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BIOCHEMICAL AND IMMUNOCHEMICAL EVIDENCE FOR THE INDUCTION OF AN ETHANOL-INDUCIBLE CYTOCHROME P-450 ISOZYME IN MALE SYRIAN GOLDEN HAMSTERS*

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Abstract—The effects of ethanol and of phenobarbital pretreatment on hamster microsomal metabolism of aniline and *p*-nitrophenol have been investigated. Hydroxylation of both compounds was increased over 2-fold by ethanol pretreatment, whereas phenobarbital pretreatment had little effect on either activity. Ethanol pretreatment had no effect on the specific content of total cytochrome P-450, while phenobarbital pretreatment increased the specific content 1.6-fold. Comparison of the specific activities for aniline hydroxylation and *p*-nitrophenol hydroxylation of individual microsomal samples from control, ethanol-pretreated and phenobarbital-pretreated animals showed a high degree of correlation ($r^2 = 0.98$) consistent with the involvement of the same site for catalysis of these two compounds. Antibody to rabbit ethanol-inducible cytochrome P-450 (isozyme 3a) inhibited over 80% of the aniline (high affinity) and *p*-nitrophenol hydroxylase activities of microsomes from ethanol-treated hamsters. A comparison of the antibody-inhibitable rates for both hydroxylase activities with microsomes from untreated, ethanol- or phenobarbital-pretreated hamsters suggested that an isozyme homologous to rabbit isozyme 3a (hamster cytochrome P-450alc) was induced in hamsters about 3.5-fold by ethanol and was unaffected by phenobarbital. The induction of hamster cytochrome P-450alc was confirmed by immunoblot analysis of hamster microsomes. A single protein with a molecular weight of approximately 54,000 was recognized by antibody to the rabbit isozyme. Quantification of the immunoblots demonstrated that the hamster isozyme was increased about 3-fold, in good agreement with the induction determined by a comparison of the antibody-inhibitable rates. The results indicated that, although there was no change in the total spectrally observable cytochrome P-450, there was a marked change in the distribution of the isozymes of cytochrome P-450, with an increase in the alcohol-inducible form after 28-day ethanol consumption by chow-fed hamsters. This isozyme can be readily monitored by either high-affinity aniline or *p*-nitrophenol hydroxylation or by Western immunoblot analysis and appears to be the ethanol-inducible form of cytochrome P-450 in hamsters.

Previous studies from our laboratory and others demonstrated that chronic consumption of ethanol by hamsters in either liquid diet form or as ethanol–water mixtures causes marked alterations in microsomal metabolism [1–6]. Both methods of ethanol delivery cause induction of aniline hydroxylation and *p*-nitroanisole *O*-demethylase activity [4]. In liquid diet-fed animals, the specific content of total cytochrome P-450 was increased significantly by ethanol [1, 3, 4], whereas chow-fed animals receiving ethanol in their drinking water showed no consistent increase in the specific content of total microsomal cytochrome P-450 [4–6]. Thus, it was not clear if the observed increases in microsomal metabolism in chow-fed animals were due to induction of a specific cytochrome P-450 isozyme, activation of a specific pathway, or inhibition of a diversionary metabolic pathway.

Chronic ethanol consumption by rabbits and rats has been shown to result in the induction of a unique isozyme of cytochrome P-450 which has been purified and characterized from ethanol-treated rabbits [7], and more recently from ethanol-treated rats [8]. Many of the changes in hepatic xenobiotic metabolism after ethanol treatment can be attributed to the activity of the induced enzyme, cytochrome P-450alc [9]. Antibody to rabbit cytochrome P-450alc (isozyme 3a) has been used to demonstrate that the enzyme was associated with the low K_m *N*-nitrosodimethylamine demethylase in rabbits as well as rats, and acetone-pretreated guinea pigs [10]. Reconstitution of rat cytochrome P-450j [11] and the inhibition of the microsomal demethylase activity by monoclonal antibody raised against cytochrome P-450et (apparently identical to P-450j and homologous to rabbit isozyme 3a) confirmed these results [12]. Cytochrome P-450alc is the predominant catalyst for aniline hydroxylation in microsomes from rats and rabbits after ethanol treatment [8, 13]. It is the purpose of this paper to demonstrate that

* The term cytochrome P-450 refers to all of the isozymes present in the hepatic preparations. The ethanol-inducible isozyme from rabbits is designated form or isozyme 3a and from rats as P-450j. The immunochemically homologous isozyme from all species will be referred to as P-450alc, although it is recognized that the primary structures are not identical.

