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Kinetic Isotope Effects on Cytochrome P-450-catalyzed Oxidation Reactions

EVIDENCE FOR THE IRREVERSIBLE FORMATION OF AN ACTIVATED OXYGEN INTERMEDIATE OF CYTOCHROME P-448*

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Substitution of deuterium for hydrogen on the α-carbon of 7-ethoxycoumarin results in an intrinsic isotope effect of approximately 14 during the cytochrome P-448-catalyzed *O*-deethylation of this substrate (G. T. Miwa, J. S. Walsh, and A. Y. H. Lu (1984) *J. Biol. Chem.* 259, 3000–3004). This dramatic decrease in the C—H bond cleavage rate does not, however, alter the rate of 7-ethoxycoumarin disappearance or the rates of NADPH and oxygen consumption indicating that the catalytic turnover of this enzyme is unaffected. Moreover, hydrogen peroxide formation and the concentrations of an oxycytochrome P-448 complex (λ_{max} = 440 nm) are also unchanged demonstrating that the steady state concentrations of various oxy intermediates of cytochrome P-448 are also unchanged.

An inescapable conclusion from these data is that an irreversible step exists between the formation of these intermediates and the oxidation of the substrate. These data are in agreement with the view that an irreversible cleavage of the dioxygen bond precedes substrate oxidation. Moreover, the oxidatively competent form of the cytochrome must then be committed to substrate oxidation. The latter conclusion is substantiated from high performance liquid chromatography studies which demonstrate the formation of a second metabolite, 6-hydroxy-7-ethoxycoumarin, from the deuterated substrate arising from the metabolic switching away from the *O*-ethyl group to the aromatic ring of this substrate.

The oxidation of xenobiotics by the cytochrome P-450-containing monooxygenase system involves a number of steps and the formation of several intermediates (1). In order to understand the detailed mechanism of this system, each step in the catalytic cycle must be elucidated. This has prompted extensive studies which have characterized, in detail, the substrate binding to oxidized P-450,[1] the first electron reduction of the cytochrome by NADPH-cytochrome P-450 reductase, and the binding of molecular oxygen to the reduced P-450-substrate complex (1). These studies have added to the detailed understanding about steps occurring in the early stages of the P-450 catalytic cycle. However, details about steps contained in the latter half of the catalytic cycle, such as the activation of molecular oxygen and the addition of oxygen to the substrate, are much less well understood.

Since the C—H bond cleavage step must occur after oxygen activation, the study of primary kinetic isotope effects may make possible the elucidation of mechanistic features around this step. To this end, we have previously studied the kinetic isotope effects for *N*-demethylation reactions (2–4) and in the preceding article we reported the extension of these studies to *O*-deethylation reactions catalyzed by this enzyme system (5). In that study, the substitution of deuterium for the hydrogen undergoing oxidative cleavage was shown to reduce the bond cleavage rate by approximately 12- to 14-fold for P-450 isozymes purified from the liver of rats induced with either PB or MC.

In this paper, the consequences of this rate perturbation are examined. The rate of formation of a previously unreported metabolite, 6-hydroxy-7-ethoxycoumarin (Table I, compound **VI**), is greater from the deuterated substrate than from the non-deuterated substrate without affecting the overall turnover of cytochrome or the steady state levels of various intermediates in the catalytic cycle. These data provide the first evidence for the existence of an irreversible kinetic step preceding substrate oxidation.

EXPERIMENTAL PROCEDURES

Substrates—7-Ethoxycoumarin (Table I, compound **I**) was purchased from Aldrich or prepared from 7-hydroxycoumarin, **V**, and ethyl iodide (Aldrich). All isotopically labeled compounds were synthesized as detailed by Walsh *et al.* (6). 7-[1,1-$^2H_2$]Ethoxycoumarin, **II**, was prepared from [1,1-$^2H_2$]ethyl iodide (Aldrich) and 7-hydroxycoumarin; isotopic purity, >95%, mass spectrum: *m/z* (m^+) 192. 7-[1-^{14}C]Ethoxycoumarin, **III**, was synthesized from [1-^{14}C]ethyl iodide (New England Nuclear) and 7-hydroxycoumarin; radiochemical purity, 99.3%. 7-[1-^{14}C-1,1-$^2H_2$]Ethoxycoumarin, **IV**, was prepared from 7-hydroxycoumarin and [1-^{14}C-1,1-$^2H_2$]ethyl tosylate, in turn prepared from [1-^{14}C-1,1-$^2H_2$]ethanol obtained through $LiAl^2H_4$ (Merck) reduction of [1-^{14}C]acetic anhydride (New England Nuclear); radiochemical purity, 99.4%. An authentic sample of the 7-[1,1-$^2H_2$]ethoxy-6-hydroxycoumarin metabolite, **VI**, was prepared by alkaline potassium persulfate oxidation of **II**.

