On the Mechanism of Action of Cytochrome P450: Evaluation of Hydrogen Abstraction in Oxygen-Dependent Alcohol Oxidation†

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ABSTRACT: The mechanism of oxidation of primary and secondary benzylic alcohols to the corresponding carbonyl compounds by purified rabbit liver cytochrome P450 forms 2B4 and 2E1 in a reconstituted enzyme system has been examined by linear free energy relationships, intramolecular and steady-state deuterium isotope effects, and the incorporation of an O_2-derived oxygen atom or solvent-derived deuterium. The k_{cat} and K_m values were found to be relatively insensitive to the presence of electronic perturbations at the para position. The Hammett reaction constants for the oxidation of benzyl alcohols by P450s 2B4 and 2E1 are −0.46 and −0.37, respectively, and with 1-phenylethyl alcohols the corresponding reaction constants are −1.41 and −1.19, respectively. With [1-$^2H_1$]benzyl alcohol, P450s 2B4 and 2E1 show similar intramolecular deuterium isotope effects of 2.6 and 2.8, respectively, whereas with [1-$^2H_2$]benzyl alcohol under steady-state conditions, the deuterium isotope effects on the catalytic constants are 2.8 and 1.3, respectively. No significant isotope effect on the catalytic constant was noted for either form of P450 with 1-phenylethyl alcohol. In D_2O, acetophenone formed by either form of P450 from 1-phenylethyl alcohol does not contain a deuterium atom at the methyl group, whereas under an atmosphere of $^{18}O_2$ approximately 30% of the labeled oxygen is incorporated into the carbonyl group with either form of the cytochrome. The results are consistent with a mechanism that involves stepwise oxidation of the alcohol to a carbon radical α to the alcohol function, followed by oxygen rebound to yield the *gem*-diol, dehydration of which gives the carbonyl product. However, the rate-determining step is dependent on the alcohol substrate and the form of cytochrome P450 that is examined. Carbon–hydrogen bond cleavage in benzyl alcohol is clearly rate-limiting with P450 2B4 and partially rate-limiting with P450 2E1, whereas in 1-phenylethyl alcohol this step is not rate-limiting with either cytochrome.

P450[1] heme proteins constitute a class of highly versatile biological catalysts that utilize molecular oxygen and NADPH to oxidize diverse organic compounds of endobiotic and xenobiotic origin (Coon et al., 1992). The various reactions result in the insertion of an atom of molecular oxygen into a hydroxylated product or in other types of oxidation at functional groups such as amines, ethers, esters, aldehydes, and alcohols (Guengerich, 1987). A general mechanistic scheme developed for such P450-catalyzed reactions accounts for various aspects of the catalytic cycle and for the insertion of a molecular oxygen-derived oxygen atom into the oxidized product (White & Coon, 1980). In the oxidation of alcohols to carbonyl products, however, some exceptions to the predicted incorporation of an atom of molecular oxygen into the carbonyl product have been observed by various laboratories. Partial or complete lack of incorporation of O_2-derived oxygen into the carbonyl product has been observed that is apparently not explainable by exchange with water (Akhtar et al., 1982; Cheng & Schenkman, 1983; Suhara et al., 1984; Wood et al., 1988). This has resulted in various mechanistic hypotheses for the oxidation of alcohols by P450 such as oxidative dehydrogenation (Cheng & Schenkman, 1983; Wood et al., 1988) or stereospecific dehydration of a transient *gem*-diol such that the inserted oxygen is specifically lost (Suhara et al., 1984). Ekström et al. (1987) have reported that cleavage of the C_1–H bond of ethanol appears to be a rate-determining step in catalysis by the ethanol-inducible form of P450.

We have chosen to examine the oxidation of benzylic alcohols by P450 2E1 and 2B4 as a mechanistic model since reactions at benzylic positions are sensitive to electronic perturbations by substituents on the aromatic ring, and the magnitude of this effect on the rate constant is a useful indicator of the intermediate generated at the benzylic position (Jaffe, 1953). Two further advantages are the large extinction coefficients of benzaldehydes and acetophenones that permit sensitive quantitative analysis by reversed-phase HPLC and the enolization rate, hydration equilibrium, and electron impact mass fragmentation pattern of acetophenone that permit a sensitive measurement of the incorporation of a solvent proton at the methyl group or of an $^{18}O_2$-derived oxygen atom into the carbonyl group. In this study, we have determined (a) the linear free energy relationship for the oxidation of a series of para-substituted benzyl and 1-phenylethyl alcohols to the aldehydes and ketones, respectively; (b) the intramolecular deuterium isotope effect for the oxidation of benzyl alcohol to benzaldehyde; (c) the steady-state deuterium isotope effect on the catalytic constants for the oxidation of benzyl and 1-phenylethyl alcohols to benzaldehyde and acetophenone, respectively; and (d) the incorporation into acetophenone of a solvent-derived proton at the methyl group or an $^{18}O_2$-derived oxygen atom at the carbonyl group.

Our results establish that the oxidation of benzyl alcohols by P450 2B4 proceeds by the rate-determining formation of

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[1] Abbreviations: P450, cytochrome P450; reductase, NADPH-cytochrome P450 reductase; DLPC, dilauroylglyceryl-3-phosphorylcholine; HPLC, high-pressure liquid chromatography. P450 2B4 and P450 2E1 are the currently recommended names (Nelson et al., 1993) for the rabbit liver microsomal isoforms originally designated LM_2 and LM_{3a}, respectively.

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a benzylic radical as an intermediate and that oxidative desaturation of 1-phenylethyl alcohol to an enol is not an intermediate with either P450 2B4 or 2E1. Our findings also indicate that both forms of P450 oxidize primary or secondary benzylic alcohols to the corresponding carbonyl compounds by the same sequence of reactions involving an intermediate benzyl radical and oxygen rebound to form the *gem*-diol, dehydration of which yields the carbonyl compounds. However, the rate-limiting step in the overall reaction is dependent on the alcohol substrate as well as the isozyme of P450.

MATERIALS AND METHODS

Substrates and Reagents. NADPH and DLPC were obtained from Sigma and Calbiochem, respectively. Sodium borodeuteride, lithium aluminum deuteride, and primary and secondary benzyl alcohols were obtained from Aldrich. The commercially obtained alcohols were examined for contamination by the corresponding carbonyl compounds and were redistilled or recrystallized from 40% hot aqueous ethanol when necessary. Other alcohols were synthesized as described below from aldehydes or ketones obtained from Aldrich. $^{18}O_2$ of 98% isotopic purity was obtained from Cambridge Isotope Laboratories.

