0026-895X/86/040370-09$02.00/0

MOLECULAR PHARMACOLOGY, 30:370–378

Immunochemical Identification of Cytochrome P-450 Isozyme 3a (P-450$_{ALC}$) in Rabbit Nasal and Kidney Microsomes and Evidence for Differential Induction by Alcohol

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Received May 12, 1986; Accepted July 14, 1986

SUMMARY

Polyclonal and monoclonal antibodies to rabbit liver microsomal alcohol-inducible cytochrome P-450 isozyme 3a (P-450$_{ALC}$) were used to examine the tissue distribution of the cytochrome. Isozyme 3a or an immunochemically indistinguishable variant of this protein was detected on immunoblots of kidney and nasal mucosa microsomes, but not of microsomes prepared from brain, lung, adrenal, heart, intestine, ovary, spleen, testis, or uterus from untreated or ethanol-treated rabbits. The presence of isozyme 3a was also indicated by inhibition by anti-3a IgG of microsomal aniline hydroxylation and butanol oxidation. The identity of isozyme 3a was further substantiated by peptide-mapping analysis of the immunoaffinity-purified proteins. The amount of isozyme 3a was increased in kidney, but not in nasal microsomes, by chronic ethanol treatment. The induction of isozyme 3a in the kidney was reflected in a more than 2-fold increase in the total rates and a 7-fold increase in the isozyme 3a-dependent rates of aniline and butanol metabolism. Based on immunoblot quantitation, the specific content of isozyme 3a is about 10 pmol/mg of protein in kidney and 80 pmol/mg of protein in nasal microsomes of untreated rabbits. After ethanol treatment of the animals, the content increases to 50 pmol/mg of protein in kidney but is unchanged in nasal microsomes. The presence of isozyme 3a may play a significant role in the toxicity of foreign compounds.

The P-450-dependent monooxygenase system has been detected in many tissues, including skin, kidney, intestine, gonads, adrenals, placenta, lung, brain, nasal mucosa, and bladder (1–4), and the involvement of extrahepatic P-450 in the bioactivation of acetaminophen (5), phenacetin (6), hexamethylphosphoramide (3), *N'*-nitrosonornicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (7), and *N*-nitrosodiethylamine (8) has been proposed. The content of the cytochrome in these tissues is generally much lower than in liver. Extrahepatic tissues may possess forms of P-450 distinct from those found in liver, or may have the same or similar forms in different proportions. A knowledge of the distribution and quantity of a particular P-450 isozyme in extrahepatic tissues is required, therefore, to understand the tissue-specific metabolism and toxicity of exogenous chemicals. For example, rabbit liver microsomal P-450 form 5 is highly specific for the metabolism of several aromatic amines (9). Form 5 is also found in rabbit bladder mucosa (4), where it amounts to about 20% of the total P-450 and is responsible for the higher rate of 2-aminofluorene metabolism found in this location than in hepatic microsomes.

This research was supported by Grant AA-06221 from the National Institute on Alcohol Abuse and Alcoholism.

A unique form of P-450, designated P-450$_{ALC}$ or isozyme 3a, was isolated from liver microsomes of ethanol-treated rabbits and characterized in this laboratory several years ago (10, 11). The purified cytochrome exhibits the highest activity of six liver P-450 isozymes toward ethanol and other alcohols as well as toward aniline (11), oxidizes acetaminophen to a reactive intermediate (12), functions as the low K_M *N*-nitrosodimethylamine demethylase (13), and catalyzes the hydroxylation of *N*-nitroso-2,6-dimethylmorpholine (14). These findings suggest that this enzyme could play a particularly important role in the metabolic activation of foreign compounds in extrahepatic tissues.

In the present study, the tissue distribution of P-450 form 3a was investigated. The criteria used for its identification in a given tissue were cross-reactivity with both monoclonal and polyclonal antibodies prepared against purified hepatic isozyme 3a and inhibition of microsomal aniline and butanol oxidation activities by the polyclonal antibody. These results provide evidence for an identical or highly similar cross-reacting protein and also establish the presence of the catalytically competent enzyme. Our findings show that P-450 form 3a is present in microsomes prepared from kidney and nasal mucosa, but not from brain, lung, adrenal, heart, intestine, ovary, spleen, testis, and uterus, from both untreated and ethanol-treated rabbits.

ABBREVIATIONS: P-450, cytochrome P-450; SDS, sodium dodecyl sulfate; ELISA, enzyme-linked immunosorbent assay; TBS, 10 mM Tris-chloride buffer, pH 7.4, containing 0.9% NaCl.

Materials and Methods

Preparation of microsomes. New Zealand White rabbits (2.0–2.5 kg in weight) were untreated or given 10% (v/v) ethanol in the drinking water for 14 days. Males were used as a source of all tissues except for uterus and ovary. All animals were fasted for 12–14 hr before they were killed, and tissues were removed within 5 min and rinsed with ice-cold 0.1 M Tris-acetate buffer, pH 7.4, containing 150 mM KCl and 1 mM EDTA. All subsequent steps were carried out at 0–4°. Tissues from four to six rabbits were pooled, and pyrophosphate-washed microsomes were prepared as previously described (15) except that all extrahepatic tissues were disrupted with a Potter-Elvehjem-type homogenizer. The whole brain was used as a source of microsomes, and the nasal mucosa used was obtained from both the ethmoturbinate and the septum.

Preparation of monoclonal antibodies. Four female BALB/c mice, 4 weeks in age, were immunized with purified P-450 isozyme 3a by the procedure described by Rothwell *et al.* (16). Spleen cells of immunized mice were fused with the nonsecreting myeloma X63-Ag8.653 as described by Kearney *et al.* (17). Parental culture supernatant fractions were screened for antigen recognition and for cross-reaction with other purified P-450 isozymes by ELISA as indicated below. Five hybridomas were subcloned by limited dilution and grown in culture. Antibodies in the cell culture supernatant solution were tested for immunoglobulin subclass with subclass-specific secondary antibodies. Antibody-containing ascites fluid was generated by injection of pristane-primed BALB/c mice with about 1×10^6 hybridoma cells per animal. IgG produced by a hybridoma cell line designated as 1H.11 was purified from ascites fluid by precipitation twice with ammonium sulfate (50% saturation) (18). The pellet was resuspended in 20 mM Tris-chloride buffer, pH 8.0, containing 30 mM NaCl (buffer A), and dialyzed against the same buffer. The sample was diluted to 2.5 mg of protein/ml and applied in the amount of 8–10 mg of protein/ml of resin to a Whatman DE52-cellulose column (1.5 × 10 cm), at 4°, previously equilibrated with buffer A. The protein was eluted with a 150-ml linear gradient from 20 mM Tris-chloride buffer, pH 8.0, containing 30 mM NaCl, to 40 mM Tris-chloride buffer, pH 8.0, containing 300 mM NaCl. Fractions that contained antibody were pooled, concentrated, and dialyzed against buffer A. The protein concentrations of the final samples were determined from the absorbance at 280 nm with use of an extinction coefficient of 13 for a 1% solution (18).

