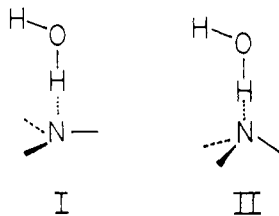


are symbolized as  $B$  and  $B^*$ , respectively. The energy difference between the amine-water complex which is produced subsequent to photon emission (and which presumably represents the equilibrium structure of the *excited* amine-water complex) and the "normal" ground-state amine-water complex is symbolized by  $E_X$ . A Förster cycle analysis of these state energies indicates that

$$E_{fA} + E_{inv} = B^* + E_{fC} + E_X - B$$

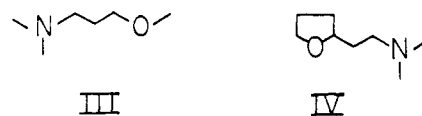
Since each of these terms can be measured (or estimated) except for  $E_X$ , a value of this quantity can be determined. It should be realized that  $E_X$ , ordinarily represented as a repulsion energy in exciplex and excimer systems (because the ground states of these species are dissociative), need not necessarily be very large. That is, the energy of the amine-water complex produced after photon emission may be nearly equal to that of the ground-state amine-water hydrate.

On the basis of the fluorescence energies of free and water-complexed TEA, the binding energies of the ground and electronically excited state TEA-water complexes, and the use of a value of 8 kcal/mol as the inversion barrier for TEA,<sup>26</sup>  $E_X$  is estimated to be ca. 13 kcal/mol. Data for the other amines suggest similar values for  $E_X$ . This value seems rather large if one assumes that the structure of the excited-state TEA-H<sub>2</sub>O complex involves the water molecule with the O-H bond directed along the C<sub>3</sub> axis of the (planar) amine, I. The 13 kcal assigned to  $E_X$  represents the energy of this structure (in which, however, excitation energy is lost) *relative* to the (ground-state) TEA-H<sub>2</sub>O hydrate II. In

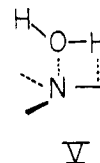


(26) (a) Bushweller, C. H.; Fleischman, S. H.; Grady, G. L.; McGoff, P.; Rithner, C. D.; Whalon, M. R.; Brennan, J. G.; Marcantonio, R. P.; Domingue, R. P. *J. Am. Chem. Soc.* **1982**, *104*, 6224. (b) Fleischman, S. H.; Bushweller, C. H. *J. Comput. Chem.* **1985**, *6*, 249.

discussing the structure of the excited-state TEA-ethanol complex, Köhler proposed that the O atom is coordinated to the quasicationic N atom core of the Rydberg excited TEA molecule.<sup>15</sup> Were this to be true, it fails to explain why a donor such as tetrahydrofuran shows no evidence of complex formation with electronically excited TEA. Likewise, intramolecular complexation in amino ethers III and IV is not observed.<sup>7</sup> Thus only protic species (H<sub>2</sub>O, D<sub>2</sub>O, ROH) appear to form emissive stoichiometric complexes with electronically excited saturated tertiary amines.



In order to rationalize the relatively large repulsion energy assigned for the TEA-H<sub>2</sub>O system, we propose that a very different bonding arrangement exists in the excited-state complex relative to the ground-state hydrate. One such possibility is the four-center structure, V, shown below. The electrostatic reorg-



anization of the amine in the Rydberg excited state results in a quasicationic N atom core which coordinates with the O atom in H<sub>2</sub>O (and presumably also in ethanol). Additional stability is achieved by the electrostatic attraction of the sterically unencumbered H atom with the quasianionic (nucleophilic) periphery of the amine, such as the C atoms. Model calculations using appropriately extended basis orbitals on structures such as V are needed to assess the reasonableness of this suggestion.

**Acknowledgment.** The donors of the Petroleum Research Fund, administered by the American Chemical Society, are acknowledged for partial support of this research.

## Metabolic Switching in Cytochrome P-450<sub>cam</sub>: Deuterium Isotope Effects on Regiospecificity and the Monooxygenase/Oxidase Ratio

William M. Atkins and Stephen G. Sligar\*

Contribution from the Departments of Biochemistry and Chemistry, University of Illinois, Urbana, Illinois 61801. Received September 29, 1986

