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Identification of P-450$_{ALC}$ in Microsomes from Alcohol Dehydrogenase-Deficient Deermice: Contribution to Ethanol Elimination *in Vivo*[1]

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Isozyme 3a of rabbit hepatic cytochrome P-450, also termed P-450$_{ALC}$, was previously isolated and characterized and was shown to be induced 3- to 5-fold by exposure to ethanol. In the present study, antibody against rabbit P-450$_{ALC}$ was used to identify a homologous protein in alcohol dehydrogenase-negative (ADH^-) and -positive (ADH^+) deermice, *Peromyscus maniculatus*. The antibody reacts with a single protein having an apparent molecular weight of 52,000 on immunoblots of hepatic microsomes from untreated and ethanol-treated deermice from both strains. The level of the homologous protein was about 2-fold greater in microsomes from naive ADH^- than from naive ADH^+ animals. Ethanol treatment induced the protein about 3-fold in the ADH^+ strain and about 4-fold in the ADH^- strain. The antibody to rabbit P-450$_{ALC}$ inhibited the microsomal metabolism of ethanol and aniline. The homologous protein, termed deermouse P-450$_{ALC}$, catalyzed from 70 to 80% of the oxidation of ethanol and about 90% of the hydroxylation of aniline by microsomes from both strains after ethanol treatment. The antibody-inhibited portion of the microsomal activities, which are attributable to the P-450$_{ALC}$ homolog, increased about 3-fold upon ethanol treatment in the ADH^+ strain and about 4-fold in the ADH^- strain, in excellent agreement with the results from immunoblots. The total microsomal P-450 content and the rate of ethanol oxidation were induced 1.4-fold and 2.2-fold, respectively, by ethanol in the ADH^+ strain and 1.9-fold and 3.3-fold, respectively, in the ADH^- strain. Thus, the total microsomal P-450 content and ethanol oxidation underestimate the induction of the P-450$_{ALC}$ homolog in both strains. A comparison of the rates of microsomal ethanol oxidation *in vitro* with rates of ethanol elimination *in vivo* indicates that deermouse P-450$_{ALC}$ could account optimally for 3 and 8% of total ethanol elimination in naive ADH^+ and ADH^- strains, respectively. After chronic ethanol treatment, P-450$_{ALC}$ could account maximally for 8% of the total ethanol elimination in the ADH^+ strain and 22% in the ADH^- strain. Further, cytochrome P-450$_{ALC}$ appears to be responsible for about one-half of the increase in the rate of ethanol elimination *in vivo* after chronic treatment with ethanol. These results indicate that the contribution of P-450$_{ALC}$ to ethanol oxidation in the deermouse is relatively small. Desferrioxamine had no effect on rates of ethanol uptake by perfused livers from ADH-negative deermice, indicating that ethanol oxidation by a hydroxyl radical-mediated mechanism was not involved in ethanol metabolism in this mutant. Peroxisomal β-oxidation capacity was increased 40% over control values by ethanol treatment, consistent with the hypothesis that the increase in ethanol elimination in the ADH-negative deermouse is mediated predominantly via catalase-H_2O_2.

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Ethanol administration induces a form of hepatic cytochrome P-450 which was purified from ethanol-treated rabbits and characterized (1, 2). The electrophoretically homogeneous enzyme, termed P-450 isozyme 3a or P-450$_{ALC}$,[5] exhibits a preference for the oxidation of alcohols (2), aniline (2), CCl_4 (3), p-nitrophenol (4), acetone (5), and N-nitrosodimethylamine (6), activities which have been attributed to an ethanol-inducible isozyme in microsomes (7). P-450$_{ALC}$ is responsible for 70 to 80% of the alcohol oxidation catalyzed by liver microsomes from ethanol-treated rabbits (8), and has been identified as the low-K_m N-nitrosodimethylamine demethylase (9). A homologous isozyme (P-450j) was recently isolated from ethanol-treated rats (10, 11).

In 1980, Burnett and Felder (12) described a strain of deermice, *Peromyscus maniculatus*, which lacks alcohol dehydrogenase yet eliminates ethanol. The ADH$^+$ animals are known to exhibit normal liver ADH activity, while the ADH$^-$ animals have no detectable ADH activity or cross-reactive protein to anti-ADH antisera (13, 14). More recently, Shigeta *et al.* (15) reported that induction of total microsomal P-450 occurs in both the ADH$^+$ and ADH$^-$ strains after chronic administration of ethanol in a liquid diet. Concomitant with the increase in P-450, chronic ethanol treatment increased microsomal ethanol oxidation as well as ethanol elimination studied at high ethanol concentrations *in vivo*. Shigeta *et al.* (15) concluded that microsomal ethanol oxidation was responsible for a substantial portion of the adaptive increase in ethanol elimination in ADH$^-$ deermice, presumably as a result of the induction of a unique isozyme of P-450.