ELISA. This assay was performed according to the method of Keren (19) in 96-well polystyrene microtiter plates. The purified P-450 isozyme (0.04 μg of protein in 0.1 ml of 50 mM sodium carbonate buffer, pH 9.6) was added to the wells and incubated overnight at 4°. Washes and subsequent dilutions were done with 10 mM phosphate buffer, pH 7.4, containing 0.9% NaCl, 0.05% Tween 20, and 0.02% sodium azide. Culture media, ascites fluid, and purified antibodies were either diluted or added directly in a volume of 0.1 ml and incubated for 4–6 hr at room temperature with gentle shaking. The plates were washed four times and incubated with 0.1 ml of a 1/2500 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG for 18 hr at room temperature. After four more washes, 0.1% *p*-nitrophenyl phosphate was added in 50 mM carbonate buffer, pH 9.6, containing 1 mM $MgCl_2$. The absorbance of the product at 405 nm was determined with a Titertech ELISA plate reader.

Immunoblots. Microsomes from various sources were submitted to SDS-polyacrylamide slab gel electrophoresis, transferred electrophoretically to nitrocellulose sheets, and immunochemically stained with anti-P-450 3a IgG as described (20), except that 3% (w/v) bovine serum albumin was included in all IgG solutions. When the monoclonal antibody, 1H.11, was used, the nitrocellulose sheets were incubated at 37° for 60 min in TBS containing 0.05% (v/v) Tween 20. After two washes with TBS, the sheets were incubated overnight at room temperature with 2–10 μg/ml of 1H.11 IgG in TBS containing 3% (w/v) nonfat dry milk (Carnation brand) (21). The sheets were then washed as described for polyclonal antibody (20) and incubated for 90 min at room temperature with a 1/2000 dilution of peroxidase-conjugated goat anti-mouse IgG in TBS containing 1% (w/v) bovine serum albumin. Peroxidase activity was detected as described for polyclonal IgG (20). For quantitation, the staining intensity was compared with that of standard samples of hepatic microsomal P-450 isozyme 3a run on each gel.

Immunopurification. Purified 1H.11 was coupled to Affi-Gel 10 at a ratio of 8.5 mg of IgG/ml of resin according to the method of Staehelin *et al.* (22). P-450 isozyme 3a was immunopurified from microsomes by a slight modification of the method of Reubi *et al.* (23) for isozyme 3b. Kidney and liver microsomal suspensions (14 mg of protein/ml) or nasal microsomes (4 mg of protein/ml) from ethanol-treated rabbits were solubilized with 0.6 mg of cholate and 1.2 mg of Tergitol NP-10 per mg of protein; the detergents were added as 10% stock solutions. A 100-μl aliquot of 1H.11 coupled to Affi-Gel was washed with 10 mM Tris-chloride buffer, pH 6.8, containing 20% glycerol, 0.1 mM EDTA, 0.2% sodium cholate, and 1.0% Tergitol NP-10 in a 1.5-ml Microfuge tube, and then incubated with end-over-end mixing overnight at 4° with solubilized liver, kidney, or nasal microsomes in the amount of 7, 28, and 5 mg of protein, respectively. The unbound fraction was removed after centrifugation and the gel was washed five times with the buffer mixture described above but also containing 1 M KCl, and five times with glass-distilled water to remove the detergents. Bound protein was eluted from the gel by 150–300 μl of 10 mM Tris-chloride buffer, pH 6.8, containing 20% glycerol, 2% SDS, and 0.1 mM EDTA. Bromphenol blue and β-mercaptoethanol were added to the samples prior to electrophoresis.

Peptide mapping. Immunopurified P-450 isozyme 3a from liver and kidney and conventionally purified isozyme 3a (10), suspended in the elution buffer used for affinity chromatography, were dialyzed at room temperature for 20 hr against four changes of 300 volumes of 20% ethanol in 10 mM Tris-chloride buffer, pH 6.8, to remove SDS. Ethanol was removed by dialysis against 300 volumes of 10 mM Tris-chloride buffer, pH 6.8, for an additional 4 hr. This procedure reduced the SDS concentration to less than 0.1% with no precipitation of the protein. Proteolysis by papain was carried out at 37° for 16 hr, after

TABLE 1

Levels of total P-450 and NADPH-P-450 reductase in microsomes from untreated and ethanol-treated rabbits

Microsomes from tissues of untreated and ethanol-treated rabbits were analyzed for total P-450 and NADPH-P-450 reductase activity as described in Materials and Methods. The tissues from four to six animals were pooled for the preparation of microsomes. To calculate the reductase content, it was assumed that this enzyme in kidney and nasal microsomes has the same turnover number toward cytochrome *c* as does the purified hepatic enzyme. All values represent the mean of two to four determinations, with a standard deviation less than 10%.

Tissue	Treatment	P-450 specific content	Reductase specific content	Estimated molar ratio, P-450/reductase
		nmol/mg protein	*nmol/mg protein*	
Liver	None	1.80	0.057	32
Liver	Ethanol	1.76	0.052	34
Kidney	None	0.10	0.004	25
Kidney	Ethanol	0.18	0.008	22
Nasal mucosa	None	0.75	0.097	8
Nasal mucosa	Ethanol	0.55	0.094	6

