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Review

Alcohol-inducible cytochrome P-450 ($P\text{-}450_{ALC}$)*

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Abstract. Of the family of P-450 cytochromes occurring in rabbit liver microsomes, only isozyme 3a ($P\text{-}450_{ALC}$) is induced by alcohol administration and is effective in catalyzing the reaction: ethanol $+0_2+NADPH+H^+\rightarrow$ acetaldehyde $+2\,H_2O+NADP^+$. As judged by immunochemical quantitation, $P\text{-}450_{ALC}$ is also induced in the animals by other diverse agents, including imidazole, trichlorethylene, acetone, pyrazole, and isoniazid. Evidence has been obtained for the occurrence of a protein immunochemically related to $P\text{-}450_{ALC}$ in human liver microsomes and of a similar alcohol-inducible protein in the rat and in the normal and alcohol dehydrogenase-deficient deermouse. $P\text{-}450_{ALC}$ catalyzes the activation of foreign compounds such as acetaminophen, various nitrosamines, and carbon tetrachloride and is therefore believed to play an important role in the enhanced toxicity of these substances accompanying alcohol administration

Key words: Cytochrome P-450 – Toxicity – Induction – Ethanol – Butanol – Acetaminophen – Nitrosamines – Cocaine – Carbon tetrachloride

Introduction

A role for cytochrome P-450 in the oxidation of ethanol and other aliphatic alcohols was controversial until the isolation in this laboratory of a unique isozyme of P-450 from liver microsomes of ethanol-treated rabbits (Koop et al. 1982). This P-450 was designated as isozyme 3a on the basis of its electrophoretic behavior relative to that of the previously known isozymes, and it is also referred to as $P\text{-}450_{ALC}$ to indicate that alcohol is both an inducer and a substrate. This alcohol-oxidizing enzyme system is designated as APO for "alcohol P-450-oxygenase", a term that suitably indicates the nature of the oxygenating catalyst and is comparable to ADH for alcohol dehydrogenase (Coon and Koop 1985). The term APO has the advantages of indicating that alcohols in general are substrates, not just ethanol, that the P-450 family of catalysts is involved, and that the mechanism is one of oxygenation. The term "microsomal ethanol oxidizing system" proved useful in the early days of investigation of this pathway (Lieber and DeCarli 1970) but has the drawbacks of emphasizing microsomes (whereas P-450 also occurs in mitochondria and nuclei), of designating ethanol as the substrate whereas the system has much broader substrate specificity, and, of greater concern, of referring to a biochemically undefined oxidizing system. Designation by a term that specifies P-450 brings to mind the chemical and physical properties of this unique family of heme proteins and its role in the metabolism of an almost unlimited number of lipids and other xenobiotics as well as alcohols. No attempt has been made in this brief review to cover the extensive literature on the general effects of chronic alcohol administration on the toxicity of other chemicals (cf. Mezey 1976; Lieber et al. 1979), but rather to emphasize the central role played by cytochrome P-450 and in particular $P\text{-}450_{ALC}$.

Characterization of $P\text{-}450_{ALC}$, including substrate specificity

Several criteria indicate that the electrophoretically homogeneous ethanol-inducible cytochrome, which has a minimal molecular weight of 51 000, is distinct from the other isozymes of P-450 (Koop et al. 1982). As judged spectrally, $P\text{-}450_{ALC}$ is high spin in the oxidized state, as is form 4, but differs in that the spin state is unperturbed by nonionic detergents. The absolute spectrum of the ferrous carbonyl complex is red-shifted as compared to that of forms 2, 3b, 3c, 4, and 6 and exhibits a maximum at 452 nm. The amino acid composition is different from that of the other isozymes, and both the NH_2- and COOH-terminal sequences are distinct; $P\text{-}450_{ALC}$ has an amino-terminal Ala residue followed in turn by a largely hydrophobic sequence, Val-Leu-Gly-Iso-Thr-Val-Ala-Leu-Leu-Gly-Trp-Met-Val-Iso-Leu-Leu-Phe-Iso-Ser-Val-Trp-, and a carboxyl-terminal Leu residue. Peptide mapping by sodium dodecyl sulfate-polyacrylamide gel electrophoresis following treatment with papain, chymotrypsin, or *Staphylococcus aureus* V_8 protease and by high performance liquid chromatography following trypsinolysis indicated that this enzyme is a unique gene product.

