0026-895X/90/040489-08$02.00/0

MOLECULAR PHARMACOLOGY, 37:489-496

Immunochemical Characterization of Multiple Forms of Cytochrome P-450 in Rabbit Nasal Microsomes and Evidence for Tissue-Specific Expression of P-450s NMa and NMb

XINXIN DING and MINOR J. COON

Department of Biological Chemistry, Medical School, The University of Michigan, Ann Arbor, Michigan 48109-0606

Received September 11, 1989; Accepted December 22, 1989

SUMMARY

Two unique forms of cytochrome P-450 (P-450), designated NMa and NMb, were recently isolated in this laboratory from nasal microsomes of rabbits. In the present study, polyclonal antibodies to the purified nasal cytochromes were prepared. Immunochemical analysis with specific rabbit anti-NMa and sheep anti-NMb antibodies indicated that P-450 isozymes identical to or having a high structural homology with NMa are present in both olfactory and respiratory mucosa, as well as in liver, but NMb was detected only in the olfactory mucosa. Neither form was detected in other tissues examined, including brain, esophageal mucosa, heart, intestinal mucosa, kidney, and lung. The specific occurrence of NMb in the olfactory mucosa was further substantiated by the detection and specific inhibition by anti-NMb of the formation of unique NMb-dependent metabolites of testosterone in olfactory microsomes but not in microsomes from liver or respiratory mucosa. Similar experiments with antibodies to previously purified rabbit hepatic P-450 isozymes indicated that not all of the hepatic cytochromes are expressed in the nasal tissues. Thus, P-450 isozymes structurally homologous to hepatic forms 2, 3a, and 4, but not 3b and 6, were found in the olfactory mucosa. On the other hand, only form 2 was detected in the respiratory mucosa. Immunoquantitation experiments revealed that NMa and NMb are the major P-450 forms in olfactory microsomes, whereas NMa and P-450 form 2 (or its homolog) constitute the major portion of the respiratory nasal microsomal P-450. The level of NMa in the liver is relatively low, accounting for less than 3% of total microsomal P-450 in this tissue. In addition, evidence is provided that NMa is the major catalyst in the dealkylation of two nasal carcinogens, hexamethylphosphoramide and phenacetin, in both olfactory and respiratory nasal microsomes.

The nasal epithelium is recognized as a potential first line of defense of the lung against inhaled xenobiotics. Numerous volatile compounds are readily absorbed by the nasal mucosa (1). In addition, many nonvolatile materials, including environmental pollutants, can also reach the nasal mucosa, probably by adsorption onto small particles in the air flow that deposit on the nasal epithelium (2, 3). Thus, whereas the liver provides protection against ingested foreign compounds by a "first pass clearance," the nasal epithelium may protect the lung by removing undesirable chemicals in the inhaled air. The compounds absorbed by the nasal mucosa are actively metabolized *in situ*, sometimes detoxified but in many cases activated to become more toxic or carcinogenic (4–7), which makes the nasal passage a potential target organ for the toxicity of many air-borne xenobiotics. Nasal cancer has been reported to occur in experimental animals after inhalation exposure to a variety of important industrial chemicals, including formaldehyde (8), bis(chloromethyl)ether (9), hexamethylphosphoramide (10), phenylglycidyl ether (11), epichlorohydrin (12), and vinyl chloride (13). Many other chemicals including various *N*-nitroso compounds such as *N*-nitrosodiethylamine (14) and the tobacco-specific nitrosamines *N'*-nitrosonornicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (15), environmental pollutants such as benzo(*a*)pyrene (16), and therapeutic agents such as PA (17) have also been shown to induce nasal tumors in animals.

Recently, the biotransformation of foreign compounds in the nasal mucosa has been studied extensively. Numerous compounds have been shown to be metabolized *in vitro* by the nasal P-450-dependent monooxygenase system, some examples of which include nasal decongestants, essences, anesthetics, alcohols, nicotine, and cocaine, as well as many known nasal carcinogens (18). A number of these compounds are also metabolized *in vivo* in the nasal mucosa, as demonstrated by whole-body autoradiography (4) and other techniques (7). The results from these studies indicate that the nasal mucosa is in

This research was supported by Grant DK-10339 from the National Institutes of Health.

ABBREVIATIONS: PA, phenacetin; P-450, cytochrome P-450; HMPA, hexamethylphosphoramide; NMa and NMb, nasal microsomal cytochrome P-450 forms a and b, respectively; TBS, 20 mM Tris·HCl buffer, pH 7.4, containing 0.9% NaCl; HPLC, high pressure liquid chromatography.

many cases much more active (on a per mg of protein basis) than the other organs, including liver, in the metabolism of foreign compounds both *in vitro* and *in vivo*. This remarkable metabolic activity may contribute to the tissue-specific toxicity of many nasal carcinogens. In this context, the nasal mucosa may represent a very useful model for studying the relationship between P-450 and chemical carcinogenesis.

The isozyme composition of the nasal P-450 monooxygenase system has not been studied extensively, although it has received increasing attention in recent years. The total P-450 content of nasal microsomes was found to be relatively high, especially in the olfactory mucosa, which is second only to the liver (19, 20). In addition, the level of NADPH-cytochrome P-450 reductase is also quite high (20, 21). Our previous immunochemical study (20) indicated that P-450 isozymes[1] 2, 3a, and 4, but not 3b or 6, are present in the olfactory mucosa of untreated rabbits. More recently, two unique P-450 isozymes, termed NMa and NMb, were purified to electrophoretic homogeneity from rabbit olfactory microsomes (22). These new forms of P-450 are distinct from all other P-450 cytochromes described to date. In reconstituted systems, NMa was found to be very active in the metabolism of three nasal carcinogens, whereas NMb was found to form unique testosterone metabolites. These findings led us to propose that NMa may play a crucial role in the metabolism and toxicity of xenobiotics in the nasal mucosa, whereas NMb may be important in the metabolism of endogenous substances (22).

In the present study, antibodies to purified NMa and NMb were prepared and used to examine the tissue distribution of these forms. Our findings indicate that NMa, or an immunochemically and electrophoretically indistinguishable isozyme, is present in microsomes prepared from liver and from both olfactory and respiratory nasal mucosa. In contrast, NMb is detectable only in olfactory microsomes. Neither form of P-450 was detected in other tissues examined. In addition, evidence is presented for the differential expression of P-450 isozymes 2, 3a, and 4 in olfactory and respiratory mucosa.