Materials—NADPH and horseradish peroxidase were purchased from Sigma and 3-(*p*-hydroxyphenyl)propionic acid was obtained from Aldrich. Dilauroyl phosphatidylcholine was purchased from Serdary Research Laboratories (Ontario, Canada) and hydrogen peroxide was obtained from Mallinckrodt. All other biochemicals were purchased from Sigma.

Enzymes—Liver microsomes were obtained from PB- and MC-

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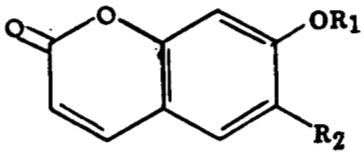
[1] The abbreviations used are: P-450, generic name for the family of cytochrome P-450 isozymes; 7-EC, 7-ethoxycoumarin; other structure abbreviations are given in Table I; PB, phenobarbital; MC, 3-methylcholanthrene; PB P-450, major liver isozyme isolated from phenobarbital-induced rats; P-448, major liver isozyme of cytochrome P-450 isolated from 3-methylcholanthrene-induced rats; HPLC, high pressure liquid chromatography.

TABLE I

Structure of substrates and products



	R_1	R_2
Substrates		
I	CH_2CH_3	H
II	$C^2H_2CH_3$	H
III	$^{14}CH_2CH_3$	H
IV	$^{14}C^2H_2CH_3$	H
Products		
V	H	H
VI	$C^2H_2CH_3$	OH
VII	$^{14}CH_2CH_3$	OH
VIII	$^{14}C^2H_2CH_3$	OH

induced male Long-Evans rats (PB, 75 mg/kg intraperitoneally for 4 days; MC, 25 mg/kg intraperitoneally for 4 days) and MC-induced male Goldon Syrian hamsters (25 mg/kg intraperitoneally for 4 days) by standard methods (7). Hepatic PB P-450 and MC P-448 were purified from rat liver microsomal preparations as described by West *et al.* (8).

NADPH-cytochrome P-450 reductase was isolated from PB-induced Long-Evans rats as described by Yasukochi and Masters (9). One unit of reductase activity is defined as the amount of enzyme catalyzing the reduction of cytochrome *c* at an initial rate of 1 nmol/min at 26 °C under the condition of Phillips and Langdon (10). Cytochrome *c* peroxidase was purified from bakers' yeast according to the procedure of Yonetani and Ray (11).

7-Ethoxycoumarin O-Deethylation Assay—The *O*-deethylation activity of 7-ethoxycoumarin was measured fluorimetrically by following the formation of 7-hydroxycoumarin according to the method of Ullrich and Weber (12) with some modifications (13). A typical incubation mixture consisted of 0.075 nmol of cytochrome P-448, 250 units of NADPH-cytochrome P-450 reductase, 30 μg of dilauroyl phosphatidylcholine, 0.25 μmol of 7-ethoxycoumarin, and 100 μmol of potassium phosphate buffer (pH 7.5) in a final volume of 1 ml. The reaction was started by addition of NADPH (150 nmol), and the mixture was incubated for 5 to 10 min at 23 °C. The reaction was terminated by addition of 0.15 ml of 15% trichloroacetic acid, and 7-hydroxycoumarin was extracted with 2 ml of chloroform. After vigorous shaking for 10 min and centrifugation for 10 min, 1 ml of the chloroform layer was collected. 7-Hydroxycoumarin was recovered from the chloroform extract by addition of 3 ml of 10 mM NaOH containing 1 M NaCl. The 7-hydroxycoumarin was measured by the fluorescence intensity of this alkaline extract with an excitation of 368 nm and an emission of 456 nm, and the concentrations were calculated from the standard curve prepared by adding known amounts of 7-hydroxycoumarin to the standard reaction mixture.