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consumption of ethanol in the drinking water by chow-fed hamsters results in the induction of an alcohol-inducible isozyme of cytochrome P-450 which is homologous to rabbit isozyme 3a.

MATERIALS AND METHODS

Chemicals. Aniline, *p*-nitrophenol, 4-chloro-1-naphthol, nicotinamide adenine dinucleotide phosphate (monosodium salt), nicotinamide adenine dinucleotide phosphate (reduced form, Type X), Tricine (*N*-tris-hydroxymethyl-methyl glycine), Tris(tris-hydroxymethyl-amino-methane), glucose-6-phosphate (monosodium salt), and glucose-6-phosphate dehydrogenase (Type XII) were obtained from the Sigma Chemical Co. (St. Louis, MO); *p*-aminophenol was from Fisher Scientific (Pittsburgh, PA). Sucrose (special enzyme grade) was from Schwarz/Mann (Orangeburg, NY); rabbit antisheep IgG and sheep peroxidase antiperoxidase were from Cooper Biomedical (Malvern, PA); and electrophoresis chemicals were from BioRad Laboratories (Richmond, CA).

Animals and treatment. Six-week-old male LVG Syrian golden hamsters (*Mesocricetus auratus*) were obtained from the Charles River Breeding Laboratories, Lakeview Colony, Newfield, NJ. Animals were housed in regulation stainless steel cages and given free access to NIH-07 lab chow (Ziegler Brothers, Gardners, PA) and tap water. At 9 weeks of age, one group was given free access to 18.5% ethanol–water in place of drinking water, as described previously [14]. At 12 weeks of age, a second group of animals was given daily intraperitoneal injections of phenobarbital (80 mg/kg body wt/day in saline) for 4 consecutive days as described previously [2]. Control and pretreated animals were killed by decapitation at 13 weeks of age.

Subcellular fractionation and biochemical analysis. Livers were rapidly removed, weighed, minced, rinsed three times with 10 vol. of ice-cold SET (0.3 M sucrose, 0.5 mM EDTA, and 5 mM Tricine, pH 7.4), and suspended in 5 vol. of the same buffer. Homogenization of livers and microsome isolation were performed as described previously [2]. The standard assay mix for the high-affinity form of aniline hydroxylase contained in a final volume of 1.0 ml: 0.1 μmol aniline, 0.125 μmol $NADP^+$, 2.5 μmol glucose-6-phosphate, 50 μmol Tris-acetate buffer (pH 8.0), 4 units glucose-6-phosphate dehydrogenase, and 0.5 to 2 mg microsomal protein. Complete assay mixtures minus microsomes were preincubated for 5 min at 37°. Reactions were initiated by the addition of microsomes. Reactions were quenched following 15-min incubations by the addition of 1.0 ml of 10% trichloroacetic acid and analyzed for *p*-aminophenol as described previously [2]. *p*-Nitrophenol hydroxylase activity was determined in 1.0-ml reaction mixtures containing: 100 μmol potassium phosphate buffer (pH 6.8), 0.10 μmol *p*-nitrophenol, 1 μmol ascorbate and 0.2 mg microsomal protein. Reaction mixtures were preincubated for 3 min at 37° and then initiated by the addition of 1 μmol NADPH. Reactions were quenched by the addition of 0.5 ml of 0.6 N perchloric acid and analyzed for 4-nitrocatechol as described previously [15].