Materials and Methods

Preparation of microsomes. New Zealand White male rabbits (2.0–2.5 kg in weight) were fasted for 12–14 hr before they were killed by pentobarbital injection. Liver and extrahepatic tissues, including brain, heart, lung, kidney, esophageal mucosa, nasal maxillary turbinals, the rear upper portion of the nasal septum, and the nasal ethmoturbinals were removed by disection, placed promptly in liquid nitrogen, and stored at −70° for up to 3 months. Frozen tissues from 3 to 12 rabbits were combined, washed with ice-cold 0.1 M Tris-acetate buffer, pH 7.4, containing 0.15 M KCl and 1.0 mM EDTA, and disrupted with a Brinkman Polytron Model PT 10/35 homogenizer. Pyrophosphate-washed microsomes were prepared as previously described (23). The nasal maxillary turbinals were used for the preparation of nasal respiratory microsomes, whereas the ethmoturbinals and the septa were combined for the preparation of olfactory microsomes. Intestinal mucosal cells were obtained from the segment of jejunum (about 20 cm in length) immediately distal to the pyloric valve, according to the technique of Weiser (24) as modified by Bonkovsky *et al.* (25), and the microsomes were then prepared (25).

Antibody production. Antibody to P-450 NMa was produced by immunization of mature male New Zealand White rabbits. Each animal was given an intradermal injection on the lower back of 50 μg of NMa in 0.5 ml of emulsified Freund's complete adjuvant (DIFCO Laboratories, Detroit, MI) and then injections at approximately 2-week intervals of 50 μg of NMa in 0.5 ml of incomplete Freund's adjuvant (GIBCO, Grand Island, NY) for about 3 months. Blood was collected 7 days after the third and subsequent immunizations and tested for antibody specificity by immunoblotting. Antibodies to NMb were produced by immunization of yearling female sheep, according to a procedure described previously for the production of antibody to P-450 form 3a (26), except that 125 μg of purified NMb were used for each injection. Monoclonal antibody to purified P-450 form 3a (1H.11) and monospecific goat antibody to purified P-450 form 2 were prepared as described earlier (20, 27). Polyclonal antibodies to purified P-450 form 3a (26) and to form 4 were produced by Dr. Dennis R. Koop of Case-Western Reserve University, by immunization of yearling female sheep.

Sheep anti-NMb antibody was made monospecific by a method described by Thomas *et al.* (28). Briefly, the IgG fraction was prepared from the immune serum by ammonium sulfate precipitation and DEAE-cellulose column chromatography (27), and an aliquot was applied to an immunosorbent affinity column packed with Affi-gel 10 (Bio-Rad) that was coupled to proteins of the 7–14% polyethylene glycol fraction of solubilized rabbit hepatic microsomes. The column effluent with absorbance at 280 nm was pooled and tested for specificity toward NMb by immunoblot analysis. The IgG concentration was determined spectrally with an absorption coefficient at 280 nm of 13.5 cm^{-1} for a 1% solution (26).

Gel electrophoresis and immunoblot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out using a discontinuous buffer system (29) in 7.5% polyacrylamide gels of 0.75-mm thickness. After electrophoresis, proteins were visualized by staining with silver (30) or were transferred to nitrocellulose sheets (31) that were then either treated with India ink for total protein staining (32) or incubated with an antibody for immunoblot analysis. The immunoblotting procedure described below represents a modification of the methods reported in previous studies (20, 33). The nitrocellulose sheets were incubated overnight at 4° with polyclonal sheep, goat, or rabbit anti-P-450 antibodies in TBS containing 3% bovine serum albumin or at room temperature with monoclonal mouse anti-3a IgG in TBS. The sheets were washed with TBS containing 0.05% Tween-20 (Bio-Rad), incubated for 1 hr at room temperature with a 1:500 dilution of peroxidase-conjugated rabbit anti-sheep, rabbit anti-goat, sheep anti-rabbit, or goat anti-mouse IgG, respectively, washed again, and then incubated for 40 min at room temperature with a 1:2000 dilution of peroxidase-conjugated sheep anti-rabbit, rabbit anti-sheep, or rabbit anti-goat IgG, respectively. Color development was carried out using 4-chloro-1-naphthol (Sigma) as a substrate for the peroxidase (34). Immunoquantitation was performed with a Zeiss soft laser densitometer, as previously described (33), with the use of purified antigens as standards.

Assay methods for catalytic activity. Oxidation of testosterone was assayed as previously described by van der Hoeven (35) and Wood *et al.* (36). The reaction mixtures contained 50 μmol of potassium phosphate buffer, pH 7.4, 140 nmol of testosterone, and either 0.2 nmol of microsomal P-450 or a reconstituted P-450 system containing 0.05 nmol of NMb, 0.15 nmol of rabbit NADPH-cytochrome P-450 reductase, and 15 μg of dilauroylglyceryl-3-phosphorylcholine in a sonicated aqueous suspension. Testosterone was added to individual assay tubes in a mixture of methanol (200 μl), chloroform (400 μl), and polyethylene glycol (two drops). The volatile solvents were evaporated under a stream of nitrogen before the addition of other components of the incubation mixture. In experiments to determine the effect of anti-NMb on testosterone oxidation, IgG was added to the incubation mixture at a level of 9 mg of protein/nmol of microsomal P-450. All reactions were initiated by addition of 2 μmol of NADPH, to give a final volume of 1.0 ml, and were conducted at 37° for 30 min. The reaction mixtures were extracted with methylene chloride, the extract

[1] The trivial names for individual rabbit hepatic P-450 isozymes and the corresponding designations as recommended by Nebert *et al.* (49) are as follows: form 2, IIB4; form 3a, IIE1; form 3b, IIC3; form 3c, IIIA6; form 4, IA2; form 5, IVB1; and form 6, IA1.

was taken to dryness, and the residue was then dissolved in 300 μl of methanol/water (50:50). Aliquots (20 to 40 μl) of the testosterone metabolites were analyzed by HPLC, using a 5-μm C18 reverse phase column from Supelco (150 × 4.6 mm i.d.) with a liquid chromatographic system as described previously (22). The metabolites were eluted isocratically with a methanol/water/acetonitrile (47.5:42.5:10) mobile phase. The formaldehyde formed from HMPA *N*-demethylation was measured by a fluorometric modification (37) of the method of Nash (38), and the formation of acetaldehyde from *O*-deethylation of PA was determined by gas chromatography of the headspace gas of the reaction mixture, as described previously for alcohol oxidation assays (26).

Other methods. Purified hepatic and nasal microsomal forms of P-450 and NADPH-cytochrome P-450 reductase were isolated as described previously (22). Protein was determined by the method of Lowry *et al.* (39), and the concentration of total P-450 in microsomal suspensions was determined according to the method of Omura and Sato (40). NADPH-cytochrome P-450 reductase activity was measured by the reduction of cytochrome *c*, and the amount of reductase was calculated using a turnover number of 4030 for this substrate with the purified enzyme (41). It was assumed that this value is the same for the reductase in all tissues examined.