$P\text{-}450_{ALC}$, isolated from hepatic microsomes of rabbits treated chronically with ethanol, was found to have a unique substrate specificity when compared with isozymes 2, 3b, 3c, and 4 (Morgan et al. 1982). This form has unusually high activity in both the *p*-hydroxylation of aniline and the oxidation of alcohols to aldehydes and ketones, which is reflected in the increased activities of these substrates in microsomes from ethanol-treated rabbits as com-

* Dedicated to Professor Dr. Herbert Remmer on the occasion of his 65th birthday

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pared to microsomes from untreated animals or those administered phenobarbital or 5,6-benzoflavone. The ethanol-oxidizing activity of P-450ALC, which requires the presence of NADPH and NADPH-cytochrome P-450 reductase and is stimulated by the presence of phospholipid, was shown not to be due to contaminating catalase or an NAD- or NADP-dependent alcohol dehydrogenase. This isozyme catalyzes the oxidation of methanol, 1-propanol, and 1-butanol as well as ethanol; the relationship between the apparent K_M values for the alcohols and their respective octanol-water partition coefficients is in accord with the presumed hydrophobic nature of the P-450 binding site. Whereas typical substrates of isozyme 2 are known to be metabolized with the stoichiometry predicted of a monooxygenase reaction, with P-450ALC the sum of acetaldehyde formed from ethanol and of hydrogen peroxide generated is inadequate to account for the NADPH and oxygen consumed (Gorsky et al. 1984). Free hydroxyl radicals are suggested to mediate the slow oxidation of ethanol in the presence of the reductase alone but not the much faster rate catalyzed by P-450 isozyme 3a. The results obtained, however, do not rule out the involvement of hydroxyl radical equivalent generated and bound at the active site of the cytochrome.

Sheep antibodies raised against P-450ALC were used to determine the role of this enzyme in the microsomal oxidation of alcohols and the *p*-hydroxylation of aniline (Koop et al. 1984). P-450 isozymes 2, 3b, 3c, 4, and 6 do not crossreact with anti-3a IgG as judged by Ouchterlony double diffusion, and radioimmunoassays indicate a crossreactivity of less than 1%. Greater than 90% of the activity of purified P-450ALC toward aniline, ethanol, *n*-butanol, and *n*-pentanol is inhibited by the antibody in the reconstituted system. The catalytic activity of liver microsomes from control or ethanol-treated rabbits is unaffected by the addition of either desferrioxamine (up to 1.0 mM) or EDTA (0.1 mM), suggesting 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 microsomal activity. The addition of anti-P-450ALC IgG to hepatic microsomes from ethanol-treated rabbits inhibits the metabolism of ethanol, *n*-butanol, *n*-pentanol, and aniline by about 75, 70, 80, and 60%, respectively, while the inhibition of the activity of microsomes from control animals is only about half as great. The rate of microsomal H_2O_2 formation is inhibited to a lesser extent than the formation of acetaldehyde, thus suggesting that the antibody acts to prevent the direct oxidation of ethanol by this cytochrome. Under conditions where purified NADPH-cytochrome P-450 reductase-catalyzed substrate oxidation is minimal, the P-450 isozymes other than P-450ALC have low but significant activity toward the four substrates examined. The residual activity at maximal concentrations of the antibody probably represents the sum of the activities of other P-450 isozymes present in the microsomal preparations. The results thus support the conclusion that the enhanced monooxygenase activity of liver microsomal membranes from ethanol-treated animals represents catalysis by P-450ALC.

Evidence for induction of the alcohol-oxygenating cytochrome P-450 by diverse agents

The somewhat surprising finding has been made that P-450ALC is induced in animals treated with agents other than ethanol, for example, imidazole. What might be called "imidazole-P-450ALC" and "ethanol-P-450ALC" were isolated from rabbits treated with these respective compounds and shown to exhibit the same chromatographic characteristics and to have identical electrophoretic mobilities upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Koop and Coon 1984). Furthermore, the two protein preparations have the same absorbance maxima and absorption coefficients in the oxidized, reduced, and reduced-CO states. A single immunoprecipitin band exhibiting complete identity was observed upon reaction of imidazole-P-450ALC and ethanol-P-450ALC with the immunoglobulin G fraction from sheep immunized with the latter protein, as indicated by the Ouchterlony analysis shown in Fig. 1. The amino acid composition and first ten residues of the amino terminus of the two protein preparations are the same, as are the high performance liquid chromatographic maps of the peptides obtained upon cleavage with trypsin, *S. aureus* V_8 protease, or Lys C endoproteinase. Furthermore, these two P-450 preparations have very similar activities in the oxidation of ethanol to acetaldehyde and the *p*-hydroxylation of aniline. As an inducer, imidazole has the advantage over ethanol of being less variable in its effects and requiring a shorter period of treatment. From the resulting liver microsomes one can readily isolate, in addition to P-450 isozymes 3a (ALC) and 6, isozymes 3c and 4 as well as epoxide hydrolase.

