Cytochrome P-450 Dependent Ethanol Oxidation. Kinetic Isotope Effects and Absence of Stereoselectivity†

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ABSTRACT: Deuterium isotope effects [$^D(V/K)$] and stereoselectivity of ethanol oxidation in cytochrome P-450 containing systems and in the xanthine–xanthine oxidase system were compared with those of yeast alcohol dehydrogenase. The isotope effects were determined by using both a noncompetitive method, including incubation of unlabeled or [1,1-$^2H_2$]ethanol at various concentrations, and a competitive method, where 1:1 mixtures of [1-^{13}C]- and [$^2H_6$]ethanol or [2,2,2-$^2H_3$]- and [1,1-$^2H_2$]ethanol were incubated and the acetaldehyde formed was analyzed by gas chromatography/mass spectrometry. The $^D(V/K)$ isotope effects of the cytochrome P-450 dependent ethanol oxidation were about 4 with liver microsomes from imidazole-, phenobarbital- or acetone-treated rabbits or with microsomes from acetone- or ethanol-treated rats. Similar isotope effects were reached with reconstituted membranes containing the rabbit ethanol-inducible cytochrome P-450 (LMeb), whereas control rat microsomes and membranes containing rabbit phenobarbital-inducible P-450 LM_2 oxidized the alcohol with $^D(V/K)$ of about 2.8 and 1.8, respectively. Addition of Fe^{III}EDTA either to microsomes from phenobarbital-treated rabbits or to membranes containing P-450 LMeb significantly lowered the isotope effect, which approached that of the xanthine–xanthine oxidase system (1.4), whereas desferrioxamine had no significant effect. Incubations of all cytochrome P-450 containing systems or the xanthine–xanthine oxidase systems with (1*R*)- and (1*S*)-[1-^{2}H]ethanol, revealed, taking the isotope effects into account, that 44–66% of the ethanol oxidized had lost the 1-*pro-R* hydrogen. The data indicate that cytochrome P-450 dependent ethanol oxidation is not stereospecific and that cleavage of the C_1–H bond appears to be a rate-determining step in the catalysis by the ethanol-inducible form of P-450. The contribution of hydroxyl radicals in ethanol oxidation by the various enzymic systems is discussed.

Increasing evidence has during recent years accumulated for the participation of cytochrome P-450 in the hepatic oxidation of ethanol (Orme-Johnson & Ziegler, 1965; Lieber & DeCarli, 1970; Ohnishi & Lieber, 1977; Mungikar et al., 1980; Ingelman-Sundberg & Johansson, 1981, 1984; Morgan et al., 1982). In particular, specific forms of cytochrome P-450 inducible by ethanol appear to be of greatest significance in this respect (Koop et al., 1982; Ingelman-Sundberg & Jörnvall, 1984; Ingelman-Sundberg & Johansson, 1984; Ryan et al., 1986; Wrighton et al., 1986; Song et al., 1986; Khani et al., 1987). The amount of ethanol-inducible cytochrome P-450 in liver microsomes from, e.g., ethanol-, imidazole- or acetone-induced rabbit, is 5–20% of the total microsomal cytochrome P-450 (Ingelman-Sundberg & Jörnvall, 1984; Koop et al., 1985), and this type of P-450 has been shown to catalyze the NADPH-dependent[1] oxidation of ethanol in reconstituted systems (Morgan et al., 1982; Ingelman-Sundberg & Johansson, 1984) and in microsomes, as revealed by the inhibition of the reaction by antibodies toward this type of P-450 (Koop et al., 1984).[2]

The mechanism(s) of the cytochrome P-450 dependent ethanol oxidation is (are) as yet not elucidated. The alcohol is an effective scavenger of hydroxyl radicals (Adams et al., 1965). Thus, any microsomal production of hydroxyl radicals, particularly in the presence of chelated iron, might contribute to the oxidation of the alcohol (Ingelman-Sundberg & Johansson, 1981, 1984; Krikun et al., 1984). The •OH-dependent oxidation of ethanol in microsomes is, however, inhibited to a great extent by desferrioxamine (Krikun et al., 1984). Addition of very small amounts of EDTA-chelated Fe^{3+} to reconstituted vesicles containing the ethanol-inducible rabbit form of P-450 [P-450 LMeb (Ingelman-Sundberg & Johansson, 1984), probably identical with P-450 3a (Koop et al., 1982)], in the presence of ethanol, specifically enhances the rate of hydroxyl radical dependent acetaldehyde production with a concomitant decrease of the hydrogen peroxide production (Ingelman-Sundberg & Johansson, 1984). In contrast, other types of P-450 are not as sensitive to Fe^{III}EDTA in this respect (Ingelman-Sundberg & Johansson, 1984). On the basis of these findings and the fact that hydroxyl radical scavengers inhibited the P-450 LMeb-catalyzed reaction even in the absence of Fe^{III}EDTA, a hydroxyl radical dependent hydroxylation scheme for the enzyme was suggested (Ingelman-Sundberg & Johansson, 1984).

An oxidative mechanism involving the action of free hydroxyl radicals is supposed to lack stereoselectivity and to exhibit a similar extent of isotope effect as systems known to oxidize ethanol with •OH as the form of active oxygen. In line with this argument, the stereoselectivity and isotope effects of cytochrome P-450 dependent ethanol oxidation have been determined in systems containing various forms of P-450 and compared to those of the xanthine–xanthine oxidase system

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[1] Abbreviations: XOD, xanthine oxidase; TCA, trichloroacetic acid; GC/MS, gas chromatography/mass spectrometry; NADPH, reduced nicotinamide adenine dinucleotide phosphate; EDTA, ethylenediaminetetraacetic acid.