Materials. Peroxidase-conjugated IgG fractions were from Cappel Laboratories. The source of testosterone derivatives has been described previously (22); 15β-hydroxytestosterone was a gift from Dr. T. A. van der Hoeven (Albany Medical College).

Results

Immunochemical evidence for tissue-specific distribution of P-450 NMa and NMb. P-450 NMa and NMb purified from rabbit olfactory nasal microsomes are separable by sodium dodecyl sulfate-polyacrylamide gel electrophoresis despite their rather similar mobilities (22), and the same is true of the hepatic P-450 forms 2 and 3a. However, as shown in Fig. 1, NMa comigrates with purified hepatic form 2, as does NMb with form 3a. Polyclonal antibodies to purified NMa and NMb were prepared in rabbits and sheep, respectively, and used in immunoblotting experiments to examine the tissue distribution of these cytochromes. As may be seen in Fig. 2, Experiment 1, anti-NMa immune serum recognized purified NMa (Fig. 2, Experiment 1, *lane a*) and detected a band with the same electrophoretic mobility as that of NMa in microsomal samples from olfactory nasal mucosa (Fig. 2, Experiment 1, *lane b*), liver (Fig. 2, Experiment 1, *lane c*), and respiratory nasal mucosa (Fig. 2, Experiment 1, *lane d*), but not in pulmonary microsomes (Fig. 2, Experiment 1, *lane e*). The specificity of the antibody is demonstrated by the observation that a single band was detected in both the olfactory and hepatic microsomes and that isozyme 2, which is a major form in lung microsomes (42), was not recognized (Fig. 2, Experiment 1, *lane e*). A very faint band with apparently higher molecular weight than NMa was observed in microsomes from respiratory nasal mucosa (Fig. 2, Experiment 1, *lane d*) and lung (Fig. 2, Experiment 1, *lane e*) and in olfactory microsomes as well when larger amounts of microsomal protein were applied (not shown). An immunochemical relationship of this higher band to NMa was ruled out, however, because this band was also detected when preimmune rabbit serum was used in place of the immune serum (data not shown).

The sheep immunoserum to NMb had much higher titer than that of rabbit anti-NMa (see Fig. 2, Experiment 2, *lanes b* and *h*), but it displayed weak cross-reaction with several other isozymes including, in the order of decreasing affinity, NMa (Fig. 2, Experiment 2, *lane e*), hepatic isozymes 2 (Fig. 2, Experiment 2, *lane j*), 3b (Fig. 2, Experiment 2, *lane a*), and 3a (Fig. 2, Experiment 2, *lane j*). The reactions with isozymes 3a and 3b were barely detectable in this experiment. It appears that the lower band seen in hepatic (Fig. 2, Experiment 2, *lanes c* and *d*) and olfactory microsomes (Fig. 2, Experiment 2, *lane i*), as well as the band detected in respiratory microsomes (Fig. 2, Experiment 2, *lanes f* and g), represents NMa and/or isozyme 2. However, despite the weak cross-reactivity, no band with the same molecular weight as NMb was detected in either hepatic (Fig. 2, Experiment 2, *lanes c* and d) or respiratory nasal microsomes (Fig. 2, Experiment 2, *lanes f* and *g*). In experiments not presented, the upper band seen in hepatic microsomes (Fig. 2, Experiment 2, *lanes c* and d) comigrated with purified isozyme 3b but ran slower than purified NMb. Similar results were obtained with hepatic microsomes from rabbits treated with acetone or phenobarbital (not shown). The specific occurrence of NMb in the olfactory mucosa was further demonstrated by immunoblot analysis with monospecific anti-NMb IgG (Fig. 2, Experiment 3).

Thus, we conclude that NMa or an immunochemically and electrophoretically indistinguishable isozyme is expressed in both olfactory and respiratory nasal mucosa, as well as in liver of male rabbits (and also in female rabbits, in results not shown). In contrast, NMb is expressed at detectable levels only in olfactory microsomes. In additional experiments not presented, microsomes prepared from male and female brain, heart, kidney, and lung, as well as male esophageal mucosa and intestinal mucosal cells, were subjected to immunoblot analysis with anti-NMa and anti-NMb. Neither of these forms of P-450 was detected in these tissues with up to 40 μg of microsomal

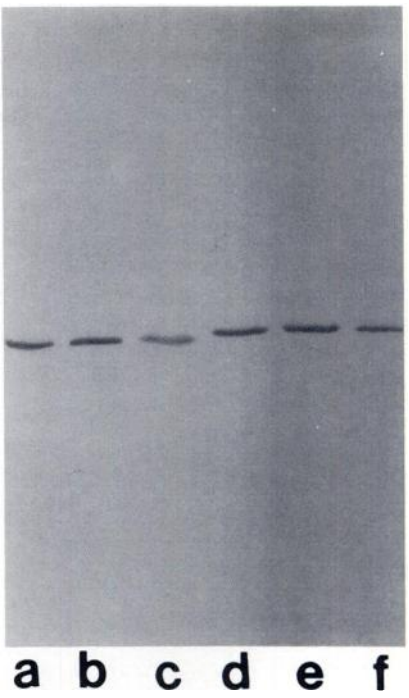


Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified P-450 isozymes. Protein samples were subjected to electrophoresis in the discontinuous buffer system, in a 7.5% polyacrylamide gel, and stained with silver. Migration was from *top* to *bottom*. The samples, with the amounts of protein indicated, are as follows: *lane a*, isozyme 2 (75 ng); *lane b*, isozyme 2 (50 ng) and NMa (70 ng); *lane c*, NMa (110 ng); *lane d*, NMb (110 ng); *lane e*, NMb (70 ng) and isozyme 3a (50 ng); and *lane f*, isozyme 3a (75 ng).