Synthesis of para-Substituted and Deuterium-Labeled Benzyl and 1-Phenylethyl Alcohols. To a solution of the aldehyde or ketone (50 mmol in 100 mL of 20% aqueous ethanol), sodium borohydride (100 mmol) was added, and the reaction mixture was stirred overnight at room temperature. The solution was then concentrated at room temperature under reduced pressure, diluted with 50 mL of water, and extracted twice with 25-mL portions of CH_2Cl_2. After the combined extract had been dried over anhydrous sodium sulfate, the solvent was removed under reduced pressure, and the residue was vacuum distilled (at 18 mmHg) to yield the desired alcohol in an overall yield that ranged from 60 to 80%. In all cases, the alcohol contained less than 0.01% of the starting aldehyde or ketone as determined by HPLC. For the synthesis of [1-$^2H_1$]-1-phenylethanol and [1-$^2H_1$]benzyl alcohol, the method was essentially as above, except that sodium borodeuteride (98% isotopic purity, from Aldrich) was used in a 2.5-fold equivalent excess and the acetophenone or benzaldehyde was 5.0 M in 20% ethanol. For the synthesis of [1-$^2H_2$]-benzyl alcohol, benzoic acid (24.6 mmol) was dissolved in 25 mL of dry ether that had been freshly distilled from lithium aluminum hydride, and the solution was injected into 250 mL of dry ether containing lithium aluminum deuteride (23.8 mmol, 98% isotopic purity, from Aldrich). The mixture was maintained under an atmosphere of dry nitrogen and stirred overnight at room temperature, after which 100 mL of ice-cold 2 N HCl was added to decompose the excess reductant. The ether layer was separated, washed once with saturated aqueous sodium bicarbonate, and dried over anhydrous sodium sulfate. The ether was then removed under reduced pressure at room temperature, and the residue was vacuum distilled (at 18 mmHg) to yield the labeled alcohol in 60% yield, based on the starting benzoic acid.

Enzymes. P450 forms 2B4 and 2E1 and the reductase were purified from rabbit liver by methods previously described by this laboratory (Coon et al., 1978; French & Coon, 1979; Koop et al., 1982). The individual preparations were homogeneous as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the specific contents were 15.7, 18.8, and 12.2–13.1 nmol/mg of protein, respectively. Stock solutions of these enzymes were 70.5, 24.0, and 53.0 μM, respectively.

Reconstitution and Enzyme Assay. For all the experiments reported herein, the stock solutions of reductase and P450 were mixed in a 1:1 molar ratio, distributed into sufficient tubes for each assay, and maintained at −20 °C until used. A typical steady-state kinetic assay was as follows. In a final volume of 0.5 mL, the reaction mixture contained 25 μmol of potassium phosphate buffer, pH 7.4, 30 μg of freshly dispersed DLPC, 0.1 nmol of P450 2E1 or 0.2 nmol of P450 2B4 with an equimolar amount of the reductase, substrate at the appropriate concentration, and 0.5 μmol of NADPH as the final addition. After incubation for 20 min at 30 °C, 0.25 mL of 6% perchloric acid was added, and the mixture was kept on ice for 30 min prior to centrifugation at 5000 rpm for 10 min. A 50-μL aliquot of the supernatant solution was then analyzed by HPLC. Each substrate concentration was assayed in duplicate along with a blank from which the NADPH was omitted. Under these assay conditions, product formation was found to be linear for 45 min. The rate of the reaction was also found to be linear with respect to the level of P450 in the range of 0.05–0.7 nmol for a period of 20 min (data not shown).

HPLC Analysis. Quantitative analysis of the enzymatically formed aldehyde or ketone was done with a Waters μBondapack C-18 reversed-phase analytical column with use of an automated HPLC system consisting of a Waters WISP Model 710 autosampler, a Model 600 solvent delivery system, and a Model 480 UV/visible detector set at the appropriate wavelength maximum of the carbonyl product, and a Hewlett-Packard Model 3600 integrator. An isocratic solvent system consisting of acetonitrile and water containing 0.1% trifluoroacetic acid was used for all determinations, with the concentration of acetonitrile adjusted so that the retention time of the aldehyde or ketone was between 7 and 9 min. Solvent mixtures that led to elution of the product earlier than 6 min caused it to appear as a shoulder on the front of the NADP/NADPH peak, frequently resulting in incorrect recognition of the peak for automated integration. Elution times longer than 10 min were also undesirable, since the product was eluted as a broad peak, resulting in decreased sensitivity. Standards of the carbonyl product were run with each assay in the range from 20 to 600 pmol; in this range, the integrated area of the peak was found to be directly proportional to the amount of the standard. Typically, the lower limit of accurate product quantitation was 30 pmol. Each sample was injected in duplicate, and the mean integration value was used to quantitate the carbonyl product.

Intramolecular Deuterium Isotope Effect with [1-$^2H_1$]-Benzyl Alcohol Determined by GC/MS. A typical reaction mixture contained 50 μmol of potassium phosphate buffer, pH 7.4, 60 μg of DLPC, 0.5 nmol of P450 form 2E1 or 1.0 nmol of form 2B4 reconstituted with reductase in a 1:1 molar ratio as described, 1.5 or 6.0 μmol of [1-$^2H_1$]benzyl alcohol for reactions with 2E1 or 2B4, respectively, and 5 μmol of NADPH in a final volume of 1.0 mL. The mixture was incubated at 30 °C for 2 h, after which time it was extracted by vigorous mixing with 2 mL of CH_2Cl_2. The CH_2Cl_2 layer was dried over anhydrous sodium sulfate, reduced to a volume of approximately 0.5 mL under a stream of nitrogen, and applied to an analytical silica gel HPLC column (25 × 0.4 cm) previously equilibrated with CH_2Cl_2. The column was treated with CH_2Cl_2 at a flow rate of 1 mL/min, and the fraction eluted between 4.0 and 6.0 min was collected and concentrated to approximately 20 μL under a stream of dry nitrogen. (Benzaldehyde and benzyl alcohol standards were eluted at 4.5 and 12.2 min, respectively, under these conditions.) A 4-μL aliquot was injected onto a 30-m DB-5 fused silica capillary column (1.0-μm film thickness, 0.32 μm i.d., J&W Scientific) in a Finnigan gas chromatograph. The

Table 1: Components Required and Effect of Catalase and Superoxide Dismutase on Oxidation of Benzyl Alcohol by P450 2E1

system	activity [nmol min^{-1} (nmol of P450)$^{-1}$]
complete[a]	3.12 ± 0.02
cytochrome P450 omitted	0.0
reductase omitted	0.0
NADPH omitted	0.0
complete + catalase (10 units)	3.20 ± 0.04
complete + catalase (100 units)	3.05 ± 0.04
complete + superoxide dismutase (180 units)	3.20 ± 0.05]
complete + superoxide dismutase (720 units)	3.10 ± 0.04

[a] The complete system was as described in the text, with 1.5 μmol of benzyl alcohol. In the other experiments, the components indicated were selectively omitted or added to the complete system.

splitless injector was maintained at 200 °C, and helium was used as the carrier gas at a head pressure of 10.0 psi. The column temperature was maintained at 50 °C for 2 min and then raised to 275 °C at 10 °C/min. Benzaldehyde was eluted at 7.4 min under these conditions. The gas chromatograph was attached to a Finnigan 4021 mass spectrometer operating at 70 eV. Data aquisition and processing were done with the Finnigan INCOS data system.

