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Immunochemical Evidence for a Role of Cytochrome *P*-450 in Liver Microsomal Ethanol Oxidation[1]

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Antibodies to cytochrome *P*-450 isozyme 3a, the ethanol-inducible isozyme in rabbit liver, were used to determine the role of this enzyme in the microsomal oxidation of alcohols and the *p*-hydroxylation of aniline. *P*-450 isozymes, 2, 3b, 3c, 4, and 6 did not crossreact with anti-3a IgG as judged by Ouchterlony double diffusion, and radioimmunoassays indicated a crossreactivity of less than 1%. Greater than 90% of the activity of purified form 3a toward aniline, ethanol, *n*-butanol, and *n*-pentanol was inhibited by the antibody in the reconstituted system. The catalytic activity of liver microsomes from control or ethanol-treated rabbits was 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 did not make a significant contribution to the microsomal activity. The addition of anti-3a IgG to hepatic microsomes from ethanol-treated rabbits inhibited 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 was only about one-half as great. The rate of microsomal H_2O_2 formation was inhibited to a lesser extent than the formation of acetaldehyde, thus suggesting that the antibody was acting to prevent the direct oxidation of ethanol by form 3a. Under conditions where purified NADPH-cytochrome *P*-450 reductase-catalyzed substrate oxidations was minimal, the *P*-450 isozymes other than 3a had low but significant activity toward the four substrates examined. The residual activity at maximal concentrations of the antibody most likely represents the sum of the activities of *P*-450 isozymes other than 3a present in the microsomal preparations. The results thus indicate that the enhanced monooxygenase activity of liver microsomes from ethanol-treated animals represents catalysis by *P*-450 isozyme 3a.

The role of cytochrome *P*-450 in the oxidation of ethanol and other alcohols has been the subject of much debate. Several laboratories have shown that a reconstituted system containing a purified isozyme of $P\text{-}450_{LM}$[3] induced by phenobarbital and NADPH-cytochrome *P*-450 reductase can oxidize ethanol to acetaldehyde, but conclusions about the participation of *P*-450 in these systems have varied (1–4). Chronic ethanol treatment of rats enhances microsomal ethanol oxidation, an effect that has been attributed to a possible increase in a previously unrecognized form of *P*-450 (5–8). It has recently been suggested that, after alcohol

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[3] Abbreviation used: $P\text{-}450_{LM}$, liver microsomal cytochrome *P*-450. The isozymes from rabbit are numbered according to their relative electrophoretic mobilities; the ethanol-inducible isozyme is referred to as $P\text{-}450_{LM3a}$, or simply as form or isozyme 3a.

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treatment, an increase also occurs in the production of an oxidizing species equivalent to the hydroxyl radical (9). In addition to causing an enhanced oxidation of ethanol, chronic alcohol consumption has been linked to a number of diseases, many of which result from the metabolic activation of specific compounds (10–12). Extensive studies have been conducted on the changes that occur in the biotransformation of xenobiotics after chronic alcohol intake, and, as in the case of ethanol oxidation, the participation of an ethanol-inducible *P*-450 has been invoked (13).

We have reported the isolation and characterization of a unique form of *P*-450, isozyme 3a, from the livers of rabbits treated chronically with ethanol (14, 15), and have shown that the purified enyzme catalyzes the oxidation of alcohols and the *p*-hydroxylation of aniline much more rapidly than do the other purified isozymes from rabbits (15). We have also presented evidence that isozyme 3a catalyzes the activation of acetaminophen to an intermediate capable of forming a conjugate with reduced glutathione (16). Such results strongly suggest that this isozyme is specifically responsible for the increased activity toward these substrates in microsomes from ethanol-treated rabbits (14, 15). In the present paper we have used antibody to purified isozyme 3a to demonstrate that this isozyme is responsible for most of the aniline hydroxylation and alcohol oxidation activity of microsomes from ethanol-treated rabbits, and catalyzes a smaller but significant portion of these activities in microsomes from untreated animals as well. The inhibition by the antibody is not the result of decreased H_2O_2 production since a hydroxyl radical-dependent pathway makes an insignificant contribution to substrate oxidations under our experimental conditions.

EXPERIMENTAL PROCEDURES

Preparation of microsomes and purification of P-450 isozymes. Adult New Zealand male rabbits (2.0 to 2.5 kg in weight) were given drinking water containing 10% ethanol (v/v) for 1 week followed by 5% ethanol (v/v) for an additional 2 weeks, with free access to Purina rabbit chow. Phenobarbital was given as a 0.1% solution (w/v) in the drinking water for 7 days as previously described (17), and isosafrole (150 mg in corn oil/kg body wt) and imidazole (200 mg of an aqueous solution neutralized with HCl/kg body wt) were administered by intraperitoneal injection as previously described (14, 18). Pyrophosphate-washed liver microsomes were prepared (14, 18) and stored at a final protein concentration of about 30 mg/ml in 100 mM Tris–acetate buffer, pH 7.4, containing 0.1 mM EDTA and 20% glycerol, at −70°C. No signficant loss of *P*-450, measured spectrally or catalytically, occurred over a 6-month period under these conditions.