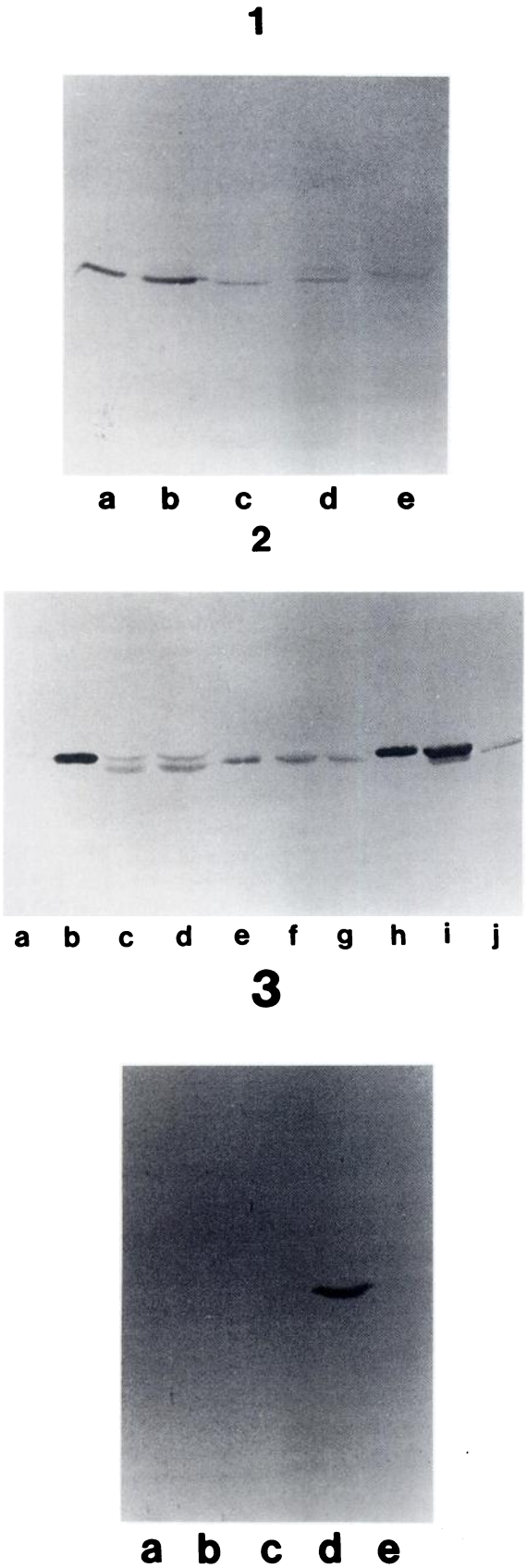


Fig. 2. Immunoblot analysis of various P-450 samples with anti-NMa and anti-NMb. P-450 samples were subjected to electrophoresis in 7.5% polyacrylamide gels, transferred to nitrocellulose sheets, and immunochemically stained with rabbit anti-NMa antiserum (1:400 dilution, Experiment 1), sheep anti-NMb antiserum (1:2000 dilution, Experiment 2), or monospecific sheep anti-NMb IgG (5 μg/ml, Experiment 3), as described in Materials and Methods. The amounts of protein are as follows. Experiment 1: *lane a*, purified NMa (40 ng); *lanes b*, *c*, and *d*, microsomes from olfactory mucosa, liver and respiratory mucosa, respectively (5 μg); and *lane e*, lung microsomes (20 μg). Experiment 2: *lane a*, *b*, and *e*, purified hepatic isozymes 3b, NMb, and NMa, respectively (70 ng); *lanes c* and *d*, liver microsomes (5 and 10 μg, respectively); *lanes f* and *g*, respiratory microsomes (20 and 10 μg, respectively); *lane h*, purified NMb (35 ng); *lane i*, olfactory microsomes (5 μg); and *lane j*, purified isozyme 2 (70 ng) and purified 3a (140 ng). Experiment 3: *lane a*, kidney microsomes (20 μg); *lanes b*, *c*, *d*, and *e*, microsomes from respiratory mucosa (10 μg), lung (15 μg), olfactory mucosa (5 μg), and liver (10 μg), respectively.

protein applied per sample well and a detection limit of less than 50 fmol of purified NMa or NMb.

Differential expression of other P-450 isozymes in olfactory and respiratory nasal mucosa. Immunoblot analysis of nasal microsomes from the olfactory and respiratory mucosa was carried out with antibodies to purified hepatic isozymes 2, 3a, and 4, as shown in Fig. 3. A band with the same mobility as purified isozyme 2 was detected in both olfactory and respiratory microsomes with goat antibody to purified isozyme 2; this antibody does not cross-react with NMa or with other hepatic isozymes (data not shown). It should be noted that the intensity of the band detected in respiratory microsomes is much greater than that in olfactory microsomes. The relative abundance of isozyme 2 (or a closely related form) in the nasal tissues agrees with the rate of benzphetamine *N*-demethylation, a reaction in which this form of P-450 is especially active (43). The turnover number for formaldehyde formation from this drug in respiratory microsomes is larger than that in olfactory microsomes (4.6 versus 2.5 nmol/min/mg of protein), even though the total P-450 content in the latter source is much higher (see Table 1).

Isozyme 3a, the alcohol-inducible form of P-450 first purified from liver (44), has been identified in rabbit nasal mucosa in previous studies (20). Differential expression of this isozyme in different regions of the nasal cavity was found in the present study with use of a monoclonal anti-3a that does not cross react with either NMa or NMb. As shown in Fig. 3, Experiment 2, isozyme 3a was detected in olfactory but not in respiratory

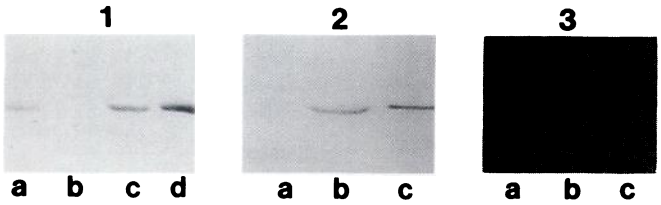


Fig. 3. Immunoblot analysis of olfactory and respiratory nasal microsomes with antibodies to purified hepatic P-450 isozymes 2, 3a, and 4. P-450 samples were subjected to electrophoresis, transferred to nitrocellulose sheets, and examined immunochemically with goat anti-isozyme 2 IgG (50 μg of protein/ml), monoclonal mouse anti-isozyme 3a IgG (20 μg of protein/ml), or sheep anti-isozyme 4 IgG (5 μg of protein/ml), as described in Materials and Methods. Experiment 1: *lane a*, olfactory microsomes (10 μg of protein); *lane b*, purified hepatic isozyme 4 (50 ng); *lane c*, respiratory microsomes (15 μg); and *lane d*, purified hepatic isozyme 2 as a standard (50 ng). Experiment 2: *lane a*, respiratory microsomes (30 μg); *lane b*, olfactory microsomes (20 μg); and *lane c*, purified hepatic isozyme 3a (5 ng). Experiment 3: *lane a*, respiratory microsomes (15 μg); *lane b*, purified hepatic isozyme 4 (50 ng); and *lane c*, olfactory microsomes (10 μg).

microsomes. Similar results were obtained with a polyclonal anti-3a antibody (data not shown).

Differential expression of isozyme 4 was also observed, as shown in Fig. 3, Experiment 3. With anti-isozyme 4 antibody, a band was detected in the olfactory microsomes that migrated at the same position as purified isozyme 4, but such a band was not detected in respiratory microsomes. The immunochemical identification of isozyme 4 in rabbit olfactory mucosa has been reported in a previous study (20). It is interesting to note that this P-450 isozyme has not been found in any other extrahepatic tissues (45). The sheep antibody used in this experiment has a strong cross-reaction with isozyme 6 (data not shown); therefore, the data also demonstrate that isozyme 6 is not present at a detectable level in either of the nasal tissues. In experiments not presented, isozyme 3b, which is recognized by polyclonal anti-3a antibody, was not detected in either of the nasal preparations.

