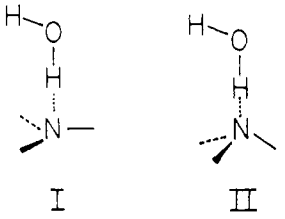
are symbolized as B and B^*, respectively. The energy difference between the amine–water complex which is produced subsequent to photon emission (and which presumably represents the equilibrium structure of the *excited* amine–water complex) and the "normal" ground-state amine–water complex is symbolized 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.

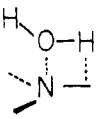
On the basis of the fluorescence energies of free and water-complexed TEA, the binding energies of the ground and electronically excited state TEA–water complexes, and the use of a value of 8 kcal/mol as the inversion barrier for TEA,[26] E_X is estimated to be ca. 13 kcal/mol. Data for the other amines suggest similar values for E_X. This value seems rather large if one assumes that the structure of the excited-state TEA–H_2O complex involves the water molecule with the O–H bond directed along the C_3 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_2O hydrate II. In 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.[15] 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.[7] Thus only protic species (H_2O, D_2O, ROH) appear to form emissive stoichiometric complexes with electronically excited saturated tertiary amines.



I II

III IV

In order to rationalize the relatively large repulsion energy assigned for the TEA–H_2O 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 reorganization of the amine in the Rydberg excited state results in a quasicationic N atom core which coordinates with the O atom in H_2O (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.



V

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Metabolic Switching in Cytochrome P-450$_{cam}$: Deuterium Isotope Effects on Regiospecificity and the Monooxygenase/Oxidase Ratio

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Abstract: Cytochrome P-450$_{cam}$, 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 deuteriation 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$_{cam}$. Metabolism of specifically deuteriated 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$_{cam}$ results from the two-electron reduction of a single oxygen–iron intermediate, $[FeO]^{3+}$.

Cytochrome P-450$_{cam}$, an extensively characterized monooxygenase derived from *Pseudomonas putida*, catalyzes the regio- and stereospecific hydroxylation of the monoterpene camphor to afford 5-*exo*-hydroxycamphor as the sole product.[1] In this tightly

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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.[2] 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_2O_2 was not an intermediate in this oxidase activity. Others have also reported a 2:1 stoichiometry of NADPH/O_2 in various liver microsomal systems and have proposed direct reduction of O_2 to H_2O[3,4] 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.[5] 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.[6] 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.[7] reported that with the hepatic phenobarbitol and 3-methylcholanthrene induced cytochromes P-450, placement of deuterium at the α-carbon of 7-ethoxycoumarin results in formation of a second metabolite in addition to the normal O-deethylated product. Similarly, Jones et al.[8] recently described how this type of metabolic switching could be used to determine an intrinsic isotope effect for hydroxylation of octane by the rat phenobarbitol induced isozyme P-450$_b$. Also, Dawson and co-workers demonstrated the formation of 9-hydroxycamphor from 5,5-*gem*-difluorocamphor by P-450$_{cam}$.[10]

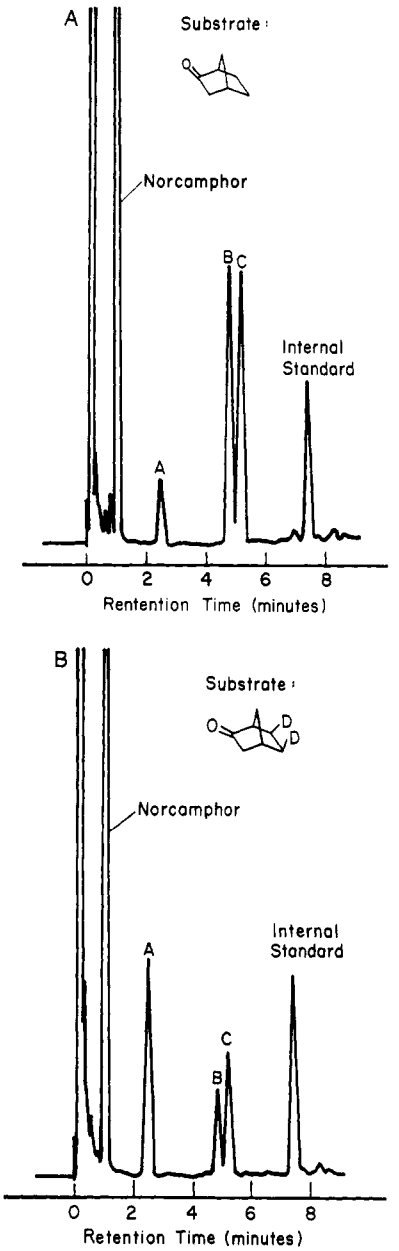

Figure 1. Gas chromatogram of norcamphor metabolites. Enzyme turnovers and gas chromatography were performed as described in the Experimental Section. After completion of the reaction, and prior to extraction, a known amount of internal standard (3-*endo*-bromocamphor) was added: (A) metabolite profile obtained from the norcamphor substrate; (B) metabolite profile obtained from 5,6-*exo*,*exo*-norcamphor-5,6-d_2.