P-450 cytochromes were purified to electrophoretic homogeneity, by procedures already described (14, 17–19), from rabbits treated with phenobarbital (for isozyme 2), isosafrole (for isozyme 4), or imidazole (for isozymes 3a, 3c, and 6), or untreated (for isozyme 3b). Isozyme 3a isolated from imidazole-treated rabbits is identical to the cytochrome obtained from ethanol-treated rabbits, as shown by catalytic, structural, and immunochemical methods (18). The specific contents (nmol P-450/mg protein) of the preparations used were as follows: isozyme 2, 19.8; isozyme 3a, 16.0 to 20.7; isozyme 3b, 18.9; isozyme 3c, 16.0; isozyme 4, 19.5; and isozyme 6, 16.2. Cytochrome *P*-450 reductase purified from liver microsomes obtained from phenobarbital-treated rabbits (20) catalyzed the reduction of 56 μmol cytochrome c min^{-1} mg $protein^{-1}$. The final preparation was dialyzed four times against 100 vol 120 mM potassium phosphate buffer, pH 7.4, containing 10% glycerol, to remove EDTA which was present throughout the purification procedure.

Antibody production. Antibody to isozyme 3a was produced by immunization of yearling female sheep. Each animal was treated initially with 500 μg of purified isozyme 3a in 1.5 ml emulsified Freund's complete adjuvant containing 2.5 mg of killed tuberculosis mycobacterium. Subsequent treatments did not include the bacterium. The animals were injected intradermally in the flanks at 4-week intervals, and 500 ml of blood was collected by jugular venipuncture 2 and 3 weeks following the third and subsequent immunizations. Antibody titer and specificity were optimal 6 to 7 months after the initial immunization. The IgG fraction from immune or preimmune sera was isolated by ammonium sulfate precipitation and DEAE-cellulose column chromatography (21). The final IgG preparations were concentrated to approximately 30 mg protein/ml by use of an Amicon Ultrafiltration cell with a PM-30 membrane. The IgG concentration was determined spectrally with an absorption coefficient at 280 nm of 13.5 cm^{-1} for a 1% solution (22).

Radioiodination of P-450 isozyme 3a. *P*-450 isozyme 3a was radioiodinated by a modification of the Hunter-Greenwood (23) Chloramine-T method. The protein (6.9 μg), 1.0 mCi $Na^{125}I$, and 40 μl 0.5 M potassium phosphate buffer, pH 7.4, were placed in

a 0.5-dram vial fitted with a multidose septum. The iodination was initiated by the addition of 6 μg Chloramine-T in 30 μl 0.1 M potassium phosphate buffer, pH 7.4 (buffer A). The mixture was shaken for 1 min at room temperature, and the reaction was then quenched by the addition of 2 μg sodium metabisulfite in 10 μl buffer A. The reaction mixture was transferred to a Bio-Rad P-60 column (0.6 × 22 cm) that had been equilibrated with buffer A containing 5.0% bovine serum albumin. Elution was carried out with buffer A, and the iodinated *P*-450 obtained in the void volume was immediately diluted to approximately 40,000 cpm/100 μl with 0.1 M potassium phosphate buffer, pH 7.4, containing 0.01% sodium merthiolate and 0.1% pigskin gelatin (buffer B) plus 20% glycerol. For nine iodinations the average incorporation of radioactivity into the protein was 38% and the average estimated specific activity was 57.8 μCi/μg.