Quantitation of the protein components of the monooxygenase system in nasal mucosa. The much lower molar ratio of total P-450 to NADPH-cytochrome P-450 reductase in nasal than in hepatic microsomes was reported previously (20). As shown in Table 1, this ratio was identical for the two nasal preparations, despite the difference in total P-450 content. Also given is the specific content of individual P-450 cytochromes as determined by quantitative immunoblot analysis. NMa and NMb represent the major P-450 isozymes in olfactory microsomes, with specific contents of 0.25 and 0.30 nmol/mg of microsomal protein, respectively. Similar to the situation in hepatic microsomes, the level of isozyme 4 (or an immunochemically indistinguishable isozyme) is also quite high in the olfactory tissue; on the other hand, the level of isozyme 2 is low, accounting for less than 5% of the total P-450. The specific content of isozyme 3a is very low, being less than 1% of the total P-450 in this tissue, as determined with the monoclonal anti-3a. It should be noted that the combined content of NMa, NMb, and isozyme 4 somewhat exceeds the spectroscopically determined content of total microsomal P-450, which apparently indicates that part of the P-450 is present as the apocytochrome or reflects possible inaccuracy in the immunoblot quantitation procedure.

The level of NMa in the respiratory microsomes is much lower than that in olfactory microsomes, although it accounts for 26% of the total P-450. On the other hand, the level of isozyme 2 is higher in the respiratory than in the olfactory tissue. These two cytochromes appear to represent the major P-450 isozymes in the respiratory mucosa. The content of NMa in hepatic microsomes is similar to that in respiratory microsomes but, as expected from previous purification studies (22), this isozyme represents less than 3% of the total P-450. Preliminary experiments with microsomes from rabbits treated with ethanol, acetone, imidazole, phenobarbital, or isosafrole indicate that the level of NMa in hepatic microsomes is not increased by these classical P-450 inducers (data not shown).

Tissue-specific formation of NMb-dependent testosterone metabolites. The differential expression of P-450 isozymes in the nasal and hepatic microsomes is believed to account largely for the tissue-specific metabolic profiles toward a variety of P-450 substrates, such as testosterone. As shown in Fig. 4A, more than 10 different metabolites of testosterone were formed by olfactory microsomes. Among these, 15α-, 15β-, 6β-, 16α-, 16β- and 11β-hydroxytestosterone, as well as androstenedione, were identified based on comigration with standards in the HPLC analysis. The peak labeled with an *asterisk* in Fig. 4A was previously tentatively identified as 19-hydroxytestosterone based on HPLC analysis (22), but the different chromatographic conditions used in the present study resulted in separation of this metabolite from the authentic 19-hydroxy compound. The identity of this and the other metabolites is being studied by gas chromatography-mass spectrometry analysis. In experiments not presented, only the first peak (solvent front) and the substrate peak were observed when the reaction was quenched before the addition of NADPH.

The effect of anti-NMb on the olfactory microsomal metabolism of testosterone was examined, as shown in Fig. 4B. Anti-NMb IgG, which inhibited more than 90% of the activity of purified NMb in reconstituted systems with HMPA as substrate (data not shown), was included in the incubation mixture at a level of 9 mg of protein/nmol of P-450. As indicated by the *arrowheads* in Fig. 4B, the formation of at least six metabolites was inhibited, including 15α-, 16α-, 16β-, and 11β-hydroxytestosterone, as well as the unknown product indicated by the *asterisk* and a metabolite that ran close to 15β-hydroxytestosterone; these metabolites correspond in retention time to the products formed by purified NMb in reconstituted systems (Fig. 4C). Interestingly, the magnitudes of the other peaks in Fig. 4B, such as those corresponding to 6β-hydroxytestosterone and androstenedione, were not affected, indicating that the inhibition is specific to NMb-catalyzed reactions.

The respiratory nasal microsomes are not as active as the olfactory microsomes in testosterone metabolism (see Fig. 5A). This is indicated by the lower magnitudes of all peaks, including 15β-, 6β-, and 16α-hydroxytestosterone as well as androstenedione and the unidentified metabolites between 16α-hydroxytestosterone and androstenedione. It is not clear whether the

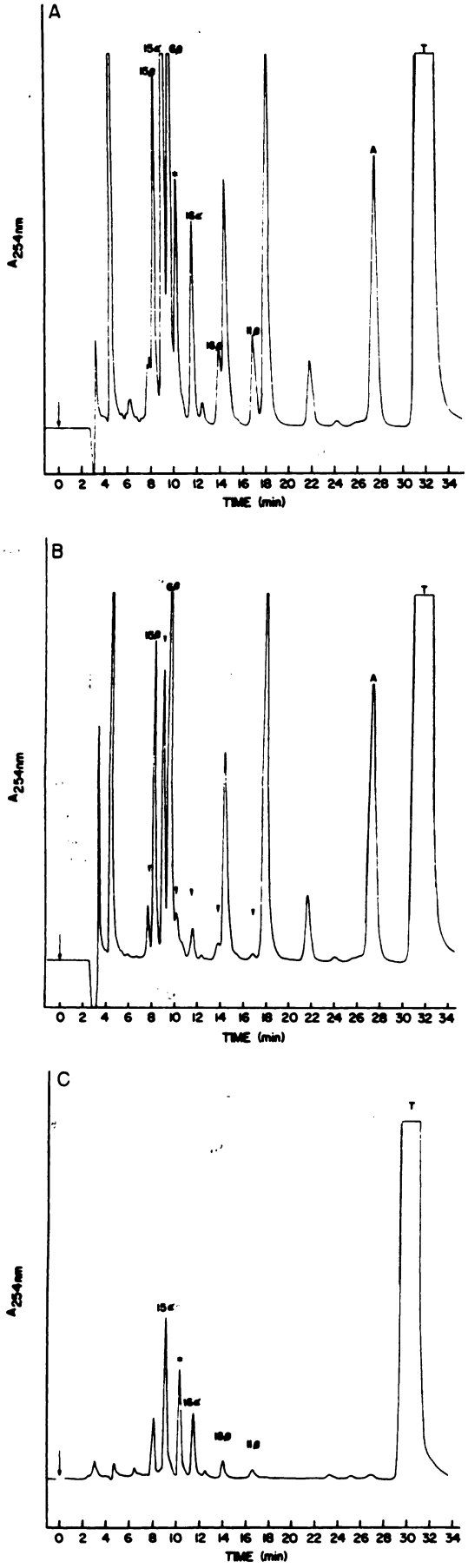
TABLE 1

Level of components of the microsomal monooxygenase system in hepatic and nasal tissues

Microsomes were prepared from tissues pooled from 9 to 12 rabbits. The levels of individual P-450 isozymes were determined by quantitative immunoblot analysis, as described in Materials and Methods. The immunochemical results are an average of duplicate or triplicate experiments, in each of which the microsomal sample and the standard purified P-450 were compared at four or more protein concentrations. The values for the reductase and total P-450 are an average of four experiments.