Due to the multiplicity of microsomal P-450's and the overlapping substrate preference of the isozyme, it is difficult to attribute a microsomal activity to a particular form of P-450 unless specific inhibitors are available. The preparation of inhibitory antibodies specific for one isozyme provides a means to establish the role of a particular P-450 isozyme in microsomal catalysis. Antibody to P-450$_{ALC}$ was characterized (8, 16) and was found to cross-react with hepatic microsomes from other species (5, 9). A direct comparison of immunoblots demonstrated that antibody to the rabbit 3a isozyme cross-reacts with P-450j in microsomes from ethanol-treated rats (10). In the present study, we used antibody to rabbit P-450$_{ALC}$ to demonstrate that an immunochemically homologous protein is present in liver microsomes of the deermouse and determined the extent to which ethanol treatment alters the relative concentration and activity of this protein in both the ADH$^+$ and ADH$^-$ strains. Further, the quantitative contribution of this isozyme to ethanol elimination *in vivo* was estimated.

MATERIALS AND METHODS

Preparation of microsomes from deermice. Male ADH$^+$ and ADH$^-$ deermice were obtained from a colony maintained at the University of North Carolina which was established from breeding pairs kindly provided by Dr. Michael Felder, University of South Carolina. Adult deermice (15–20 g in weight) were fed either a control diet or an ethanol-containing diet described by Lieber and DeCarli (17) for 3 to

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[5] Abbreviations used: P-450$_{LM}$, liver microsomal cytochrome P-450; ADH, alcohol dehydrogenase; SDS, sodium dodecyl sulfate. The isozymes of rabbit P-450$_{LM}$ are numbered according to their relative electrophoretic mobilities. The ethanol-inducible cytochrome is designated as form or isozyme 3a according to this system or as P-450$_{ALC}$ to indicate that alcohol is an inducer and a substrate. The immunochemically homologous protein in other species is also called P-450$_{ALC}$, but with the understanding that the amino acid sequences need not be completely identical. These hemoproteins are termed forms II. E.1. in the standard nomenclature.

5 weeks prior to measurement of blood ethanol clearance or preparation of liver homogenates and microsomes. The concentration of ethanol in the diet was initially 0.5% (w/v) and was increased gradually to 2.5% (w/v) over the first week of treatment. The microsomal fraction was prepared by differential centrifugation, and the initial 100,000*g* pellets were resuspended in 0.1 M sodium pyrophosphate buffer, pH 7.4, containing 1 mM EDTA and recentrifuged at 100,000*g* for 1 h. The washed pellets were suspended at a protein concentration of 25–40 mg/ml in 100 mM Tris-acetate buffer, pH 7.4, containing 20% glycerol, and stored at −70°C for up to 9 months with no loss of activity. The protein concentration was determined by the method of Lowry *et al* (18) and *P*-450 content was determined by difference spectroscopy after solubilization with 0.33% (v/v) Tergitol NP-10 (19). Liver weights ranged from 4 to 5% of the total body weight.

Gel electrophoresis and Western blots. Hepatic microsomal proteins were separated by electrophoresis in 7.5% sodium dodecyl sulfate-polyacrylamide gels as described by Laemmli (20). The gels were stained with silver (21) or the proteins were transferred electrophoretically to nitrocellulose (16). Nitrocellulose sheets containing protein samples from electrophoretic transfer or direct application were incubated overnight at 4°C in 10 mM Tris-Cl buffer, pH 7.4/0.15 M NaCl (Tris/NaCl) containing 1% (w/v) bovine serum albumin. The sheets were washed twice with Tris/NaCl, anti-3a IgG (1 μg/ml) was added, and incubation was carried out for 1 h at room temperature. The sheets were washed five times with Tris/NaCl containing 0.05% (v/v) Tween 20, followed by two washes with Tris/NaCl, and then incubated in a 1:300 dilution of rabbit anti-sheep IgG (Cappel Laboratories, Cochranville, PA) in Tris/NaCl. After 30 min at room temperature, washing was again carried out as just described. Peroxidase-conjugated sheep anti-rabbit IgG (Cappel Laboratories) at 1:5000 dilution was incubated with nitrocellulose for 30 min at room temperature, followed by washing as described above. The location of peroxidase activity was detected with 4-chloro-1-naphthol and H_2O_2 as described by Nielsen *et al.* (46). The reaction was quenched at the end of 30 min by placing the nitrocellulose in water. The sheets were dried and the intensity of the stain was quantified with a Zeiss soft-laser densitometer.