Radioimmunoassay. The serum from immunized sheep (in buffer B containing 0.05 M EDTA) was used at a dilution of 1:1,000,000. Standard isozyme 3a and other forms of *P*-450 for crossreactivity studies were prepared for each experiment by dilution in buffer B. All procedures employed donkey anti-sheep antisera to precipitate the sheep anti-isozyme 3a–radioiodinated isozyme 3a complex and thus permit its separation from the unbound labeled enzyme. Preimmune sheep serum in buffer B was added to each tube to ensure sufficient precipitation. In triplicate experiments, standard isozyme 3a (0.5–32 ng protein) or other isozymes of *P*-450 (1–10,000 ng) were added to tubes, followed by 200 μl of the antibody preparation, 100 μl of ^{125}I-isozyme 3a (approximately 40,000 cpm), and sufficient buffer B to bring the final volume to 0.8 ml. Quadruplicate total count tubes (containing 100 μl of ^{125}I-isozyme 3a), background tubes (containing 100 μl of ^{125}I-isozyme 3a and buffer B), and buffer control tubes (zero-standard, containing 100 μl of ^{125}I-isozyme 3a, 200 μl of antibody, and buffer B) were included. The assay mixtures were incubated at 4°C overnight. To precipitate the bound complex, 250 μl of donkey anti-sheep antisera (80 μl serum/ml buffer B containing 2.5% polyethylene glycol 6000) was added. The mixtures were incubated for 30 min at room temperature, and then centrifuged at 800*g* for 20 min. The supernatant solution was poured off, and the resulting pellet was washed with 3 ml 0.01 M sodium phosphate buffer, pH 7.4, containing 0.14 M NaCl and 0.01% sodium merthiolate, and was centrifuged at 800*g* for 20 min. The tubes containing the pellets were centrifuged at 200*g* for 2 min and counted for 1 min in a Searle 1185 gamma counter, and the results were analyzed by computer as described elsewhere (24).

Catalytic assays. The enzyme system was reconstituted from purified isozymes of *P*-450, NADPH-cytochrome *P*-450 reductase, and sonicated dilauroylglyceryl-3-phosphorylcholine. The composition of the individual reaction mixtures is given in the figure and table legends. In all cases, the reactions were initiated by the addition of NADPH, and the rates of product formation were corrected for values obtained in control experiments in which the reactions were quenched prior to the addition of NADPH. All reactions were run at 30°C for times which represented the initial rate of product formation. The *p*-hydroxylation of aniline was measured colorimetrically as described by Mieyal *et al.* (25). The oxidation of ethanol, *n*-butanol, and *n*-pentanol was determined by gas chromatography of the headspace gas of the reaction mixtures; the reactions were carried out in 3.5-ml sealed serum vials and were quenched by the addition of 0.2 ml 35% perchloric acid. After the addition of an internal standard to each vial, 1.0 ml of the headspace gas was removed with a Hamilton gas-tight syringe after incubation at 60°C for 20 min. Separation of the product was obtained with use of a Varian Model 3700 gas chromatograph fitted with an 80/100 Carbopack B/5% Carbowax 20 M glass column (6 ft × 2 mm) and a flame ionization detector. The carrier gas was nitrogen at a flow rate of 25 ml/min. The separation of acetaldehyde, ethanol, and butyraldehyde (internal standard) was accomplished with a temperature program from 70 to 100°C at 10°C/min beginning 2 min after injection of the sample; butyraldehyde, *n*-butanol, and propionaldehyde (internal standard) were separated with a temperature program from 100 to 180°C at 10°C/min beginning 2 min after injection; and valeraldehyde, *n*-pentanol, and butyraldehyde (internal standard) were separated with a temperature program from 100 to 210°C at 10°C/min beginning 2 min after injection of the sample. The amount of product was determined by comparison of the peak area ratios (area of product/area of internal standard) of the samples with standards included in each assay.

Hydrogen peroxide was determined colorimetrically by the ferrithiocyanate method (26). Mixtures of iron-EDTA were prepared by the addition of a twofold molar excess of EDTA to ferrous ammonium sulfate (1, 2). All buffer solutions and glass-distilled water were passed through a Chelex-100 column to remove any contaminating iron.

Materials. Na^{125}I, carrier free, was obtained from New England Nuclear, Chelex-100 from Bio-Rad, and the 80/100 Carbopack B/5% Carbowax 20 M column from Supelco. Desferrioxamine (desferyl mesylate) was a gift from Ciba-Geigy Corporation. Alcohols and aldehydes obtained from Aldrich were greater than 99% pure as judged by gas chromatography. Aniline was distilled twice under nitrogen before use. The source of other chemicals has been described elsewhere (14, 15, 19).