[2] I. Johansson, G. Ekström, B. Scholte, D. Puzycki, H. Jörnvall, and M. Ingelman-Sundberg, submitted for publication to *Biochemistry*.

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[cf. Beauchamp and Fridovich (1970)].

Experimental Procedures

Materials

[1,1-$^2H_2$]Ethanol (99.6% ^2H) and [2,2,2-$^2H_3$]ethanol (99.0% ^2H) were obtained from Alfred Hempel GmbH & Co. (Düsseldorf, Germany), [$^2H_6$]ethanol (99% ^2H) was obtained from Merck AG (Darmstadt, Germany), and [1-^{13}C]ethanol (90% ^{13}C) was obtained from Merck Sharp and Dohme (Montreal, Canada). (1*R*)-[1-^{2}H]Ethanol and (1*S*)-[1-^{2}H]-ethanol were prepared and purified as previously described (Cronholm & Fors, 1976). The isotopic composition of the alcohols used was analyzed by GC/MS of the 3,5-dinitrobenzoates (Cronholm, 1985). In addition, the chiral monodeuterioethanols and 1:1 mixtures of [1,1-$^2H_2$]- and [2,2,2-$^2H_3$]ethanol as well as of [$^2H_6$]- and [1-^{13}C]ethanol were oxidized by yeast alcohol dehydrogenase (Simon & Medina, 1968). The alcohol (20 mM) was incubated for 30 min at 37 °C in 2 mL of 35 mM sodium phosphate buffer, pH 7.7, containing 0.1 M pyruvate, 1 mM NAD^+, 0.6 mg of yeast alcohol dehydrogenase (300 units/mg, Boehringer Mannheim), and 0.1 mg of lactate dehydrogenase (300 units/mg, Boehringer Mannheim). Product analysis was carried out as described below. The analysis revealed that 20.7% of the *R* form and 9.3% of the *S* form were unlabeled, whereas 75.2% and 4.1%, respectively, were labeled only in the 1-*pro-R* position and 0.4% and 81.8%, respectively, were labeled only in the 1-*pro-S* position. Dideuteriated molecules constituted 3.8% of the *R* form and 4.7% of the *S* form. NADPH, xanthine, and xanthine oxidase (XOD), grade III (specific activity 1.1 units/mg), were purchased from Sigma (St. Louis, MO), EDTA was from Fluka (Buchs, Switzerland), and Chelex 100 was from Bio-Rad Laboratories (Richmond, CA). Dimethylformamide, analytical grade, obtained from BDH Chemicals Ltd. (Poole, England), was distilled over 2,4-dinitrophenylhydrazine prior to use. *n*-Hexane was purified and distilled (Schwartz & Parks, 1961). Dinitrophenylhydrazine was obtained from Merck AG and recrystallized from ethanol. Desferrioxamine was from Ciba Geigy (Basel, Switzerland). Sodium azide and semicarbazide were purchased from Merck AG.

Methods

Preparation of Liver Microsomes. Male rabbits (2.5–3 kg), obtained from a local farm, were injected ip with either imidazole (200 mg/kg for 3 consecutive days) or phenobarbital (70 mg/kg for 2 days) or were given acetone [3% (v/v) in the drinking water for 3 weeks]. Male Sprague-Dawley rats (150–180 g) were fed the liquid diet that provides 36% of the energy from ethanol described by DeCarli and Lieber (1967). The diet (Bioserv, Inc., Frenchtown, NJ) was given ad libitum for 2 weeks. Other groups of rats were injected intragastrically with acetone (5 mL/kg for 2 days) as previously described (Johansson et al., 1985). Rabbit liver microsomes were prepared in 0.25 M sucrose and washed in 1.14% KCl, whereas rat liver microsomes were isolated in 10 mM Na^+/K^+ phosphate buffer (Johansson et al., 1985). The microsomes were suspended in 50 mM potassium phosphate buffer, pH 7.4, yielding a protein concentration of about 50 mg/mL. The cytochrome P-450 contents in microsomes from control and acetone- and ethanol-treated rats were 0.7 ± 0.1, 1.3 ± 0.1, and 1.3 ± 0.2 nmol/mg, respectively. The contents of the apoprotein of the ethanol-inducible cytochrome P-450 form [P-450j (Ryan et al., 1986)] in these microsomes were, according to radial immunodiffusion experiments,[3] 0.05 ± 0.03, 0.45 ± 0.12, and 0.45 ± 0.03 nmol/mg, respectively. The concentrations of cytochrome P-450 in liver microsomes from acetone-, phenobarbital-, and imidazole-treated rabbits were 1.6, 2.1 ± 0.3, and 1.6 ± 0.2 nmol/mg, respectively, and the apoprotein levels of the ethanol-inducible P-450 form (P-450 LMeb) were 0.35, 0.005 ± 0.003, and 0.43 ± 0.10 nmol/mg, respectively. NADPH–cytochrome P-450 reductase, cytochrome P-450 LM_2, and the ethanol- and acetone-inducible form of rabbit liver microsomal cytochrome P-450 (P-450 LMeb) were purified as described previously (Ingelman-Sundberg & Johansson, 1984). Reconstituted phospholipid vesicles containing microsomal phospholipids, cytochrome P-450, and NADPH–cytochrome P-450 reductase at a molar ratio of 1200:1:0.4 were prepared by the cholate gel filtration technique (Ingelman-Sundberg & Glaumann, 1980) in Chelex 100 treated 15 mM potassium phosphate buffer, pH 7.4, containing 50 mM potassium chloride.