7-Ethoxycoumarin Metabolite Profile—When the total metabolite profile of 7-ethoxycoumarin was examined, the chloroform extract from the reaction mixture was concentrated under a stream of nitrogen gas and chromatographed by HPLC on a Zorbax ODS (Dupont) analytical column using an 8-min linear gradient from 40% methanol/water (v/v) to 100% methanol (14). Both substrate and metabolites were monitored by UV absorption at 330 nm.

Assays for Hydrogen Peroxide—Hydrogen peroxide formation was determined by three different methods. In most assays, the fluorescence method of Zaitsu and Ohkuma (15) was used. As hydrogen peroxide in the terminated reaction mixture was not extracted by chloroform, 0.5 ml of the aqueous layer in the chloroform extraction was added to a mixture of 0.2 ml of 7.5 mM 3-(*p*-hydroxyphenyl)propionic acid, 2 ml of 0.15 M Tris-HCl buffer (pH 8.5), and 0.1 ml of 2 units/ml of horseradish peroxidase. After 10 min, the fluorescence emission intensity was measured at 404 nm with an excitation at 320 nm. The recovery of hydrogen peroxide was determined by addition of a known amount of hydrogen peroxide to the reaction mixture. Hydrogen peroxide formation was also measured by cytochrome *c* peroxidase which forms a stable complex stoichiometrically with hydrogen peroxide and by an oxidase meter (Yellow Springs Instrument Co. model 25) equipped with hydrogen peroxide electrode. The formation of cytochrome *c* peroxidase-hydrogen peroxide complex was calculated from the hydrogen peroxide difference spectrum ($A_{324-314\ nm}$) according to Yonetani (16) using an extinction coefficient of 42 $mM^{-1}\ cm^{-1}$. Both methods gave the same results as that obtained by the fluorescence method, confirming the reliability of these three methods.

Oxygen Consumption and NADPH Oxidase Assays—Oxygen consumption was determined by using an oxygraph (Gilson model 5-6H) equipped with a Clark oxygen electrode. NADPH oxidation was determined by following the decrease of absorbance at 338 nm instead of 340 nm because 7-ethoxycoumarin and 7-hydroxycoumarin have the same molar extinction coefficient at 338 nm while NADPH shows the same absorbance ($E_{338\ nm} = 6.26\ mM^{-1}\ cm^{-1}$) at both wavelengths (17).

Steady State Levels of the Oxycytochrome P-450 Complex—The steady state levels of the oxy-P-450 complex in microsomes during 7-ethoxycoumarin metabolism were determined by the method of Werringloer and Kawano (18) using $E_{440-500\ nm} = 42\ mM^{-1}\ cm^{-1}$. For this assay, various sources of microsomes (2.5 mg of protein) were added to the standard reaction mixture for the 7-ethoxycoumarin *O*-deethylation assay except that 0.4 mM NADH was also included to reduce the cytochrome b_5. The suspension was divided between two cuvettes and a base-line was recorded. NADPH (0.2 mM) was added to the sample cuvette, and the difference spectrum was recorded immediately.

Other Assays—The content of cytochrome P-450 in microsomes was determined according to Omura and Sato (19) using the absorption coefficient of 91 $mM^{-1}\ cm^{-1}$. Protein concentrations were determined by the method of Lowry *et al.* (20) using bovine serum albumin as a standard.

Mass Spectra—Electron impact mass spectra of underivatized samples of the isotopically labeled ethoxycoumarin substrates, 7-[1,1-$^2H_2$]ethoxy-6-hydroxycoumarin metabolite and standard, **VI**, were obtained on a LKB model 9000 mass spectrometer. The operating conditions were: 70 eV ionization potential, 50 μA filament current, 3.5 kV acceleration potential, and 250 °C source temperature. The samples were introduced into the source by way of the direct insertion probe.

NMR Analysis—Proton NMR were obtained on a Varian 300-MHz superconducting spectrometer equipped with a Fourier transform accessory. Samples were dissolved in $CDCl_3$ and chemical shifts (parts per million) are presented relative to trimethylsilane.