Source of microsomes	NADPH-cytochrome P-450 reductase content	Cytochrome P-450 content					
		Total	NMa	NMb	2	3a	4
		nmol/mg of protein					
Liver	0.060	2.1 (35)[a]	0.05	ND[b]	0.12	0.16	0.73
Nasal mucosa, olfactory	0.086	0.60 (7)	0.25	0.30	0.03	<0.01	0.19
Nasal mucosa, respiratory	0.031	0.23 (7)	0.06	ND	0.08	ND	ND

[a] Numbers in parentheses indicate the molar ratio of total P-450 to reductase.
[b] ND, not detectable.



peak between 15β- and 6β-hydroxytestosterone corresponds to 15α-hydroxytestosterone; however, no peaks corresponding to the unknown product (Fig. 4) or 11β-hydroxytestosterone were found. The absence of the unknown product as well as of the other NMb-dependent metabolites, except for 16α-hydroxytestosterone, was also apparent in incubations with hepatic microsomes (Fig. 5B). Furthermore, the addition of anti-NMb to the incubation mixture did not result in any changes in the metabolic profile with either respiratory or hepatic microsomes (data not shown). Thus, neither NMb apoprotein nor NMb-dependent catalytic activity could be detected in microsomes from these two tissues.

Immunochemical evidence that NMa is the major catalyst in HMPA and PA metabolism in nasal microsomes. The role of P-450 isozymes in microsomal metabolism of the two nasal carcinogens was examined by antibody inhibition experiments with goat anti-isozyme 2 and sheep anti-isozyme 3a IgG. The latter (which was used in place of anti-NMa, a noninhibitory antibody preparation) was shown to cross-react with NMa on immunoblots and to inhibit about 50% of the butanol oxidation activity of purified NMa in a reconstituted system at a ratio of 10 mg of IgG protein/nmol of P-450 (data not shown). This antibody is useful for estimating the contribution of NMa to the nasal microsomal metabolism of HMPA and PA because isozyme 3a, the authentic antigen, is very poorly active toward these substrates in reconstituted systems (22), and the level of isozyme 3a is either not detectable or very low in the nasal tissues. It should be noted, though, that the NMa-dependent activity determined in this way may be underestimated, considering the partial inhibition observed with purified NMa. Thus, as shown in Table 2, NMa catalyzes at least 50 to 60% of HMPA and PA metabolism in both olfactory and respiratory microsomes. A very small contribution from NMb in the olfactory microsomes to these reactions was evident (data not shown). In addition, isozyme 2, which is a major P-450 in the respiratory mucosa and is very active toward HMPA in reconstituted systems (22), also makes a significant contribution to HMPA metabolism in respiratory but not in olfactory microsomes. In experiments not presented, hepatic microsomes displayed turnover numbers that are 5 to 10 times lower than that of nasal microsomes with HMPA and PA and the contribution of NMa is rather small. No increase in nasal or hepatic microsomal metabolism of HMPA or PA was observed after treatment of rabbits with acetone (data not shown), which confirms that the inhibition by anti-3a observed in nasal tissues is not due to its effect on isozyme 3a.

Fig. 4. Effect of anti-NMb on the metabolism of testosterone by olfactory microsomes. Metabolite formation was determined following incubation of 140 μM testosterone with 0.2 μM olfactory nasal microsomal P-450 in the absence (A) or presence (B) of anti-NMb IgG (1.8 mg of protein/ml) or upon incubation with purified NMb in a reconstituted system (C). The procedures were as described in Materials and Methods. HPLC profiles were obtained with a Supelco reverse phase C18 column with use of a methanol/water/acetonitrile mobile phase. The samples (20 μl each) were injected, isocratic elution was carried out at a flow rate of 0.5 ml/min, and the absorbance at 254 nm was monitored at a sensitivity of 0.05 absorbance units, full scale. The *arrow* at 0 min indicates the time of sample injection. *A* and *T*, androstenedione and testosterone, respectively; the remaining abbreviations represent various monohydroxy derivatives of testosterone. The *asterisks* in A and C indicate an unidentified metabolite that is formed specifically by NMb, and the *arrowheads* in B indicate the location of the NMb-dependent metabolites, which were at a decreased level as compared with A.

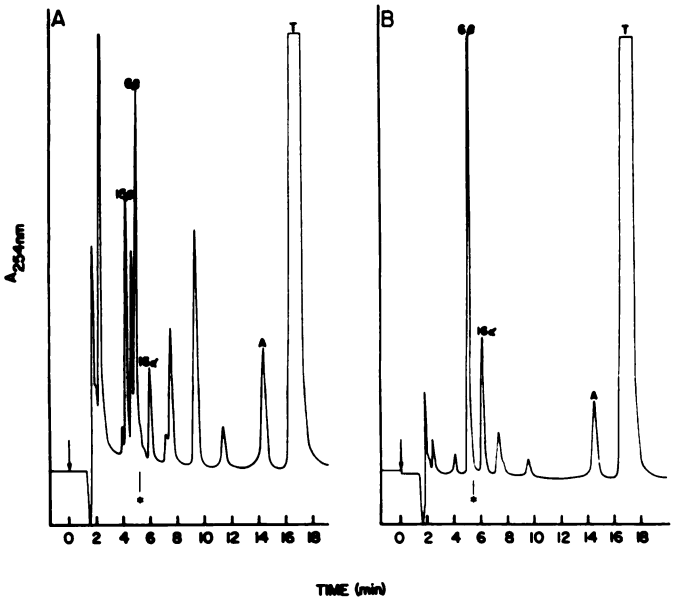


Fig. 5. HPLC profile of testosterone metabolites formed by microsomes from respiratory mucosa and liver. The metabolites were formed by incubation of 0.2 μM respiratory (A) or hepatic microsomal P-450 (B) with 140 μM testosterone. Analytical conditions were as described in the legend to Fig. 4, except that samples of 40 μl were injected, and elution was carried out at a flow rate of 1.0 ml/min. For comparison, the *asterisk* and *vertical line* indicate where the unidentified testosterone metabolite formed by NMb (see Fig. 4) would have eluted. Abbreviations are as in Fig. 4.