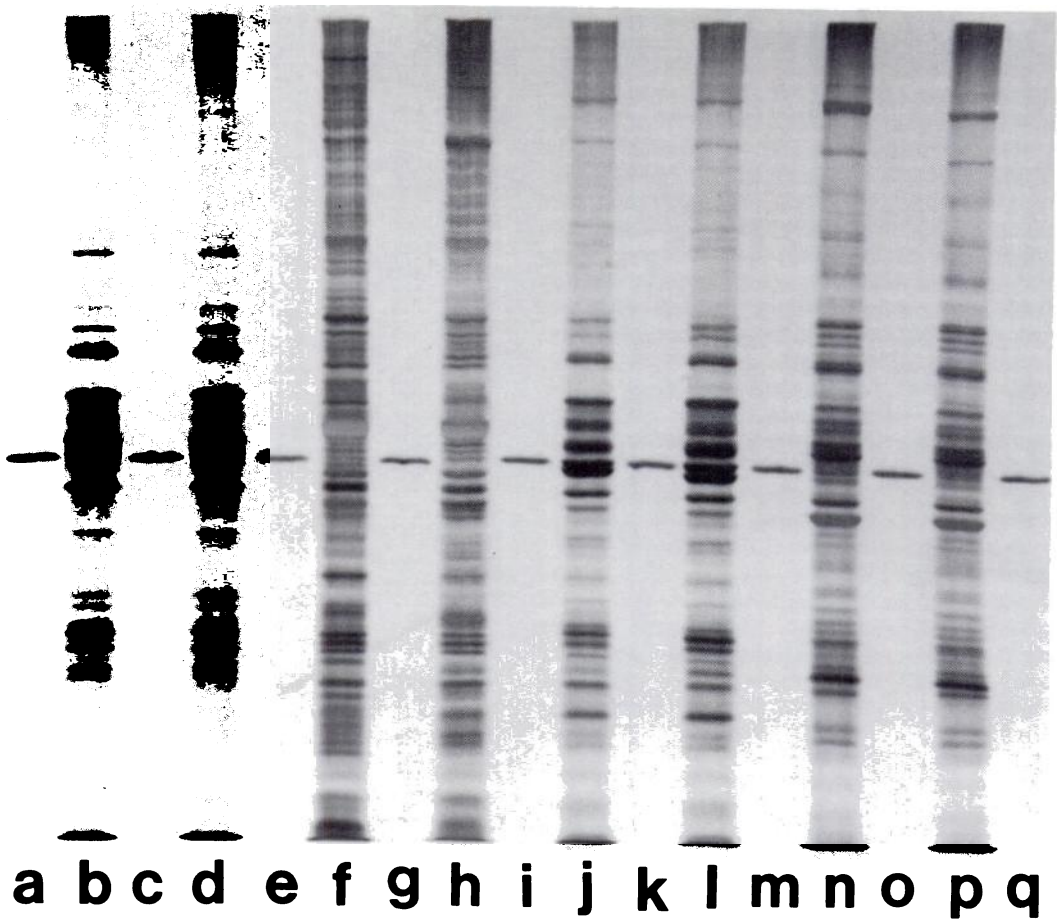

Fig. 1. SDS-polyacrylamide slab gel electrophoresis of hepatic and extrahepatic microsomes. Purified liver P-450 isozyme 3a and microsomes from untreated or ethanol-treated rabbit hepatic or extrahepatic tissues were submitted to electrophoresis on polyacrylamide gels and stained with silver. The samples examined were as follows, with the lane and amount of protein submitted to electrophoresis indicated in parentheses. Purified liver P-450 isozyme 3a (alternating lanes starting with *a*, 0.1 μg). Microsomes from untreated animals: liver (*d*, 2 μg), brain (*h*, 6.7 μg), nasal mucosa (*l*, 3.3 μg), and kidney (*p*, 5 μg). Microsomes from ethanol-treated animals: liver (*b*, 2 μg), brain (*f*, 6.7 μg), nasal mucosa (*j*, 3.3 μg), and kidney (*n*, 5 μg).

which the reaction mixtures were frozen in a dry ice/acetone bath and lyophilized. The peptides were redissolved in 100 μl of 62.5 mM Tris-chloride buffer, pH 6.8, containing 10% glycerol, 1% SDS, 0.001% pyronin Y, and 5% (v/v) mercaptoethanol, and boiled for 3 min, and 25 μl were taken for analysis. Peptides were separated on a 12.5% acrylamide slab gel and electrophoretically transferred to nitrocellulose sheets. The sheets were stained immunochemically with polyclonal anti-P-450-3a IgG as described earlier (20).

Other methods. Butanol and aniline oxidation were assayed as previously described (24). Total microsomal P-450 was quantified from the reduced-CO difference spectrum after solubilization with 0.3% Tergitol NP-10 (10, 25). NADPH-P-450 reductase activity was measured by the reduction of cytochrome *c*, and the amount of reductase was calculated from the known turnover number with this substrate, 4030 (26). Protein was determined by the method of Lowry *et al.* (27), and SDS concentrations were measured according to the method of Waite and Wang (28). SDS-polyacrylamide slab gel electrophoresis was performed according to the method of Laemmli (29), in 0.4 × 0.075 × 10-cm lanes of 7.5% acrylamide gel unless otherwise specified. The protein in the gels was detected by silver staining (30).

Materials. BALB/c mice were obtained from Charles River Breeding Laboratories, Wilmington, MA; 96-well microtiter plates were from Dynatech, and pristane was from Aldrich. Alkaline phosphatase-conjugated goat anti-mouse IgG and peroxidase-conjugated goat anti-mouse IgG were from Boehringer Mannheim Biochemicals. Disodium *p*-nitrophenyl phosphate was from Sigma Chemical Co. and Affi-Gel 10 was from Bio-Rad; desferrioxamine was a gift from Ciba-Geigy Corp. The source of other chemicals has been described elsewhere (10, 11, 20, 24).

Results

The levels of P-450 and of NADPH-P-450 reductase in microsomes from kidney, liver, and nasal mucosa of untreated and ethanol-treated rabbits are compared in Table 1. Ethanol treatment had no effect on the microsomal P-450 or reductase content in all three tissues. The lack of an increase in total P-450 after ethanol administration to rabbits was reported previously for liver microsomes (20). In kidney microsomes, the P-450 content and the reductase activity are much lower than in liver microsomes, but the molar ratio of P-450 to reductase is only slightly lower than in liver microsomes. The nasal microsomes have a relatively high P-450 content for a nonhepatic source, being about one-third of that in liver microsomes, and have very high levels of the reductase as judged by its activity in cytochrome *c* reduction and by immunoblot analysis with anti-reductase IgG (data not shown). As a result, the molar ratio of P-450 to reductase in microsomes from nasal mucosa is much lower than that in microsomes from the other two tissues.

The electrophoretic profile of proteins in microsomes prepared from brain, kidney, liver, and nasal mucosa from both