RESULTS

Steady State Levels of the Oxy-P-450 Complex—Previous studies have established that deuterium substitution of the α-hydrogen that undergoes bond cleavage during the *O*-deethylation of 7-EC results in a deuterium isotope effect on V_m, but not on K_m, with various purified P-450 and microsomal systems (5). Since deuterium substitution selectively reduces the rate of the bond cleavage step, which occurs late in the catalytic cycle of the enzyme, an accumulation of intermediate P-450 complexes formed prior to this step would be expected during the oxidation of the deuterium-labeled substrate, **II**. The steady state levels of one of these intermediates, the oxy-P-450, can be easily measured by the method of Werringloer and Kawano (18). As shown in Table II, the steady state levels of the oxy-P-450 complex in the metabolism of 7-EC as well as the observed deuterium isotope effects of the reaction are dependent on the sources of the microsomal preparations. Although the observed isotope effects in the *O*-deethylation reaction were significant in all microsomal preparations, there are no differences in the steady state levels of oxy-P-450 complexes in the reaction with **I** or **II** demonstrating the lack of further accumulation of the oxy-P-450 complex with the deuterated substrate. The steady state level of the oxy-P-450 complex does, however, change as a function of the pH of the reaction mixture (data not shown) as previously reported (18, 21).

Stoichiometry Studies of 7-Ethoxycoumarin Metabolism with the Cytochrome P-448-reconstituted System—Since the slower rate of *O*-deethylation following deuterium substitu-

TABLE II

Steady state levels of oxycytochrome P-450 complexes in the metabolism of isotopically labeled 7-ethoxycoumarin

Various sources of microsomal preparations were used. Assay conditions are described under "Experimental Procedures."

Microsomal preparation	Fraction of total P-450 as oxycytochrome P-450		Isotope effect (v_H/v_D[a])
	Substrate I	Substrate II	
	%		
MC-treated rats	11	10	2.14
PB-treated rats	23	23	3.85
MC-treated hamsters	46	45	5.46

[a] Isotope effects are expressed as relative rates at 0.25 mM substrate concentrations.

TABLE III

Stoichiometry of 7-ethoxycoumarin metabolism with a cytochrome P-448-reconstituted system

Assay conditions are described under "Experimental Procedures."

Substrate	ΔNADPH oxidation	ΔO_2 consumption	Δ7-Hydroxycoumarin formation	ΔH_2O_2 formation
	nmol/5 min/reaction mixture			
I	−14.4	−13.2	+10.7	+3.55
II	−13.2	−12.4	+4.49	+3.27
v_H/v_D	1.09	1.06	2.38	1.09

tion cannot be explained by the increase in the oxy-P-450 level, stoichiometry experiments were carried out in order to determine the consequences of the primary isotope effect on the P-450 reaction cycle. A reconstituted monooxygenase system containing P-448 was used in all subsequent experiments. As shown in Table III, deuterium-labeled 7-EC, **II**, was oxidatively *O*-deethylated to 7-hydroxycoumarin at a slower rate than the unlabeled substrate, **I**, giving rise to an observed primary deuterium isotope effect of 2.38. However, the formation of hydrogen peroxide, a product of uncoupling of the intermediate complexes of P-450 and O_2 (22, 23) was virtually identical for both compounds. This result indicates that deuterium substitution does not induce the further accumulation or the uncoupling of the P-450-O_2 complexes which are known to generate H_2O_2. Moreover, these results are consistent with those obtained on the steady state level of oxy-P-450 complexes in the metabolism of 7-EC. Table III also shows that there are no differences in the utilization of NADPH and oxygen with the two substrates, indicating that deuterium substitution has not altered the overall oxidase activity of the system. These results suggest that an alternate metabolite of 7-ethoxycoumarin must be formed to explain the discrepancy in the material balance observed with the deuterated substrate.

HPLC Analysis of 7-Ethoxycoumarin Metabolites—In order to determine the disposition of 7-EC during metabolism the total metabolite profile was examined by reverse phase HPLC (Fig. 1). When the nondeuterated ^{14}C-substrate, **III**, was incubated with the purified P-448 system in the presence of NADPH, the HPLC profile monitored by UV absorption showed that 7-hydroxycoumarin was formed predominantly (retention time of 9.0 min) along with a trace amount of another metabolite (retention time of 9.7 min) (Fig. 1, *upper left*). The radiochromatogram (Fig. 1, *lower left*) demonstrated that the radiolabel was retained in the metabolite that was formed in trace quantity. On the other hand, when the deuterated ^{14}C-substrate, **IV**, was incubated the formation of 7-hydroxycoumarin was decreased approximately 2-fold consistent with the isotope effect of 2.38 determined fluorimet-