Incubation Conditions. Incubations with ethanol were carried out in 25-mL stoppered tubes containing 5-mL tubes with reagent for derivatization of the acetaldehyde formed (Ingelman-Sundberg & Johansson, 1981). Conditions for ethanol oxidation linear with time were established for all enzyme sources. All incubation mixtures had a final volume of 2 mL and contained 50 mM ethanol in chelexed 50 mM potassium phosphate buffer, pH 7.4. Reaction mixtures with microsomes contained 0.5 mM sodium azide. All incubations were carried out at 37 °C. Conditions for incubations aimed for GC/MS analysis were the following. Rat liver microsomes were incubated for 6–10 min with material corresponding to 2–4 mg of protein. Rabbit liver microsomes corresponding to 4 mg of protein were incubated for 30 min. Reaction mixtures in the xanthine oxidase system contained 0.1 unit of XOD, 0.35 mg of xanthine, and 10 mM EDTA and were incubated for 60 min. The reconstituted cytochrome P-450 system contained vesicles corresponding to 0.5 nmol of P-450 and 1.5 mg of NADPH and were incubated for 60 min. Kinetic analyses of the ethanol oxidation were carried out for 10 min with rabbit liver microsomes corresponding to 1.5 mg of microsomal protein or with reconstituted vesicles corresponding to 0.15 nmol of P-450. Alternatively, the reaction mixtures contained 0.01 unit of XOD, 0.15 mg xanthine, and 10 mM EDTA, and the incubations were carried out for 15 min. Control incubations were performed by adding NADPH or XOD after TCA.

Product Analysis. All incubations were terminated by the addition of 200 μL of 6 M TCA. The reaction vessels were subsequently incubated at 37 °C for 2.5 h (kinetic analysis) or shaken overnight at room temperature (GC/MS analysis). In the former case, the 5-mL inner tube contained 15 mM semicarbazide in 160 mM potassium phosphate buffer, pH 7.0, and the absorbance of this solution at 224 nm was registered. In the latter case, 5 mM 2,4-dinitrophenylhydrazine in 1 mL of dimethylformamide and 5 μg of butyraldehyde, as internal standard, were present in the inner tubes. The contents of these tubes were transferred with 1 mL of redistilled water to 5-mL stoppered tubes. The dinitrophenylhydrazones were extracted twice with 2 mL of *n*-hexane, and the pooled hexane phases were washed with 2 mL of redistilled water and dried with anhydrous sodium sulfate. After evaporation of the solvent under nitrogen at 40 °C, the residues were dissolved in 100 μL of hexane and subjected to GC/MS analysis using an LKB 9000 gas chromatograph/mass spec-

[3] I. Johansson, E. Wiersma, and M. Ingelman-Sundberg, unpublished observations.

trometer equipped with a fused-silica capillary column having a chemically bonded poly(dimethylsiloxane) phase, CP Sil 5CB (Chrompack, Middelburg, The Netherlands). The column temperature was 200 °C. Mass spectra were recorded at an electron energy of 22.5 eV and an ion source temperature of 290 °C by using repetitive accelerating voltage scanning over the molecular ion regions m/z 222.5–230.5 (acetaldehyde) or m/z 250.5–258.5 (butyraldehyde) in 0.5 s. The values were bunched (Axelson et al., 1974), and the area of the central 30% of the mass spectrometric peak was used in the calculation of the mean of 10 spectra. The relative abundance of molecules having 0–4 ^2H atoms or a ^{13}C atom was determined by comparisons with spectra of a reference compound (Biemann, 1962). Data storage and calculations were done on ND 100 computers (Norsk Data A. S., Oslo, Norway).

Evaluation of Hydrogen Exchange. Hexadeuteriated ethanol was oxidized by yeast alcohol dehydrogenase, and the deuteriated product was analyzed by GC/MS after derivatization with 2,4-dinitrophenylhydrazine. In preliminary experiments, the derivative was formed under the commonly used acidic conditions in 2 M hydrochloric acid (Shriner & Fuson, 1946) or under neutral conditions in dimethylformamide as described above. Analysis of the deuterium content revealed that after reaction in dimethylformamide the acetaldehyde was deuteriated to 98.0%, compared to 53.4% during reaction under acidic conditions. The extent of hydrogen exchange was checked after each experiment performed with the enzymic systems and corrected for accordingly [cf. Cronholm et al. (1974)]. It was usually less than 3% and in all cases less than 10%.

Calculations of the Isotope Effect and the Stereoselectivity. The kinetic isotope effect was measured by competitive and noncompetitive methods (Northrop, 1977). In the competitive method the ratio between the rates of oxidation of two differently labeled ethanols was measured by analysis of the product by capillary GC/MS. [1-^{13}C]Ethanol and [$^2H_6$]-ethanol or [2,2,2-$^2H_3$]ethanol and [1,1-$^2H_2$]ethanol in 1:1 molar ratios were used. Since the extent of conversion was small (less than 3%), the isotope effect, $^D(V/K)$, could be estimated as the ratio between [$^{13}C_1$]- and [$^2H_4$]acetaldehyde and between [$^2H_3$]- and [$^2H_1$]acetaldehyde, respectively (Northrop, 1977, 1981). The experiments were carried out with differently labeled ethanol in order to decrease errors due to interferences during oxidation or analysis, e.g., hydrogen exchange. In the noncompetitive method the rates to be compared were measured separately, with and without deuterium substitution into the ethanol, at eight different concentrations. The V_{max} and K_m values were determined according to the method of Lineweaver and Burk (1934), and the isotope effect, $^D(V/K)$, was determined as the ratio $(V_{max}/K_m)_H/(V_{max}/K_m)_D$. Student's t test was used for statistical evaluation, and p values <0.02 were considered significant.