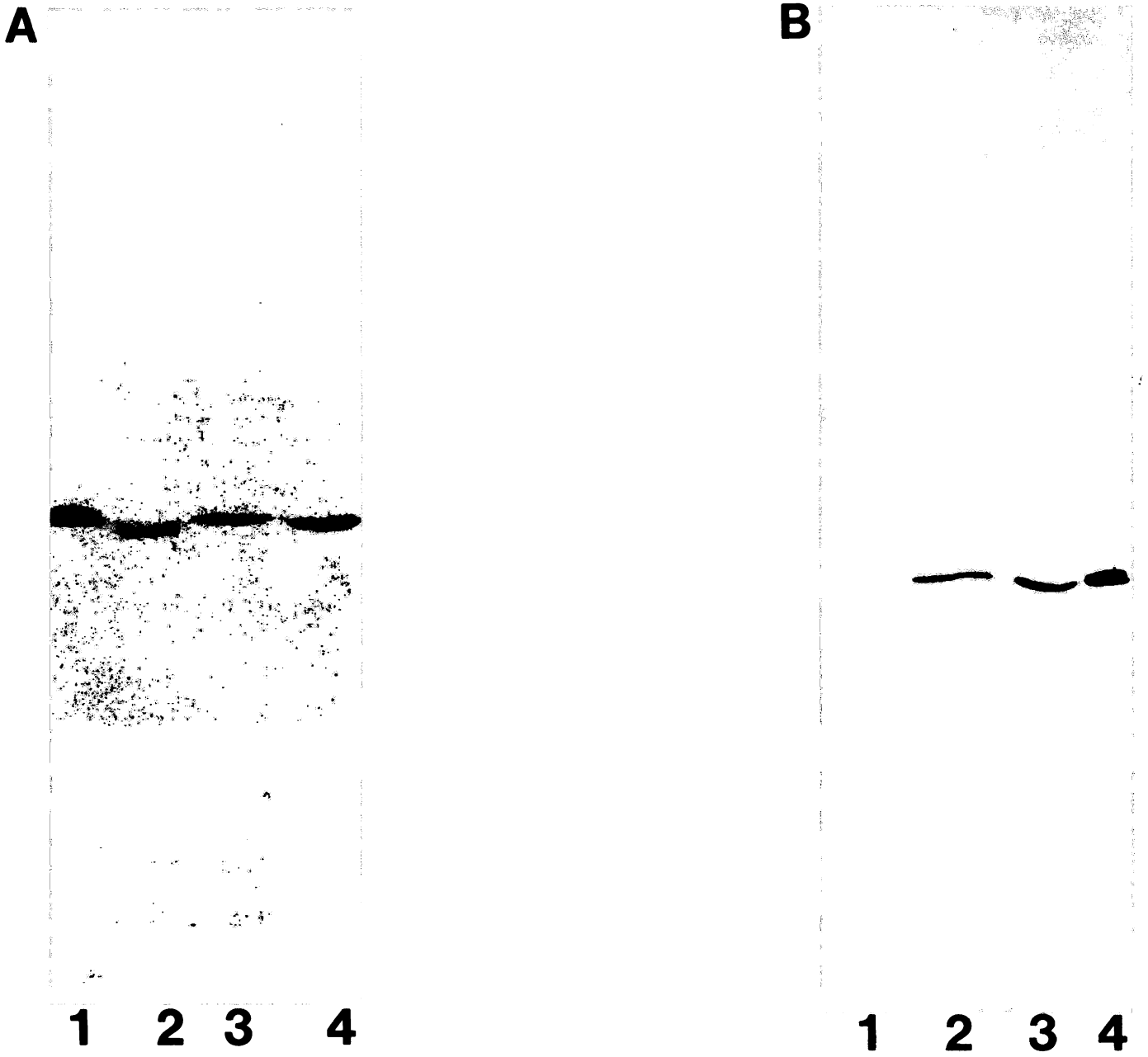

Fig. 2. Immunoblot analysis of kidney and nasal microsomes for presence of P-450 isozyme 3a. Purified hepatic P-450 isozyme 3a and microsomal preparations from ethanol-treated rabbits were submitted to electrophoresis in 7.5% acrylamide gels and electrophoretically transferred to nitrocellulose sheets as described in Materials and Methods. For immunochemical detection of proteins, the nitrocellulose sheets were treated with either polyclonal anti-3a IgG (2 μg/ml) in the experiment in A, or with monoclonal anti-3a IgG (5 μg/ml) in the experiment in B, and staining was then carried out (20). The samples, with amounts of protein indicated in parentheses, were as follows. A: *lane 1*, purified P-450 isozyme 3a (0.075 μg); *lane 2*, nasal microsomes (35μg); *lane 3*, liver microsomes (2 μg); and *lane 4*, kidney microsomes (20 μg). B: *lane 1*, nasal microsomes (36 μg); *lane 2*, liver microsomes (1.8 μg); *lane 3*, kidney microsomes (15 μg); and *lane 4*, purified P-450 isozyme 3a (0.05 μg).

untreated and ethanol-treated adult rabbits is shown in Fig. 1. A protein with the same mobility as purified hepatic P-450 isozyme 3a was present in microsomal samples from all sources. However, in such experiments it was not possible to detect quantitative differences due to ethanol administration.

An immunoblot analysis of microsomes from liver, kidney, and nasal mucosa of ethanol-treated rabbits using both polyclonal and monoclonal antibodies is shown in Fig. 2. Similar results not presented were obtained with microsomes from untreated animals. The polyclonal anti-3a antibody was used previously to quantitate the induction of P-450 isozyme 3a in liver microsomes by diverse chemical agents (20). As shown in Fig. 2A, a single band with the same electrophoretic mobility as purified hepatic P-450 isozyme 3a was stained with the use of polyclonal antibody in microsomes from liver and kidney, whereas a band with slightly greater mobility was stained in the microsomes from nasal mucosa. The amount of isozyme 3a identified immunochemically was much less than expected from the intensity of the protein band observed on gels after silver staining (Fig. 1, *lanes j* and *l*). The low intensity and a mobility slightly greater than that of the purified enzyme seen in Fig. 2 suggest that only the leading edge of the silver-stained band

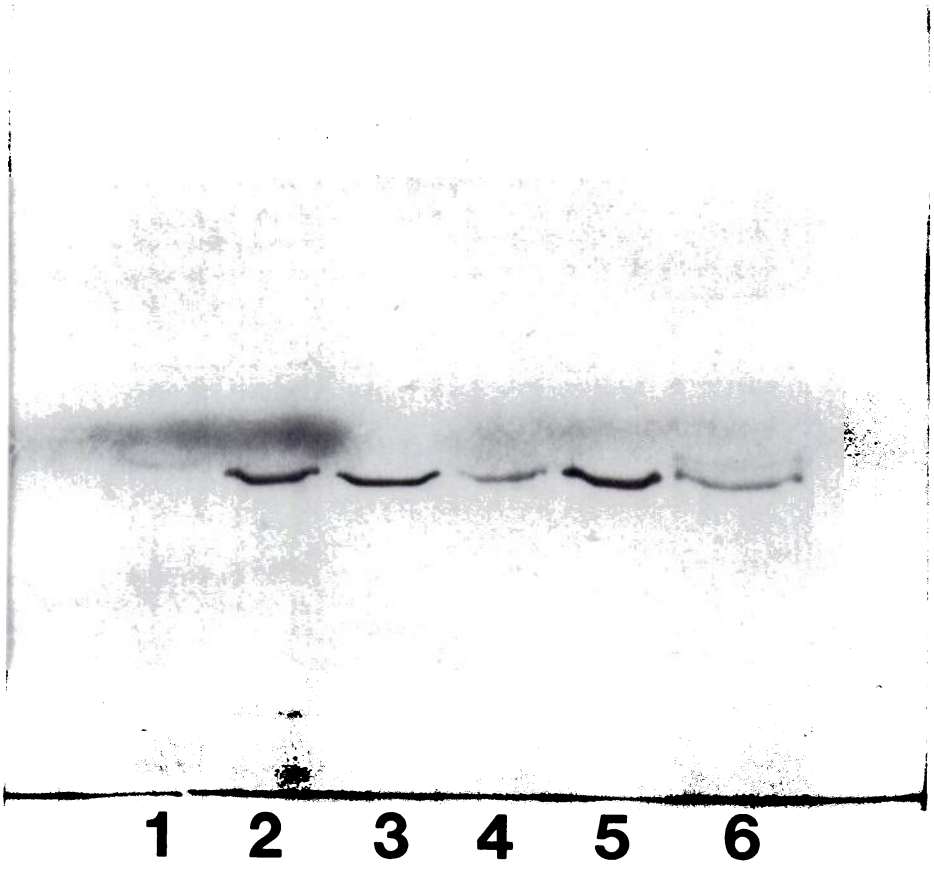

Fig. 3. Slab gel electrophoresis of immunoaffinity-purified P-450 isozyme 3a from kidney, liver, and nasal microsomes of ethanol-treated rabbits. The cytochrome was eluted from the affinity matrix, submitted to electrophoresis in 8% polyacrylamide gel, and stained with silver. P-450 isozyme 3a purified from liver microsomes by the published procedure (10) was included as a standard (*lanes 3* and *5*). *Lane 1* was a control in which microsomes were omitted, and *lanes 2, 4,* and *6* contained the immunopurified enzyme from kidney, liver, and nasal mucosa, respectively.