Microsomal assays. The oxidation of ethanol was determined in capped vessels in 1.0 ml reaction mixtures containing 50 mM potassium phosphate buffer, pH 7.4, 1 mM sodium azide, 100 mM ethanol, microsomes (0.2 mg of protein), and 0.5 mM desferrioxamine. The mixtures were preincubated at 37°C for 3 min, and NADPH (1.0 mM, final concentration) was added. After a 10-min incubation at 37°C, reactions were terminated by the addition of 0.2 ml of 35% perchloric acid. Butyraldehyde was added as an internal standard, and acetaldehyde was measured by gas chromatography of the head space gas as described in detail elsewhere (8). In preliminary experiments, desferrioxamine was found to inhibit the ethanol oxidation activity of microsomal preparations from untreated and ethanol-treated deermice by 2 to 13%. Accordingly, desferrioxamine (0.5 mM) was included in all reaction mixtures to prevent ethanol oxidation due to Fenton-type reactions (8, 22–24). Where indicated, purified sheep anti-3a IgG or preimmune sheep IgG was added so the total concentration of IgG was 2.0 mg of protein/ml. All rates were corrected for zero-time blanks in which the reactions were stopped with perchloric acid prior to the addition of NADPH. *p*-Aminophenol produced from aniline hydroxylation was determined as described by Mieyal and Blumer (25). The reaction mixtures were identical to those described for the experiments with ethanol, except that aniline (2.5 mM) served as the substrate and the reactions were terminated with 0.30 ml of 20% trichloroacetic acid. All reactions were linear with respect to protein concentration and length of incubation.

Determination of ethanol elimination. Ethanol was administered ip and ethanol elimination was calculated from measurements of breath ethanol taken every 15–30 min for about 3 h as described in detail elsewhere (26). Briefly, blood ethanol concentrations were calculated from measurements of breath ethanol and rates of ethanol elimination were calculated by using standard procedures (27). Individual deermice were forced to breathe in a 2.75-ml closed vessel for 17 s to ensure maximal equilibration between breath and vapor in the chamber. After equilibration, a 1.0-ml sample of exhaled breath was analyzed for ethanol by injection into a Hewlett-Packard Model 5720 gas chromatograph equipped with a carbowax 60/80 column and a flame ionization detector. The operation parameters were: oven, 110°C; detector, 315°C; injection port, 300°C; and carrier gas flow, 80 ml/min. A peak corresponding to ethanol with a retention time of about 50 s was compared with ethanol standards (28). Ethanol concentrations in blood correlated linearly with breath ethanol.

Determination of rates of peroxisomal β-oxidation and urate oxidase. Rates of peroxisomal β-oxidation of palmitate were measured as described by Inestrosa *et al* (29). Briefly, livers from ADH-negative deermice fed either the control or ethanol-containing diet were homogenized (1:5) in 0.25 M sucrose. Thirteen milligrams of tissue was incubated for 10 min at 37°C in 3 ml of 100 mM Tris-Cl, pH 8.3, containing 100 mM methanol, 0.1 mM CoA, 2.5 mM ATP, 5 mM $MgCl_2$, 200 μM NAD^+, 33 mM nicotinamide, 0.9 mg/ml bovine serum albumin, 0.01% (w/v) Triton X-100, 6.6 mM semicarbazide, and 1 mM sodium palmitate. Reactions were terminated with 0.3 ml of 40% trichloro-

acetic acid and the formaldehyde formed from the oxidation of methanol was measured colorimetrically by the method of Nash (30). Zero-time blanks (all components were present after the addition of acid at time zero) were less than 10% of the measured values. Rates of peroxisomal β-oxidation were calculated from rates of formaldehyde formation per unit time and the amount of tissue employed.

Urate oxidase activity was determined by incubating 0.2 ml of homogenate in 1.0 ml of 100 mM Tris-Cl, pH 8.3, containing 1 mM KCN. At 25°C the disappearance of O_2 was measured after the addition of urate (final concentration, 0.1 mM). Rates were calculated from rates of O_2 uptake per unit time and the amount of tissue employed.

Perfusion of livers from ADH-negative deermice. Livers from ADH-negative deermice were perfused with Krebs-Henseleit buffer, pH 7.4, 37°C in a recirculating system as described elsewhere (31). Deermice were anesthetized with 50 mg/kg pentobarbital, the abdomen was opened, and a cannula (PE 10 tubing) was secured in the portal vein with the flow stopped. The chest was opened rapidly and the heart was cut. Flow was then initiated at a rate of about 4 ml/min/g liver. The aorta was ligated below the kidney and the ascending vena cava was severed close to the diaphram. The liver was then removed surgically from the body. An outflow cannula was secured into the vena cava by using the diaphram for support and flow was increased to 6 to 8 ml/min/g. Under these conditions, oxygen uptake was constant and LDH was not released into the perfusate for at least 2 h of perfusion.