The stereoselectivity was measured after oxidation of either (1*S*)-[1-^2H]ethanol or (1*R*)-[1-^2H]ethanol by various enzyme systems. The ratio between unlabeled and deuteriated acetaldehyde was determined by GC/MS. The ratio was corrected for contaminating acetaldehyde remaining in the solvents and reagents even after purification. This amount was quantified by using butyraldehyde as internal standard and subsequently subtracted from the experimental values. The corrected ratio (r) was used together with the isotopic composition of the monodeuterioethanols (cf. Materials) and the different isotope effects [$^D(V/K)$] in the calculation of the stereoselectivity given as the probability of removal of a 1-*pro-R* hydrogen (S_R) in experiments with (1*R*)-[1-^2H]ethanol (eq 1) or (1*S*)-[1-^2H]-ethanol (eq 2). The constants in these equations represent

$$r = \frac{75.2S_R/^D(V/K) + 0.42(1 - S_R)/^D(V/K) + 20.7}{75.2(1 - S_R) + 0.42S_R + 3.8/^D(V/K)} \quad (1)$$

$$r = \frac{4.10S_R/^D(V/K) + 81.8(1 - S_R)/^D(V/K) + 9.3}{4.10(1 - S_R) + 81.8S_R + 4.7/^D(V/K)} \quad (2)$$

the relative amount of substrate (ethanol) that was unlabeled or carried deuterium in the 1-*pro-R* or 1-*pro-S* position or in both these positions (cf. Materials). The equations were derived under the assumption that the probability of removal of deuterium was equal to the corresponding probability of removal of protium divided by $^D(V/K)$, irrespective of, e.g., secondary isotope effects.

Results

Kinetic Deuterium Isotope Effects in Ethanol Oxidation. The kinetic isotope effect, $^D(V/K)$, of cytochrome P-450 dependent ethanol oxidation in liver microsomes and reconstituted systems was measured by a noncompetitive and a competitive method [cf. Northrop (1977)]. Some Lineweaver–Burk plots obtained with the noncompetitive method are depicted in Figure 1 and mass spectra of the products formed in the competitive method are shown in Figure 2. The results, including those reached with the xanthine–xanthine oxidase system or yeast alcohol dehydrogenase, are summarized in Table I. When liver microsomes from control rats were used, a $^D(V/K)$ isotope effect of about 2.8 was reached. This was significantly ($p < 0.01$) lower than the isotope effect of 3.7 reached with microsomes isolated from acetone-treated rats. The isotope effect obtained with microsomes from ethanol-treated rats was 3.5. The rate of ethanol oxidation in microsomes from acetone- or ethanol-treated rats was 3-fold higher than in control microsomes. The ^DK and ^DV isotope effects were calculated from the results of the noncompetitive method to 0.51 and 1.8, respectively, with microsomes from imidazole-treated rabbits.

In control experiments it was found that desferrioxamine at maximum concentration (1 mM) inhibited the rate of ethanol oxidation in microsomes from acetone-treated rats by 30%. Desferrioxamine at 100 μM concentration did not significantly affect the observed $^D(V/K)$ isotope effect in this type of microsome (Table I). This indicates that a major portion of the overall reaction is insensitive to nonheme iron.

The kinetic deuterium isotope effect using liver microsomes isolated from phenobarbital-, imidazole-, or acetone-treated rabbits was generally about 4. Addition of 10 μM $Fe^{III}EDTA$ to microsomes from phenobarbital-treated rabbits increased the rate of P-450 dependent ethanol oxidation by a factor of 4.5 and significantly decreased the $^D(V/K)$ isotope effect to about 2.

The isotope effects reached in rabbit liver microsomes were compared with those obtained with reconstituted membrane vesicles containing the major form of cytochrome P-450 isolated from microsomes of imidazole- or phenobarbital-treated rabbits. Ethanol oxidation catalyzed by imidazole- and ethanol-inducible cytochrome P-450 (P-450 LMeb) showed a higher isotope effect (3.5) than the corresponding reaction catalyzed by phenobarbital-inducible P-450 LM_2 (1.8). Addition of $Fe^{III}EDTA$ (3 or 10 μM, respectively) caused a 5–6-fold increase in the rate of ethanol oxidation in both cases and caused a significant decrease in the isotope effect of the P-450 LMeb catalyzed reaction to 2.1. The P-450 LM_2 dependent reaction was essentially unaffected in this sense. For

Table I: Kinetic Deuterium Isotope Effects [$^D(V/K)$] of Ethanol Oxidation in Various Enzymic Systems[a]

enzyme system[b]	types of ethanol isotopes used: competitive method: $^2H_6$ and 1-^{13}C	competitive method: 1,1-$^2H_2$ and 2,2,2-$^2H_3$	noncompetitive method: 1,1-$^2H_2$ and unlabeled	v[c]
rabbit liver microsomes				
imidazole (4)	3.9 ± 0.6 (11)	3.9 ± 0.4 (11)	3.7 (2)	4.5
phenobarbital (4)	3.3 ± 0.5 (16)	3.6 ± 0.6 (11)	3.2 (2)	2.0
phenobarbital + FeEDTA (10 μM) (1)	2.0 ± 0.1[d] (4)	2.7 ± 0.5[d] (4)		9.0
acetone (1)	3.9 ± 0.4 (4)	4.4 ± 0.4 (4)		5.5
control (1)	2.8 (2)	3.6 (2)		2.6
cytochrome P-450				
LMeb	3.5 ± 0.8 (6)	3.4 ± 0.6 (6)	4.0 (2)	4.5
LMeb + FeEDTA (3 μM)	1.8 ± 0.3[e] (4)	2.4 ± 0.4[f] (4)	1.4 (2)	22
LM_2	2.1 ± 0.6[g] (4)	1.5 ± 0.1[h] (3)		0.9
LM_2 + FeEDTA (10 μM)	1.4 ± 0.2 (4)	1.6 ± 0.2 (4)	2.4 (2)	5.5
rat liver microsomes				
control (3)	3.0 ± 0.5 (7)	2.7 ± 0.2 (7)		4.3
acetone (4)	3.6 ± 0.1[i] (10)	3.7 ± 0.5[i] (10)		13.8
acetone + desferrioxamine (100 μM)	5.5 ± 0.5 (4)	4.3 ± 1.0 (4)		11.5
ethanol (4)	3.5 ± 0.3 (5)	3.5 ± 0.2 (5)		12.9
xanthine–xanthine oxidase	1.4 ± 0.1 (4)	1.4 ± 0.2 (4)	1.9 (2)	
yeast alcohol dehydrogenase	0.9 ± 0.03 (6)	1.1 ± 0.1 (9)		