RESULTS

Cytochrome *P*-450 isozyme 3a isolated from hepatic microsomes of rabbits treated chronically with ethanol was shown earlier to be distinct from isozymes 2, 3b, 3c, 4, and 6 by spectral, catalytic, and structural criteria (14, 15). The crossreactivity of these cytochromes with antibodies produced in sheep against purified form 3a is shown in Fig. 1. The results of the radioimmunoassay with ^{125}I-labeled isozyme 3a indicate that the immune sera had a high specificity for isozyme 3a. The amount of unlabeled *P*-450 that inhibited precipitation of labeled isozyme 3a by 50% was 2.7 ng 3a, 350 ng form 3b, 620 ng form 6, 2.9 μg form 3c, 4.8 μg form 4, and greater than 10 μg form 2. The maximal crossreactivity observed with the anti-3a antibody was calculated from the ratio of these 50% points [cf. (27)] to be only 0.77% with form 3b and 0.44% with form 6. The other isozymes exhibited even less than 0.1% crossreactivity with anti-3a antibody. The very low but significant level of crossreactivity may indicate the presence of identical antigenic sites on the proteins, but we cannot eliminate the possibility that the preparations of isozyme 3b and 6 used contained less than 1% isozyme 3a as a contaminant. Electrophoretic examination of extremely high loads of form 6 followed by silver staining of the gel (28) revealed the presence of a trace contaminant with a similar electrophoretic mobility to that of form 3a (results not shown). A similar electrophoretic analysis of form 3b was not possible due to the almost identical mobilities of forms 3a and 3b in the gel. The results of inhibition experiments with forms 3b and 6 and the anti-3a IgG also could not distinguish between true crossreactivity and minor contamination by isozyme 3a. Ouchterlony double-diffusion experiments also revealed no crossreaction with the other isozymes (results not shown), and the antibody gave a single precipitin line with liver microsomes from ethanol-treated rabbits that suggested it was reacting with a single antigen (18).

Antisera from three different sheep exhibited very similar specificity for isozyme 3a as judged by radioimmunoassay. However, one was not inhibitory, a second showed partial inhibitions, and the third showed good inhibition of isozyme 3a-catalyzed reactions. The effect of the third antibody preparation on the catalytic activities of purified form 3a in the reconstituted system is shown in Fig. 2. Increasing concentrations of anti-3a IgG had a similar effect on the turnover of the four substrates (aniline, ethanol, *n*-butanol, and *n*-pentanol) examined, and at the highest level of antibody all activities were inhibited by greater than 90%.

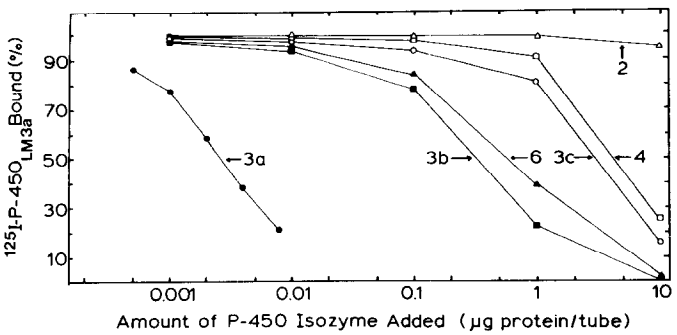

FIG. 1. Crossreactivity of purified *P*-450 isozymes with antibody to *P*-450$_{LM3a}$. The crossreactivities of the various cytochromes were determined by radioimmunoassay as described under Experimental Procedures. The ordinate represents the amount of radioiodinated isozyme 3a bound in the presence of unlabeled enzyme as the percentage of the amount bound in the absence of unlabeled enzyme. The position of the arrow designating each curve (except for isozyme 2) represents the amount of unlabeled enzyme needed to inhibit by 50% the binding of labeled isozyme 3a.

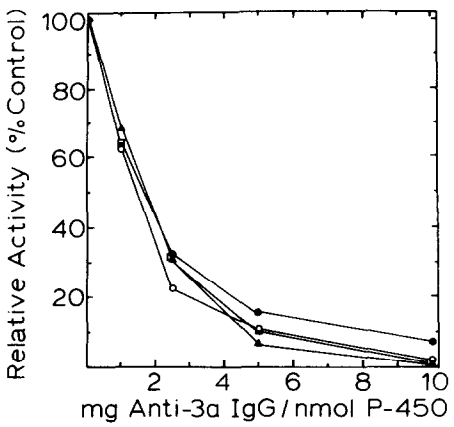

FIG. 2. Inhibition of catalytic activities of purified isozyme 3a by anti-3a IgG. The reaction mixtures contained 50 mM potassium phosphate buffer, pH 7.6, 0.1 μM *P*-450 isozyme 3a, 0.3 μM NADPH-cytochrome *P*-450 reductase, 30 μg/ml dilauroylglyceryl-3-phosphorylcholine, 1 mM NADPH, substrate (100 mM ethanol, ●; 30 mM *n*-butanol, ○; 10 mM *n*-pentanol, □; or 2.5 mM aniline, ▲), and anti-isozyme 3a IgG or preimmune IgG. The level of the preimmune IgG was adjusted so that the total amount of IgG was constant (10 mg protein/nmol isozyme 3a) in all incubations. The control (uninhibited) rate, expressed as turnover number for each substrate, was ethanol, 5.1 min^{-1}; butanol, 11.5 min^{-1}; pentanol, 13.8 min^{-1}; and aniline, 8.4 min^{-1}.

The inhibition was not due to a nonspecific effect of IgG, since the total concentration was kept constant by the addition of IgG isolated from the serum of preimmune sheep.