Other procedures and materials. P-450 isozyme 3a was purified from acetone-treated rabbits (1, 5). Antibody to the purified isozyme was raised in sheep and purified as described previously (8). All water and buffers used in the microsomal assay mixtures were passed through Chelex-100 columns. Rabbit anti-sheep IgG and sheep peroxidase-antiperoxidase were obtained from Cooper Biomedical. Desferrioxamine (desferal mesylate) was a gift from the Ciba-Geigy Corp. Sodium palmitate, NAD^+, nicotinamide, ATP, enzymes, and bovine serum albumin were from Sigma Chemical Co. (St. Louis, MO). The source of other materials has been described elsewhere (1, 8, 16).

RESULTS

Identification of deermouse P-450$_{ALC}$. Treatment of ADH^+ and ADH^- deermice with ethanol results in the induction of cytochrome P-450. In the present study, exposure to ethanol increased the specific content of liver microsomal P-450 in ADH^+ deermice about 1.4-fold (to 1.40 $\pm$ 0.10 nmol of P-450/mg of protein) compared to the level in untreated animals (1.03 $\pm$ 0.09). The effect of ethanol was greater in ADH^- deermice, where the content of P-450 was increased to 2.80 $\pm$ 0.41 as compared to 1.08 $\pm$ 0.06 nmol/mg of protein in the control animals.

When proteins from microsomes isolated from control and ethanol-treated ADH^+ and ADH^- deermice were separated by SDS-polyacrylamide gel electrophoresis, a protein with an apparent molecular weight of about 52,000 was observed (data not shown). When the proteins were transferred to nitrocellulose and stained immunochemically with antibody specific for rabbit P-450$_{ALC}$, a single band having the same mobility was observed in all four microsomal preparations studied (Fig. 1), results similar to other studies where isozyme 3a or a homolog was identified with the anti-3a antibody (8, 9, 10).

Since the deermouse isozyme was not available as a standard, direct quantification of this protein in microsomes was not possible. Therefore, the relative increase in the deermouse protein was established by comparison of the staining intensity of increasing concentrations of each microsomal preparation on the same electrophoretic blot (Fig. 2). Preliminary data established the protein concentration ranges that gave a linear staining response for each microsomal preparation. The relative slopes of the lines (see Fig. 2 legend) are indicative of the concentration of the deermouse protein cross-reacting with anti-rabbit P-450$_{ALC}$. Ethanol treatment resulted in a 2.6-fold increase in the content of this protein in ADH^+ and a 3.8-fold increase in ADH^- deermice. However, the total cytochrome P-450 content of the microsomal preparations was increased only 1.3-fold by ethanol in the ADH^+ strain and 1.9-fold in the ADH^- strain. Such a difference, which was also observed upon the administration of ethanol and other agents to rabbits (16), may be due to repressed synthesis of one or more P-450 isozymes in the endoplasmic reticulum.

In order to determine whether the deermouse protein is catalytically similar to rabbit P-450$_{ALC}$, the effect of anti-P-

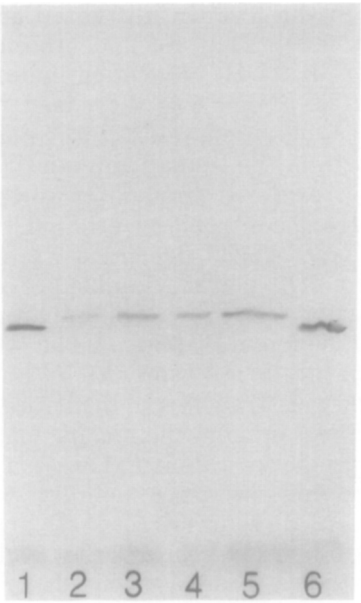

FIG. 1. Immunochemical staining of hepatic microsomes from deermice. Hepatic microsomes (2.0 μg of protein) from untreated or ethanol-treated ADH$^+$ and ADH$^-$ deermice and purified rabbit liver P-450$_{ALC}$ (0.1 μg of protein) were submitted to electrophoresis in 7.5% SDS-polyacrylamide gels as described under Materials and Methods. The following samples were loaded on the gel: lanes 1 and 6, rabbit P-450$_{ALC}$; lane 2, microsomes from untreated ADH$^+$ animals; lane 3, microsomes from ethanol-treated ADH$^+$ animals; lane 4, microsomes from untreated ADH$^-$ animals; and lane 5, microsomes from ethanol-treated ADH$^-$ animals. The proteins were transferred electrophoretically to nitrocellulose and stained immunochemically with anti-3a antibody as described previously (16).

