as suggested from the reconstitution experiments, then the microsomal rate could also be limited by the extent of the interaction of cytochrome $b_5$ with isozyme 3a. Johnson and co-workers (32, 33) reported that similar correlation analyses of the rabbit isozyme 1-catalyzed 21-hydroxylation of progesterone and the 2-hydroxylation of 17$\beta$-estradiol gave turnover numbers similar to those obtained with a reconstituted system. The near-zero intercept of the regression line in Fig. 5 is consistent with the low or negligible activity of purified isozymes 2, 3b, 3c, 4, and 6. The inhibition by anti-3a IgG of greater than 95% of the hydroxylase activity of all the rabbit microsomal preparations examined affirms the role of isozyme 3a as the *p*-nitrophenol hydroxylase.

Reinke and Moyer (19) proposed that *p*-nitrophenol hydroxylation could be used to monitor the induction of ethanol-inducible P-450. Antibodies to isozyme 3a were used to demonstrate that the ethanol-inducible isozyme is the low $K_m$ *N*-nitrosodimethylamine demethylase (17) and the acetone hydroxylase (16) in rabbit microsomes. In addition, the antibodies to the rabbit enzyme exhibited species cross-reactivity and inhibited the *N*-nitrosodimethylamine demethylase activity in microsomes from untreated and ethanol-treated rats (17) and the acetone hydroxylase activity of microsomes from acetone-treated rats (16). These results provided the first direct evidence that an ethanol-inducible isozyme is responsible for these two activities in rat microsomes; support for this in previous reports was indirect, being based solely on the inducibility of the activities by ethanol treatment (5–12). The results of immunoblots of rat microsomes and the direct comparison of isozyme 3a and P-450$_j$ (34), which was purified to homogeneity from ethanol-treated rats, indicate that P-450$_j$ is induced by ethanol and is the 3a-homologue in the rat (35). In preliminary experiments anti-3a IgG inhibited 90% of the *p*-nitrophenol hydroxylase activity of microsomes from ethanol- or acetone-treated rats. Thus, in both the rat and the rabbit, *p*-nitrophenol hydroxylation provides an excellent indicator of the induction of the ethanol-inducible isozyme.

The toxicological significance of isozyme 3a-catalyzed catechol formation remains to be determined as the specificity of this reaction is extended to other phenols. Catechols are readily oxidized to semiquinones and quinones that are, in general, sufficiently electrophilic to be toxic metabolites (20). Phenols are not readily metabolized to catechols since they undergo extensive conjugation reactions. However, ethanol treatment results in an inhibition of glucuronide conjugation by decreasing the availability of UDP-glucuronic acid (36) while inducing isozyme 3a. Under these conditions, it is possible that phenols are more extensively metabolized to catechols. For example, ethanol treatment enhances both benzene metabolism (11, 12) and toxicity (37), whereas other inducers, such as phenobarbital and 3-methylcholanthrene, only enhance metabolism (38). A concomitant decrease in conjugation of phenol and an increase in catechol formation catalyzed by isozyme 3a could explain these observations. Billings (39) demonstrated that intact hepatocytes from phenobarbital-treated rats produce catechol from benzene via the dihydrodiol rather than pehnol. It would be of interest to determine whether the mechanism of catechol formation shifts from a dihydrodiol pathway to a phenol pathway after ethanol treatment.

**Acknowledgments**

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