Total cytochrome P-450 was determined by the method of McLean and Day [16], using an extinction coefficient of 91 mM^{-1} for the absorbance difference between 450 and 490 nm. Protein was determined by the method of Lowry *et al.* [17]. Kinetic and spectral analyses were performed on a Varian Cary model 210 UV/VIS spectrophotometer. Statistical significance was evaluated using Student's two-tailed *t*-test.

Electrophoretic methods and immunoblots. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis was performed according to the method of Laemmli [18] in a separating gel containing 7.5% acrylamide. Protein was detected by silver staining as described by Wray *et al.* [19]. The procedure for electrophoretic transfer of microsomal proteins and purified isozymes to nitrocellulose, the purification of sheep anti-isozyme 3a IgG, and immunochemical staining with anti-3a IgG have been described in detail elsewhere [13, 20]. Immunoblots were quantified by determining the relative staining intensity with an LKB soft laser densitometer.

RESULTS

The effects of 28-day ethanol consumption and phenobarbital pretreatment on hamster microsomal aniline hydroxylase (high-affinity form), *p*-nitrophenol hydroxylase, and total specific content of cytochrome P-450 are presented in Table 1. Previous studies from our laboratory have shown that there are two forms of aniline hydroxylase which can be distinguished by kinetic analysis into a high-affinity and low-affinity form [2]; the high-affinity form is inducible by ethanol in both chow-fed and liquid

Table 1. Differential effects of ethanol and phenobarbital pretreatments on aniline and *p*-nitrophenol hydroxylase activities and cytochrome P-450 content

Pretreatment	Aniline hydroxylase ($nmol \cdot min^{-1} \cdot mg^{-1}$)	*p*-Nitrophenol hydroxylase ($nmol \cdot min^{-1} \cdot mg^{-1}$)	Cytochrome P-450 ($nmol \cdot mg^{-1}$)
Control	0.54 ± 0.04 (5)	3.47 ± 0.21 (5)	1.04 ± 0.03 (5)
Ethanol	1.14 ± 0.11*(5)	7.50 ± 0.86*(5)	0.95 ± 0.05 (5)
Phenobarbital	0.69 ± 0.02†(5)	4.09 ± 0.21†(4)	1.66 ± 0.05*(5)

All values are means ± SE. Values in parentheses are the number of separate microsomal preparations.
* $P \leq 0.01$.
† $P \leq 0.05$.

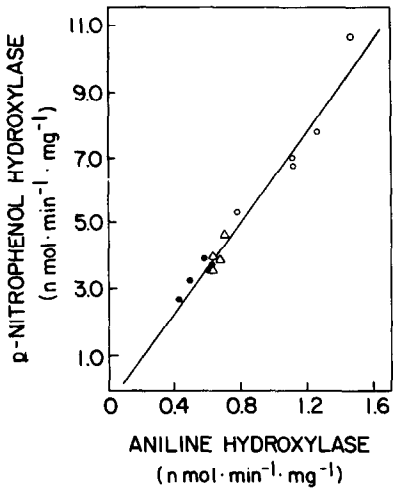

Fig. 1. Comparison of hamster liver microsomal aniline and *p*-nitrophenol hydroxylase activities. Enzymatic activities of control (●—●), ethanol (○—○), and phenobarbital (△—△) pretreated microsomes were determined as described in Materials and Methods.

diet-fed hamsters [4]. Phenobarbital pretreatment has been shown to increase slightly both the high- and low-affinity forms of aniline hydroxylase, whereas 3-methylcholanthrene or Aroclor 1254 pretreatment increases the activity of the low-affinity form [2]. The hydroxylation of *p*-nitrophenol to 4-nitrocatechol is increased by ethanol pretreatment in both rats and rabbits [15, 21]. The principal catalyst for this reaction in rabbit microsomes was shown to be isozyme 3a [15]. Ethanol consumption by hamsters resulted in a marked increase in this activity as well. The specific content of total cytochrome P-450, as determined spectrally, was not increased by ethanol pretreatment whereas phenobarbital markedly increased it.