[a] The isotope effects were determined competitively and noncompetitively. In the former case, GC/MS analysis of the product ratio was carried out after oxidation of mixtures (1:1) of [1-^{13}C]ethanol and [$^2H_6$]ethanol or [2,2,2-$^2H_3$]ethanol and [1,1-$^2H_2$]ethanol. The values are mean ± SD, and the number of experiments is given in parentheses. The enzyme incubations were performed with two different enzyme preparations. In the noncompetitive method, V_{max} and K_m values were determined for eight different concentrations of unlabeled ethanol or [1,1-$^2H_2$]ethanol as substrate. Mean values of two experiments with two different membrane preparations are given. [b] The number of animals is indicated in parentheses. [c] v denotes the mean rate of ethanol oxidation in the various enzyme systems expressed as nmol per nmol of P-450 per min. [d] Significantly different ($p < 0.01$) compared to phenobarbital microsomes from the same phenobarbital-treated animal incubated in the absence of Fe^{III}EDTA. [e] Significantly different ($p < 0.01$) compared to P-450 LMeb incubated in the absence of Fe^{III}EDTA. [f] Significantly different ($p < 0.02$) compared to P-450 LMeb incubated in the absence of Fe^{III}EDTA. [g] Significantly different ($p < 0.02$) compared to P-450 LMeb catalyzed ethanol oxidation. [h] Significantly different ($p < 0.01$) compared to P-450 LMeb catalyzed ethanol oxidation. [i] Significantly different ($p < 0.01$) compared to control microsomes.

the xanthine–xanthine oxidase system, a $^D(V/K)$ isotope effect of 1.4 was observed, whereas with yeast alcohol dehydrogenase, in the absence of coupling reagents for acetaldehyde, it was only about 1.0. The absence of isotope effect was expected since ethanol and acetaldehyde were rapidly interconverted as evident from analysis of ethanol isolated from incubations with [1,1-$^2H_2$]- and [2,2,2-$^2H_3$]ethanol [cf. Cronholm (1985) and Palm et al. (1968)].

The ^DV and ^DK isotope effects calculated from results of incubations according to the noncompetitive method with reconstituted systems containing P-450 LM_2 (+Fe^{III}EDTA) were 1.3 and 0.6, respectively. The similar isotope effects reached with P-450 LMeb in the absence of Fe^{III}EDTA were 0.66 and 0.16, respectively (cf. Figure 1).

Stereoselectivity of Cytochrome P-450 Dependent Ethanol Oxidation. The xanthine–xanthine oxidase system and rabbit and rat liver microsomes as well as reconstituted membranes containing either ethanol-inducible P-450 LMeb or phenobarbital-inducible P-450 LM_2 (the enzymes in the presence or in the absence of 3 or 10 μM Fe^{III}EDTA, respectively) were incubated with chiral [1-^{2}H]ethanols. The acetaldehyde produced was analyzed by GC/MS, and partial mass spectra were recorded (Figure 3). To avoid experimental errors due to contaminations and loss of deuterium by exchange, the stereoselectivity was determined for both (1*R*)- and (1*S*)-[1-^{2}H]ethanol. The isotope effects were quantified and taken into account when the stereoselectivity was calculated (cf. Methods). The stereoselectivity given as the probability of the removal of a 1-*pro-R* hydrogen atom from (1*R*)-[1-^{2}H]ethanol in all enzymic systems was 52–74% (Table II). The corresponding values with (1*S*)-[1-^{2}H]ethanol as substrate were 31–59% (Table II), and the mean values from experiments with the two isotopes were 44–66%. In a similar experimental approach, the corresponding value for the stereoselective removal of the 1-*pro-R* hydrogen atom from (1*R*)-[1-^{2}H]ethanol was 100% when ethanol oxidation was carried out in catalase-dependent cytosolic systems.[4] Accordingly, our results suggest that there is no stereospecific action of cytochrome P-450 in liver microsomes from control and acetone- or ethanol-treated rats, in microsomes from control and imidazole- or acetone-treated rabbits, or in reconstituted systems con-

Table II: Stereoselectivity in the Oxidation of either (1*R*)-[1-^{2}H]Ethanol or (1*S*)-[1-^{2}H]Ethanol by Different Enzyme Systems[a]

enzyme system	(1*R*)-[1-^{2}H]-ethanol S_R (%)	(1*S*)-[1-^{2}H]-ethanol S_R (%)	mean
rat liver microsomes			
control	74.2	58.8	66
acetone	62.7	53.1	58
acetone + desferrioxamine (100 μM)	56.2	46.3	51
ethanol	58.0	48.2	53
rabbit liver microsomes			
control	67.8	54.8	61
imidazole	62.4	46.2	54
acetone	62.1	53.5	58
cytochrome P-450			
LMeb	65.0	44.6	55
LMeb + FeEDTA (3 μM)	57.6	30.9	44
LM_2	69.7	49.2	59
LM_2 + FeEDTA (10 μM)	74.1	37.1	56
xanthine–xanthine oxidase	52.0	46.4	49

[a] The ratio between unlabeled and deuteriated acetaldehyde was measured by GC/MS as described under Experimental Procedures. The stereoselectivity is expressed as the probability of the removal of a 1-*pro-R* hydrogen (S_R) in experiments performed with (1*R*)-[1-^{2}H]-ethanol or (1*S*)-[1-^{2}H]ethanol. The figures represent mean values for two to four experiments.