Solvent Deuterium Incorporation into Acetophenone. A reaction mixture similar to that described above for determination of the intramolecular isotope effect with benzyl alcohol was used, except that the medium was 92% deuterium oxide and the substrate was 1-phenylethanol at a concentration of 3.0 or 6.0 mM with P450 2E1 or 2B4, respectively. A comparable reaction mixture lacking NADPH and the substrate but containing 1.5 μmol of acetophenone served as a control for the exchange of deuterium with the methyl hydrogens. Acetophenone was isolated by HPLC on silica gel and analyzed by GC/MS as described earlier for benzaldehyde. The retention times of acetophenone and 1-phenylethanol on the silica gel column were 6.5 and 14.0 min, respectively; the retention time of acetophenone on the DB-5 fused silica capillary column was 8.5 min.

Incorporation of Oxygen from $^{18}O_2$ into Acetophenone. A typical 10-mL reaction mixture contained 0.5 mmol of potassium phosphate buffer, pH 7.4, 0.6 mg of DLPC, 60 μmol of 1-phenylethanol, and 5.0 nmol of P450 2B4 or 2.5 nmol of P450 2E1 reconstituted with reductase as described. The mixtures were made anaerobic by repeated purging with oxygen-free nitrogen, $^{18}O_2$ was then introduced, and NADPH (0.1 mmol) was injected as an aqueous anaerobic solution to initiate the reaction. The incubation was at 30 °C for 2.5 h, after which time the reaction mixtures were extracted with two 5-mL aliquots of CH_2Cl_2. The extract was dried over anhydrous sodium sulfate and then evaporated under reduced pressure to 0.5 mL, and the acetophenone was purified by silica gel chromatography and analyzed by GC/MS as described above. To determine the extent of $^{16}O_2$ isotope dilution that might have occurred under the experimental conditions, the hydroxylation of toluene to benzyl alcohol by P450 2B4 was determined. GC/MS analysis of the resulting benzyl alcohol showed 96% ^{18}O incorporation (data not given), indicating that insignificant isotopic dilution took place under the experimental conditions used.

RESULTS

Components Required. The requirements for the oxidation of benzyl alcohol to benzaldehyde by P450 2E1 in the reconstituted system and the effect of catalase and superoxide dismutase on the reaction are shown in Table 1. The formation

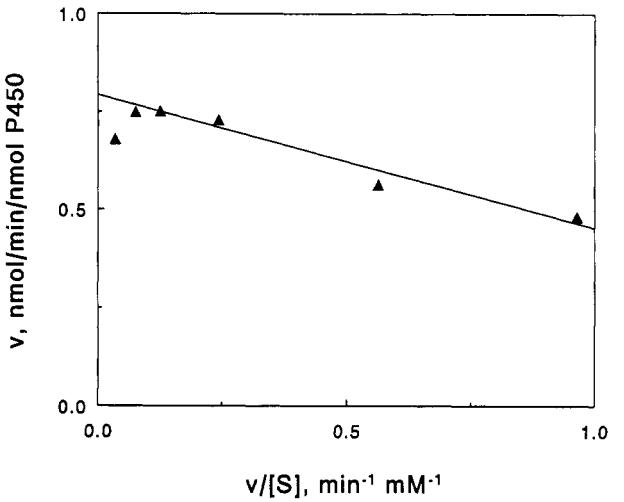


FIGURE 1: Typical Woolf–Augustinsson–Hofstee plot as shown for *p*-fluorophenylethyl alcohol with P450 2B4, from which kinetic constants were obtained. The plot indicates the inhibition observed at the highest substrate concentrations. K_m and k_{cat} values were obtained from those concentrations at which substrate inhibition was not observed.

of benzaldehyde is dependent on P450, the reductase, and NADPH; omission of any of these components results in no observable oxidation of the alcohol. Catalase and superoxide dismutase have no effect on product formation, indicating that hydrogen peroxide and superoxide, which are produced in the reconstituted enzyme system (White & Coon, 1980), are not involved in a nonenzymatic, Fenton-type reaction with the alcohol. Similar results were obtained with all of the other alcohols examined in this study, including the experiments with P450 2B4, thus showing that alcohol oxidation occurs within the catalytic site of the cytochrome.

Steady-State Kinetics and Linear Free Energy Correlation Analysis. The activities of benzyl alcohol and seven para-substituted derivatives and of 1-phenylethanol and eight para-substituted derivatives were examined in each case at six concentrations, the range of which depended on the form of P450 being studied. With P450 2B4, the substrate concentrations varied from 0.3 to 10.0 mM, and with 2E1 they were from 0.1 to 3.0 mM. The kinetic constants were obtained from linear regression analysis of the initial rates fitted to Lineweaver–Burk and Woolf–Augustinson–Hofstee plots (Segel, 1975); the latter is shown in Figure 1 for *p*-fluorophenylethyl alcohol. Some substrates showed inhibition at high concentrations with both cytochromes. Accordingly, such results were not included in the determination of kinetic constants, but at least four data points were used for each value calculated. Both plots gave correlation coefficients greater than 0.99, and the kinetic constants obtained by the two analytical methods were in good agreement. Tables 2 and 3 summarize the steady-state kinetic constants determined for the oxidation of the series of benzyl alcohols and the series of 1-phenylethyl alcohols, respectively, by both P450 2B4 and P450 2E1. The K_m values vary from 0.11 to 7.3 mM for the benzyl alcohols and from 0.05 to 7.5 mM for the phenylethyl alcohols, with no obvious correlation with the partition coefficient, the P450 used, or the rate of oxidation. The k_{cat} values were used for linear free energy correlation analysis as shown in Figure 2A for the oxidation of para-substituted benzyl alcohols and in Figure 2B for the oxidation of para-substituted 1-phenylethyl alcohols by the two cytochromes. The reaction constants for the oxidation of benzyl alcohols obtained from these plots are –0.46 (correlation coefficient = –0.59) and –0.37 (correlation coefficient = –0.60) with P450 2B4 and 2E1, respectively. With 1-phenylethyl alcohols, the cor-