**Abstract:** Cytochrome P-450<sub>cam</sub>, isolated and purified to homogeneity from the soil bacterium *Pseudomonas putida*, has been shown to catalyze the hydroxylation of the substrate analogue norcamphor to form three distinct products and yields: 5-*exo*-hydroxynorcamphor (45%), 6-*exo*-hydroxynorcamphor (47%), and 3-*exo*-hydroxynorcamphor (8%). Specific deuteration of the norcamphor skeleton at the 5-, 6-, and 3-positions drastically alters this product distribution, indicating a substantial deuterium isotope effect. When the sum total of all oxygenated products formed in the presence of norcamphor is compared to the number of reducing equivalents consumed in the reaction (NADH), a striking unaccountability of electrons is observed. These are shown to reside in excess water produced by the four-electron reduction of atmospheric dioxygen by P-450<sub>cam</sub>. Metabolism of specifically deuterated norcamphor demonstrates a deuterium isotope effect on the branching ratio of substrate hydroxylation to excess water production and suggests that this oxidase activity of P-450<sub>cam</sub> results from the two-electron reduction of a single oxygen-iron intermediate, [FeO]<sup>3+</sup>.

Cytochrome P-450<sub>cam</sub>, an extensively characterized mono- and stereospecific hydroxylation of the monoterpene camphor to

coupled reaction cycle, two reducing equivalents originating in NADH are transferred sequentially to the cytochrome via putidaredoxin reductase and putidaredoxin with the subsequent O—O bond scission of molecular oxygen. Nearly all reducing equivalents are accounted for by substrate hydroxylation and monooxygenase derived water, and with minimal production of hydrogen peroxide. In contrast, the cytochrome P-450 systems isolated from the hepatic endoplasmic reticulum are much less tightly coupled both *in vivo* as well as in reconstituted reactions. Here, as much as half of the pyridine nucleotide supplied reducing equivalents are used to generate hydrogen peroxide. Recently, Gorsky et al. demonstrated an additional fate for the electrons and molecular oxygen with several of these hepatic cytochrome P-450's.<sup>2</sup> They demonstrated that these liver microsomal isozymes were also capable of performing an oxidase-type reduction of atmospheric dioxygen, requiring four electrons and presumably generating 2 equiv of water. Evidence suggested that H<sub>2</sub>O<sub>2</sub> was not an intermediate in this oxidase activity. Others have also reported a 2:1 stoichiometry of NADPH/O<sub>2</sub> in various liver microsomal systems and have proposed direct reduction of O<sub>2</sub> to H<sub>2</sub>O<sup>3,4</sup> with the efficacy of this oxidase activity varying with isozyme and organic substrate. Although investigations have established the existence of a cytochrome oxidase activity in these cytochrome P-450 systems, the mechanism of input of the additional two reducing equivalents into the normal monooxygenase reaction cycle has remained undetermined.

An independent focus of investigation within the P-450 fields has involved the use of deuterium isotope effects to characterize the mechanism of C—H bond cleavage, the intermediacy of a substrate radical, and the nature and identity of the hydrogen abstracting species. At least two types of metabolic switching have been observed upon substitution of deuterium at an oxidizable carbon. Gelb et al.<sup>5</sup> found that both the 5-exo and the 5-endo hydrogens of the camphor skeleton were candidates for abstraction and that deuterium substitution at either position altered the exo/endo selectivity of hydrogen removal without affecting the rigid specificity of exo oxygen rebound. White et al.<sup>6</sup> found that the stereospecificity of hydrogen abstraction as well as oxygen rebound was affected by deuterium substitution on phenylethane. A second type of metabolic switching, in which new metabolic products have been obtained, has also been observed. Harada et al.<sup>7</sup> reported that with the hepatic phenobarbital and 3-methylcholanthrene induced cytochromes P-450, placement of deuterium at the  $\alpha$ -carbon of 7-ethoxycoumarin results in formation of a second metabolite in addition to the normal O-deethylated product. Similarly, Jones et al.<sup>8</sup> recently described how this type of metabolic switching could be used to determine an intrinsic isotope effect for hydroxylation of octane by the rat phenobarbital induced isozyme P-450<sub>b</sub>. Also, Dawson and co-workers demonstrated the formation of 9-hydroxycamphor from 5,5-*gem*-difluorocamphor by P-450<sub>cam</sub>.<sup>10</sup>

(1) Sligar, S. G.; Murray, R. I. *Cytochrome P-450 Structure, Mechanism and Biochemistry*; Ortiz de Montellano, P. R., Ed.; Plenum: New York, 1986; pp 443–479.

(2) Gorsky, L. D.; Koop, D. R.; Coon, M. J. *J. Biol. Chem.* **1984**, *259*, 6812.

(3) Staudt, H.; Lichtenberger, F.; Ullrich, V. *Eur. J. Biochem.* **1974**, *46*, 99.

(4) Zhukov, A. A.; Archakov, A. I. *Biochem. Biophys. Res. Commun.* **1982**, *109*, 813.