The most inhibitory antibody was used to determine the relative activity of isozyme 3a toward the same substrates in hepatic microsomes. The effect of adding increasing concentrations of anti-3a IgG to hepatic microsomes from untreated or ethanol-treated rabbits is shown in Fig. 3. Chronic ethanol treatment resulted in an increase in the microsomal oxidation of ethanol, *n*-butanol, and *n*-pentanol, and the *p*-hydroxylation of aniline. The antibody inhibited the metabolism of all four substrates, although the extent of inhibition was dependent on the source of the microsomes. When microsomes from ethanol-treated animals were used, the activities were inhibited between 60% (aniline) and 80% (*n*-pentanol), while the activities of microsomes from untreated rabbits were inhibited only 20% (aniline) to 40% (*n*-pentanol). With all four substrates, when the form 3a-dependent activity was inhibited by the antibody, the absolute rate with microsomes from untreated and ethanol-treated rabbits was almost the same, suggesting that the residual activity was the sum of that of the other isozymes present. Furthermore, the large difference in inhibition observed with microsomes from control and ethanol-treated rabbits with each sub-

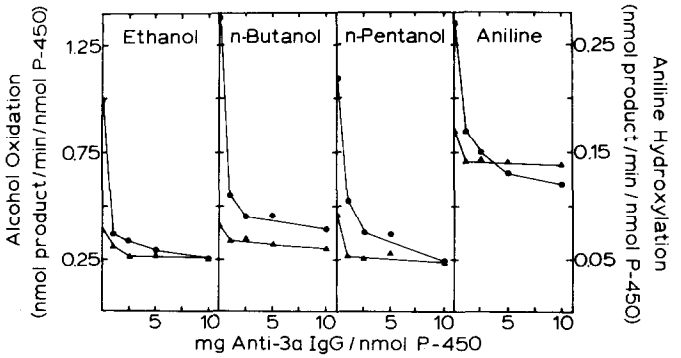

FIG. 3. Effect of anti-3a IgG on liver microsomal alcohol oxidation and aniline hydroxylation. The reaction mixtures contained 50 mM potassium phosphate buffer, pH 7.6, substrates at the same concentrations as in Fig. 2, 1 mM NaN_3, 1 mM NADPH, anti-3a IgG with the level of the preimmune sheep IgG adjusted so that the total IgG concentration remained constant (10 mg protein/nmol *P*-450), and microsomes from control rabbits (0.27 mg protein) or ethanol-treated rabbits (0.33 mg protein) providing 1 nmol total *P*-450. The results presented for each substrate are with microsomes from control (▲) or ethanol-treated (●) rabbits.

strate suggests that more isozyme 3a is present after chronic ethanol treatment, and supports the hypothesis that this cytochrome is responsible for the increase in the microsomal activity after such exposure (15).

In the presence of an iron chelate which is able to catalyze ethanol oxidation, the H_2O_2 produced by liver microsomes will be partitioned between that participating in the iron-catalyzed reaction and that which will be detectable, the partition being constant for a given set of incubation conditions. If all of the microsomal ethanol oxidation were the result of an H_2O_2-iron chelate-catalyzed reaction, then the inhibition of H_2O_2 formation by anti-3a IgG should be paralleled by an equal inhibition of ethanol oxidation, assuming that the antibody did not affect the partition of the H_2O_2. The results of such an experiment are shown in Fig. 4. At optimal concentrations of anti-3a IgG with microsomes from untreated rabbits, the rate of H_2O_2 production was inhibited by 0.07 nmol min^{-1} nmol *P*-450^{-1}, while ethanol oxidation was inhibited by 0.13 nmol min^{-1} nmol *P*-450^{-1}. Similarly, when microsomes from ethanol-treated rabbits were used in the assay, the rate of H_2O_2 formation was inhibited by 0.37 nmol min^{-1} nmol *P*-450^{-1}, while ethanol oxidation was inhibited by 0.80 nmol min^{-1} nmol *P*-450^{-1}. In both cases, the rate of inhibition of ethanol oxidation was about two times greater than the inhibition of H_2O_2 production. Thus, these results suggest that the effect of the antibody in decreasing ethanol oxidation was not simply due to the inhibition of microsomal H_2O_2 formation.