Immunochemical evidence for the induction of P-450ALC by other agents, including trichloroethylene, acetone, pyrazole, and isoniazid has recently been reported (Koop et al. 1985). We adopted the dot-blot methodology described by Domin et al. (1984) to quantify P-450ALC in

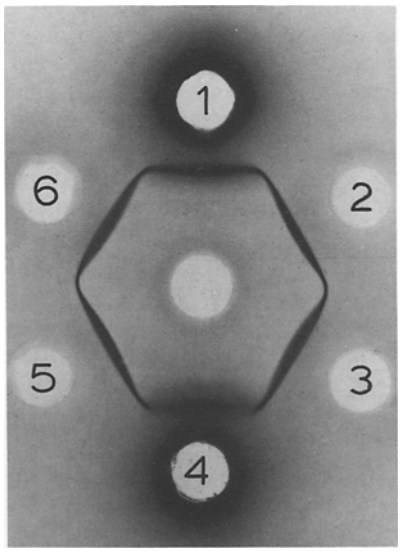

Fig. 1. Immunochemical comparison of P-450ALC purified from ethanol- and imidazole-treated rabbits, taken from Koop and Coon 1984. Ouchterlony double-diffusion analysis was carried out in 0.9% agarose gels. Microsomes were solubilized with 1 mg cholate per mg protein. The center well was loaded with 250 µg anti-P-450ALC IgG. Wells *1* and *4* were loaded with 200 µg microsomal protein from imidazole- and ethanol-treated rabbits, respectively; wells *2* and *5* were loaded with 6 µg imidazole-P-450ALC; and wells *3* and *6* were loaded with 6 µg ethanol-P-450ALC. Diffusion was allowed to proceed at 4' for 48 h in a moist chamber, and staining was then carried out with aqueous Coomassie blue

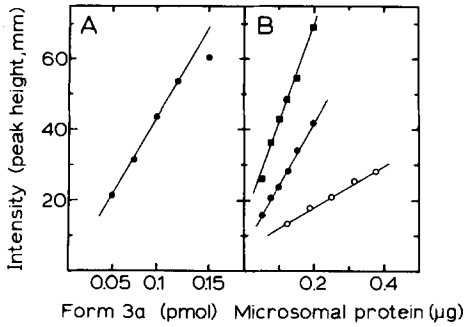

Fig. 2A, B. Dot immunoblot analysis of purified P-450 and microsomal samples, taken from Koop et al. (1985). The purified cytochrome or hepatic microsomes were directly applied to nitrocellulose and immunochemically stained with anti-P-450ALC IgG. The peak heights of soft-laser densitometer scans were measured directly. **A** Peak heights vs the amount of purified cytochrome applied. **B** Peak heights vs the amount of microsomal protein from untreated (○), ethanol-treated (●), or imidazole-treated rabbits (■)

various microsomal samples. The range of linearity of the assay was very narrow. Therefore, each of the microsomal preparations was examined at five or more different concentrations, and the amount of isozyme 3a was calculated from the slope obtained in a plot of the amount of microsomal protein versus staining intensity. A typical set of results for purified P-450ALC and for microsomes from untreated, ethanol-treated, and imidazole-treated rabbits is shown in Fig. 2.