Table 2: Steady-State Kinetic Constants for Oxidation of para-Substituted Benzyl Alcohols by P450s 2E1 and 2B4[a]

		P450 2E1		P450 2B4	
para-substituent	Hammett constant (σ^o_p)	k_{cat} (min^{-1})	K_m (mM)	k_{cat} (min^{-1})	K_m (mM)
OCH_3	–0.27	4.20	1.00	1.20	4.31
CH_3	–0.17	3.37	0.31	0.79	0.33
H	0.00	3.59	0.45	3.38	7.28
F	0.06	2.89	0.32	0.61	1.28
Br	0.23	2.08	0.13	0.75	0.13
Cl	0.23	2.37	0.11	0.52	0.13
CN	0.66	2.31	0.69	0.49	1.48
NO_2	0.78	2.90	0.43	0.73	1.90

[a] The k_{cat} and K_m values were obtained from Lineweaver–Burk and Woolf–Augustinsson–Hofstee plots and are the mean of two or three separate determinations. The Hammett substituent constants are taken from Hansch (1973).

Table 3: Steady-State Kinetic Constants for Oxidation of para-Substituted 1-Phenylethyl Alcohols by P450s 2E1 and 2B4[a]

		P450 2E1		P450 2B4	
para-substituent	Hammett constant (σ^o_p)	k_{cat} (min^{-1})	K_m (mM)	k_{cat} (min^{-1})	K_m (mM)
OCH_3	–0.27	3.50	0.64	3.46	3.12
CH_3	–0.17	3.38	0.44	4.37	0.72
H	0.00	4.04	0.49	6.03	1.88
F	0.06	2.35	0.26	2.28	0.65
Br	0.23	0.92	0.06	1.59	0.06
Cl	0.23	1.76	0.05	1.93	0.27
COOH	0.45	0.62	7.54	0.31	6.09
CN	0.66	1.19	0.42	0.97	1.19
NO_2	0.78	1.06	0.13	1.00	2.31

[a] The k_{cat} and K_m values were obtained from Lineweaver–Burk and Woolf–Augustinsson–Hofstee plots and are the mean of two or three separate determinations. The Hammett substituent constants are taken from Hansch (1973).

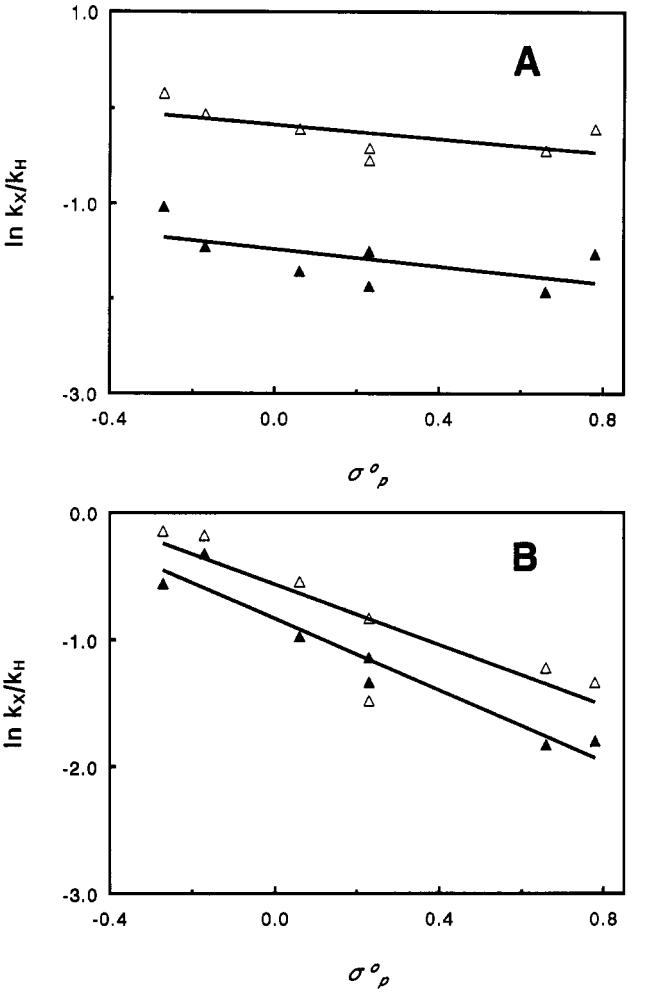


FIGURE 2: Linear free energy correlation diagrams for the P450 2E1-catalyzed (△) and P450 2B4-catalyzed (▲) oxidation of benzyl alcohols (panel A) and of 1-phenylethyl alcohols (panel B). Ln k_X/k_H was calculated from the rate constants shown in Tables 2 and 3, and values for σ^o_p were obtained from Hansch (1973).

responding reaction constants are –1.41 (correlation coefficient = –0.96) and –1.19 (correlation coefficient = –0.85), respectively. The results with *p*-carboxy-1-phenylethyl alcohol deviated significantly from linearity and were excluded from linear regression analysis of the data. The significance of the reaction constants in connection with the isotope effects to be presented is discussed below.

Deuterium Isotope Effect with Benzyl and Phenylethyl Alcohols. Table 4 shows the isotope effect on the steady-state parameters for the oxidation of benzyl alcohol as compared to its 1-dideuterio derivative and of 1-phenylethyl alcohol as compared to its 1-monodeuterio derivative by P450 2B4 and P450 2E1. With phenylethyl alcohol and either cytochrome no isotope effect on the catalytic constant was observed, suggesting that, with these enzymes, cleavage of the benzylic carbon–hydrogen bond is not rate-determining in the overall reaction. With the dideuterio benzyl alcohol, however, a significant isotope effect was obtained with both of the cytochromes. The effect on the catalytic constant in the case of P450 2B4 is 2.8, indicating that carbon–hydrogen bond breakage contributes to the enzymatic rate-limiting process. In contrast, the isotope effect on this parameter with P450 2E1 is 1.3, suggesting that hydrogen abstraction is only partially rate-limiting in the overall reaction. With respect to the K_m values, a significant isotope effect was seen only with P450 2B4 and benzyl alcohol; the cause of the 1.6-fold increase has not been studied in detail.