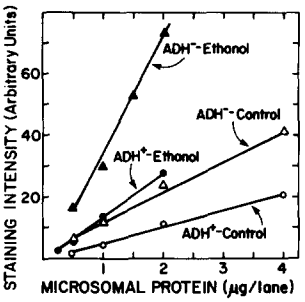

FIG. 2. Induction of P-450$_{ALC}$ in ethanol-treated deermice as judged by immunoblotting with use of anti-rabbit P-450$_{ALC}$. Increasing concentrations of hepatic microsomes from untreated and ethanol-treated ADH$^+$ and ADH$^-$ deermice were submitted to electrophoresis and then transferred onto nitrocellulose. After immunochemical staining with antibody to rabbit P-450$_{ALC}$, the intensity of the stain was quantified by densitometry. The slopes (arbitrary units/μg of microsomal protein) were determined by linear regression analysis and were: untreated ADH$^+$ (○), 5.5; ethanol-treated ADH$^+$ (●), 14.6; untreated ADH$^-$ (△), 10.0; and ethanol-treated ADH$^-$ (▲), 38.6.

450$_{ALC}$ IgG on the microsomal metabolism of aniline and ethanol was determined (Fig. 3). The oxidation of both substrates was inhibited 85 to 90% by anti-3a IgG at a level of about 4.5 mg of anti-3a IgG/nmol P-450. At optimal antibody concentrations, the oxidation of ethanol by microsomes from untreated ADH$^+$ and ADH$^-$ deermice was approximately equal; the antibody inhibited 50 to 75% of the ethanol oxidation in microsomes from ADH$^+$ and ADH$^-$ strains, respectively (Fig. 4). Ethanol treatment increased the ethanol oxidation activity of microsomes from the ADH$^+$ strain by 2.2-fold and the ADH$^-$ strain by 3.3-fold. Anti-3a IgG inhibited the increase in ethanol metabolism due to chronic ethanol treatment 73 to 82% in both strains. The residual activity ob-

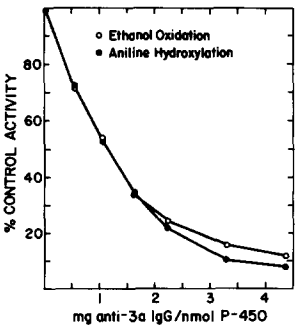

FIG. 3. Effect of anti-rabbit P-450$_{ALC}$ on the metabolism of aniline and ethanol by deermouse hepatic microsomes. Increasing amounts of anti-rabbit P-450$_{ALC}$ were added as indicated to reaction mixtures containing microsomes (0.2 mg of protein; 0.46 nmol of P-450) from ethanol-treated ADH$^-$ deermice. The total IgG level was kept constant at 2.0 mg of protein by the addition of preimmune IgG. The other components were as described under Materials and Methods, and the reactions were allowed to proceed for 10 min at 37°C. Control rates for aniline and ethanol oxidation were 11.4 and 37.5 nmol/min/mg of protein, respectively.

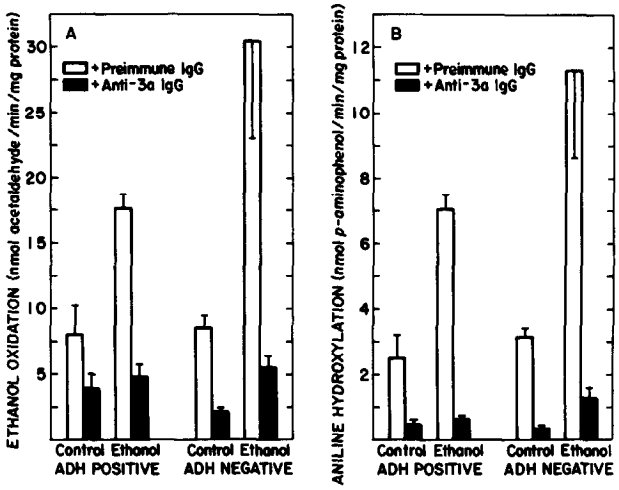

FIG. 4. Effect of anti-rabbit P-450$_{ALC}$ IgG on the oxidation of ethanol and aniline by microsomes from untreated and ethanol-treated deermice. The incubation mixtures contained anti-3a IgG or preimmune IgG (2 mg of protein) and other components as described under Materials and Methods. (A) Results obtained with ethanol as substrate; (B) results with aniline as substrate with the same microsomal preparations. The values are expressed as means ± SE of three different microsomal preparations each prepared by pooling the livers from four to six deermice.

served in the presence of anti-3a IgG was about the same with all of the microsomal samples studied except the untreated ADH preparation, which was significantly lower than the other three. The results of aniline hydroxylation paralleled those obtained with ethanol, although the two substrates are oxidized at significantly different rates. The ratio of ethanol to aniline activity was 2.75 ± 0.09 for 12 different deermouse microsomal preparations studied from untreated and ethanol-treated deermice. Anti-3a IgG proved to be a slightly more effective inhibitor of aniline hydroxylation than ethanol oxidation, decreasing the rates in microsomes from untreated and ethanol-treated deermice by 80 to 90%. The residual activity most likely represents catalysis by other isozymes present in the microsomal preparations.