The residual activity remaining at the highest concentration of anti-3a IgG could be the result of direct catalysis by isozymes of *P*-450 other than 3a, or could be due to H_2O_2-dependent, hydroxyl radical-mediated oxidation of ethanol effected by trace iron-chelates in the system. Even though the water and buffer were treated with Chelex-100, residual iron could still be present in the incubation mixtures (2, 9). In order to determine the contribution of this pathway in our system, the effect

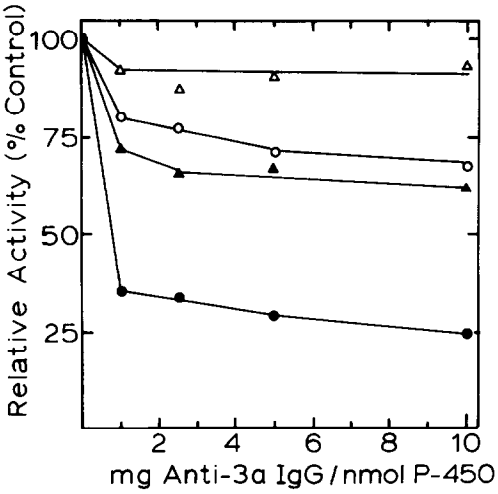

FIG. 4. Effect of anti-3a IgG on H_2O_2 formation and ethanol oxidation with liver microsomes from control and ethanol-treated rabbits. Reaction mixtures contained 50 mM potassium phosphate buffer, pH 7.6, 100 mM ethanol, 1 mM NaN_3, 1 mM NADPH, microsomal protein equivalent to 1 nmol total *P*-450 from control (0.27 mg protein) or ethanol-treated rabbits (0.33 mg protein), and anti-3a IgG or preimmune sheep IgG with the preimmune IgG concentration adjusted so the total concentration remained constant at 10 mg protein/nmol *P*-450. Two sets of experiments were carried out; for measurement of ethanol oxidation the reactions were quenched with perchloric acid, while for the determination of H_2O_2 they were quenched with trichloroacetic acid. The control activities (100%), expressed as turnover numbers, obtained with microsomes from untreated animals were H_2O_2 formation, 0.80 min^{-1} (△); and ethanol oxidation, 0.40 min^{-1} (▲); Those with microsomes from ethanol-treated rabbits were H_2O_2 formation, 1.09 min^{-1} (○); and ethanol oxidation, 1.0 min^{-1} (●).

of the addition of desferrioxamine or EDTA was examined, with the results presented in Table I. A control experiment showed that the rates of ethanol oxidation by microsomes suspended in the presence or absence of 0.1 mM EDTA were not significantly different. The addition of up to 1 mM desferrioxamine (with preincubation times from 5 to 20 min) had only a very slight effect on the rate of ethanol oxidation by microsomes from ethanol-treated rabbits. Similarly, the addition of 0.1 mM EDTA resulted in only a very slight stimulation of ethanol oxidation

TABLE I

EFFECT OF VARIOUS AGENTS ON MICROSOMAL ETHANOL OXIDATION[a]

Addition to system	Activity (nmol acetaldehyde min^{-1} nmol $P\text{-}450^{-1}$)
None	1.06 (100)
Desferrioxamine (0.1 mM)	1.05 (99)
Desferrioxamine (0.5 mM)	1.02 (96)
Desferrioxamine (1.0 mM)	1.02 (96)
EDTA (0.1 mM)	1.15 (108)
EDTA (0.1 mM) + desferrioxamine (0.5 mM)	1.10 (104)
Fe-EDTA (7.5 μM)	2.26 (213)
Fe-EDTA (7.5 μM) + desferrioxamine (0.5 mM)	1.59 (150)
Fe-EDTA (7.5 μM) + desferrioxamine (1.0 mM)	1.45 (137)
Anti-3a IgG (10 mg of protein)	0.25 (24)

[a] The reaction mixtures (1.0 ml final volume) contained 50 mM potassium phosphate buffer, pH 7.6, 100 mM ethanol, microsomes from ethanol-treated rabbits equivalent to 1 nmol *P*-450 (0.3 mg of protein), 1 mM NaN_3, 1 mM NADPH, and other additions as indicated. Reactions were initiated with NADPH, and were quenched by the addition of 0.2 ml 35% perchloric acid. Zero-time blanks contained all components, but the NADPH was added after the reaction was quenched with perchloric acid. The values in parentheses are the percentage of the rate with no addition to the system.

and, when EDTA and desferrioxamine were added together, the rate was not significantly changed. Similar results, not shown here, were obtained with microsomes from untreated animals. The modest effect of EDTA and desferrioxamine suggests that very low levels of trace iron may be present in the incubation mixtures. With Fe-EDTA present, the rate was stimulated about twofold, and the further addition of desferrioxamine returned the rate to nearly control levels, which confirmed the efficacy of the desferrioxamine as an iron-complexing agent. These results are similar to those of Cederbaum and co-workers (1, 2, 29, 30). Finally, the combination of anti-3a IgG and desferrioxamine had no greater effect than the antibody alone.