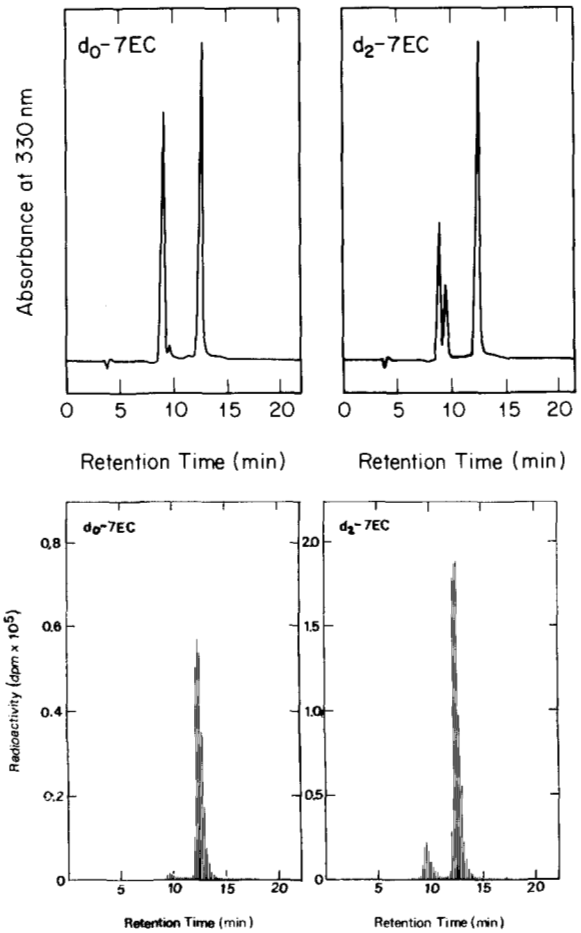


FIG. 1. **HPLC profile of the metabolites of isotopically labeled 7-EC substrates.** The deuterated (d_2) or nondeuterated (d_0) 7-[1-^{14}C]ethoxycoumarin (0.5 mM) were incubated with a reconstituted cytochrome P-448 system consisting of 0.3 nmol of cytochrome P-448, 1000 units of reductase, and 30 μg of dilauroyl phosphatidylcholine in the presence of 3 mM NADPH at 37 °C for 5 min. The metabolites were extracted into chloroform and were concentrated under a stream of dry nitrogen. The concentrated metabolites were analyzed by HPLC under the conditions described under "Experimental Procedures." The UV absorbance (*upper portions*) was monitored at 330 nm. Fractions were collected every 12 s and the radioactivity quantitated by liquid scintillation (*lower portions*). The retention times are approximately 9.0 min for 7-hydroxycoumarin, **V**, 9.7 min for 6-hydroxy-7-ethoxycoumarin, **VII** or **VIII**, and 12.5 min for 7-ethoxycoumarin, **III** or **IV**.

rically (Table III). Concomitantly, a greater amount of the minor metabolite was formed with a retention time of 9.7 min (Fig. 1, *upper right*). The greater quantity of the minor metabolite could also be confirmed from the radiochromatogram (Fig. 1, *lower right*).

Identification of a Second Metabolite—The second metabolite was purified from an incubation mixture containing **II** by repetitive HPLC under the conditions described under "Experimental Procedures." The electron impact mass spectrum (Fig. 2, *right*) revealed that this metabolite had a molecular ion 16 amu larger than the substrate, **II** (*left*). Moreover, this 16 amu difference was preserved in the two major fragment ions occurring at *m/z* 150 and 178 suggesting that hydroxylation had occurred on the aromatic ring. The fragmentation of the metabolite is analogous to that observed for the substrate and is due to the sequential loss of ethylene and CO as depicted in Fig. 2.