The induction of deermouse P-450$_{ALC}$ by chronic ethanol treatment can be monitored by the increase in the staining intensity of the homolog on immunoblots of hepatic microsomes and by the increase in the antibody-inhibited rate of ethanol or aniline oxidation. Such data are shown in Table I for one group of microsomal preparations. The P-450$_{ALC}$-dependent rates were calculated by subtracting the rate obtained in the presence of anti-rabbit P-450$_{ALC}$ from the rate obtained in the presence of preimmune IgG. Very similar values for the extent of induction of this cytochrome were obtained by both immunochemical staining and by the catalytic rates. The cytochrome is induced about 2.9-fold by ethanol in the ADH$^+$ and 3.9-fold in the ADH$^-$ strain. The results also show that the constitutive levels of P-450$_{ALC}$ are about 1.6- to 1.8-fold higher in microsomes from naive ADH$^-$ than from naive ADH$^+$ deermice.

Contribution of deermouse P-450$_{ALC}$ to ethanol elimination in vivo. If one assumes that the microsomal system is operating under optimal conditions *in vitro*, a maximal contribution of P-450$_{ALC}$ to the overall rate of alcohol oxidation *in vivo* can be estimated. Basal rates of ethanol elimination in this study were similar to those reported previously by others (15, 26, 32). The contributions of the total microsomal rate and the P-450$_{ALC}$-dependent rate to hepatic ethanol elimination are summarized in Table II. In untreated ADH$^+$ deermice, only about 7% of the ethanol

TABLE I

INDUCTION OF P-450$_{ALC}$ IN DEERMOUSE MICROSOMES

Treatment	Relative P-450$_{ALC}$ content determined by immunostaining (units/μg protein)	P-450$_{ALC}$-dependent substrate oxidation (nmol product/min/mg protein)	
		Ethanol	Aniline
ADH$^+$ strain			
None	5.5	4.5	2.0
Ethanol	14.6 (2.7)	13.7 (3.0)	5.7 (2.8)
ADH$^-$ strain			
None	10.0	8.0	3.1
Ethanol	38.6 (3.9)	33.1 (4.1)	11.5 (3.7)

Note. The values for the relative P-450$_{ALC}$ content, based on immunochemical staining, are the slopes taken from Fig. 2, and the P-450$_{ALC}$-dependent rates were calculated as described in the text. The results are from one microsomal preparation in each treatment group made from the pooled livers from four to six deermice. The values in parentheses indicate the fold increase due to ethanol treatment relative to control group.

elimination could be accounted for by microsomes and about one-half of that amount appeared to be P-450$_{ALC}$-dependent. Upon induction of cytochrome P-450$_{ALC}$ by ethanol treatment, the deermouse P-450$_{ALC}$-dependent activity was about 8% of the total ethanol elimination. In untreated ADH$^-$ deermice, the microsomal rate could account for about 10% of total ethanol elimination while the P-450$_{ALC}$-dependent value was about 8%. After ethanol treatment of ADH$^-$ animals, however, approximately 22% of the total ethanol elimination was catalyzed by P-450$_{ALC}$.

To address the question of the involve-

TABLE II

COMPARISON OF THE MICROSOMAL AND P-450$_{ALC}$-DEPENDENT RATES OF ETHANOL OXIDATION WITH ETHANOL ELIMINATION *in Vivo*

Treatment	Ethanol elimination *in vivo*	Ethanol oxidation (mmol/kg/h)	
		Microsomal	P-450$_{ALC}$-dependent
ADH-positive			
Control	14.8 ± 1.3	1.0 ± 3.0 (7)	0.5 ± 0.1 (3)
Ethanol-treated	19.5 ± 3.2	2.1 ± 0.1 (11)	1.5 ± 0.1 (8)
ADH-negative			
Control	9.9 ± 0.5	1.0 ± 0.1 (10)	0.8 ± 0.1 (8)
Ethanol-treated	13.4 ± 1.4	3.7 ± 1.0 (28)	3.0 ± 0.9 (22)

Note. Deermice were injected ip with ethanol (2.0 g/kg) and breath samples were taken repeatedly to determine blood ethanol concentrations as described in detail elsewhere (26). The liver weight:body weight ratio was 0.04 and was not affected by ethanol treatment. It was assumed that livers contained 50 mg of microsomal protein/g liver (41). Rates of oxidation of ethanol by microsomes were measured in the presence and absence of anti-P-450$_{ALC}$ IgG and the rates due to deermouse P-450$_{ALC}$ were calculated as described in the text. Results are expressed as means ± SE. Rates of ethanol elimination *in vivo* were obtained with six to nine individual deermice in each group, and the rates of microsomal ethanol oxidation were obtained with three separate microsomal preparations made from livers from four to six deermice. The numbers in parentheses represent the maximal contribution of the microsomal system P-450$_{ALC}$ to the total rate of ethanol elimination.