We have previously reported that the rabbit liver isozymes of *P*-450 as well as NADPH-cytochrome *P*-450 reductase catalyze the oxidation of ethanol and that the reductase-dependent activity appears to involve $O_2^{\cdot}$, as judged by its sensitivity to superoxide dismutase (15). Similar results were subsequently reported for the rat liver *P*-450 reductase, and it was suggested that the reaction involved the production of hydroxyl radicals by an iron-catalyzed Haber–Weiss reaction (1, 2). Thus, the removal of traces of iron and EDTA from the reaction mixtures should eliminate this side reaction and permit more reliable measurement of the *P*-450-catalyzed rate. As shown in Table II, even under conditions where EDTA was removed from the system and the water and buffers had been treated with Chelex-100, the reductase still catalyzed a very low rate of alcohol oxidation and aniline hydroxylation, which probably reflects the small amount of trace iron still present (1, 2). The rate of ethanol oxidation by the reductase is about one-fifth the rate we reported previously (15), and represents the formation of 1 nmol acetaldehyde during a 20-min incubation, about the lower limit of detection in the gas chromatographic assay utilized. The addition of desferrioxamine to a 3a-reconstituted system resulted in about a 5% inhibition of ethanol oxidation (results not shown). This degree of inhibition is similar to the reductase-dependent rate, suggesting that the small residual activity of isozyme 3a in the presence of optimal anti-3a IgG (Fig. 2) may actually reflect

TABLE II

CATALYTIC ACTIVITY OF SIX PURIFIED P-450 ISOZYMES AND P-450 REDUCTASE TOWARD ANILINE, n-BUTANOL, AND n-PENTANOL[a]

Substrate	Turnover number of P-450 isozyme or reductase (nmol product min^{-1} nmol $enzyme^{-1}$)						
	2	3a	3b	3c	4	6	Reductase
Aniline	1.47	8.31	0.76	0.06	0.72	2.54	0.03
Ethanol	0.85	7.72	0.48	0.58	1.14	2.41	0.17
n-Butanol	2.04	12.0	0.44	0.25	3.02	3.46	0.08
n-Pentanol	4.43	13.0	0.54	0.25	2.32	2.61	0.05

[a] Reaction mixtures contained 50 mM potassium phosphate buffer, pH 7.6, 0.1 μM $P\text{-}450_{LM}$, 0.3 μM NADPH-cytochrome P-450 reductase, 30 μg/ml dilauroyglyceryl-3-phosphorylcholine, 1 mM NADPH, and the substrate (ethanol, 100 mM; aniline, 2.5 mM; n-butanol, 30 mM; or n-pentanol, 10 mM). The mixtures with P-450 reductase were identical except that P-450 was omitted. The values given represent the averages of duplicates from a typical experiment. The rates are expressed as nanomoles of product per minute per nanomole P-450 or, in the absence of P-450, per nanomole of the reductase.

the activity of the reductase present. Under conditions where the level of an iron-catalyzed reaction is minimal, the isozymes other than 3a still display significant activity toward aniline, ethanol, n-butanol, and n-pentanol. The activities of isozymes, 2, 4, and 6, although 3 to 10 times lower than that of isozyme 3a, could make a significant contribution to the activity remaining at the highest levels of anti-3a IgG used. These results, coupled with the lack of an effect of added EDTA or desferrioxamine, strongly suggest that, in the microsomal system, the oxidation of ethanol, butanol, and pentanol, and the hydroxylation of aniline are dependent on the monooxygenase activity of the isozymes of P-450, and do not involve the participation of microsomally generated H_2O_2 in an iron chelate-catalyzed reaction.

DISCUSSION

The increase in hepatic microsomal ethanol oxidation after chronic ethanol treatment has been attributed to an increase in the catalytic activity of an ethanol-inducible system containing cytochrome P-450 (5, 31–33). The isolation of a unique rabbit liver cytochrome P-450, isozyme 3a, following ethanol treatment, as well as the observation that this enzyme exhibits the greatest activity of the isozymes tested toward alcohols and aniline, is consistent with this hypothesis (14, 15). As demonstrated in the present paper, antibody specific for isozyme 3a inhibited essentially all of the activity of this cytochrome toward three alcohols and aniline. The antibody also inhibited the isozyme 3a-dependent metabolism in microsomes from control and ethanol-treated animals. The degree of inhibition was dependent on the source of microsomes; the activity of microsomes from ethanol-treated rabbits was inhibited about threefold more than that of microsomes from untreated rabbits. Furthermore, at optimal concentrations of the antibody the residual rates of the two different microsomal preparations were nearly identical, indicating that, in the absence of isozyme 3a, the sum of the remaining activity of the other isozymes in the two different microsomal preparations was similar with respect to alcohols and aniline. These results confirm the hypothesis that an increase in the activity of isozyme 3a is reponsible for enhanced microsomal alcohol oxidation after ethanol treatment. Whether an increase occurred in the total amount of isozyme 3a or in its catalytic efficiency cannot be determined from the present results, but the

antibody could be used to quantify the level of 3a using a variety of immunochemical techniques (34–36). These methods are currently being developed in our laboratory.