Intramolecular Deuterium Isotope Effects with Benzyl Alcohol. The procedure used for examining the intramolecular isotope effect for the oxidation of monodeuterio benzyl alcohol is given in Materials and Methods. The mass fragmentation pattern of benzaldehyde at 70 eV shows a molecular ion peak (m/z = 106) and an $(M - 1)^+$ ion peak (m/z = 105) of approximately the same intensity (Figure 3, panel A). With deuterium at the aldehydic position, produced in the enzymatic oxidation of the dideuterio benzyl alcohol, the fragmentation pattern has a molecular ion peak at m/z = 107 and an $(M - 2)^+$ peak at m/z = 105 of about the same intensity (panel B). The loss of one mass unit from benzaldehyde and two units from deuteriobenzaldehyde indicates that in this mass region the fragmentation pattern arises from the loss of the aldehydic hydrogen. The small m/z = 106 peak obtained with [^{2}H]benzaldehyde with an intensity approximately 8% of that of the m/z = 107 peak is due to the inherent contribution by ^{13}C (5.6%) and by the residual hydrogen present from the lithium aluminum deuteride (98% isotopic purity) used in the synthesis of [1-$^2H_2$]benzyl alcohol. Thus, in a mixture of benzaldehyde and deuteriobenzaldehyde, the ratio of the corrected signal intensities at m/z = 106 and 107 gives the relative abundance of the two species in the mixture. As shown by the scheme in Figure 4, benzaldehyde formed by enzymatic oxidation of (±) [1-$^2H_1$]benzyl alcohol by P450 would contain hydrogen and deuterium at the aldehydic position in amounts proportional to the rates of C–D and C–H bond cleavage, respectively. Therefore, the ratio of the corrected peak intensities at m/z = 107 and 106 corresponds to the intramolecular deuterium isotope effect. Panels C and D of Figure 3 show the relevant ion mass fragments of benzaldehyde

Table 4: Steady-State Kinetic Deuterium Isotope Effects on Oxidation of Benzyl Alcohol and 1-Phenylethyl Alcohol by P450 Cytochromes 2E1 and 2B4[a]

	P450 2E1				P450 2B4			
substrate	k_{cat} (min^{-1})	k_{cat} (H)/k_{cat} (D)	K_m (mM)	K_m (H)/K_m (D)	k_{cat} (min^{-1})	k_{cat} (H)/k_{cat} (D)	K_m (mM)	K_m (H)/K_m (D)
benzyl alcohol	4.26		0.54		3.22		7.82	
[1-$^2H_2$]benzyl alcohol	3.33	1.3	0.49	1.1	1.15	2.8	4.93	1.6
1-phenylethyl alcohol	3.95		0.50		5.86		1.87	
[1-$^2H_1$]-1-phenylethyl alcohol	4.28	0.9	0.53	0.9	5.38	1.1	1.68	1.1

[a] The k_{cat} and K_m values were obtained from Lineweaver–Burk and Woolf–Augustinsson–Hofstee plots and are the mean of two or three separate determinations.

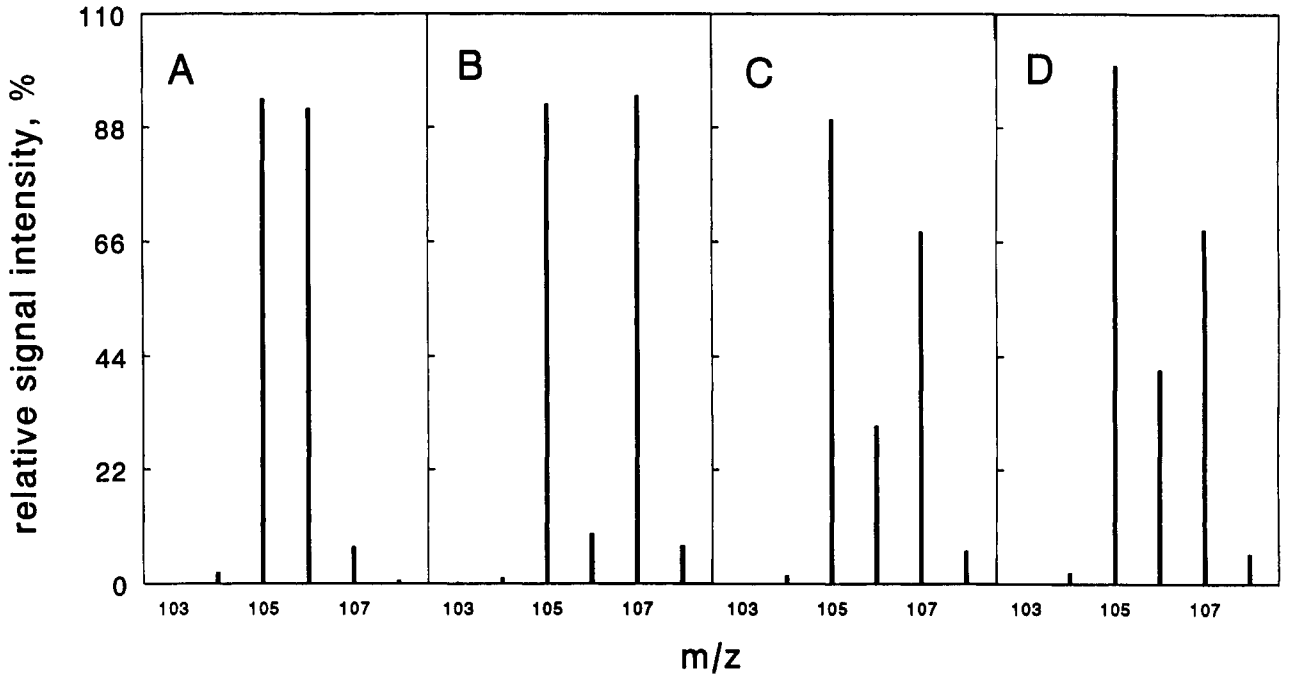


FIGURE 3: Mass spectral fragmentation pattern for benzaldehyde in the region from m/z = 102 to 109. Panel A, benzaldehyde; panel B, [1-$^2H_1$]benzaldehyde derived from P450 2B4-catalyzed oxidation of [1-$^2H_2$]benzyl alcohol; panel C, benzaldehyde obtained from P450 2E1-catalyzed oxidation of [1-$^2H_1$]benzyl alcohol; and panel D, benzaldehyde obtained from P450 2B4-catalyzed oxidation of [1-$^2H_1$]benzyl alcohol.

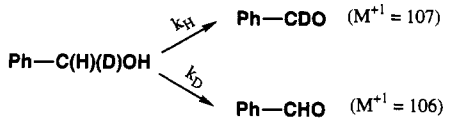


FIGURE 4: Molecular ion peak (m/z value) expected in mass fragmentation pattern of benzaldehyde formed by loss of hydrogen (k_H) or of deuterium (k_D) from [1-$^2H_1$]benzyl alcohol.

formed from (±) [1-$^2H_1$]benzyl alcohol by P450 2B4 and P450 2E1, respectively. The similar and relatively small magnitude of the intramolecular isotope effect for benzyl alcohol oxidation by 2E1 and 2B4, 2.8 and 2.6, respectively, suggests a comparable geometry for bond cleavage by the two forms of the cytochrome, but the significance with respect to transition states, as discussed by More O'Ferrall (1970) for chemical models, is not clear.