corresponded to isozyme 3a in nasal microsomes. This was confirmed by the observation that purified hepatic P-450 isozyme 3a added to nasal microsomes had a mobility identical to that of the protein stained immunochemically (results not shown). Immunoblot analysis with use of the monoclonal anti-3a antibody gave similar results, as shown in Fig. 2B. The specificity of the 1H.11 antibody is demonstrated in Fig. 2B, *lane 2*, where a single band was recognized in hepatic microsomes. This cross-reacting band had the same mobility as the purified P-450 isozyme 3a (Fig. 2B, *lane 4*). The purified 1H·11 monoclonal IgG was known, from experiments not presented here, not to cross-react with purified P-450 isozymes 2, 3b, 3c, 4, or 6, or with epoxide hydrolase, when assayed by ELISA with the IgG at the level of 5 μg/well. The results from the immunoblots thus indicate that isozyme 3a is present in rabbit kidney and nasal mucosa. However, at the same concentration of anti-3a polyclonal antibody, isozyme 3a was not detected in immunoblots of rabbit brain, lung, adrenal, heart, intestine, ovary, spleen, testis, or uterus microsomes with up to 90 μg of microsomal protein present per sample well (results not shown). The limit of detection of form 3a is about 0.1 pmol. Although brain microsomes contain a protein with the same electrophoretic mobility as P-450 isozyme 3a, as shown in Fig. 1, we conclude from the immunoblotting experiments that it is a different protein. It should be noted that the experiments in Fig. 2 establish qualitatively the occurrence of isozyme 3a in nasal and kidney microsomes and that the relatively light staining of the nasal protein as compared to the kidney protein is atypical. A complete quantitative comparison is presented below (see Fig. 5).

The identification of P-450 isozyme 3a in kidney and nasal microsomes was further substantiated by immunoaffinity chromatography. The 1H.11 monoclonal IgG coupled to Affi-Gel 10 was used to purify P-450 isozyme 3a from solubilized kidney, liver, and nasal microsomes obtained from ethanol-treated rabbits. From kidney and liver microsomes a single protein band was observed on polyacrylamide gels with the same mobility as hepatic P-450 isozyme 3a purified in the conventional fashion, as shown in Fig. 3. The chief protein obtained from nasal microsomes also corresponded to P-450 3a, but the sample contained an additional faint band with a slightly higher apparent minimal molecular weight than that of isozyme 3a (Fig. 3, *lane 6*). This faint band is probably the heavy chain of IgG since it also appears in the control experiment in which microsomes were omitted (Fig. 3, *lane 1*).

The immunopurified cytochrome from kidney and liver, as well as hepatic P-450 isozyme 3a purified by conventional procedures (10), was submitted to limited proteolysis by papain. Insufficient purified nasal P-450 was available for such experiments. The peptides formed were separated by electrophoresis on a 12.5% polyacrylamide slab gel, transferred to nitrocellulose sheets, and immunochemically stained with the use of polyclonal anti-3a antibody. As shown in Fig. 4, the peptide maps of the immunopurified P-450 isozyme 3a from kidney (Fig. 4, *lane 3*) and from liver (Fig. 4, *lane 1*) gave the same peptide patterns, suggesting that these cytochromes are highly similar, if not identical, in structure. These patterns are essentially identical to that obtained from the conventionally purified hepatic cytochrome (Fig. 4, *lane 2*) except for the difference in the extent of digestion, as indicated by differences in the relative intensities of the various peptides. For some reason not yet understood, this protein may be somewhat more resistant to proteolysis after immunoaffinity chromatography.

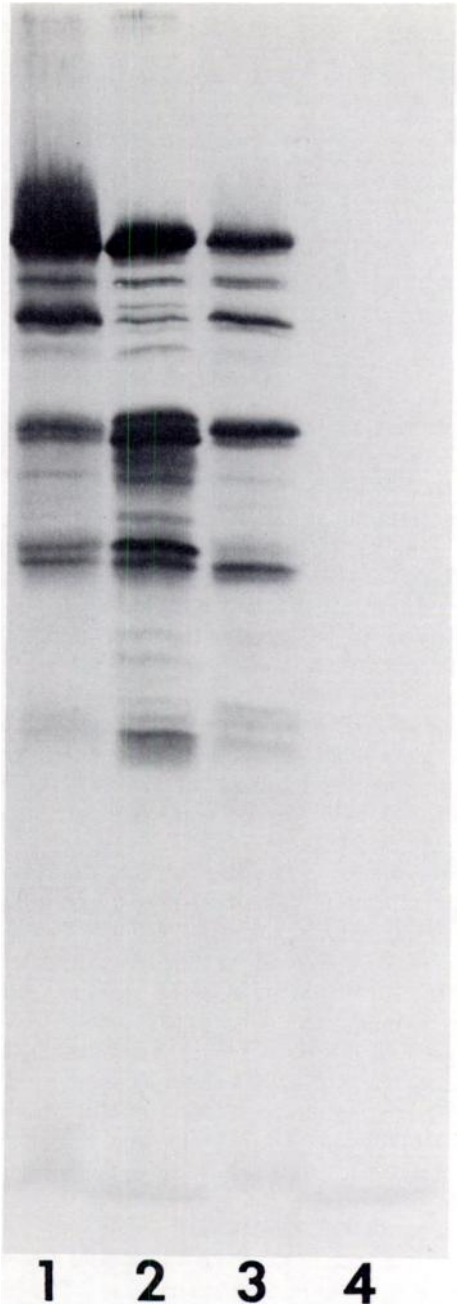

Fig. 4. Peptide maps of P-450 isozyme 3a preparations from liver and kidney. The reaction mixtures contained, in a final volume of 1.0 ml, 10 μg of the purified cytochrome (10) with 0.14 μg of papain, 10 μg of the immunopurified cytochrome from liver microsomes with 2.8 μg of papain, or 3 μg of the immunopurified cytochrome from kidney microsomes with 4.6 μg of papain. The peptides generated were resolved on a 12.5% polyacrylamide gel and immunochemically stained with polyclonal anti-3a IgG. The results are as follows: *lane 1*, immunopurified liver cytochrome; *lane 2*, conventionally purified liver cytochrome; *lane 3*, immunopurified kidney cytochrome; and *lane 4*, control with papain (4.6 μg) present but no cytochrome.