When the specific activity of the high-affinity form of aniline hydroxylase was plotted against the specific activity of *p*-nitrophenol hydroxylase activity from control, ethanol-pretreated, and phenobarbital-pretreated hamsters, a strong positive correlation (r^2 = 0.98) was observed (Fig. 1). These data suggest that the two substrates share a common site for catalysis.

The induction of both aniline and *p*-nitrophenol hydroxylase activities by ethanol consumption was suggestive that an isozyme homologous to rabbit isozyme 3a was being induced even though there was no observable difference in total cytochrome P-450 content. Antibody to rabbit cytochrome P-450alc exhibits good species crossreactivity, inhibiting cytochrome P-450alc-dependent activities in rats, mice, and guinea pigs [10, 22]. A direct comparison of the isozyme inducible by ethanol in rats (P-450j) and rabbits (isozyme 3a) demonstrated that antibody to isozyme 3a recognized P-450j on immunoblots and was as effective an inhibitor of P-450j in microsomes [8]. The effect of isozyme 3a antibody on the hamster microsomal-dependent metabolism of aniline and *p*-nitrophenol is shown in Fig. 2, A and B. Aniline hydroxylase activity was inhibited by 74, 60 and 87% with microsomes from untreated, phenobarbital- and ethanol-treated hamsters respectively. Similar inhibition of *p*-nitrophenol hydroxylase activity was also observed. The antibody-inhibited rate can be defined as that portion of the total microsomal activity which is attributable to the hamster cytochrome P-450alc. A comparison of the increase in the antibody-inhibitable rate indicates that PB treatment had very little effect on the activity of cytochrome P-450alc, but ethanol increased the cytochrome P-450alc-dependent rate of aniline hydroxylation 3.5-fold and *p*-nitrophenol hydroxylation, 4.2-fold. The good agreement of these values is consistent with the

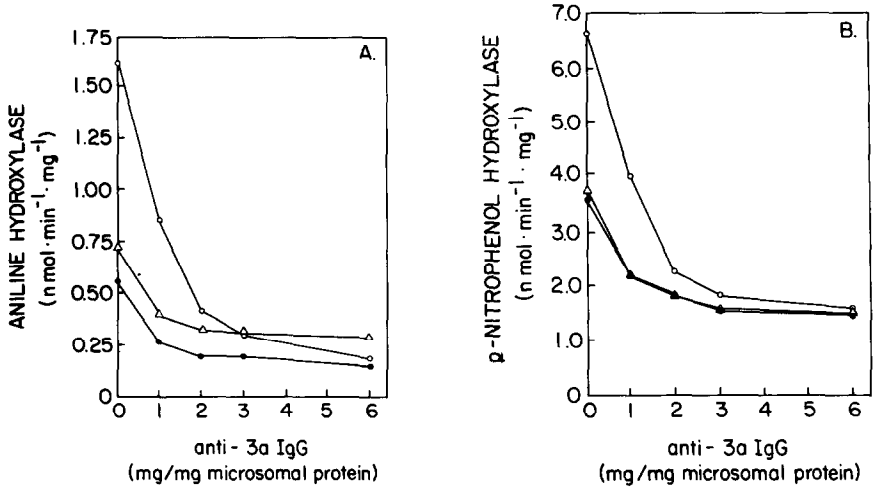

Fig. 2. Effect of antibody to rabbit isozyme 3a on hamster microsomal hydroxylation of aniline and *p*-nitrophenol. Microsomes were preincubated with the indicated amounts of anti-3a IgG for 3–5 min at 37°. Enzymatic activities were determined as described in Materials and Methods. Panel A: Effect of anti-3a IgG on high-affinity aniline hydroxylase activity of microsomes from control (●—●), ethanol (○—○) or PB (△—△) pretreated hamsters. Panel B: Effect of anti-3a IgG on *p*-nitrophenol hydrolase activity of microsomes from control (●—●), ethanol (○—○) or PB (△—△) pretreated hamsters. The addition of equivalent amounts of preimmune IgG had no effect on either activity.