Isotope Incorporation into Acetophenone from Solvent or Molecular Oxygen in Enzymatic Oxidation of 1-Phenylethyl Alcohol. The ability of P450 to catalyze oxidative desaturation reactions (Nagata et al., 1986; Rettie et al., 1987, 1988) suggests the possibility of such a reaction with 1-phenylethyl alcohol to form the enol as a route to the carbonyl product. With this alcohol such a mechanism would result in the uptake of a solvent-derived proton into the methyl group of acetophenone. Accordingly, acetophenone formed enzymatically in 92% deuterium oxide from 1-phenylethyl alcohol was examined for deuterium uptake. The expectation was that incorporation of an atom of deuterium into the enzymatically formed acetophenone would cause the m/z = 121 ion signal intensity to increase by approximately 92% and the m/z = 120 intensity to decrease by an equal amount. However, as shown in Figure 5, the intensity of m/z = 121 was not significantly increased relative to that of the molecular ion peak at m/z = 120. The results indicate that an oxidative desaturation mechanism does not operate with either form of P450 examined.

The possible incorporation of an atom of $^{18}O_2$ into acetophenone was determined to establish whether a *gem*-diol is formed as an intermediate in the oxidation of 1-phenylethyl alcohol by either form of P450. (The rapid exchange of oxygen at the aldehyde function precluded the estimation of ^{18}O incorporation into benzaldehyde formed from benzyl alcohol.) Figure 6 shows the relevant mass fragmentation region of acetophenone. The extent of oxygen incorporation was determined from the ion intensities at m/z 122 and 120 and at m/z 107 and 105. The results indicate that with P450 forms 2B4 and 2E1 the incorporation of oxygen from $^{18}O_2$ into the carbonyl group was 32 ± 2 and 29 ± 2%, respectively. A control experiment with acetophenone in $H_2{}^{18}O$ under similar conditions showed incorporation of 14 ± 2% ^{18}O due to solvent exchange; thus, it is obvious that the observed $^{18}O_2$ incorporation into the product would have been greater without loss by such an exchange.

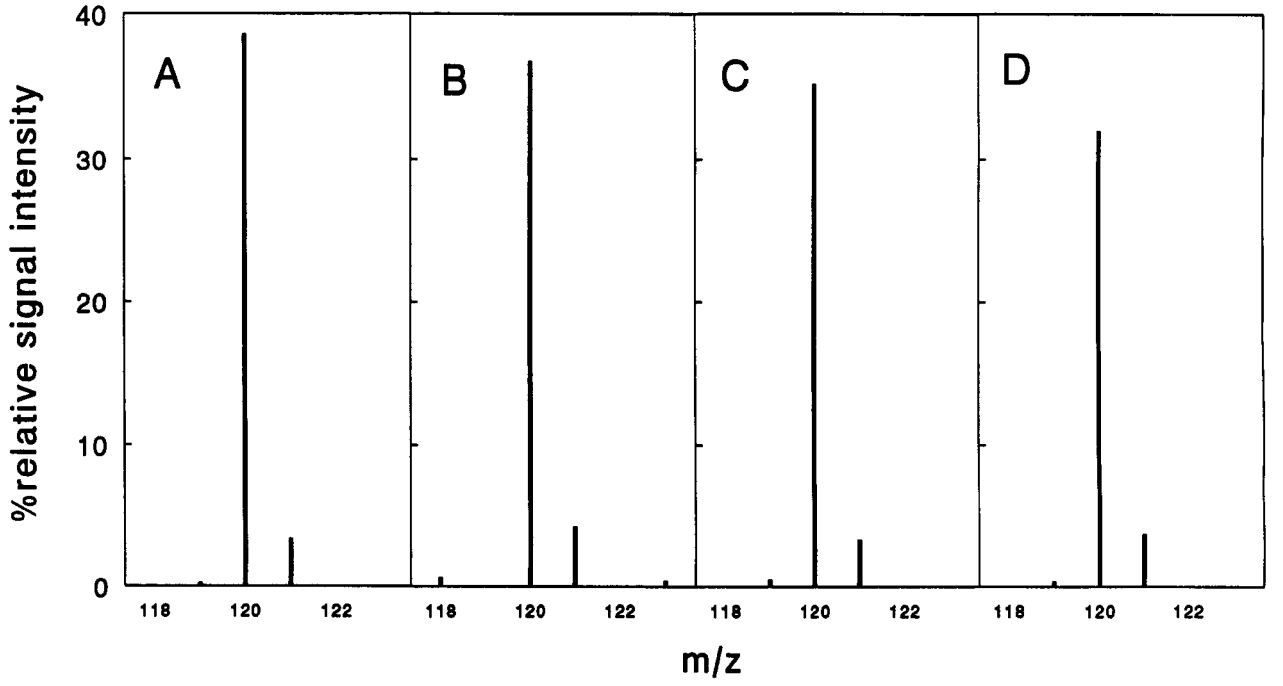


FIGURE 5: Mass spectral fragmentation pattern for acetophenone in the molecular ion region. Panel A, authentic standard; panels B and C, acetophenone produced from 1-phenylethanol oxidation by P450 2B4 or P450 2E1, respectively, in D_2O; panel D, control experiment with P450 2B4 (or 2E1) present but with NADPH and 1-phenylethanol omitted and 1.5 μmol of acetophenone added.