[4] C. Norsten, G. Ekström, T. Cronholm, M. Ingelman-Sundberg, J. Handler, and R. G. Thurman, unpublished observations.

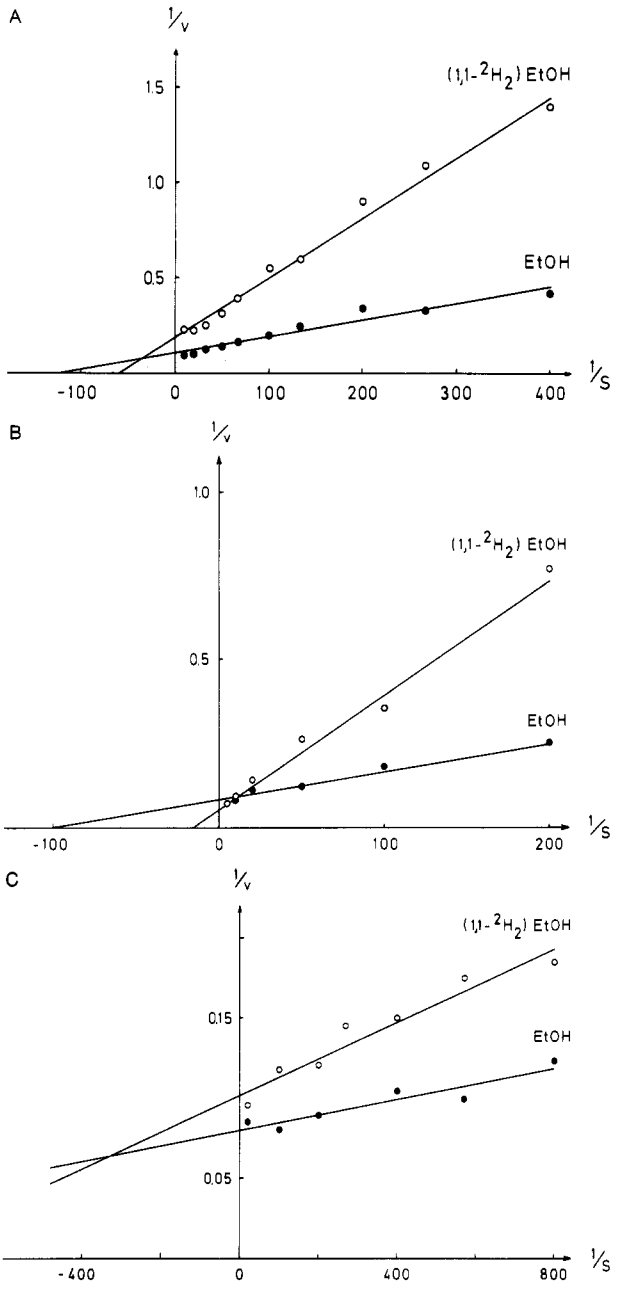

FIGURE 1: Lineweaver–Burk plots of cytochrome P-450 dependent oxidation of unlabeled and 1,1-$^2H_2$-labeled ethanol in liver microsomes from imidazole-treated rabbits (A) and in reconstituted membrane vesicles containing either cytochrome P-450 LMeb (B) or cytochrome P-450 LM_2 (C). The latter type of incubation system contained 10 μM $Fe^{III}EDTA$. Incubation conditions were as described under Experimental Procedures. The relative standard deviations of the slopes were <10% (A), <24% (B), and <22% (C). The relative standard deviations of the intercepts were <15% (A), <67% (B), and <8% (C). Statistical analysis (t test) of the slopes revealed significant differences between deuteriated and nondeuteriated substrates with $p < 0.001$ in all cases. Similar analysis of the intercepts revealed $p < 0.001$ in (A) and (C) and $p < 0.05$ in (B). v is given in nmol/(nmol·min), and S is the substrate concentration in M.

taining rabbit liver ethanol-inducible or phenobarbital-inducible cytochrome P-450. In addition, the xanthine–xanthine oxidase system apparently does not oxidize the alcohol in a stereospecific manner.

Discussion

The results presented indicate that the cytochrome P-450 dependent oxidation of ethanol in either rat or rabbit liver microsomes as well as in reconstituted membranes containing different forms of purified rabbit liver cytochrome P-450 does not exhibit any stereoselectivity. The results are in contrast to those reached in alcohol dehydrogenase containing systems (Loewus et al., 1953) and in catalase-dependent systems (Corral et al., 1974; Gang et al., 1973), where, in both cases, a pronounced stereoselectivity for the 1-*pro-R* hydrogen is observed. Even the ethanol-inducible form of cytochrome P-450 (P-450 LMeb) did not exhibit any preference for the 1-*pro-S* or 1-*pro-R* hydrogen atom. As expected, no stereoselectivity in the reaction was reached in the hydroxyl radical dependent xanthine–xanthine oxidase system, and addition of $Fe^{III}EDTA$ or desferrioxamine to microsomes or reconstituted systems did not have any effect on the stereoselectivity of cytochrome P-450 dependent ethanol oxidation.

Previous investigations by Gang et al. (1973) and Corrall et al. (1975) indicated that oxidation of ethanol by rat liver microsomes was stereospecific in the same manner as alcohol dehydrogenase. However, as also pointed out by Krikun and Cederbaum (1984), it appears that these measurements did not reflect the action of cytochrome P-450 in the microsomes, since catalase was not inhibited in these instances.