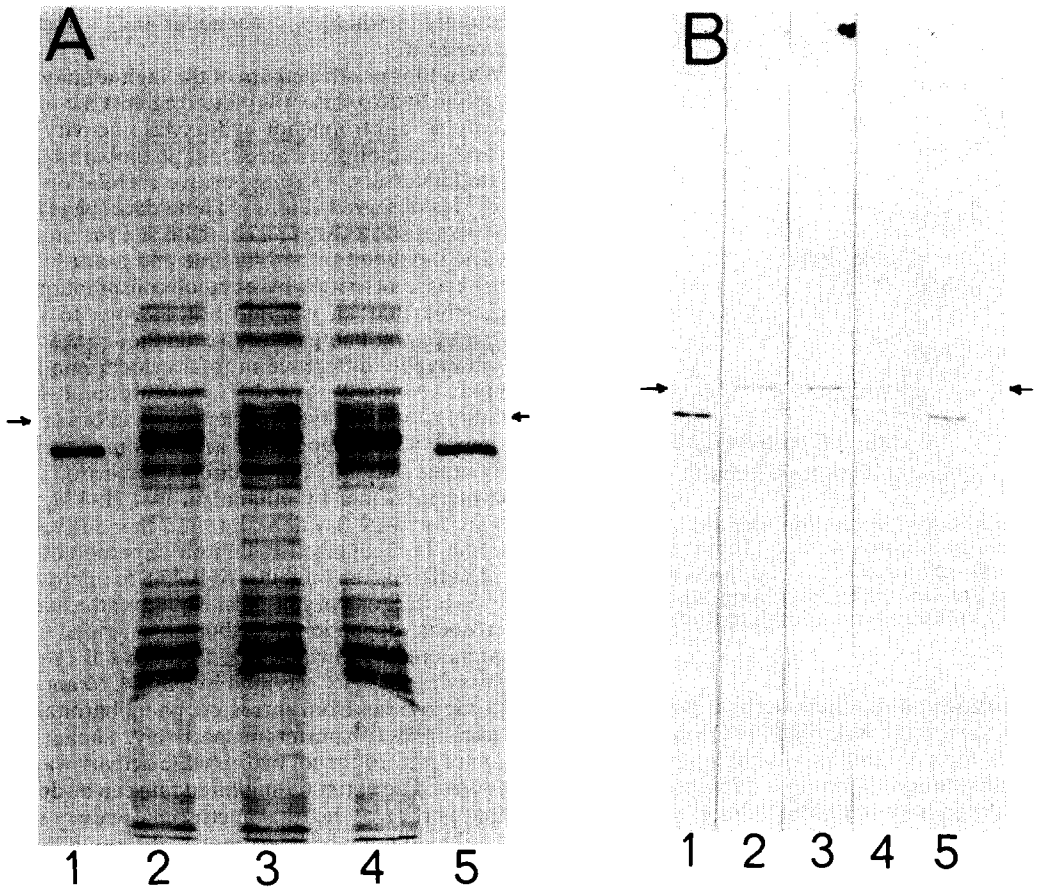

Fig. 3. SDS–polyacrylamide gel electrophoresis and immunoblot analysis of hamster hepatic microsomes. Panel A: Hamster microsomes from control (lane 2, 2 μg), ethanol- (lane 3, 2 μg) or PB- (lane 4, 2 μg) treated hamsters and purified rabbit isozyme 3a (lanes 1 and 5, 1 pmol) were electrophoresed in 7.5% acrylamide gels and stained with silver [19]. The position of the protein induced by ethanol at about 54,000 daltons is indicated by the arrow. Panel B: Hepatic microsomes from control (lane 2, 2 μg), ethanol- (lane 3, 2 μg) or PB- (lane 4, 2 μg) treated hamsters and purified rabbit isozyme 3a (lanes 1 and 5, 1 pmol) were submitted to SDS–polyacrylamide gel electrophoresis, electrophoretically transferred to nitrocellulose, and stained with anti-3a IgG. The samples are representative of the results obtained when increasing concentrations of each microsomal preparation were run on the same gel.

correlation between the two activities shown in Fig. 1 and indicates that cytochrome P-450alc is the predominant catalyst for both reactions in ethanol-treated hamsters.