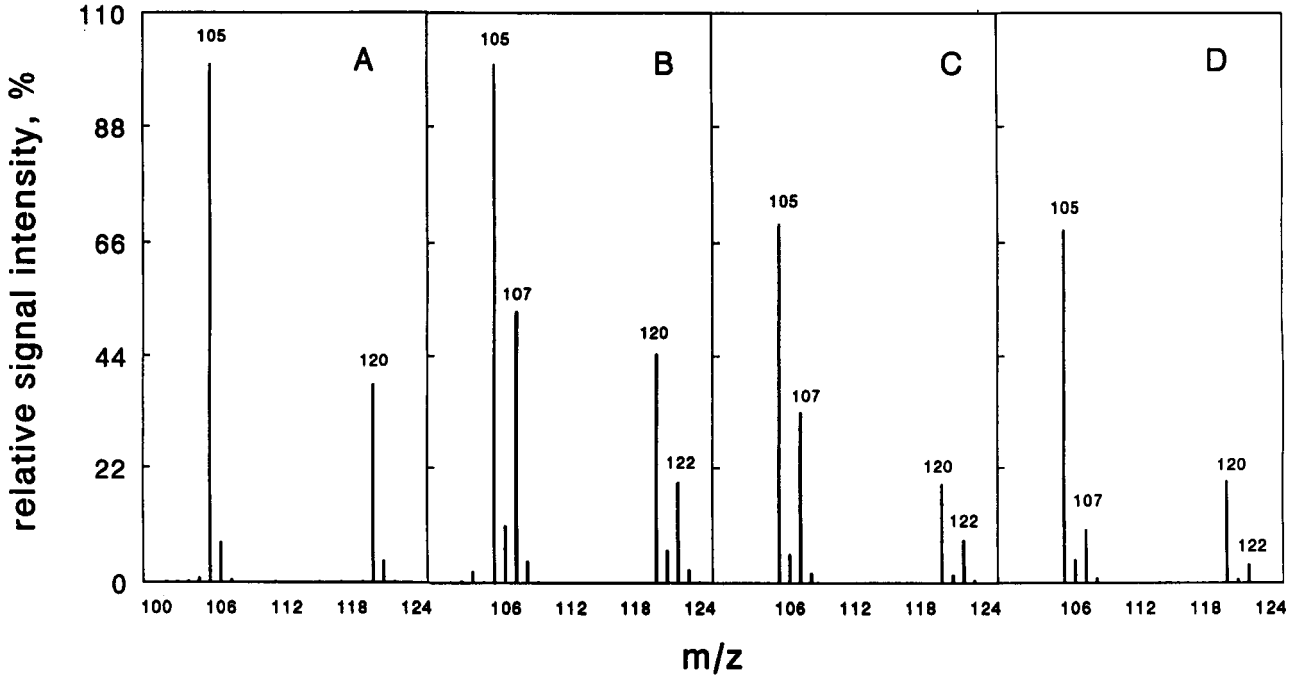


FIGURE 6: $^{18}O_2$-derived oxygen incorporation into the carbonyl group of acetophenone, determined from the mass spectral fragmentation pattern in the region between m/z = 100 and 125. Acetophenone standard, panel A; acetophenone obtained from oxidation of 1-phenylethyl alcohol under an atmosphere of $^{18}O_2$ by P450 2E1 or 2B4, panels B and C, respectively; acetophenone (1.5 μmol) incubated in $H_2{}^{18}O$ under conditions similar to those of the enzymatic reaction, but with NADPH and 1-phenylethanol omitted, panel D.

DISCUSSION

As with certain other types of reactions catalyzed by P450 in microsomal membranes, alcohol oxidation is accomplished by numerous isozymes with overlapping substrate specificity. Purified ethanol-inducible P450 2E1 was originally shown to be more active than four other forms of the cytochrome in the oxidation of ethanol as well as other aliphatic alcohols (Morgan et al., 1982). Additional substrates were subsequently identified (Koop & Coon, 1986), including benzyl alcohols, which are particularly useful for mechanistic studies, as briefly reported several years ago (Vaz & Coon, 1989). The activities vary somewhat with the substrate examined, but P450s 2E1 and 2B4 are about equally active with unsubstituted benzyl alcohol, and 2B4 has 50% greater activity than 2E1 with unsubstituted 1-phenylethanol.

For chemical reactions where the rate-limiting step is at a benzylic carbon, the sign and magnitude of the reaction constant can distinguish the anionic, free radical, or cationic character of the intermediate developed in the transition state (Hammett, 1940). Correlation of the catalytic constants with the Hammett substituent constants for the oxidation of benzylic alcohols examined in this study gave reaction constants between −0.37 and −1.41 with both forms of P450. These small negative values indicate that the slow step in the enzymatic reaction,

while being relatively insensitive to electronic perturbation on the aromatic ring, involves an electron-deficient species. The lack of deviation from linearity between Hammett σ°_{p} values from –0.27 to +0.78 suggests a single transition state for the oxidation of these alcohols. The correlation diagrams in this study have been limited to the Hammett substituent constant σ°_{p}. A further detailed description of the active-site mechanics involved in benzylic alcohol oxidations might be obtained from consideration of other substituent constants (Hansch, 1973) as reported for the hydroxylation of toluene by P450 (White & McCarthy, 1986), but is beyond the scope of this study.

Deuterium isotope effects, a useful means of examining the cleavage of carbon–hydrogen bonds and the extent to which such cleavages are enzymatically rate-limiting, have been used previously to study various P450-catalyzed reactions (Foster et al., 1974; Groves et al., 1978; Miwa et al., 1980, 1983, 1984; Gelb et al., 1982; Burka et al., 1985). The intramolecular deuterium isotope effect determined for the oxidation of benzyl alcohol in the present study showed comparable values for both forms of P450; in contrast, the steady-state isotope effect on the catalytic constant was dependent on the form of P450 examined. The small deuterium isotope effect of 1.3 on the catalytic constant observed for 2E1, as compared to the intramolecular isotope effect of 2.8, indicates that cleavage of the carbon–hydrogen bond contributes only partially to the overall rate-limiting process for this form of P450. With 2B4, the isotope effect on the catalytic constant and the intramolecular isotope effect are similar, 2.8 and 2.6 respectively. Thus, for this cytochrome, cleavage of the benzylic carbon–hydrogen bond is clearly the enzymatic rate-limiting step. It may be noted that the magnitude of the intramolecular isotope effect observed for both enzymes in this study is comparable to the deuterium isotope effects of 2.3 observed for the Ce^{4+} ion oxidation of secondary alcohols to ketones (Mathur et al., 1985) and of 2.6 for the 2B4-catalyzed oxidation of toluene to benzyl alcohol (White & McCarthy, 1986). In nonenzymatic reactions, small kinetic isotope effects have been attributed to asymmetric or nonlinear bond stretching in the transition state (More O'Ferrall, 1970; Kwart & Latimore, 1971; Pryor & Kneipp, 1971; Bethell et al., 1981). These reactions are thought to involve rate-limiting hydrogen atom abstraction. Therefore, the small intramolecular kinetic isotope effect observed in this study may reflect an inherent asymmetry of bond stretching in the transition state.