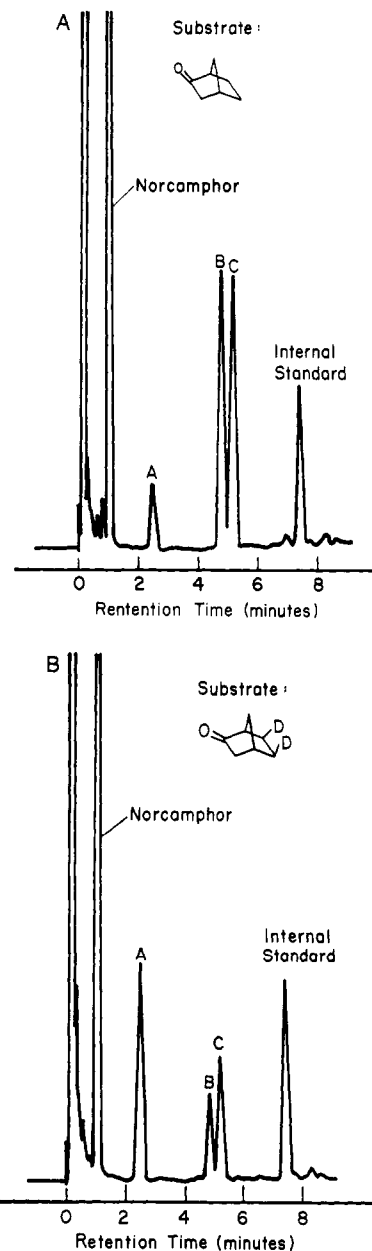
(5) Gelb, M. H.; Heimbrook, D. C.; Malkonen, P.; Sligar, S. G. *Biochemistry* **1982**, *21*, 370.

(6) White, R. E.; Miller, J. P.; Faureau, L. V.; Bhattacharyya, A. *J. Am. Chem. Soc.* **1986**, *108*, 6024.

(7) Harada, N.; Miwa, G. T.; Walsh, J. S.; Lu, A. Y. *J. Biol. Chem.* **1984**, *259*, 3005.

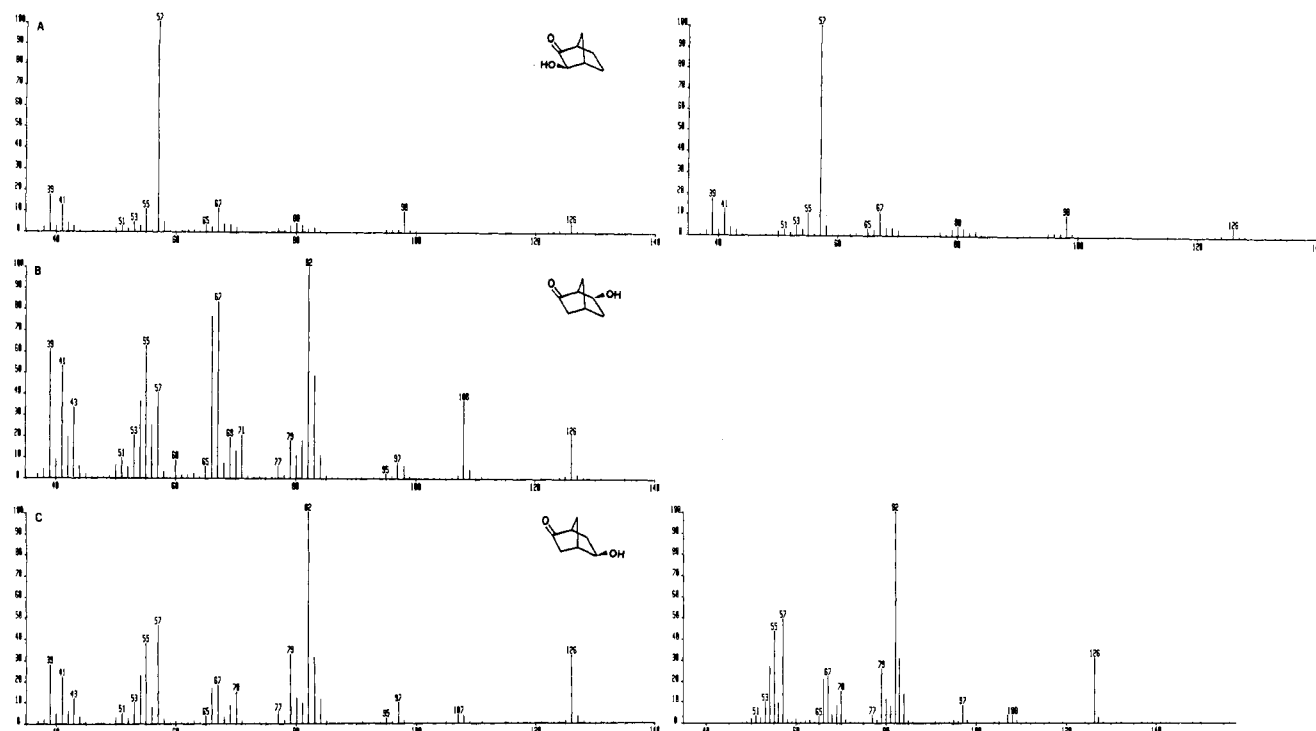
(8) Jones, J. P.; Korzekwa, K. R.; Rettie, A. E.; Trager, W. F. *J. Am. Chem. Soc.* **1986**, *108*, 7074.