Since treatment of rabbits with a variety of foreign compounds results in an increase in the oxidation of alcohols (as well as in aniline hydroxylation), as shown earlier, we expected to find a correlation between the level of P-450ALC and the rate of alcohol oxidation in the different preparations of liver microsomes. A series of experiments designed to address this questin was undertaken with the results shown in Table 1. Hepatic microsomes were isolated from untreated rabbits and from rabbits administered pyrazole, isoniazid, ethanol, acetone, imidazole, or trichloroethylene. The pyrophosphate-washed microsomal preparations were examined for their total content of cytochrome P-450, content of P-450ALC, and total butanol oxidation activity, as well as butanol oxidation activity inhibited by anti-P-450ALC antibody and therefore attributable to the action of this particular cytochrome. Butanol was selected as the test substrate because the turnover numbers are higher than with ethanol and the zero-time blanks are much lower for butyraldehyde than acetaldehyde. Treatment of the animals with each of the agents resulted in an increase in the P-450ALC content (expressed as nmol/mg protein), whereas an increase in total P-450 content was observed only with pyrazole and imidazole treatment. P-450ALC represents about 5% of the total P-450 in the control animals and is increased to as high as about 27% by acetone treatment. Under the conditions used, isoniazid gave the smallest relative increase in this cytochrome (about 2-fold), whereas imidazole and acetone were the most effective inducers, giving about 4.5- and 4.9-fold increases, respectively. The increase in total butanol oxidation activity in microsomal suspensions underestimates the extent of induction of this isozyme. For example, a comparison of the specific activity of microsomes from untreated animals with those after isoniazid and ethanol treatment (expressed as nmol per min per mg protein) showed increases of 1.2- and 1.7-fold, respectively. However, when the P-450ALC-dependent rates were compared, increases of about 2.7- and 4.0-fold were calculated for these two agents. This may be explained by the presence of other isozymes of P-450, each of which makes a small but significant contribution to butanol oxidation (Koop et al. 1984). Also shown in Table 1 is the ratio of the increase in P-450ALC activity to the increase in the P-450ALC content. The values are similar (1.1–1.3) for the different inducers administered with the exception of acetone, which gave a ratio of 1.7. It should be emphasized that the effects of the various agents on factors other than P-450 which could influence the catalytic activity, such as the level of NADPH-cytochrome P-450 reductase or the phospholipid composition of the membrane, may be somewhat different for the various inducers tested.

The molecular mechanisms by which levels of P-450ALC are elevated have not been defined. Induction of the protein could occur as a result of an increase in the level of translatable mRNA, stabilization of the existing mRNA, an increase in translation of existing mRNA, or a decrease in the rate of degradation of the isozyme. Direct analysis with cDNA probes indicates that, in the rat, treatment with phenobarbital or 3-methylcholanthrene increases the

Table 1. Various agents as inducers of liver microsomal P-450ALC[a]

Agent administered	P-450 content			Butanol oxidation activity			% P-450ALC activity/ % P-450ALC content
	Total, nmol/mg of protein	P-450ALC, nmol/mg of protein	%	Total, nmol/min/mg of protein	P-450ALC-dependent, nmol/min/mg of protein	%	
None	2.80±0.22	0.15±0.01	100	1.45±0.16	0.34±0.03	100	1.0
Isoniazid	2.00±0.17	0.32±0.01	213	1.80±0.28	0.93±0.08	274	1.3
Trichloroethylene	2.74±0.21	0.39±0.08	260	2.21±0.30	1.03±0.23	303	1.2
Pyrazole	3.41±0.17	0.41±0.09	273	2.21±0.33	0.98±0.28	288	1.1
Ethanol	2.45±0.13	0.47±0.04	313	2.41±0.16	1.38±0.16	406	1.3
Imidazole	3.46±0.41	0.67±0.02	447	3.02±0.28	1.95±0.20	574	1.3
Acetone	2.71±0.24	0.73±0.16	487	3.88±0.61	2.74±0.66	806	1.7

[a] Hepatic microsomes were isolated from each animal, total P-450 content was determined, and P-450ALC was quantified immunochemically by the dot-blot method. The P-450ALC-dependent rate of butanol oxidation was obtained by subtraction of the rate of butanol oxidation obtained in the presence of optimal levels of anti-P-450ALC IgG from the rate obtained in the presence of the same concentration of preimmune sheep IgG. All results are given as the mean ± SEM for each group. The data are taken from Koop et al. (1985)

Table 2. NDMA demethylase activity of different P-450 isozymes[a]

NDMA (mM)	P-450 isozymes; (nmol HCHO/min/nmol P-450)					
	2	3a	3b	3c	4	6
0.1	< 0.03	0.25	< 0.03	< 0.03	< 0.03	< 0.03
4.0	0.10	5.89	0.03	0.08	< 0.03	0.31
10	0.36	6.68	0.17	0.22	0.18	0.72
100	2.21	6.71	1.45	0.79	2.01	4.35

[a] The reaction mixture contained 0.1 nmol of a P-450 isozyme, 0.63 unit NADPH-cytochrome P-450 reductase, 7.5 μg dilauroylglyceryl-3-phosphorylcholine, and different concentrations of NDMA in a final volume of 0.25 ml. The data are taken from Yang et al. (1985 a)

synthesis of specific mRNA (Adesnik et al. 1981; Morville et al. 1983). Whether the induction of P-450ALC involves a similar mechanism or a common hormonal or metabolic change in the animal that indirectly leads to induction remains to be determined.