As reported earlier by this laboratory (24), isozyme 3a is the most active of the rabbit liver P-450s in the oxidation of alcohols and the *p*-hydroxylation of aniline. In order to confirm the presence of isozyme 3a in kidney and nasal microsomal membranes, the effect of the polyclonal antibody on these activities was determined, with the results given in Table 2. In the experiments with hepatic microsomes that were included for comparison, the rate of P-450 isozyme 3a-dependent butanol oxidation was increased from 0.71 to 3.07 nmol/min/mg of protein when the animals were treated with ethanol. This 4.3-fold induction agrees well with the value of 4.1 reported previously (20). The corresponding increase in the rate of aniline hydroxylation was 3.3-fold. It should be noted that the two activities would not necessarily be expected to increase by the same amount, since the magnitude of the induction effect is dependent in part on the relative activities of the other P-450 isozymes toward these two substrates. In contrast to hepatic microsomes, kidney microsomes exhibited quite low total and P-450 3a-dependent hydroxylation rates in the case of the control animals, and, although alcohol administration gave about 7-fold induction, the resulting activities were much lower than in liver. The results with nasal microsomes from untreated rabbits are particularly interesting in that the total and P-450 isozyme 3a-dependent rates of butanol oxidation, as compared to those in liver microsomes, were about 3.2 and 2.6 times as great, respectively. The corresponding nasal activities in aniline hydroxylation were also somewhat higher than in liver, 1.3 and 1.5 times, respectively. Furthermore, the results show another striking difference in that P-450 isozyme 3a is not induced in nasal microsomes as judged by these activity measurements. In additional experiments not presented, the presence of 1.0 mM desferrioxamine in the reaction mixtures was shown not to affect the catalytic activities of liver, kidney, and nasal microsomes. This finding suggests that reactions involving the production of hydroxyl radicals from H_2O_2 and any contaminating iron in the system do not make a significant contribution to the P-450-dependent microsomal activities, in accord with conclusions reached earlier (24).

The induction of isozyme 3a was also quantitated by the immunoblot procedure. The slopes of the individual lines plotted in Fig. 5 represent the relative content of this cytochrome in different microsomal samples. Comparison of the slopes offers a more accurate measurement of the isozyme than does the staining intensity of individual samples (31). Thus, the content of P-450 isozyme 3a was induced 3.9-fold in liver microsomes and 4.6-fold in kidney microsomes, but was not induced in nasal microsomes by ethanol treatment of the animals. Determination of the actual content of this P-450 isozyme in different samples was also possible since the staining intensity could be compared with that of a standard sample run on each gel. In liver, kidney, and nasal microsomes from untreated rabbits the specific content of isozyme 3a is about 0.15, 0.01, and 0.08 nmol/mg of protein, respectively. After ethanol treatment, the specific content increases to about 0.58 for liver microsomes and 0.05 for kidney microsomes, but is unchanged in nasal microsomes.

A comparison of the results obtained from the immunoblot analysis (Fig. 5) and from antibody inhibition experiments (Table 2) is shown in Table 3. The extent of induction of P-450 isozyme 3a determined by activity measurements agrees fairly well with the values obtained by determination of the specific content of this cytochrome. The greater variation in the results in the case of kidney microsomes may be due to the low values for the P-450 isozyme 3a-dependent activities for untreated rabbits, where a small difference could result in a large change in the calculated fold of induction. With the nasal microsomes, no significant induction was evident by either method.

TABLE 2

Inhibition by anti-P-450 3a IgG of microsomal aniline hydroxylation and butanol oxidation

Microsomal oxidation of *n*-butanol to butyraldehyde was measured by gas chromatography of the headspace gas. The reaction mixtures contained microsomes (0.2 nmol of P-450), 2 mg of anti-3a IgG or preimmune sheep IgG, 30 μmol of *n*-butanol, 50 μmol of potassium phosphate buffer, pH 7.6, and 1.0 μmol of NADPH in a final volume of 1.0 ml. The mixtures were incubated at 30° for 20 min. The isozyme 3a-dependent rates were obtained by subtracting the rate obtained in the presence of anti-3a IgG from that obtained in the presence of preimmune IgG. Reaction mixtures for aniline hydroxylation were the same as for butanol oxidation except that 2.0 μmol of NADPH were used, with 5 μmol of aniline in place of butanol. Since nasal microsomes from a single animal were inadequate in amount for the determinations to be made, microsomes from this and the other tissues were pooled from six animals. All results are given as the mean of duplicate or triplicate determinations.

Tissue	Treatment	Butanol oxidation activity			Aniline hydroxylation activity		
		Total	Isozyme 3a dependent	% Inhibition by antibody	Total	Isozyme 3a dependent	% Inhibition by antibody
		nmol/min/mg protein			*nmol/min/mg protein*		
Liver	None	1.70	0.71	42	0.45	0.15	33
Liver	Ethanol	4.11	3.07	75	0.77	0.49	64
Kidney	None	0.066	0.010	15	0.011	0.004	36
Kidney	Ethanol	0.144	0.073	51	0.046	0.026	57
Nasal	None	5.51	1.86	34	0.58	0.22	38
Nasal	Ethanol	4.82	1.83	38	0.57	0.26	46

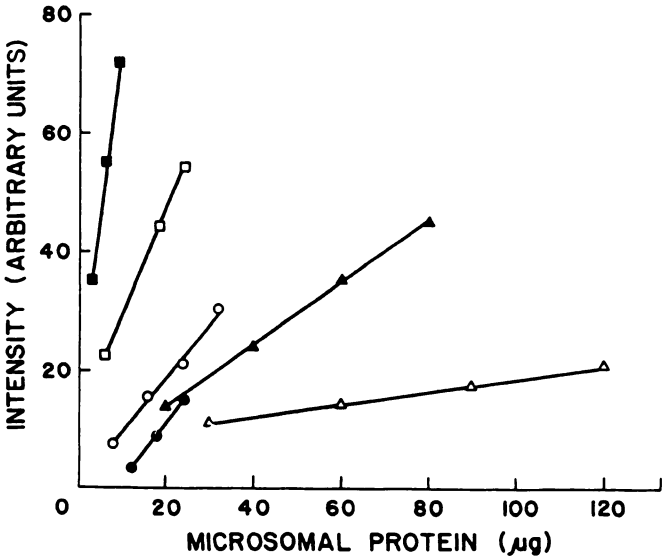

Fig. 5. Relative amount of P-450 isozyme 3a in microsomes from untreated and ethanol-treated rabbits. Pooled microsomes from six animals were submitted to electrophoresis, and the proteins were transferred to nitrocellulose sheets and immunochemically stained with polyclonal anti-3a IgG. The peak area of soft laser densitometer scans was measured and plotted versus the amount of protein loaded into individual wells. The experiments were as follows: liver microsomes from ethanol-treated (■), or untreated (□) animals; nasal microsomes from ethanol-treated (●) or untreated (○) animals; and kidney microsomes from ethanol-treated (▲) or untreated (△) animals.

Discussion

Multiple P-450s isolated from or identified in rabbit kidney microsomes include forms 2, 4, 6 (32, 33), and a constitutive form, designed P-450a, which catalyzes the ω-hydroxylation of prostaglandin A_1, as well as the ω- and (ω-1)-hydroxylation of fatty acids (33, 34). In the present report, we provide evidence for the identification of isozyme 3a in rabbit kidney. This cytochrome is present in renal tissue from untreated rabbits and is inducible by ethanol. The isozyme 3a preparations from renal and hepatic microsomes have similar primary structures as shown by peptide mapping experiments, similar immunochemical determinants as judged by the use of polyclonal and monoclonal antibodies, and similar catalytic activities toward aniline and butanol that are inhibited by the polyclonal antibody. The total P-450 content of kidney microsomes after ethanol treatment of the animals was about 10% of that of liver microsomes from untreated animals. However, the specific content of isozyme 3a in kidney microsomes from ethanol-treated rabbits was about one-third of that in liver microsomes from untreated rabbits. Kidney microsomes of untreated rabbits have only about 0.01 nmol of 3a/mg of protein, but this is increased to about 0.05 upon ethanol treatment. This would represent about 10% and 28%, respectively, of the total P-450 in kidney microsomes; such values are similar to those reported for hepatic microsomes (20). It should be noted that P-450 is not uniformly distributed in rabbit kidney (5) and that the renal microsomes used in the present study were prepared from the whole kidney.