TABLE 2

Role of P-450 isozymes in microsomal metabolism of HMPA and PA

Reaction mixtures containing 50 mM potassium phosphate buffer, pH 7.4, 0.1 μM microsomal P-450, 0.1 mM HMPA or 2 mM PA, 1 mM NADPH, and 0.5 mg of IgG protein in a final volume of 0.5 ml were incubated at 37° for 30 min. Microsomes were prepared from tissues pooled from 9 to 12 rabbits. HMPA demethylation or PA deethylation catalyzed by microsomal P-450 was determined in mixtures to which preimmune IgG or goat anti-isozyme 2 IgG was added. The dealkylation due to NMa was estimated in reaction mixtures with sheep anti-3a IgG added. The activities for the individual isozymes were calculated as the difference between the rate observed in the presence of preimmune IgG and that in the presence of immune IgG; the average of duplicate determinations is reported. Numbers in parentheses represent percentage of total microsomal activity.

Tissue	Substrate	Catalytic activity		
		Total	Due to form 2	Due to NMa
		nmol/min/nmol of P-450		
Olfactory	HMPA	6.0	0.9 (15%)	2.9 (48%)
Respiratory	HMPA	3.9	1.4 (36%)	2.3 (59%)
Olfactory	PA	13.5	1.6 (12%)	6.8 (50%)
Respiratory	PA	13.7	1.0 (7%)	8.1 (59%)

Discussion

It is well accepted that, although extrahepatic tissues may possess a subset of P-450 isozymes that are found in liver, they may also contain P-450 forms that are distinct from the hepatic isozymes. Available data indicate that exclusively extrahepatic forms, such as the steroidogenic forms of P-450 (46), are essential for physiological functions. Thus, by analogy, the specific occurrence of NMb in the olfactory mucosa suggests that this P-450 isozyme may be important for the physiological function of olfactory tissue. On the other hand, the identification of NMa in both olfactory and respiratory mucosa, as well as in liver, suggests that it is probably not restricted to olfaction-related functions. The much higher level of this isozyme in nasal tissue than in liver may reflect its primary involvement in the metabolism of inhaled chemical compounds. The broad substrate specificity and high catalytic capacity of this isozyme (22) would make it suitable for removing odorant compounds from the olfactory mucosa and, thereby, maintaining olfactory sensitivity. In this connection, Nef *et al.* (47) have proposed a role in olfactory perception for a rat nasal P-450 protein for which the cDNA has been obtained. The predicted NH_2-terminal sequence shows some similarity to that of P-450 NMb (22).

Thus, it is reasonable to speculate that all P-450s involved in xenobiotic metabolism are expressed in liver, which has a strategic location in the general circulation and a high metabolic capacity that make it a key participant in the disposition of xenobiotics entering the body through all routes. However, the unique chemical environment of a given extrahepatic tissue dictates the selective expression and relative level of individual xenobiotic-metabolizing P-450 isozymes. This is illustrated by the present study as well as by the recent report of Sabourin *et al.* (48) on the differential distribution of microsomal P-450s 2, 4, 5, and 6 in the rabbit respiratory tract. Exclusively extrahepatic isozymes such as NMb, in contrast, may be expressed to meet the physiological need of an individual tissue and, therefore, may not be required in the liver. Based on this hypothesis, we predict that further studies on a variety of extrahepatic tissues will lead to the discovery of additional unique forms of cytochrome P-450.

Acknowledgments

We are grateful to Dr. Barry G. Englund, Department of Pathology, and Dr. Todd D. Porter, Department of Biological Chemistry, The University of Michigan, for assistance is the production of antibodies to P-450 NMa and P-450 NMb.

References

1. Stott, W. T., J. C. Ramsey, and M. J. McKenna. Absorption of chemical vapors by the upper respiratory tract of rats, in *Toxicology of the Nasal Passages* (C. S. Barrow, ed.). Hemisphere Publishing Corp., Washington, D. C., 191–210 (1986).
2. Natusch, D. S., and J. R. Wallace. Urban aerosol toxicity: the influence of particle size. *Science (Wash. D. C.)* **186**:695–699 (1974).
3. Schlesinger, R. B. Comparative deposition of inhaled aerosols in experimental animals and humans: a review. *J. Toxicol. Environ. Health* **15**:197–214 (1985).
4. Brittebo, E. B., A. Castonguay, J. J. Rafter, B. Kowalski, M. Åhlman, and I. Brandt. Metabolism of xenobiotics and steroid hormones in the nasal mucosa, in *Toxicology of the Nasal Passages* (C. S. Barrow, ed.). Hemisphere Publishing Corp., Washington, D. C., 211–234 (1986).
5. Bond, J. A. Bioactivation and biotransformation of xenobiotics in rat nasal tissue, in *Toxicology of the Nasal Passages* (C. S. Barrow, ed.). Hemisphere Publishing Corp., Washington, D. C., 249–261 (1986).
6. Dahl, A. R. Possible consequences of cytochrome P-450-dependent monooxygenases in nasal tissues, in *Toxicology of the Nasal Passages* (C. S. Barrow, ed.). Hemisphere Publishing Corp., Washington, D. C., 263–273, (1986).
7. Petridou-Fischer, J., S. L. Whaley, and A. R. Dahl. *In vivo* metabolism of nasally instilled benzo(*a*)pyrene in dogs and monkeys. *Toxicology* **48**:31–40 (1988).
8. Swenberg, J. A., W. D. Kerns, R. I. Mitchell, E. J. Gralla, and K. L. Pavkov. Induction of squamous cell carcinomas of the rat nasal cavity by inhalation exposure to formaldehyde vapor. *Cancer Res.* **40**:3398–3402 (1980).
9. Leong, B. K. J., R. J. Kociba, and G. C. Jersey. A lifetime study of rats and mice exposed to vapors of bis(chloromethyl)ether. *Toxicol. Appl. Pharmacol.* **58**:269–281 (1981).
10. Lee, K. P., and H. J. Trochimowicz. Metaplastic changes of nasal respiratory epithelium in rats exposed to hexamethylphosphoramide (HMPA) by inhalation. *Am. J. Pathol.* **106**:8–19 (1982).
11. Lee, K. P., P. W. Schneider, and H. J. Trochimowicz. Morphological expression of glandular differentiation in the epidermoid nasal carcinomas induced by phenylglycidyl ether inhalation. *Am. J. Pathol.* **111**:140–148 (1983).
12. Laskin, S., A. R. Sellakumar, M. Kuschner, N. Nelson, S. La Mendola, G. M. Rusch, G. V. Katz, N. C. Dulak, and R. E. Albert. Inhalation carcinogenicity of epichlorohydrin in noninbred Sprague-Dawley rats. *J. Natl. Cancer Inst.* **65**:751–757 (1980).
13. Feron, V. J., and R. Kroes. One year time-sequence inhalation toxicity study of vinyl chloride in rats. II. Morphological changes in the respiratory tract,