Several mechanistic alternatives for the oxidation of alcohols by P450 are shown in Figure 7. A stepwise one-electron process has been assumed based on the general scheme for P450-catalyzed reactions (White & Coon, 1980). The generation of a benzylic radical can occur by hydrogen atom abstraction in path A or by electron abstraction from the oxygen (or the aromatic ring, not shown) in path B, followed by proton loss (Shono et al., 1982; Brown et al., 1972; Miwa et al., 1983). The second one-electron oxidation can occur by paths C–G. Carbonyl products derived in paths C–F do not require insertion of an atom of molecular oxygen. In contrast, path G is equivalent to the mechanism of hydrocarbon hydroxylation (White & Coon, 1980), with dehydration of the *gem*-diol to yield the carbonyl product. In the absence of exchange with solvent water, nonspecific dehydration would result in 50% incorporation of a molecular oxygen-derived oxygen atom at the carbonyl group. With benzyl alcohols, the magnitude of the intramolecular deuterium isotope effect for both forms of P450 is consistent with path A (Brown et al., 1972; Shono et al., 1982; White & McCarthy, 1986). For P450 2B4 and benzyl alcohols, the observed intramolecular and steady-state deuterium isotope effects and the reaction constant from the

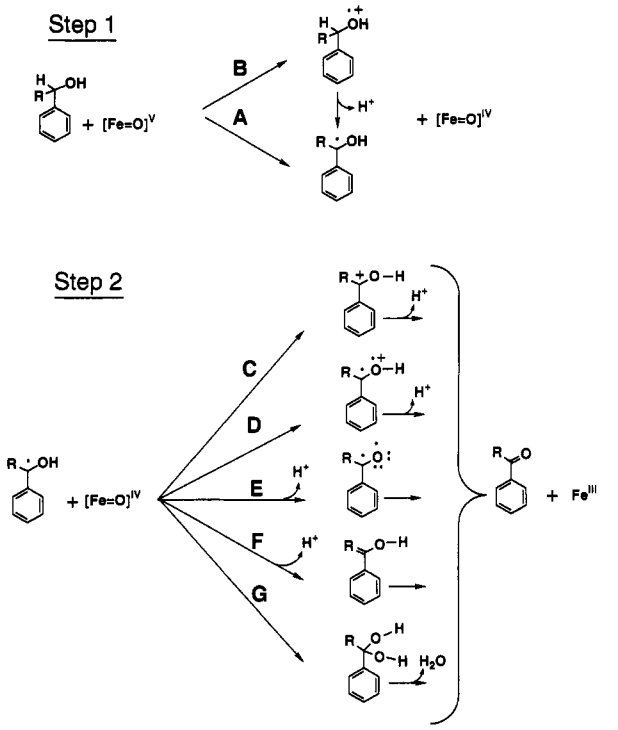
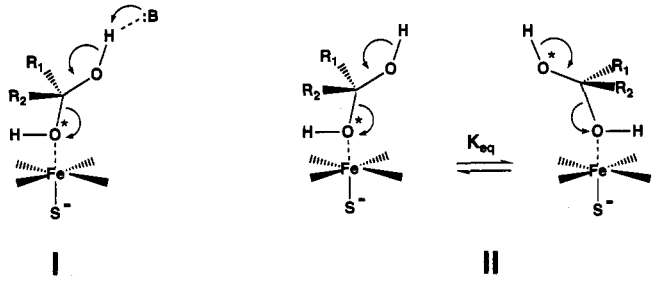


FIGURE 7: Possible paths for oxidation of a benzyl alcohol to the carbonyl product, where R represents a hydrogen atom or a methyl group.

linear free energy correlation diagram clearly establish that a rate-limiting hydrogen atom abstraction occurs at the benzylic carbon to yield a benzylic radical intermediate, whereas with P450 form 2E1 this carbon–hydrogen bond cleavage step contributes only partially to the overall rate-limiting process. If the second electron oxidation is rate-limiting, then path C to give the carbonium ion can be excluded on the basis of the low negative reaction constant of the linear free energy correlation diagram. In path D, electron abstraction from the oxygen followed by proton loss results in the product, whereas in path E hydrogen atom abstraction from the hydroxyl group yields the product. For P450 2E1 and benzyl alcohols, neither path D nor path E can be definitively excluded based on the experimental approaches used in this study. Unlike path G, paths D and E would result in no incorporation of molecular oxygen into the product; however, the rapid exchange of the aldehyde oxygen with solvent precludes the use of $^{18}O_2$ incorporation to distinguish between these alternatives. The rate-limiting step with 2E1 could be second electron abstraction, oxygen rebound, or dissociation of product from the enzyme. In this connection, it is interesting to note that the dissociation of product has been proposed as the rate-determining process in the hydroxylation of toluene to give benzyl alcohol (Ling & Hanslik, 1989).

The mechanism of the initial oxidative step with 1-phenylethyl alcohols by both forms of P450 is not as clearly evident. The intermolecular steady-state kinetic deuterium isotope effects indicated that hydrogen abstraction is not rate-limiting. In the absence of information on intramolecular isotope effects for this substrate, either path A or path B could result in a benzylic radical intermediate. The lack of deuterium incorporation into the carbonyl product excludes path F, and the incorporation of $^{18}O_2$ into acetophenone by both forms of P450 excludes paths C–F, clearly demonstrating the formation of the ketone product by a *gem*-diol intermediate as shown in path G. The rate-limiting step may then be oxygen rebound, dehydration of the nascent *gem*-diol, or its release from the enzyme. While it is unclear which step is rate-limiting in the



I II

FIGURE 8: Scheme for dehydration of *gem*-diol intermediate by a stereospecific (I) or nonstereospecific route (II). B represents a possible basic group in the protein, and O* represents the oxygen atom derived from molecular oxygen.

oxidation of 1-phenylethyl alcohols by either form of P450, it appears that the sequence of reactions resulting in the carbonyl product is similar to that for benzyl alcohols. In summary, our conclusions are in general accord with the earlier finding of Ekström et al. (1987) that P450-dependent oxidation of ethanol is subject to various extents of kinetic deuterium isotope effects, depending on the isozyme in question and the method of induction of the animals from which microsomal suspensions were obtained.

To the extent that benzylic alcohols are a useful mechanistic model for the oxidation of other alcohols by P450, the lack of oxygen incorporation into the carbonyl group in steroids as observed by various investigators (Akhtar et al., 1982; Suhara et al., 1984; Cheng & Schenkman, 1983; Wood et al., 1988) must arise from stereospecific dehydration of the nascent *gem*-diol intermediate at the active site. This may be effected as shown by the scheme in Figure 8, possibly with the aid of a basic residue in the active site, with the heme iron serving as a Lewis acid and resulting in complete loss of the molecular oxygen-derived oxygen atom. The degree of selectivity in the loss of either hydroxyl from the bound, nascent *gem*-diol intermediate would depend on the rate constant for diol dehydration and the equilibrium constant for ligand exchange at the heme iron. The constant for ligand exchange would depend largely on the freedom of movement of the substrate within the active site, resulting in varying degrees of incorporation of oxygen from $^{18}O_2$ into the carbonyl group.

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