Unlike microsomes from other extrahepatic tissues, microsomes prepared from nasal mucosa have a rather high specific content of P-450 and a high level of NADPH-P-450 reductase. These features could account for the high metabolic activity of this tissue toward numerous airborne xenobiotics. The nasal mucosa has been reported to be one of the major targets for the carcinogenicity of compounds such as hexamethylphosphoramide (35), phenacetin (36), and the tobacco-specific nitrosamines *N'*-nitrosonornicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (37). The metabolism of these compounds by nasal mucosa has also been demonstrated (3, 6, 7). The specific content of P-450 isozyme 3a in the nasal microsomes from untreated rabbits was about 0.08 nmol/mg of protein, or half of that in the liver. This would represent about 11% of the total P-450 in nasal microsomes. The content was unchanged by ethanol treatment of the animals under the conditions used. Preliminary experiments with antibodies to rabbit liver microsomal isozymes 2, 4, and 6 indicated that the major isozyme of the nasal cytochrome P-450 system in rabbits is form 4, as is the case for liver. Low levels of form 2, but not form 6, were also detected in nasal microsomes from untreated animals. Unlike the results obtained with liver and kidney, P-450 isozyme 3a is apparently not induced in nasal mucosa by ethanol treatment. The lack of induction of nasal cytochrome P-450 in rabbits by phenobarbital administration has been reported (3), and it was suggested that either the phenobarbital did not reach nasal cells in sufficient quantities or the enzymes might already be kept in an induced state by the very low levels of organic compounds present in normal air (3).

The nasal preparations used in the present study, including membranes of the nasal septum and ethmoturbinates, were mostly mucosa of the olfactory epithelium, which is composed

TABLE 3

Induction of microsomal isozyme 3a by treatment of the animals with ethanol

P-450 isozyme 3a-dependent activities, determined by inhibition with anti-3a IgG, are taken from Table 2. The relative content of the cytochrome was estimated by immunoblot quantitation. The values for the microsomes from untreated animals are taken as 100 in each case.

Source of microsomes	Agent administered to animals	P-450 isozyme 3a-dependent activity		Content of P-450 isozyme 3a
		Butanol oxidation	Aniline hydroxylation	
Liver	None	100	100	100
Liver	Ethanol	432	327	387
Kidney	None	100	100	100
Kidney	Ethanol	730	650	456
Nasal mucosa	None	100	100	100
Nasal mucosa	Ethanol	98	118	110

of a variety of different types of cells. Using immunohistochemical techniques, Voigt *et al.* (38) reported that a number of different cell types in rat olfactory epithelium contain NADPH-P-450 reductase and P-450$_{PB\text{-}B}$, the major phenobarbital-inducible isozyme of rat hepatic microsomes. However, a distinction between neuronal cells and non-neuronal supporting cells was not reported. Our preliminary experiments with the immunoblot technique have failed to detect isozymes 2, 3a, 4, or 6 in brain microsomes from untreated rabbits or from rabbits treated with known inducers of hepatic P-450 such as ethanol, imidazole, isosafrole, and phenobarbital. Since isozymes 3a and 4 are present in the microsomes prepared from the olfactory mucosa, it would be interesting to see if they can be detected in the olfactory neuronal cells.

The identification of isozyme 3a in nasal mucosa may lead to a better understanding of the mechanisms which underlie the species- and organ-specific carcinogenicity of various nitrosamines. It is known that rabbit liver isozyme 3a metabolizes *N*-nitrosodimethylamine (13, 39) and *N*-nitroso-2,6-dimethylmorpholine (14). The carcinogenicity of *N*-nitrosodiethylamine, *N'*-nitrosonornicotine, and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in nasal mucosa has been studied in other species (37), but the identity of the activating isozyme involved has not been established. However, it seems likely that P-450 isozyme 3a or a highly similar cytochrome may play this role.

Acknowledgments

We are grateful to Dr. Charles E. Rothwell, School of Public Health, The University of Michigan, for assistance in the production of monoclonal antibodies.