The 300-MHz NMR spectrum of the aromatic proton re-

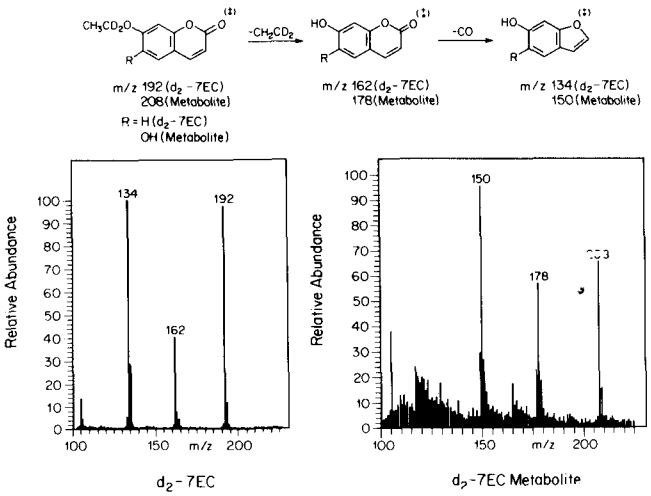


FIG. 2. **Mass spectra of deuterated 7-ethoxycoumarin (*d*$_2$-*7EC*) and its metabolite.** The deuterated 7-EC was incubated with the cytochrome P-448-reconstituted system as described under "Experimental Procedures." The new metabolite was separated from the chloroform-extracted metabolites by HPLC. Fractions containing the metabolite were pooled and electron impact MS were obtained from a 0.5-μg sample of the metabolite (*right*) and deuterated 7-EC (*left*).

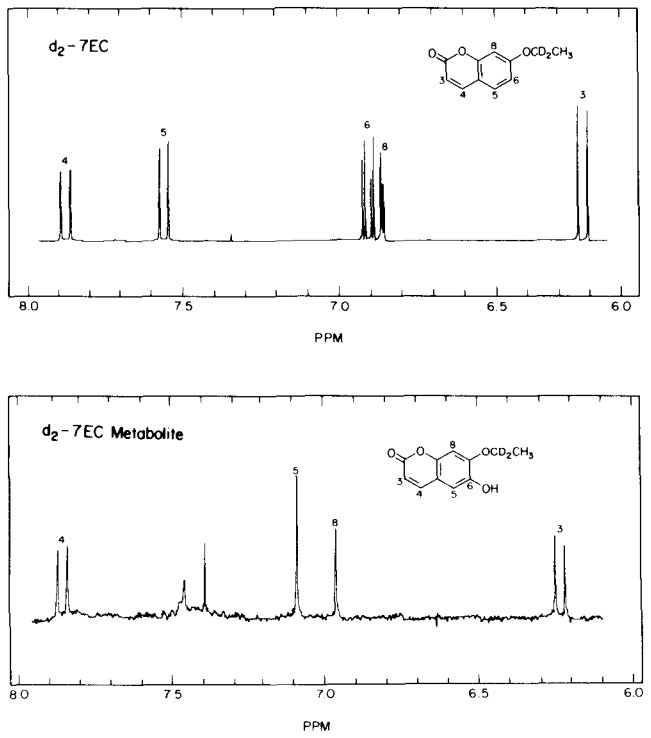


FIG. 3. **300-MHz nuclear magnetic resonance spectra of deuterated 7-ethoxycoumarin (*d*$_2$-*7EC*) and its metabolite.** The new metabolite was collected as described in the legend of Fig. 1. NMR spectra were obtained with approximately 10-μg quantities of the metabolite (*lower*) and deuterated substrate (*upper*) dissolved in deuteroacetone.

Of primary significance is the simplicity of the metabolite spectrum compared with the substrate (Fig. 3, *upper portion*). The doublets observed for C_5 and C_8 protons in the substrate have degenerated into singlets in the metabolite. Moreover, the C_6 proton doublet of doublets observed in the substrate is absent in the metabolite. These data unequivocally demonstrate that hydroxylation has occurred at C_6 on the aromatic

A synthetic standard of the metabolite, **VI**, was prepared and analyzed by MS and NMR. Both spectra were identical with those of the metabolite (data not shown), confirming the structural assignment (**VI**).

Stoichiometry of Substrate Utilization and Metabolite Formation during the Metabolism of 7-Ethoxycoumarin—A radiometric assay using ^{14}C-labeled substrates was developed in order to quantitate the disappearance of substrate and the formation of products. The substrate and the products were separated by reversed phase HPLC as shown in Fig. 1. 7-Hydroxycoumarin, 6-hydroxy-7-ethoxycoumarin, and 7-ethoxycoumarin were eluted from the HPLC with retention times of 9.0, 9.7, and 12.5 min, respectively. The consumption of 7-ethoxycoumarin and the formation of 6-hydroxy-7-ethoxycoumarin were determined from the radioactivities of the corresponding HPLC peaks (Fig. 1, *lower portion*). Other experiments demonstrated that these two compounds were quantitatively extracted into $CHCl_3$ and quantitatively recovered during HPLC. The 7-hydroxycoumarin was measured fluorimetrically. Since 7-hydroxycoumarin has no radioactivity due to the removal of ^{14}C-labeled *O*-ethyl group from the substrate, each determination can be carried out independently without mutual interference. As shown in Table IV, there are little or no differences in the consumption of **III** and **IV** demonstrating that deuterium substitution has not affected the overall turnover rate for 7-ethoxycoumarin metabolism. However, the 7-hydroxycoumarin metabolite was observed to form 1.9 times more slowly from **IV** by this assay, consistent with results obtained fluorimetrically (Table III, substrate **II**).