Klein *et al.* (9) have recently suggested that, in rats, the increase in ethanol oxidation after chronic ethanol treatment may, in part, be due to an increase in the rate of production of hydroxyl radicals. In addition, reports by Cederbaum and co-workers (1, 30) have suggested that the rat liver microsomal activity toward ethanol results from two distinct pathways, one being *P*-450 independent and involving H_2O_2 in either an iron-catalyzed Haber–Weiss reaction or an iron-catalyzed Fenton reaction, and the second being directly catalyzed by *P*-450 (1, 30). The *P*-450-independent activity was inhibited by the iron chelator desferrioxamine (1, 2, 29, 30), and added EDTA caused a stimulation of microsomal oxidation of ethanol and other hydroxyl radical scavengers.Taken together, these results suggest that about 50% of microsomal ethanol oxidation is the result of an indirect role of *P*-450, where the NADPH oxidase activity provides H_2O_2 that participates with an iron chelate to produce an oxidant equivalent to hydroxyl radicals. Thus, the inhibition we observed in the present paper with anti-3a might be the result of inhibition of microsomal electron transport and therefore of H_2O_2 formation. However, the lack of a significant effect of added EDTA or desferrioxamine suggests that, in our *in vitro* system containing rabbit liver microsomes, there is very little iron present and the mechanism proposed by Cederbaum and co-workers plays an insignificant role. Furthermore, the results presented demonstrate that the oxidation of ethanol was inhibited to a greater extent than the formation of H_2O_2 in assays with the microsomes from either control or ethanol-treated rabbits. Although it might be argued that only the H_2O_2 formation that is inhibited by the antibody is responsible for the oxidation of ethanol, this suggests that only H_2O_2 produced by isozyme 3a can participate in the Fenton-type reaction. This seems unlikely, especially in light of the fact that added H_2O_2 stimulates microsomal butanol oxidation (37), an activity not catalyzed by contaminating catalse. A recent report by Ingelman-Sundberg and Johansson (38) has confirmed that purified cytochrome *P*-450 can support an iron-EDTA-catalyzed oxidation of ethanol, and suggested that isozyme 3a [*P*-450eb in Ref. (38)] required both an iron-chelate and H_2O_2 to oxidize ethanol. We have previously reported that, in the presence of sufficient catalase to remove all H_2O_2 from the incubation mixtures, the isozyme 3a-catalyzed oxidation of butanol was not inhibited. This result, together with the lack of an effect of desferrioxamine in reconstituted and microsomal incubations and the differential rates of inhibition of H_2O_2 and acetaldehyde formation, suggests that an iron chelate is not required for the isozyme-3a catalyzed oxidation of ethanol. The difference between the present results and those of Cederbaum and co-workers and Ingelman-Sundbar and co-workers appears to reside in the level of free iron or an iron chelate present in the enzyme preparations. Our microsomal preparations are routinely washed with pyrophospahte, which may be more effective in removing any remaining iron sequestered on the microsomal membrane (39), or there may be a significant difference in the concentration of iron in the livers of rabbits and rats (40). Sinaceur *et al.* (41) have reported that the administration of desferrioxamine to rats results in a statistically significant decrease in the elimination of ethanol, suggesting that the hydroxyl radical-mediated pathway may play a role *in vivo*. However, the results of the present report strongly suggest that, in our *in vitro* system, the oxidation of ethanol is catalyzed almost exclusively by cytochrome *P*-450, with isozyme 3a accounting for about 75% of the activity in microsomes from ethanol-treated rabbits and about 40% of that in control microsomes. The residual activity represents the sum of the activities of the other *P*-450 isozymes present.

It has been estimated by various methods that cytochrome *P*-450 plays only a

minor role in the metabolism of ethanol *in vivo* (42-45), but the role of isozyme 3a (or a homologous isozyme in other species) may be greater in the oxidation of more hydrophobic alcohols that might partition into the endoplasmic reticulum membrane. In addition, the ethanol-inducible isozyme of *P*-450 has been implicated in the toxicity of many compounds, including acetaminophen (46), CCl_4 (47), and dimethylnitrosamine (10, 13). We have recently reported that purified isozyme 3a is highly effective in the activation of acetaminophen (16), and have found, in collaboration with other laboratories, that this form of *P*-450 catalyzes the demethylation of dimethylnitrosamine (48) and the hydroxylation of *N*-nitroso-2,6-dimethylmorpholine (49). Thus, this form of *P*-450 has toxicological significance, regardless of its physiological role in ethanol metabolism.

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