References

1. Burke, M. D., and S. Orrenius. Isolation and comparison of endoplasmic reticulum membranes and their mixed function oxidase activities from mammalian extrahepatic tissues. *Pharmacol. Ther.* **7**:549–599 (1979).
2. Cohn, J. A., A. P. Alvares, and A. Kappas. On the occurrence of cytochrome P-450 and aryl hydrocarbon hydroxylase activity in the rat brain. *J. Exp. Med.* **145**:1607–1611 (1977).
3. Hadley, W. M., A. R. Dahl, J. M. Benson, F. F. Hahn, and R. O. McClellan. Cytochrome P-450 dependent monooxygenases in nasal epithelial membranes: effect of phenobarbital and benzo(a)pyrene. *Proc. West. Pharmacol. Soc.* **25**:197–199 (1982).
4. Vanderslice, R. R., J. A. Boyd, T. E. Eling, and R. M. Philpot. The cytochrome P-450 monooxygenase system of rabbit bladder mucosa: enzyme components and isozyme 5-dependent metabolism of 2-aminofluorene. *Cancer Res.* **45**:5851–5858 (1985).
5. Mohandas, J., G. G. Duggin, J. S. Horvath, and D. J. Tiller. Metabolic oxidation of acetaminophen (paracetamol) mediated by cytochrome P-450 mixed-function oxidase and prostaglandin endoperoxide synthetase in rabbit kidney. *Toxicol. Appl. Pharmacol.* **61**:252–259 (1981).
6. Brittebo, E., and M. Åhlman. Studies on a nasal cavity carcinogen: metabolism and binding of phenacetin in the mucosa of the upper respiratory tract, in *Extrahepatic Drug Metabolism and Chemical Carcinogenesis* (J. Rydström, J. Montelius, and M. Bengtsson, eds). Elsevier Science Publishers B. V., Amsterdam, 619–620 (1983).
7. Brittebo, E. B., A. Castonguay, K. Furuya, and S. S. Hecht. Metabolism of tobacco-specific nitrosamines by cultured rat nasal mucosa. *Cancer Res.* **43**:4343–4348 (1983).
8. Brittebo, E. B., B. Löfberg, and H. Tjälve. Site of metabolism of *N*-nitrosodiethylamine in mice. *Chem.-Biol. Interact.* **34**:209–221 (1981).
9. Robertson, I. G. C., R. M. Philpot, E. Zeiger, and C. R. Wolf. Specificity of rabbit pulmonary cytochrome P-450 isozymes in the activation of several aromatic amines and aflatoxin B_1. *Mol. Pharmacol.* **20**:662–668 (1981).
10. Koop, D. R., E. T. Morgan, G. E. Tarr, and M. J. Coon. Purification and characterization of a unique isozyme of cytochrome P-450 from liver microsomes of ethanol-treated rabbits. *J. Biol. Chem.* **257**:8472–8480 (1982).
11. Morgan, E. T., D. R. Koop, and M. J. Coon. Catalytic activity of cytochrome P-450 isozyme 3a isolated from liver microsomes of ethanol-treated rabbits. *J. Biol. Chem.* **257**:13951–13957 (1982).
12. Morgan, E. T., D. R. Koop, and M. J. Coon. Comparison of six rabbit liver cytochrome P-450 isozymes in formation of a reactive metabolite of acetaminophen. *Biochem. Biophys. Res. Commun.* **112**:8–13 (1983).
13. Yang, C. S., Y. Y. Tu, D. R. Koop, and M. J. Coon. Metabolism of nitrosamines by purified rabbit liver cytochrome P-450 isozymes. *Cancer Res.* **45**:1140–1145 (1985).
14. Kokkinakis, D. M., D. R. Koop, D. G. Scarpelli, M. J. Coon, and P. F. Hollenberg. Metabolism of *N*-nitroso-2,6-dimethylmorpholine by isozymes of rabbit liver microsomal cytochrome P-450. *Cancer Res.* **45**:619–624 (1985).
15. Coon, M. J., T. A. van der Hoeven, S. B. Dahl, and D. A. Haugen. Two forms of liver microsomal cytochrome P-450, P-450$_{LM2}$ and P450$_{LM4}$ (rabbit liver). *Methods Enzymol.* **52**:109–117 (1978).
16. Rothwell, C. E., M. B. Khazaeli, and I. A. Bernstein. Radiometric assay for direct quantitation of rat liver cytochrome P-450b using monoclonal antibodies. *Anal. Biochem.* **149**:197–201 (1985).
17. Kearney, J. F., A. Radbruch, B. Liesegang, and K. Rajewsky. A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines. *J. Immunol.* **123**:1548–1550 (1979).
18. Thomas, P. E., L. M. Reik, D. E. Ryan, and W. Levin. Characterization of nine monoclonal antibodies against rat hepatic cytochrome P-450c. *J. Biol. Chem.* **258**:4590–4598 (1984).
19. Keren, D. F. Enzyme-linked immunosorbent assay for immunoglobulin G and immunoglobulin A antibodies to *Shigella flexneri* antigens. *Infect. Immun.* **24**:441–448 (1979).
20. Koop, D. R., B. L. Crump, G. D. Nordblom, and M. J. Coon. Immunochemical evidence for induction of the alcohol-oxidizing cytochrome P-450 of rabbit liver microsomes by diverse agents: ethanol, imidazole, trichloroethylene, acetone, pyrazole, and isoniazid. *Proc. Natl. Acad. Sci. USA* **82**:4065–4069 (1985).
21. Morgan, E. T., C. MacGeoch, and J.-Å. Gustafsson. Sexual differentiation of cytochrome P-450 in rat liver: evidence for a constitutive isozyme as the male-specific 16α-hydroxylase. *Mol. Pharmacol.* **27**:471–479 (1985).
22. Staehelin, T., D. S. Hobbs, H.-F. Kung, C.-Y. Lai, and S. Pestka. Purification and characterization of recombinant human leukocyte interferon (IFLrA) with monoclonal antibodies. *J. Biol. Chem.* **256**:9750–9754 (1981).
23. Reubi, I., K. J. Griffin, J. Raucy, and E. F. Johnson. Use of a monoclonal antibody specific for rabbit microsomal cytochrome P-450 3b to characterize the participation of this cytochrome in the microsomal 6β- and 16α-hydroxylation of progesterone. *Biochemistry* **23**:4598–4603 (1984).
24. Koop, D. R., G. D. Nordblom, and M. J. Coon. Immunochemical evidence for a role of cytochrome P-450 in liver microsomal ethanol oxidation. *Arch. Biochem. Biophys.* **235**:228–238 (1984).
25. Omura, T., and R. Sato. The carbon monoxide-binding protein of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.* **239**:2370–2378 (1964).
26. French, J. S., and M. J. Coon. Properties of NADPH-cytochrome P-450 reductase purified from rabbit liver microsomes. *Arch. Biochem. Biophys.* **195**:565–577 (1979).
27. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–272 (1951).

28. Waite, J. H., and C.-Y. Wang. Spectrophotometric measurement of dodecyl sulfate with basic fuchsin. *Anal. Biochem.* **70**:279–280 (1976).
29. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* **227**:680–685 (1970).
30. Wray, W., T. Boulikas, V. P. Wray, and R. Hancock. Silver staining of proteins in polyacrylamide gels. *Anal. Biochem.* **118**:197–203 (1981).
31. Domin, B. A., C. J. Serabjit-Singh, and R. M. Philpot. Quantitation of rabbit cytochrome P-450, form 2, in microsomal preparations bound directly to nitrocellulose paper using a modified peroxidase-immunostaining technique. *Anal. Biochem.* **136**:390–396 (1984).
32. Liem, H. H., U. Muller-Eberhard, and E. F. Johnson. Differential induction by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin of multiple forms of rabbit microsomal cytochrome P-450: evidence for tissue specificity. *Mol. Pharmacol.* **18**:565–570 (1980).
33. Kusunose, M., K. Ogita, E. Kusunose, S. Yamamoto, and K. Ichihara. Separation of multiple forms of cytochrome P-450 from rabbit kidney cortex microsomes, in *Extrahepatic Drug Metabolism and Chemical Carcinogenesis* (J. Rydström, J. Montelius, and M. Bengtsson, eds.). Elsevier Science Publishers B. V., Amsterdam, 229–230 (1983).
34. Ogita, K., E. Kusunose, S. Yamamoto, K. Ichihara, and M. Kusunose. Multiple forms of cytochrome P-450 from kidney cortex microsomes of rabbits treated with phenobarbital. *Biochem. Int.* **6**:191–198 (1983).
35. Lee, K. P., H. J. Trochimowicz, and C. F. Reinhardt. Induction of nasal tumors in rats exposed to hexamethylphosphoramide (HMPA). *Toxicologist* **1**:128 (1981).
36. Isaka, H., H. Yoshii, A. Otsuji, M. Koike, Y. Nagai, M. Koura, K. Sugiyasu, and T. Kanabayashi. Tumors of Sprague-Dawley rats induced by long-term feeding of phenacetin. *Gann* **70**:29–36 (1979).
37. Hoffmann, D., and S. S. Hecht. Nicotine-derived *N*-nitrosamines and tobacco-related cancer: current status and future directions. *Cancer Res.* **45**:935–944 (1985).
38. Voigt, J. M., F. P. Guengerich, and J. Baron. Localization of a cytochrome P-450 isozyme (cytochrome P-450 PB-B) and NADPH-cytochrome P-450 reductase in rat nasal mucosa. *Cancer Lett.* **27**:241–247 (1985).
39. Yang, C. S., D. R. Koop, T. Wang, and M. J. Coon. Immunochemical studies on the metabolism of nitrosamines by ethanol-inducible cytochrome P-450. *Biochem. Biophys. Res. Commun.* **128**:1007–1013 (1985).

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