(9) Lipscomb, J. D.; Sligar, S. G.; Namtvedt, M. J.; Gunsalus, I. C. *J. Biol. Chem.* **1976**, *251*, 1116.



**Figure 1.** Gas chromatogram of norcamphor metabolites. Enzyme turnovers and gas chromatography were performed as described in the Experimental Section. After completion of the reaction, and prior to extraction, a known amount of internal standard (3-*endo*-bromocamphor) was added: (A) metabolite profile obtained from the norcamphor substrate; (B) metabolite profile obtained from 5,6-*exo,exo*-norcamphor-5,6-*d*<sub>2</sub>.

In view of the efficient production of 5-*exo*-hydroxycamphor by the bacterial P-450<sub>cam</sub> and the tight coupling of this system, wherein there is efficient channeling of pyridine nucleotide derived reducing equivalents into monooxygenase stoichiometry, we have chosen this system to explore the metabolic fate of dioxygen and substrate under varying conditions. Herein we describe results obtained with cytochrome P-450<sub>cam</sub> in the presence of the substrate analogue norcamphor, which earlier experiments suggested to be unmetabolized by this system.<sup>9</sup> Careful analysis shows, however, that P-450<sub>cam</sub> demonstrates a prolific four-electron oxidase activity as well as substrate hydroxylation and a significant degree of metabolic switching upon deuteration of the norcamphor substrate. Most intriguing, however, is the demonstration of an isotopically sensitive branching which affects the monooxygenase/oxidase ratio and which offers compelling evidence for a common intermediate that is responsible for hydrogen abstraction and is also susceptible to a two-electron reduction to form water



**Figure 2.** Electron-impact mass spectra (70 eV) of norcamphor metabolites: (A) 3-*exo*-hydroxynorcamphor; (B) 6-*exo*-hydroxynorcamphor; (C) 5-*exo*-hydroxynorcamphor. The spectra shown in the right-hand panel are of the corresponding authentic compounds.

## Results

**Hydroxylated Products Derived from Norcamphor.** Three hydroxylated products were found to be produced from norcamphor metabolism by cytochrome P-450<sub>cam</sub>. The formation of each is strictly dependent on the presence of NADH, P-450<sub>cam</sub>, and the purified electron-transfer proteins putidaredoxin and NADH-putidaredoxin reductase. These norcamphor metabolites were identified by comparison to authentic samples with gas chromatography and mass spectral analysis. A typical GC chromatogram of the reaction products when norcamphor was used as substrate is shown in Figure 1A. The corresponding 70-eV electron impact mass spectrum of the products (A, B, C) is shown in Figure 2. The 3-*exo*-hydroxynorcamphor (A) and 5-*exo*-hydroxynorcamphor (C) produced in the reaction are identical with synthetic standards obtained as described in the Experimental Section. The third metabolite (B) has been assigned as 6-*exo*-hydroxynorcamphor based on GC mass spectral analysis and the following: (1) Metabolite B is not 5-*endo*-hydroxynorcamphor. The 5-*endo* alcohol was also prepared as described in the Experimental Section, and it is apparent from GC analysis that the 5-*endo* isomer is not present in the reaction mixture. With the GC conditions used, 5-*endo*-hydroxynorcamphor had a retention time of 5.9 min and was easily separated from any of the products observed. (2) Production of metabolite B is sensitive to deuterium substitution at the 6-position, and from selective ion monitoring mass spectral analysis, it is apparent that B undergoes nearly complete loss of one deuterium when 5,6-*exo,exo*-norcamphor-5,6-*d*<sub>2</sub> is the substrate. These results are presented in more detail in the following discussion of stereochemistry. By analogy to the situation observed for the 5-alcohol, it is reasonable to assume that the 6-alcohol that is formed with loss of 6-*exo* deuterium is arrived at with a preponderance of retention of configuration. It is unlikely that the 5-alcohol would be formed with nearly complete retention and the 6-alcohol would be formed with nearly complete inversion. This line of reasoning leads us to conclude that B is 6-*exo*-hydroxynorcamphor. Furthermore, the fragmentation pattern in the mass spectrum of B is entirely consistent with 6-*exo*-hydroxynorcamphor. It is noteworthy that both of the isomeric 7-hydroxynorcamphors were synthesized and shown to be absent from the metabolite profile of norcamphor.