The increase in the anti-3a IgG inhibitable rates of aniline and *p*-nitrophenol hydroxylation was suggestive that the concentration of the hamster homologue was increased by ethanol treatment. This conclusion was supported by quantitative immunoblot analysis. Figure 3 shows a sodium dodecyl sulfate-polyacrylamide gel (panel A) and representative immunoblots (panel B) of microsomes from untreated, PB-, and ethanol-treated hamsters. As reported by Elliott *et al.* [5], we found that ethanol treatment resulted in an increase in a protein with a molecular weight of about 54,000 (indicated by the arrow in Fig. 3A); this protein crossreacted with the antibody to rabbit cytochrome P-450alc on immunoblots (Fig. 3B). The relative increase of the cross-reacting protein was quantified by a comparison of the relative staining intensity of the homologue per μg of microsomal protein, as illustrated in Fig. 4. PB treatment had no effect on the concentration of the protein, but it was increased 3.1-fold by ethanol treatment. This value is in good agreement with the induction of hamster cytochrome P-450alc by ethanol as determined by the antibody inhibition experiments (3.1-fold by immunoblots versus 3.5-fold and 4.2-fold by aniline and *p*-nitrophenol hydroxylation, respectively) and indicates that the 54,000 molecular weight protein is hamster cytochrome P-450alc.

DISCUSSION

As initially reported by Rubin *et al.* [23], chronic ethanol consumption by rats results in proliferation of the smooth endoplasmic reticulum, increased specific content of cytochrome P-450, and increased specific activity of aniline hydroxylase. Of these three variables, it is the enhanced aniline hydroxylase

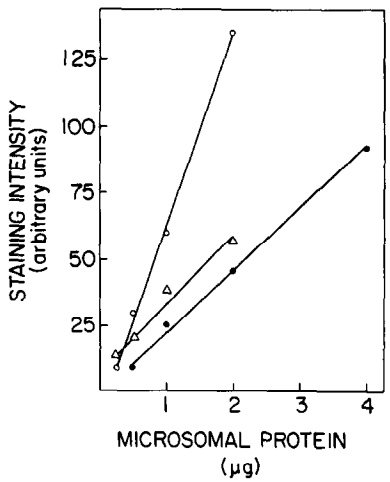

Fig. 4. Induction of hamster cytochrome P-450alc. Increasing concentrations of hamster microsomes from control, ethanol- and PB-treated hamsters were electrophoresed on the same gel and immunochemically stained with anti-3a IgG after electrophoretic transfer to nitrocellulose. The relative staining intensity of the stained band was determined by densitometry with an LKB Ultrascan laser densitometer. The slopes of the lines, as determined by linear regression analysis, are control (●, 23 units/μg protein), ethanol (○, 71 units/μg protein) and PB (△, 26 units/μg protein) treated microsomes.

activity that is consistently observed in all experimental pretreatments with ethanol which have been observed to date including vapor [24], liquid diet [23, 25, 26], and in drinking water [27, 28]. We know of no case in which documented ethanol consumption by animals does not increase aniline hydroxylase activity. Total microsomal aniline hydroxylation has been reported to be induced by all of the classical inducers of microsomal mixed-function oxygenase activity including ethanol [2, 29], whereas the induction of *p*-nitrophenol hydroxylation has been shown to be more specifically induced by conditions that result in the induction of the ethanol-inducible form of cytochrome P-450 [15, 21]. The data presented here clearly demonstrate a close correspondence between the two activities when aniline hydroxylation is carried out under limiting substrate concentrations which minimizes the contribution of other forms of cytochrome P-450. In contrast, proliferation of the smooth endoplasmic reticulum and increases in total cytochrome P-450 are not consistently observed and may be a function of experimental diet formulations rather than a direct consequence of ethanol consumption.