In the metabolism of **III** by the P-448-reconstituted system, 7-hydroxycoumarin is the major product (about 94% of total metabolites) while 6-hydroxy-7-ethoxycoumarin represents only about 5% (Table IV). In contrast, during the metabolism of **IV**, the formation of 7-hydroxycoumarin is decreased about 2-fold compared to that observed for **III** while the formation of 6-hydroxy-7-ethoxycoumarin is increased about 5-fold relative to that obtained from **III**.

DISCUSSION

Stoichiometry studies of the metabolism of deuterated and nondeuterated 7-EC provide important information about the P-448 reaction cycle. Since the C—H bond cleavage step occurs late in the catalytic cycle of this enzyme (Fig. 4, conversion of VI to I), an increased steady state concentration of various intermediate cytochrome complexes (such as IV, V, and VI in Fig. 4) would be expected when the rate of this bond cleavage is decreased as a consequence of deuterium substitution.

This perturbation did not, however, change the steady state levels of an oxy-P-448 intermediate (IV in Fig. 4) nor did it

TABLE IV

Stoichiometry of substrate utilization and product formation in the metabolism of 7-ethoxycoumarin with cytochrome P-448-reconstituted system

Deuterated and nondeuterated 7-[1-^{14}C]ethoxycoumarin were used as substrates. Substrates and products were separated by HPLC, and concentrations were determined by radioactivity and fluorescence. The assay conditions are described under "Experimental Procedures."

Substrate	Δ7-EC consumption	Δ7-Hydroxycoumarin formation	Δ6-Hydroxy-7-EC formation	Material balance
	nmol/5 min/reaction mixture			%
III	−63.1	+59.1	+2.90	98
IV	−56.3	+31.1	+14.2	80
v_H/v_D	1.12	1.90	0.20	

cause an increase in H_2O_2 production which would have been produced from higher steady state levels of intermediate complexes IV or V (Fig. 4). Thus, to the extent that the catalytic cycle has been perturbed by deuterium substitution, there is no evidence for the reversibility necessary for the accumulation of various oxy-P-448 complexes. These data are compatible with the irreversible bond cleavage of molecular oxygen during the formation of an active oxygen-P-448 species such as the oxenoid intermediate, VI, depicted in Fig. 4.

The irreversible formation of this oxenoid intermediate appears to commit this enzyme to the oxidation of the 7-EC substrate since deuterium substitution failed to slow the turnover of the enzyme as assessed by 7-EC utilization, NADPH oxidation, or O_2 consumption (Tables III and IV). The increased formation of a second metabolite, 6-hydroxy-7-ethoxycoumarin, which is normally formed in trace quantities relative to the 7-hydroxycoumarin metabolite (Table IV) demonstrates the strong commitment to catalysis of this active oxygen intermediate.

It has been previously shown that a $^DV/K$ isotope effect must be a consequence of a rapid interchange between the deuterium labeled and unlabeled substrates on the enzyme catalytic site (2). Thus, the presence of an observed $^DV/K$ isotope effect for 7-EC along with the evidence for the irreversible formation of the oxene intermediate provide compelling arguments for the reversible binding of 7-EC to this intermediate during catalysis. Consequently, these data indicate that this substrate binds to at least two forms of the cytochrome (Fig. 4, II and VI) during its catalytic cycle.