When deuterium is substituted at the 5- and 6-carbons of

**Table I.** Regiospecificity of Norcamphor Hydroxylation

substrate <sup>a</sup>	relative percent of product, %		
	3- <i>exo</i>	5- <i>exo</i>	6- <i>exo</i>
norcamphor	8	45	47
I	52	27	21
II	2	49	49
III	10	44	46

<sup>a</sup> The symbols used are I, 5,6-*exo,exo*-norcamphor-5,6-*d*<sub>2</sub>; II, norcamphor-3,3-*d*<sub>2</sub>; and III, 5,6-*exo,exo*-norcamphor-3,3,5,6-*d*<sub>4</sub>.

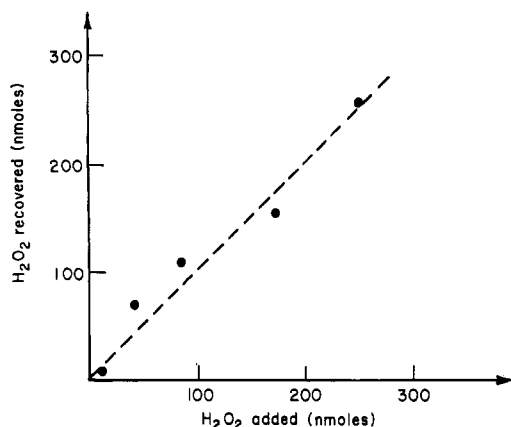
**Table II.** Stereochemistry of Norcamphor Hydroxylation<sup>a</sup>

substrate/ buffer	product	<i>d</i> <sub>2</sub> , %	<i>d</i> <sub>1</sub> , %	<i>d</i> <sub>0</sub> , %
I in H <sub>2</sub> O	5- <i>exo</i> -hydroxy	4.6 ± 0.6	94.4 ± 0.5	0.96 ± 0.09
	6- <i>exo</i> -hydroxy	2.8 ± 0.9	95.9 ± 0.9	1.1 ± 0.2
norcamphor in D <sub>2</sub> O	5- <i>exo</i> -hydroxy	0	1.93	98.07
	6- <i>exo</i> -hydroxy	0	1.59	98.43

<sup>a</sup> Deuterium content of the products was determined by ion selective monitoring GCMS. Peak intensities for ions corresponding to *m/z* 128, 127, 126, 125, and 124 were determined. For both the 5- and 6-alcohols (undeuteriated), the relative intensity of M<sup>+</sup> - 1 was less than 6% of the M<sup>+</sup>.

which results in a change in the relative and absolute yield of each product (Figure 1); no new products appear to be formed. When various combinations of deuterium are present on the substrate, the product profile changes in a corresponding fashion. These results are summarized in Table I. It is apparent that, in sharp contrast to what has been observed with camphor as substrate, there is a deuterium-dependent intramolecular isotope effect on the regioselectivity of hydroxylation. Altered regioselectivity of camphor hydroxylation is observed only when a large kinetic barrier to hydroxylation has been presented, as was demonstrated with 5,5-*gem*-difluorocamphor metabolism to 9-hydroxycamphor.<sup>10</sup>

**Stereochemistry of Norcamphor Hydroxylation.** The stereochemical course of norcamphor hydroxylation at the 5- and 6-positions was investigated by determining the deuterium content of the corresponding alcohols when 5,6-*exo,exo*-norcamphor-5,6-*d*<sub>2</sub> was metabolized. The results are summarized in Table II. Several