Role of P-450ALC in toxicological effects of various compounds

Acetaminophen (Tylenol; *p*-hydroxyacetanilide), a widely used antipyretic and analgesic drug, is normally nontoxic but in large doses is known to produce acute hepatic necrosis, and increased hepatotoxicity of the drug has been reported in human alcoholics (Proudfoot and Wright 1970; McClain et al. 1980). We have reported that isozymes 3a (P-450ALC), 4, and 6, all of which are present in significant amounts in liver microsomes from rabbits chronically administered ethanol, exhibit the highest activities in the reconstituted enzyme system in converting acetaminophen to a reactive intermediate capable of reacting with glutathione whereas isozymes 3b and 3c are 10- to 20-fold less effective, and phenobarbital-inducible isozyme 2 is essentially inactive, even in the presence of cytochrome b_5 (Morgan et al. 1983). The results obtained thus indicate that induction by ethanol of P-450ALC apparently contributes to the toxicity of acetaminophen but that other P-450 cytochromes also play a significant role.

P-450ALC is also involved in the metabolism of nitrosamines, such as *N*-nitroso-2,6-dimethylmorpholine (NNDM) (Kokkinakis et al. 1985). The *cis* isomer of NNDM, a pancreatic carcinogen for the Syrian golden hamster, is metabolized by hamster liver microsomes to yield N-nitroso(2-hydroxypropyl)(2-oxopropyl)amine (HPOP), the proximate carcinogenic metabolite, as the major product. The role of rabbit liver microsomal cytochrome P-450 in the metabolism of the *cis* isomer of NNDM was studied in the reconstituted system consisting of NADPH-cytochrome P-450 reductase, phospholipid, and one of the individual P-450s. P-450 isozyme 2, which is induced by pretreatment with phenobarbital, exhibited the highest activity for the metabolism of *cis*-NNDM. The V_{max} for the formation of HPOP was 1.78 nmol/min/nmol of the cytochrome, and the apparent K_M was 360 μM. P-450ALC also catalyzed the metabolism of NNDM to HPOP at a significant rate (0.25 nmol/min/nmol cytochrome). Of the four other isozymes (forms 3b, 3c, 4, and 6) tested in the reconstituted system, only isozymes 3b and 3c exhibited measurable activities (approximately 0.04 nmol HPOP formed/min/nmol of cytochrome).

The activation of additional nitrosamine derivatives by microsomal cytochrome P-450 isozymes was studied in the reconstituted monooxygenase system (Yang et al. 1985a). Of the cytochromes tested, P-450ALC had the highest N-nitrosodimethylamine (NDMA) demethylase activity with a K_M of 2.9 mM and V_{max} of 9.3 nmol/min/nmol P-450. The other isozymes exhibited significant activity only at high NDMA concentrations, as shown in Table 2, thus providing an interesting example where the family of P-450s shows selectivity for a xenobiotic at low substrate concentration but all forms of the enzyme contribute to its metabolism at elevated substrate concentrations. This may indicate the biological advantage of having a number of such hepatic cytochromes with overlapping activities. The evidence presented in Table 3 indicates that the methylethyl, methylbutyl, methylbenzyl, and methylphenyl analogs of NDMA also serve as substrates for the various P-450 isozymes. The cytochromes catalyze the denitrosation of nitrosamines at rates comparable to or lower than the demethylation, and the ratio of these two reactions was found to be different with different nitrosamines. For example, the ratio of the rate of nitrite formation to that of formaldehyde formation (given in Table 3), was as follows for 40 mM nitrosoamine substrate and P-450ALC: 0.13 (dimethyl), 0.71 (methylethyl), 0.63 (methylbutyl), and 0.74 (methylbenzyl); for P-450 form 2 the corresponding values were 0.28, 0.74, 0.53, and 0.88, respectively. 2-Phenylethylamine and 3-amino-1,2,4-triazole, which were believed previously to affect NDMA metabolism by mechanisms independent of P-450, were shown to be potent inhibitors of P-450-dependent NDMA demethylation. These results further establish the role of P-450 isozymes in the metabo-