ceruminous glands, brain, kidney, heart and spleen. *Toxicology* **13**:131–141 (1979).
14. Lijinsky, W. Structure-activity relations in carcinogenesis by *N*-nitroso compounds, in *Genotoxicology of N-Nitroso Compounds* (T. K. Rao, W. Lijinsky, and J. L. Epler, eds.). Plenum Press, New York, 189–231 (1984).
15. 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).
16. Thyssen, J., L. Altoff, G. Kimmerle, and U. Mohr. Inhalation studies with benzo(*a*)pyrene in Syrian golden hamsters. *J. Natl. Cancer Inst.* **66**:575–577 (1981).
17. 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).
18. Dahl, A. R. The effect of cytochrome P-450-dependent metabolism and other enzyme activities on olfaction, in *Molecular Neurobiology of the Olfactory System* (F. L. Margolis and T. V. Getchell, eds.). Plenum Press, New York, 51–70 (1988).
19. Hadley, W. M., and A. R. Dahl. Cytochrome P-450-dependent monooxygenase activity in nasal membranes of six species. *Drug Metab. Dispos.* **11**:275–276 (1983).
20. Ding, X., D. R. Koop, B. L. Crump, and M. J. Coon. Immunochemical identification of cytochrome P-450 isozyme 3a ($P\text{-}450_{ALC}$) in rabbit nasal and kidney microsomes and evidence for differential induction by alcohol. *Mol. Pharmacol.* **30**:370–378 (1986).
21. Reed, C. J., E. A. Lock, and F. De Matteis. NADPH-cytochrome P-450 reductase in olfactory epithelium. *Biochem. J.* **240**:585–592 (1986).
22. Ding, X., and M. J. Coon. Purification and characterization of two unique forms of cytochrome P-450 from rabbit nasal microsomes. *Biochemistry* **27**:8330–8337 (1988).
23. Coon, M. J., T. A. van der Hoeven, S. B. Dahl, and D. A. Haugen. Two forms of liver microsomal cytochrome P-450, $P\text{-}450_{LM2}$ and $P\text{-}450_{LM4}$ (rabbit liver). *Methods Enzymol.* **52**:109–117 (1978).
24. Weiser, M. M. Intestinal epithelial cell surface membrane glycoprotein synthesis. I. An indicator of cellular differentiation. *J. Biol. Chem.* **248**:2536–2541 (1973).
25. Bonkovsky, H. L., H. P. Hauri, U. Marti, R. Gasser, and U. A. Meyer. Cytochrome P-450 of small intestinal epithelial cells: immunochemical characterization of the increase in cytochrome P-450 caused by phenobarbital. *Gastroenterology* **88**:458–467 (1985).
26. 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).
27. Dean, W. L., and M. J. Coon. Immunochemical studies on two electrophoretically homogeneous forms of rabbit liver microsomal cytochrome P-450: $P\text{-}450_{LM2}$ and $P\text{-}450_{LM4}$. *J. Biol. Chem.* **252**:3255–3261 (1977).
28. Thomas. P. E., D. Korzeniowski, D. Ryan, and W. Levin. Preparation of monospecific antibodies against two forms of rat liver cytochrome P-450 and quantitation of these antigens in microsomes. *Arch. Biochem. Biophys.* **192**;524–532 (1979).
29. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *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. Towbin, H., T. Staehelin, and J. Gordon. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354 (1979).
32. Hancock, K., and V. C. W. Tsang. India ink staining of proteins on nitrocellulose paper. *Anal. Biochem.* **133**:157–162 (1983).
33. 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).
34. Nielson, P. J., K. L. Manchester, H. Towbin, J. Gordon, and G. Thomas. The phosphorylation of ribosomal protein S6 in rat tissues following cycloheximide injection, in diabetes, and after denervation of diaphragm: a simple immunological determination of the extent of S6 phosphorylation on protein blots. *J. Biol. Chem.* **257**:12316–12321 (1982).
35. van der Hoeven, T. Testosterone oxidation by rat liver microsomes: effects of phenobarbital pretreatment and the detection of seven metabolites by HPLC. *Biochem. Biophys. Res. Commun.* **100**:1285–1291 (1981).
36. Wood, A. W., D. E. Ryan, P. E. Thomas, and W. Levin. Regio- and stereoselective metabolism of two C19 steroids by five highly purified and reconstituted rat hepatic cytochrome P-450 isozymes. *J. Biol. Chem.* **258**:8839–8847 (1983).
37. Belman, S. The fluorimetric determination of formaldehyde. *Anal. Chim. Acta.* **29**:120–126 (1963).
38. Nash, T. The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem. J.* **55**:416–421 (1953).
39. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
40. 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).
41. 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).
42. Domin, B. A., T. R. Devereux, and R. M. Philpot. The cytochrome P-450 monooxygenase system of rabbit lung: enzyme components, activities, and induction in the nonciliated bronchiolar epithelial (Clara) cell, alveolar type II cell, and alveolar macrophage. *Mol. Pharmacol.* **30**;296–303 (1986).
43. Koop, D. R., A. V. Persson, and M. J. Coon. Properties of electrophoretically homogeneous, constitutive forms of liver microsomal cytochrome P-450. *J. Biol. CHem.* **256**:10704–10711 (1981).
44. 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).
45. Schwab, G. E., and E. F. Johnson. Enzymology of rabbit cytochrome P-450, in *Mammalian Cytochromes P-450* (F. P. Guengerich, ed.). CRC Press, Boca Raton, FL, 55–105 (1987).
46. Waterman, M. R., M. E. John, and E. R. Simpson. Regulation of synthesis and activity of cytochrome P-450 enzymes in physiological pathways, in *Cytochrome P-450* (P. R. Ortiz de Montellano, ed.). Plenum Publishing Corp., New York, 345–386 (1986).
47. Nef, P., J. Heldman, D. Lazard, T. Margalit, M. Jaye, I. Hanukoglu, and D. Lancet. Olfactory-specific cytochrome P-450: cDNA cloning of a novel neuroepithelial enzyme possibly involved in chemoreception. *J. Biol. Chem.* **264**:6780–6785 (1989).
48. Sabourin, P. J., R. E. Tynes, R. M. Philpot, S. Winquist, and A. R. Dahl. Distribution of microsomal monooxygenases in the rabbit respiratory tract. *Drug Metab. Dispos.* **16**:557–562 (1988).
49. Nebert D. W., D. R. Nelson, M. Adesnik, M. J. Coon, R. W. Estabrook, F. J. Gonzalez, F. P. Guengerich, I. C. Gunsalus, E. F. Johnson, B. Kemper, W. Levin, I. A. Phillips, R. Sato, and M. R. Waterman. The P450 superfamily: updated listing of all genes and recommended nomenclature for the chromosomal loci. *DNA* **8**:1–13 (1989).

Send reprint requests to: Minor J. Coon, Department of Biological Chemistry, Medical School, The University of Michigan, Ann Arbor, MI 48109-0606.