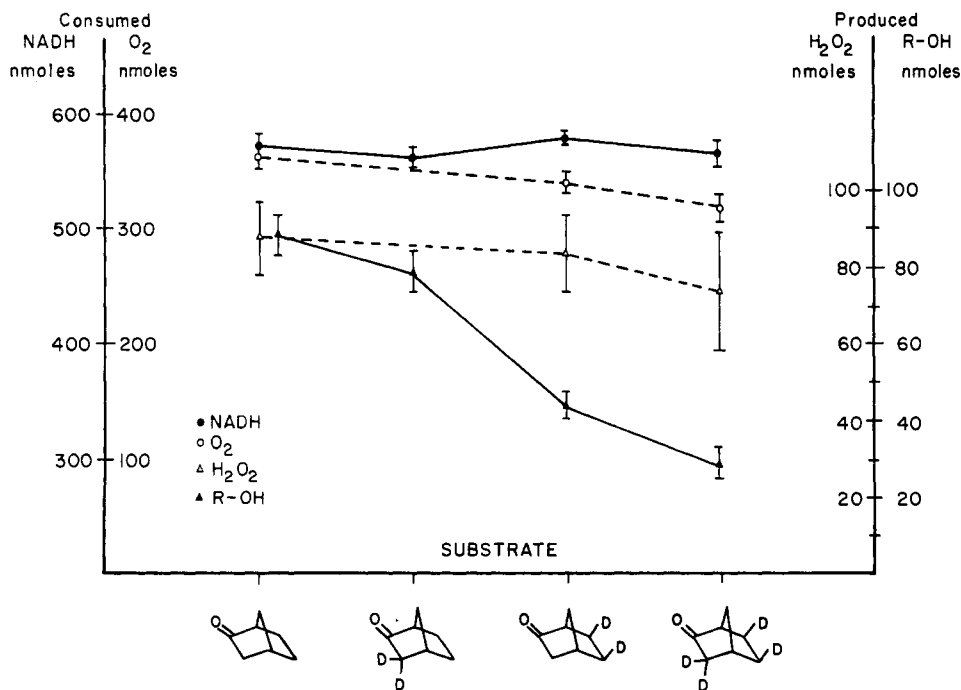
**Figure 3.** Recovery of hydrogen peroxide in the presence of norcamphor. The  $\text{H}_2\text{O}_2$  recovered after addition of varying amounts of exogenous  $\text{H}_2\text{O}_2$  was quantitated as described in the Experimental Section. The plotted values are corrected for the amount which is produced in the absence of added  $\text{H}_2\text{O}_2$  (88 nmol). The dashed line represents the theoretical yield of 100% recovery.

stereochemical course observed with camphor.<sup>5</sup> It is apparent that the 5-alcohol obtained from norcamphor results from nearly complete specificity for exo deuterium abstraction, with only minor endo abstraction followed by inversion of configuration. It is likely that due to the primary isotope effect, this stereochemical result significantly underestimates the exo specificity that occurs when protium is present at both the exo and endo face. A portion of the  $d_2$  product may simply arise from a trace amount of substrate with label at the endo position. Gelb et al.<sup>5</sup> found, in studying the metabolism of the normal substrate camphor, that with deuterium present at the endo position there was still approximately 20% endo abstraction with subsequent exo oxygen insertion. Thus, with norcamphor as substrate, P-450<sub>cam</sub> shows a drastic reduction in the fraction of 5-exo alcohol produced with inversion of configuration as compared to the substrate camphor. The deuterium data for the 6-alcohol reflect a similar preference for exo abstraction. The stereochemistry of hydrogen abstraction at the 3-position was not investigated. It is apparent from the experiment conducted in  $\text{D}_2\text{O}$ /buffer that the nearly complete loss of deuterium observed for the 5- and 6-alcohols is not due to an artefact of experimental workup. The background level of  $d_1$

product obtained in  $\text{D}_2\text{O}$  is most likely due to a minor degree of solvent exchange at the 3-position. This is supported by the observation that the 3-alcohol produced in  $\text{D}_2\text{O}$  is less than 1%  $d_1$ .

The importance of these stereochemical considerations becomes evident when the stoichiometry of norcamphor turnover as a function of substrate deuterium content is determined.