Table 3. Substrate specificity of P-450 isozymes in the demethylation of nitrosamines[a]

Substrate	P-450 isozymes; (nmol HCHO/min/nmol P-450)					
	2		3a		4	
	4 mM	40 mM	4 mM	40 mM	4 mM	40 mM
Nitrosodimethylamine	0.13	1.43	5.01	7.89	< 0.03	0.51
Nitrosomethylethylamine	0.07	1.39	0.94	1.59	0.07	0.35
Nitrosomethylbutylamine	1.73	1.91	0.23	1.17	0.10	0.32
Nitrosomethylbenzylamine	0.50	1.08	0.28	1.17	0.08	0.22
Nitrosomethylaniline	5.57	7.36	2.51	3.65	1.57	2.46

[a] The experimental conditions were the same as those for Table 2, except that 4 and 40 mM nitrosamines were used as substrates. The data are taken from Yang et al. (1985 a)

lism of nitrosamines and indicate that P-450ALC is apparently responsible for the increased NDMA metabolism associated with ethanol treatment.

The enhanced toxicity of cocaine (Smith et al. 1981) and carbon tetrachloride (Traiger and Plaa 1972) seen following chronic exposure of animals to ethanol should also be noted. Evidence for the metabolism of NDMA and carbon tetrachloride by a common isozyme of cytochrome P-450, presumably P-450ALC, has recently been reported (English and Anders 1985). Treatment of rats with pyrazole resulted in an increase in the microsomal metabolism of CCl_4 to give phosgene and in the conversion of CCl_4 to chloroform. The similarities between the induction of the low K_M NDMA demethylase and the metabolism of carbon tetrachloride to form phosgene suggest that these catalytic activities represent a common isozyme of cytochrome P-450. Evidence that P-450ALC is a highly effective catalyst of CCl_4 metabolism has recently been reported (Johansson and Ingelman-Sundberg 1985).

Evidence for a protein similar to P-450ALC in other species

The antibody to P-450ALC has been shown to be an effective inhibitor of the NDMA demethylase activity of microsomes from untreated or ethanol-treated rats, untreated mice, and untreated or acetone-treated guinea pigs (Yang et al. 1985b). In addition, the antibody inhibits the acetone and acetol hydroxylase activities of microsomes from acetone-treated rats (Koop and Casazza 1985). As shown in Fig. 3, anti-P-450ALC IgG crossreacts with one major protein band in hepatic microsomes from humans and rats. A second minor band was observed in some microsomal samples. The finding that the staining intensity of the major crossreactive protein was increased by ethanol treatment in the case of the rat suggests that the ethanol-inducible P-450 isozyme in this species corresponds to rabbit P-450ALC, but additional experiments will be required to confirm this hypothesis.

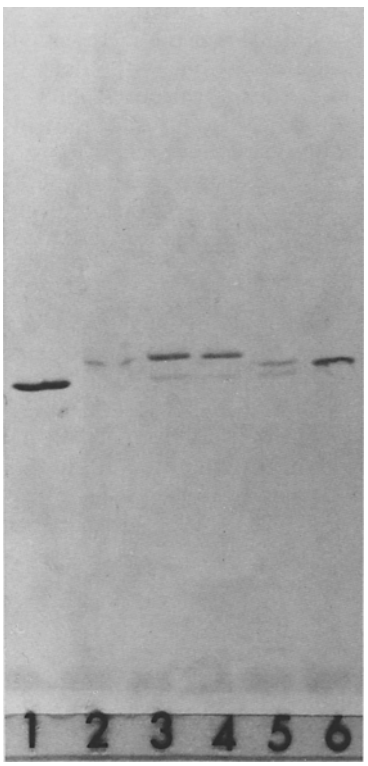

Fig. 3. Immunoblot analysis of hepatic microsomes from several species. Microsomes from human liver (kindly provided by Drs. F. P. Guengerich and L. M. Distlerath, Vanderbilt University) (lanes *2*, *3*, and *4*; 4 μg protein), untreated or ethanol-treated rats (lanes *5* and *6*, respectively; 2 μg protein); and purified rabbit P-450ALC (lane *1*; 0.1 μg protein) were submitted to polyacrylamide gel electrophoresis. The proteins were then transferred to nitrocellulose sheets, and blots were developed with anti-P-450ALC IgG

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