In many instances, cytochrome P-450 dependent hydroxylations have been reported to occur with retention of stereochemistry. This is valid for hydroxylation of, e.g., steroids (Bergström et al., 1958; Corey et al., 1958) and lauric acid (Hamberg & Björkhem, 1971). The absence of stereoselectivity in the removal of the 1-*pro-S* or 1-*pro-R* hydrogen atom from ethanol by cytochrome P-450 in the present cases thus indicates that this reaction is more unspecific in this sense and therefore compatible with a hydroxyl radical mediated oxygenation mechanism. However, the absence of stereoselectivity can be attributed to the type of substrate binding to the enzyme and must not necessarily reflect the mechanism of oxidation. Absence of stereoselectivity has been described in the action of cytochrome $P\text{-}450_{cam}$, which removes the endo or the exo hydrogen from its substrate camphor (Gelb et al., 1982). In this case, the hydroxyl group is delivered specifically to the exo position. Furthermore, the results by White et al. (1986) indicate the absence of stereospecific product formation in cytochrome P-450 LM_2 dependent hydroxylation of phenylethane.

The $^D(V/K)$ isotope effects in liver microsomes of cytochrome P-450 catalyzed ethanol oxidation were higher than reported for cytochrome P-450 dependent hydroxylation reactions in general [cf. Ortiz de Montellano (1986) and Björkhem (1977)]. This indicates that the overall rate of ethanol oxidation is determined to a large extent by the rate of the cleavage of the C_1–H bond in the alcohol. However, variations were registered depending on the type of cytochrome P-450 containing system used. These variations may reflect differences in the mechanisms of C_1–H cleavage but might also be explained by differences in the extent of commitment to catalysis in the various enzyme systems (Northrop, 1981).

Evidence has previously been presented for the involvement of free hydroxyl radicals in the oxidation of ethanol dependent on cytochrome P-450 LM_2 (Ingelman-Sundberg & Johansson, 1981). In agreement with these results, the $^D(V/K)$ isotope effects in cytochrome P-450 LM_2 containing systems were similar to those reached in the hydroxyl radical dependent xanthine–xanthine oxidase system [cf. Beauchamp and Fridovich (1970)]. Significantly higher $^D(V/K)$ isotope effects were seen on ethanol oxidation in microsomal and reconstituted systems containing ethanol-inducible cytochrome P-450. Addition of 3 μM $Fe^{III}EDTA$ to reconstituted membranes containing cytochrome P-450 LMeb caused a 5-fold increase in the rate of ethanol oxidation and a significant decrease in

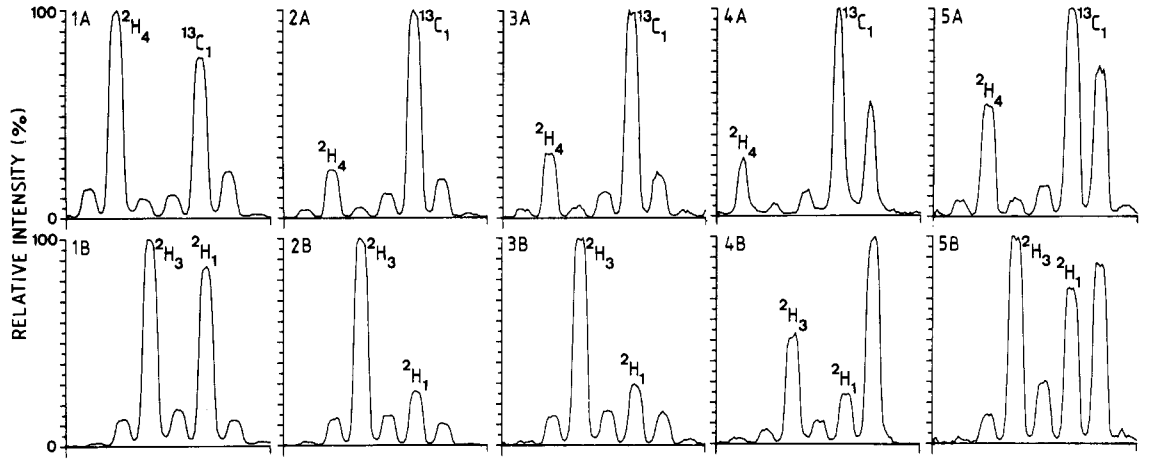

FIGURE 2: Kinetic deuterium isotope effects of ethanol oxidation as determined by GC/MS analysis of derivatized acetaldehyde. Partial mass spectra were recorded by repetitive scanning over m/z 222.5–230.5 (the region of molecular ions). The figure shows the relative abundance of acetaldehyde molecules having 0–4 ^2H atoms or a ^{13}C atom obtained from incubations of [1-^{13}C]ethanol and [$^2H_6$]ethanol (A) or [2,2,2-$^2H_3$]ethanol and [1,1-$^2H_2$]ethanol (B). The enzyme systems used were yeast alcohol dehydrogenase (1), rabbit liver microsomes from acetone-treated animals (2), rat liver microsomes from ethanol-treated animals (3), and reconstituted vesicles containing either cytochrome P-450 LMeb (4) or vesicles containing cytochrome P-450 LM_2 (5).