**Stoichiometry of Norcamphor Metabolism.** It is qualitatively apparent from Figure 1 that there is a significant reduction in the amount of hydroxylated products obtained when hydrogen is substituted with deuterium on the substrate. This result necessitated experiments in which the complete stoichiometry of this system was determined. Experiments were conducted in which the NADH and  $\text{O}_2$  consumptions were precisely quantitated along with formation of  $\text{H}_2\text{O}_2$  and hydroxynorcamphor. Conditions were employed which insured that NADH was the limiting reagent. A similar reaction utilizing camphor as a substrate was conducted for comparison. A catalase assay was employed to convert in situ produced hydrogen peroxide to oxygen and water and was found to afford quantitative recovery of exogenously added  $\text{H}_2\text{O}_2$  as demonstrated in Figure 3. The results from these measurements are summarized in Table III. When the sum totals of oxygenated products are compared to the absolute number of reducing equivalents consumed in the reaction, a striking unaccountability of electrons is observed. These are found to reside in excess water produced by four-electron reduction of dioxygen as described by Gorsky et al.<sup>2</sup> In the presence of norcamphor, P-450<sub>cam</sub> displays a remarkable degree of oxidase activity with a correspondingly less efficient monooxygenase chemistry. A striking trend is observed when additional positions of norcamphor are deuteriated (Figure 4). With incremental addition of deuterium there is a corresponding decrease in the amount of hydroxylated product obtained. This cannot be accounted for by a significant increase in the amount of  $\text{H}_2\text{O}_2$  produced, and, based on the 2:1 stoichiometry of excess NADH/excess  $\text{O}_2$ , the decrease in hydroxylation appears to coincide with an increase in oxidase-derived water production. These results demonstrate that, in addition to a deuterium isotope effect on the regioselectivity of hydroxylation, there exists an isotope-dependent switching from monooxygenase to oxidase activity at the active site of P-450<sub>cam</sub>. The near complete specificity for the exo face abstraction at the 5- and 6-positions should result in a maximal isotope effect on this metabolic switching when the 5,6-*exo,exo*-norcamphor-3,3,5,6- $d_4$  is processed by the enzyme. Placement of deuterium at the endo face would





**Table III.** Stoichiometry of Norcamphor Metabolism

substrate	catalase	consumed		produced			$\frac{\text{NADH} - [\text{H}_2\text{O}_2 + \text{ROH}]}{\text{O}_2 - [\text{H}_2\text{O}_2 + \text{ROH}]}$
		NADH, nmol	O <sub>2</sub> , nmol	H <sub>2</sub> O <sub>2</sub> , nmol	ROH, nmol	"extra H <sub>2</sub> O", <sup>a</sup> nmol	
norcamphor	-	577 ± 8	365 ± 5	88 ± 22	88 ± 12	189	2.1
	+	567 ± 6	321 ± 6		85 ± 6		
I	-	583 ± 1	338 ± 5	84 ± 22	44 ± 2	210	2.2
	+	570 ± 6	296 ± 6		42 ± 6		
II	-	562 ± 4	ND <sup>b</sup>	ND	78 ± 9	ND	
	+	571 ± 5	ND		80 ± 5		
III	-	564 ± 15	319 ± 4	74 ± 28	27 ± 4	218	2.1
	+	582 ± 4	282 ± 10		30 ± 5		
<i>d</i> -camphor	-	313 ± 8	290 ± 8	20 ± 12	290 ± 2	0	
	+	318 ± 3	280 ± 3		286 ± 9		

<sup>a</sup> Determined from samples not containing catalase. <sup>b</sup> ND = not determined.

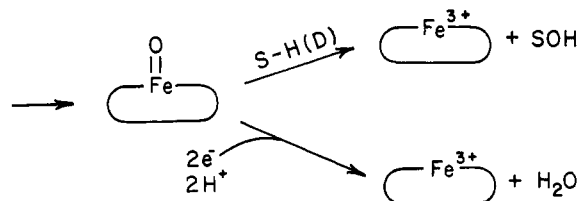
be expected to have a minimal effect, although a secondary isotope effect might be detected.

### Discussion

Several key points of departure between camphor and norcamphor metabolism by the bacterial P-450<sub>cam</sub> system have been discovered. The multiplicity of metabolic options observed in the presence of norcamphor undoubtedly results from a lack of absolute complementarity between the enzyme active site and norcamphor. High-resolution crystal structures of both the camphor bound and the substrate free enzyme have been reported.<sup>11,12</sup> Substrate specificity appears to be imposed by specific interactions including a hydrogen bond between Tyr 96 and the carbonyl moiety of camphor, van der Waals contact between Val 295 and the substrate 8,9-*gem*-dimethyl group, and between the camphor 10-methyl group and the Leu 244-Val 247 hydrophobic cleft. Furthermore, there is no substantial conformational rearrangement of the active site upon camphor binding, as indicated by a comparison between substrate free and substrate bound crystal structures. The major difference between the two structures seems to be in the amount of water present at the substrate binding pocket,<sup>12</sup> which is consistent with the finding that differential spin states imposed by various camphor analogues are correlated to the degree of solvent exposure of the active site tyrosine and the on-rate for solvent binding to the heme iron.<sup>13</sup> Obviously norcamphor possesses the ability to hydrogen bond with Tyr 96, but any hydrophobic or steric interactions involving the methyl groups of camphor with the protein would be diminished. Our findings indicate that destruction of these interactions is sufficient to allow access of the 3- and 6-positions to the [FeO]<sup>3+</sup> intermediate.