ment of hydroxyl radicals in ethanol oxidation in the ADH-negative deermouse, ethanol uptake was measured in the presence and absence of desferrioxamine. Desferrioxamine enters hepatocytes since it prevents allyl alcohol-induced damage in perfused livers from the rat (45) and the deermouse (unpublished). Basal rates of ethanol uptake of 89 ± 9 μmol/g/h were not affected by desferrioxamine (1 mM; Table III). Thus, it is concluded that hydroxyl radicals are not involved in ethanol oxidation in perfused livers from ADH-negative deermice.

Induction of peroxisomes by chronic ethanol treatment. Chronic treatment with ethanol has been reported to cause a proliferation of peroxisomes in the liver (33). Accordingly, the effect of ethanol treatment on peroxisomal H_2O_2 generating systems was studied in the ADH-negative deermouse. Five weeks of treatment with ethanol caused rates of peroxisomal β-oxidation of palmitate measured in liver homogenates *in vitro* to increase significantly above activities measured in control livers (Table IV). Peroxisomal β-oxidation was not affected significantly by the control diet under these conditions. The activity of urate oxidase, a protein located in the peroxisomal matrix, was about 70 μmol/g/h and was not affected by ethanol treatment (Table IV). Thus, it is possible that a portion of the increase in ethanol oxidation observed after chronic treatment with ethanol involves enhanced H_2O_2 production for catalase via the peroxisomal β-oxidation pathway.

TABLE III

EFFECT OF DESFERRIOXAMINE ON ETHANOL UPTAKE BY PERFUSED LIVERS FROM ADH-NEGATIVE DEERMICE

Addition	Ethanol uptake (μmol/g/h)
Ethanol	89 ± 9
Ethanol + desferrioxamine	87 ± 9

Note. Livers from ADH-negative deermice were perfused in a recirculating system (23 ml) with Krebs-Henseleit buffer (pH 7.4, 37°C) for 20 min. Ethanol (20 mM) was added and samples were taken every 30 min. Desferrioxamine (1 mM) was added prior to ethanol in some experiments. Ethanol was measured enzymatically (42) in samples of perfusate collected every 30 min. Data represent means ± SE for four livers in each group.

TABLE IV

INCREASES IN HEPATIC PEROXISOMAL β-OXIDATION CAPACITY IN ADH-NEGATIVE DEERMICE AFTER ETHANOL TREATMENT

Treatment	Weeks of treatment	Peroxisomal β-oxidation (μmol/g/h)	Urate oxidase (μmol/g/h)
Control	0	29 ± 4	70 ± 2
	5	25 ± 2	90 ± 10
Ethanol	0	29 ± 1	71 ± 3
	5	34 ± 2*	70 ± 6

Note. ADH-negative deermice were treated chronically with control or ethanol-containing diets as described under Materials and Methods. After 5 weeks of treatment, deermice were anesthetized with Ketamine and livers were removed surgically. Livers were homogenized (1:5) in 0.25 M sucrose and peroxisomal β-oxidation from palmitate and urate oxidase were measured as described under Materials and Methods. Data represent means ± SE for four livers per group.

* $P < 0.05$ as compared to corresponding time of treatment of control group.

DISCUSSION

In the present report, we have used antibodies to rabbit P-450$_{ALC}$ to demonstrate that ethanol treatment induces an immunochemical homolog to this cytochrome, which is termed deermouse P-450$_{ALC}$, in both ADH$^+$ and ADH$^-$ strains. An examination of the content of this cytochrome indicates that the ADH$^-$ strain has higher constitutive levels and that it is induced to a greater extent by ethanol in the ADH$^-$ than in the ADH$^+$ strain. In both strains, the isozyme is responsible for 70 to 80% of ethanol oxidation by microsomes after treatment of the animals with ethanol. The antibody has a parallel effect on the rates of aniline p-hydroxylation, an activity also catalyzed by rabbit P-450$_{3a}$ (2, 8) and rat P-450j (10). The increase observed in the P-450$_{ALC}$-dependent rates agreed well with the increase in the relative con-

centration of the protein measured immunochemically, which indicates that the 52,000 molecular weight protein is the $P\text{-}450_{ALC}$-homolog.