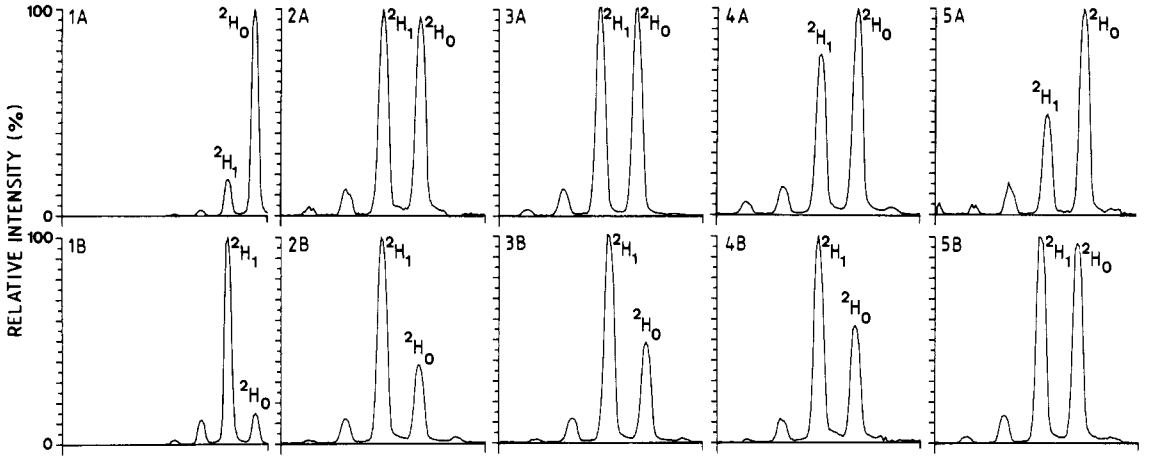

FIGURE 3: Stereoselectivity of ethanol oxidation as revealed by GC/MS analysis of acetaldehyde produced by enzymic oxidation of (1*R*)-[1-^{2}H]ethanol (A) or (1*S*)-[1-^{2}H]ethanol (B). Partial mass spectra were recorded during elution of derivatized acetaldehyde by repetitive scanning over m/z 222.5–230.5 (the region of molecular ions). The figure shows the relative abundance of molecules having 0–4 ^2H atoms. The stereoselectivity was deduced from the ^2H excess in acetaldehyde formed in incubation mixtures with yeast alcohol dehydrogenase (1), rabbit liver microsomes from acetone-treated animals (2), rat liver microsomes from ethanol-treated animals (3), and reconstituted vesicles containing either cytochrome P-450 LMeb (4) or cytochrome P-450 LM_2 (5).

the isotope effect. These results might indicate that free hydroxyl radicals are not involved in oxidizing systems dependent on ethanol-inducible cytochrome P-450 in the absence of Fe^{III}EDTA and that the increase in oxidation upon addition of Fe^{III}EDTA was due to parallel oxidation in a hydroxyl radical dependent pathway. Alternatively, the results are caused by lower commitment to catalysis when oxidation was dependent on cytochrome P-450 LM_2 and/or Fe^{III}EDTA. In view of the higher catalytic center activity of ethanol-inducible cytochrome P-450 compared to cytochrome P-450 LM_2 (cf. Table I), this would indicate higher rates of binding and dissociation of substrate to this form of P-450. Addition of Fe^{III}EDTA might enhance the rate in the C_1–H cleavage by supplying hydroxyl radicals and thus cause the commitment to catalysis to increase. Further experiments are needed to differentiate between these possibilities.

Kinetic deuterium isotope effects on the microsomal oxidation of ethanol has previously been determined in a system where catalase was not inhibited (Gang et al., 1973) and more recently in microsomes and partially purified systems from rat liver (Damgaard, 1982). In the latter study, the isotope effect was found to be small, the $^D(V/K)$ effect being 1.15 in the purified system. However, these determinations were performed under the assumption that the reaction was stereospecific and only (1*R*)-[1-^{2}H]ethanol was therefore used as substrate (Damgaard, 1982). In light of our own studies presented here, showing absence of stereoselectivity in ethanol oxidation catalyzed by rat liver microsomal cytochrome P-450 systems, it appears that the isotope effect in the study by Damgaard (1982) has been seriously underestimated since the isotope effect was assumed to equal the ratio between the oxidation of ethanols having protium and deuterium in the 1-*pro*-*R* position. Accordingly, it can be calculated that this ratio would be about 1.6 with the isotope effect of 4 observed in the present work. Furthermore, in the study of Damgaard (1982) incubations were performed in the presence of 1 mM EDTA. Since the solutions were not treated with Chelex 100 prior to use, it appears very plausible that an underestimation of the isotope effect has been made due to the contribution of Fe^{III}EDTA-dependent ethanol oxidation in the incubation systems, since the presence of Fe^{III}EDTA would have caused a decrease in the isotope effect on the reaction (cf. Table I).

Under the acidic conditions usually used for derivatization, a pronounced hydrogen exchange occurs between the 2,4-dinitrophenylhydrazone of labeled acetaldehyde and water. This source of error was eliminated in the present study by deriv-

atizing under neutral conditions in dimethylformamide, a method that was found to give only a very small exchange of hydrogen. This small error could be corrected for since the abundances of molecules having different numbers of isotopic atoms were determined separately. Furthermore, measurements were performed with a deuteriated methyl group present on both C-1 unlabeled and C-1 deuteriated ethanol. Errors due to hydrogen exchange would influence the values obtained in these measurements in opposite directions. In addition, the $^{D}(V/K)$ isotope effects measured by the competitive methods were in good agreement with those determined by the noncompetitive method.

In conclusion, the data presented in this paper indicate that the cytochrome P-450 dependent oxidation of ethanol proceeds without any stereoselectivity but is subject to various extents of kinetic deuterium isotope effects, depending on the isozyme in question. The absence of stereoselectivity in this reaction indicates that it is different from most cytochrome P-450 dependent hydroxylations of hydrocarbons. The results presented provide a basis for future investigations with isotope techniques aimed to elucidate the relative contribution of alcohol dehydrogenase, catalase, and cytochrome P-450 to the in vivo elimination of ethanol at various concentrations [cf. Damgaard (1982)].

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Registry No. P-450, 9035-51-2; EtOH, 64-17-5; AcH, 75-07-0; alcohol dehydrogenase, 9031-72-5.

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