It is also evident that the absence of these interactions has a significant effect on the stereochemical course of hydroxylation at the 5-position of norcamphor; the isosteric carbon of camphor experiences a significant degree of inversion of configuration whereas norcamphor does not. The 6-*exo* alcohol is derived with a similar stereochemical course.

The amount of norcamphor-derived alcohol obtained from a fixed pool of NADH is nearly an order of magnitude less than the analogous product obtained from camphor. When one considers the uncoupling of pyridine nucleotide utilization and the small amount of product divided among three distinct species, it is not surprising that norcamphor-derived products had previously been overlooked. These results demonstrate that in the presence of norcamphor, the majority of reduced oxygen intermediates go to produce H<sub>2</sub>O rather than H<sub>2</sub>O<sub>2</sub> or organic alcohol. Although the kinetics of H<sub>2</sub>O<sub>2</sub>, hydroxynorcamphor, and water production have not been examined in detail, preliminary data reported herein suggest that the rate of oxidase-derived water production is much faster in cytochrome P-450<sub>cam</sub> than it is with any of the hepatic cytochrome P-450's. At saturating levels of norcamphor, putidaredoxin, putidaredoxin reductase, and NADH, the rate of

**Scheme I.** Proposed Branching of Monooxygenase vs. Oxidase Activity<sup>a</sup>

<sup>a</sup> The oxidation state of the oxo-iron intermediate is not included since the exact electronic configuration of this species is not established.

NADH consumption by P-450<sub>cam</sub> is approximately 25–30 nmol of NADH/nmol of P-450/min (data not shown). Our data would suggest that the rate of oxidase-derived water production is about 10 nmol of H<sub>2</sub>O/nmol of P-450<sub>cam</sub>/min or about 33% of the total NADH consumption rate. Typical rates of NADPH consumption by various hepatic isozymes are slower than this value,<sup>14,15</sup> as inferred from rates of product formation, so oxidase-derived water production is correspondingly quite slow compared to the P-450<sub>cam</sub> system. It is especially interesting that the mononuclear metal center of cytochrome P-450<sub>cam</sub>, with no active-site acid/base groups,<sup>11,12</sup> possesses the ability to reduce molecular oxygen to water at a relatively high rate.

Deuteration of the norcamphor skeleton has novel and profound effects on the stoichiometry of this system in addition to the isotope-dependent alteration in regioselectivity. It is unlikely that substrate deuteration would have any appreciable effects on the kinetics or thermodynamics of substrate binding, electron transfer, or O<sub>2</sub> binding, although each of these is expected to be different from the corresponding parameters in the presence of camphor. Indeed, others have observed that deuteration substitution does not lead to an increase in the level of H<sub>2</sub>O<sub>2</sub> production or in the steady-state levels of oxy-P-450 intermediates.<sup>7</sup> In view of these considerations, one would expect that the only isotopically sensitive step in the P-450 reaction cycle would be hydrogen abstraction by a compound I type high-valent iron-oxo species. There is precedent for two-electron reduction of this intermediate or hydrogen atom abstraction followed by electron transfer from substrate radical in P-450 systems.<sup>16</sup> This reflects the chemical competence of the iron-oxo species toward two-electron reduction. It has been proposed that a possible point for electron input to result in an oxidase stoichiometry is at the level of this hydroxylating intermediate.<sup>2</sup> Our data indicate that the species undergoing two-electron reduction is sensitive to deuterium substitution of substrate and suggests that the [FeO]<sup>3+</sup> reduction may be mediated by NADH and associated electron-transfer partners (Scheme I). This may have some physiological significance. Production of a highly electrophilic oxidant at the active site, in

(11) Poulos, T. L.; Finzel, B. C.; Gunsalus, I. C.; Wagner, G. C.; Kraut, J. *J. Biol. Chem.* **1985**, *260*, 16122.

(12) Poulos, T. L.; Finzel, B. C.; Howard, A. *J. Biochemistry* **1986**, *25*, 53134.

(14) Koop, D. R.; Morgan, E. T.; Tarr, G. E.; Coon, M. J. *J. Biol. Chem.* **1982**, *257*, 8472.

(15) White, R. E.; McCarthy, M. B.; Egeberg, K. E.; Sligar, S. G. *Arch. Biochem. Biophys.* **1984**, *228*, 493.

# Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

## Real-Time Litigation Alerts



Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

## Advanced Docket Research



With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

## Analytics At Your Fingertips



Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

## API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

## LAW FIRMS

Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

## FINANCIAL INSTITUTIONS

Litigation and bankruptcy checks for companies and debtors.

## E-DISCOVERY AND LEGAL VENDORS

Sync your system to PACER to automate legal marketing.