Care must be taken in the interpretation of increased rates of microsomal ethanol oxidation. In addition to the *P*-450-catalyzed oxidation of ethanol, a pathway that involves H_2O_2 and a reduced iron chelate could make a significant contribution to microsomal rates *in vitro*, especially when EDTA is added to the incubation mixtures (23, 24). The relative contribution of each pathway can be determined since desferrioxamine inhibits the iron-chelate-dependent pathway but not the *P*-450-dependent pathway (22–24). Since Cederbaum and Dicker (22) have reported that 60% of the ethanol-oxidizing activity of KCl-washed microsomes from ethanol-treated rats is inhibited by desferrioxamine, an observed increase in rate does not necessarily reflect a *P*-450-specific increase in ethanol oxidation. Shigeta *et al.* (15) reported that ethanol treatment of both ADH^+ and ADH^- deermice resulted in an increase in the rate of ethanol oxidation by hepatic microsomes and that the increase paralleled an enhancement in the content of microsomal *P*-450. The components of the reactions mixtures were not detailed in their report, but the method referenced (35) utilized EDTA, which could lead to elevated rates of ethanol oxidation. As a result, the rates reported with microsomes in that study may not reflect the *P*-450-specific increase in ethanol oxidation resulting from ethanol treatment. As reported for rabbit microsomes (8), we found that removal of free iron by washing with pyrophosphate results in little or no sensitivity to desferrioxamine. Thus, the rates reported here are attributable to catalysis by *P*-450. In addition, desferrioxamine did not alter rates of ethanol uptake by perfused livers from ADH-negative deermice (Table III), supporting the hypothesis that Fenton chemistry is not involved in ethanol elimination in this mutant *in vivo*.

While the data obtained *in vitro* in the present report provide strong evidence for the induction of a $P\text{-}450_{ALC}$-homolog and establishes its role as the predominant catalyst of ethanol oxidation in microsomes from ethanol-treated deermice, they do not address the role of the enzyme in the elimination of ethanol *in vivo* directly. Since $P\text{-}450_{ALC}$ is induced by ethanol and oxidizes ethanol *in vitro*, it is not unreasonable to assume that it functions similarly *in vivo*. When the rates *in vitro* were compared with rates of ethanol elimination *in vivo*, it was estimated that $P\text{-}450_{ALC}$ accounts for only 3% of the ethanol elimination in naive ADH^+ animals and about 8% of the elimination in naive ADH^- animals (Table II). Thus cytochrome $P\text{-}450_{ALC}$ has a minor role in ethanol oxidation *in vivo*. The induction of $P\text{-}450_{ALC}$ accounts for about one-half of the increase in ethanol elimination observed in ADH^- deermice after ethanol treatment; however, even after ethanol treatment, cytochrome *P*-450 could account for only about 22 to 28% of ethanol elimination *in vivo* (Table II).

The estimation that cytochrome *P*-450 accounts maximally for about 10% of rates of ethanol elimination in naive ADH deermice (Table II) contrasts sharply to the conclusions of Aldermann *et al* (43). By using 1*R*-[^{3}H]ethanol, it was reported that the isotope effect for ethanol elimination by ADH deermice was almost identical to isotope effects of about 1.15 measured in microsomes. However, because only one stereoisomer of ethanol instead of both the *R* and *S* enantiomers was employed, it is probable that the isotope effect was underestimated (44). In fact, the isotope effect determined in microsomes of ADH deermice was between 4 and 5 (32). Thus, conclusions regarding the role of catalase or cytochrome *P*-450 cannot be assessed quantitatively in experiments which assume stereospecificity of the reaction.

Since ADH^- deermice have no ADH, it is concluded that a major portion of ethanol elimination occurs by mechanisms independent of ADH and *P*-450 in this strain. If ethanol metabolism in the ADH^- deermouse does not occur to an appreciable extent via ADH, cytochrome $P\text{-}450_{ALC}$ or hydroxyl radical-mediated mechanisms, or by respiration or excretion (15),

or by extrahepatic metabolism (34), what pathway then is responsible? Others have concluded (36) that catalase does not play a significant role in ethanol metabolism because rates of H_2O_2 generation, which are rate-limiting for the oxidation of ethanol via catalase (37), were reported to be more than an order of magnitude lower than rates of ethanol metabolism in perfused rat livers (38). However, considerable evidence from our laboratory has demonstrated that conditions exist where catalase is an important ethanol oxidase. For example, in the presence of fatty acids, the rate of H_2O_2 generation in rat liver is sufficient to support high rates of ethanol oxidation via catalase-H_2O_2 (39, 40). In the presence of physiological concentrations of albumin, long-chain fatty acids supply H_2O_2 at rates ranging from 60 to 80 μmol/g/h, values similar to rates of 4-methylpyrazole-insensitive ethanol uptake observed in the presence of fatty acids (40). Therefore, we conclude that ethanol metabolism in the ADH-negative deermouse is mediated predominantly via catalase. In support of this conclusion, we have reported recently that rates of H_2O_2 production were about 65 μmol/g/h, quite close to rates of ethanol uptake of about 60 μmol/g/h in perfused livers from ADH-negative deermice (31).

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