Northrop has developed equations that permit interpretation of isotope effect data on enzyme-catalyzed reactions (24). Equation 1 describes the relationship between the observed deuterium isotope effect, $^DV/K$, and the intrinsic isotope effect, k_H/k_D.

$$^DV/K = \frac{k_H/k_D + C_f}{1 + C_f}. \quad (1)$$

The partition ratio, C_f, is referred to as the "commitment to catalysis" by Northrop since C_f is equal to the ratio k_H/k_2 where k_H is the rate constant for the C—H bond cleavage step leading to the 7-hydroxycoumarin metabolite and k_2 is the rate constant for the nonproductive dissociation of the P-450-ethoxycoumarin complex. The metabolic switching observed in the present study is simply another manifestation of this nonproductive dissociation of the P-448-7-EC complex. This process is nonproductive, however, only in the sense that the dissociation did not lead to an isotopically sensitive step.

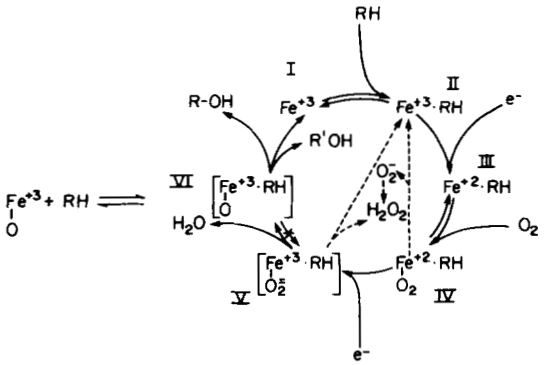


FIG. 4. **Proposed scheme for the cytochrome P-450 catalytic cycle.** RH and ROH represents the 7-EC substrate and the 7-hydroxycoumarin product, respectively. R'OH represents the 6-hydroxy-7-ethoxycoumarin metabolite. I, oxidized P-450; II, substrate-bound oxidized P-450; III, substrate-bound reduced P-450; IV, oxy-P-450 complex; V, 2-electron reduced oxy-P-450; VI, active oxygen-P-450 (oxene).

The intrinsic isotope effect (k_H/k_D = 13.5) for the *O*-deethylation of 7-EC by P-448 has been estimated from the observed deuterium and tritium isotope effect measurements (5). We had previously suggested that a relatively high "commitment to catalysis" was responsible for the 7-fold suppression of the intrinsic isotope effect (observed $^DV/K$ = 2.0) when 7-hydroxycoumarin was measured. The ratio of 7-hydroxycoumarin to 6-hydroxy-7-ethoxycoumarin formed from III is 20 (Table IV). This is approximately the value estimated earlier (5) for C_f (k_H/k_2 = 13) and demonstrates that k_2 chiefly reflects the dissociation-reassociation of 7-EC from the catalytic site required for the formation of the 6-hydroxy-7-ethoxycoumarin metabolite.

This phenomena of metabolic switching in the metabolism of deuterium-substituted compounds was first reported by Horning *et al.* (25, 26) and Jarman *et al.* (27). They observed that deuterium substitution gave rise to different ratios in the metabolites formed from caffeine, antipyrine (25, 26), and cyclophosphamides (27), and suggested the possibility that deuterium substitution on compounds metabolized by multiple alternative pathways rechanneled metabolism to pathways away from the deuterated portion of the substrate. On the other hand, Lindsay Smith and Sleath (28) have recently demonstrated that several chemical model systems of P-450, including tetraphenylporphinatoiron (III) chloride and iodosobenzene, do not give metabolic switching even though the large isotope effect observed for the *O*-demethylation of trideuteriomethylanisole (k_H/k_D = 9.0) is similar in magnitude to those reported here and by Jarman *et al.* (27). In addition, Bjorkhem (29) did not observe metabolic switching in the metabolism of deuterated lauric acid by P-450 in spite of a significant deuterium isotope effect (k_H/k_D = 3 to 4). Thus, it is not yet clear whether this metabolic switching is a general phenomena and further stoichiometry studies would be necessary with other substrates and other isozymes of P-450 before a generalization about the characteristics of the active oxygen form of cytochrome P-450 can be made.

In conclusion, the commitment to catalysis term, C_f, has been shown to reflect the partitioning of the substrate, **I**, between the *O*-deethylated product, **V**, and the ring hydroxylated product, **VII**. In addition, the actual rate reduction caused by deuterium (k_H/k_D = 13.5) could substantially be accounted for by a "metabolic switching" to a second, ring-hydroxylated metabolite (Table IV). This "metabolic switching" occurs without consequence to the steady state levels of various P-450 intermediates and the overall turnover of the enzyme. We have interpreted these data to indicate that an irreversible oxygen-oxygen bond cleavage step occurs prior to substrate oxidation.

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