It has been demonstrated that, in addition to ethanol, numerous other compounds are capable of inducing isozyme 3a in rabbits without increasing the specific content of total cytochrome P-450 and that the *p*-nitrophenol hydroxylase activities of microsomes increase in parallel with the increase in isozyme 3a [15, 20]. Similar results were obtained with ethanol-treated hamsters in the present work. Implicit in these observations is that there must be corresponding decreases in other cytochrome P-450 isozymes since the total content remains unchanged. It has been shown that ethanol consumption by chow-fed hamsters results in lowered specific activities for benzphetamine-*N*-demethylase [5, 6], 7-ethoxycoumarin *O*-deethylase [6], 7-ethoxyresorufin *O*-deethylase [4], and benzo[*a*]pyrene hydroxylase [4, 6]. Morgan *et al.* [24] have demonstrated that exposure of rats to ethanol vapors results in lowered rates of microsomal dealkylation of aminopyrine, ethylmorphine, benzphetamine, and ethoxyresorufin. Whether the differences observed in the present study are due to a decrease in the specific content of a particular cytochrome P-450 isozyme or the result of lowered cytochrome b_5 specific content [3, 4, 24] needs to be clarified. Although several of the protein bands in the 40,000 to 60,000 molecular weight region exhibit decreased silver staining intensity, their identification as cytochrome P-450 isozymes must await further studies.

The availability of antibody specific for the ethanol-inducible isozyme from rabbits has allowed for the exploration of species other than rabbit for the appearance of similar activities in ethanol-treated animals and to determine if ethanol pretreatment results in induction of a homologous isozyme. In the present study we have been able to show that two commonly utilized enzymatic analyses for microsomal drug oxidations in general, and the inductive effects of ethanol in particular, parallel each other both in terms of degree of induction by ethanol as well as the degree of inhibition by antibody specific for rabbit isozyme 3a. The degrees of induction of the two enzymatic activities are similar when expressed as a total increase in enzymatic activity or as the antibody-inhibitable rates, and these values are similar to the induction of the protein as quantified by immunoblot analysis. These data indicate that hamster cytochrome P-450alc is induced by ethanol in the drinking water and that relatively large changes in the distribution of cytochrome P-450 isozymes are possible without corresponding changes in total cytochrome P-450 content measured spectrally. These data along with earlier demonstrations of the dependency of dietary fat for the induction of total cytochrome P-450 but not aniline hydroxylation by ethanol clearly indicate that little if any significance can be ascribed to the lack of effect of any modifications of diet or inducer pretreatment on spectrally determined total cytochrome P-450 content [30–32]. Our data suggest that the inhibition of enzymatic activities by antibodies specific for particular cytochrome P-450 isozymes is a more appropriate indicator of P-450 status. Indeed, since significant changes in the distribution of cytochrome P-450 isozymes can occur with no demonstrable change in the total cytochrome P-450 content, it will be necessary to monitor each isozyme specifically by appropriate use of isozyme-specific catalytic activities, specific inhibitors of isozyme catalysis, or immunoquantitation of individual isozymes before conclusions can be reached on the consequences of any treatment regimen on microsomal mixed-function oxidase activities. The demonstration that antirabbit isozyme 3a IgG crossreacts and can inhibit the ethanol-inducible isozyme in hamsters suggests that the antibody can be used to probe the microsomal activity of other substrates which are induced by alcohol such

as *N*-nitrosopyrrolidine [1] and nicotine [33] without having to first purify the enzyme from hamsters. If this is required, the antibody can be used to monitor the purification and, as a result, will greatly facilitate these experiments. Alternatively, antibody to rabbit 3a can be utilized for immunopurification of the hamster homologue.

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