In view of the efficient production of 5-*exo*-hydroxycamphor by the bacterial P-450$_{cam}$ 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$_{cam}$ in the presence of the substrate analogue norcamphor, which earlier experiments suggested to be unmetabolized by this system.[9] Careful analysis shows, however, that P-450$_{cam}$ demonstrates a prolific four-electron oxidase activity as well as substrate hydroxylation and a significant degree of metabolic switching upon deuteriation 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 in an activity paralleling that of cytochrome oxidase.

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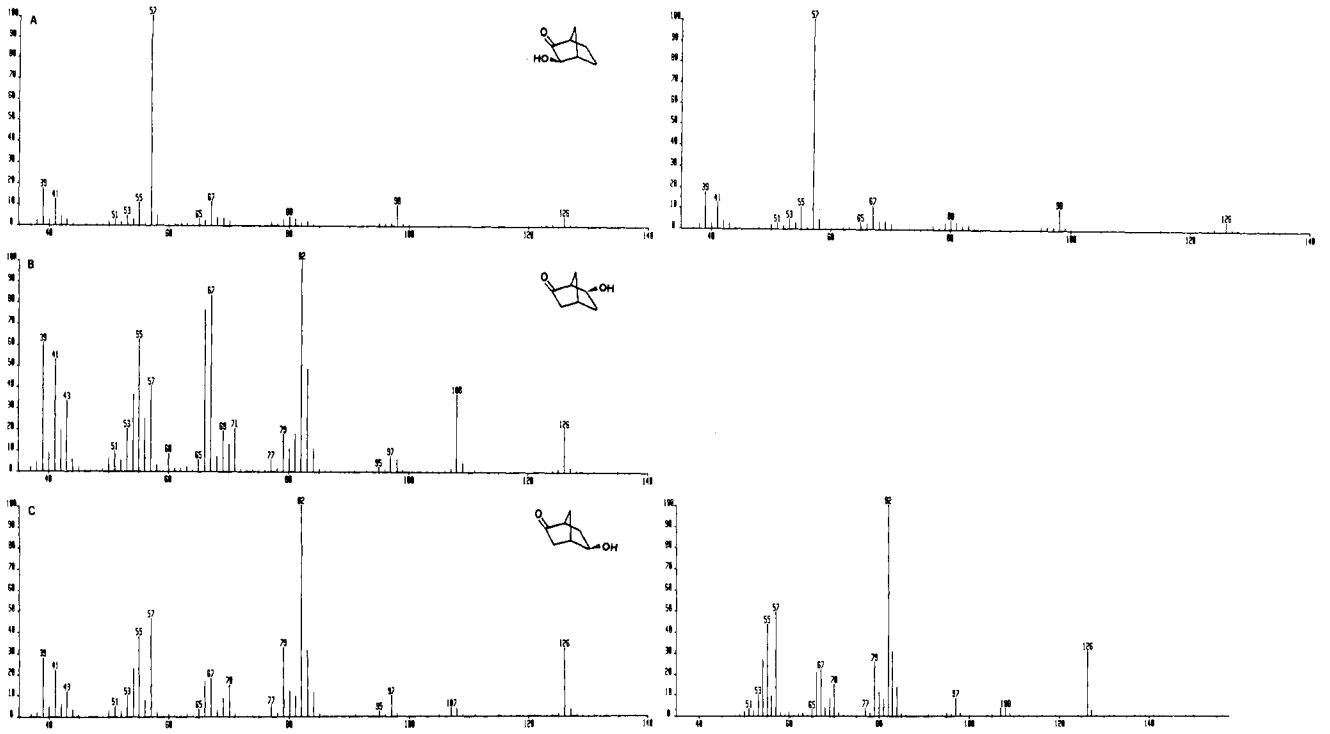

Figure 2. Electron-impact mass spectra (70 eV) of norcamphor metabolites: (A) 3-*exo*-hydroxynorcamphor; (B) 6-*exo*-hydroxynorcamphor; (C) 5-*exo*-hydroxynorcamphor. The spectra shown in the right-hand panel are of the corresponding authentic compounds.

Results

Hydroxylated Products Derived from Norcamphor. Three hydroxylated products were found to be produced from norcamphor metabolism by cytochrome P-450$_{cam}$. The formation of each is strictly dependent on the presence of NADH, P-450$_{cam}$, 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*$_2$ 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 norcamphor, there is a significant intramolecular isotope effect 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 summrized 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.[10]

Table I. Regiospecificity of Norcamphor Hydroxylation

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

[a] The symbols used are I, 5,6,-*exo*,*exo*-norcamphor-*5,6-d*$_2$; II, norcamphor-*3,3-d*$_2$; and III, 5,6-*exo*,*exo*-norcamphor-*3,3,5,6-d*$_4$.

Table II. Stereochemistry of Norcamphor Hydroxylation[a]

substrate/ buffer	product	d_2, %	d_1, %	d_0, %
I in H_2O	5-*exo*-hydroxy	4.6 ± 0.6	94.4 ± 0.5	0.96 ± 0.09
	6-*exo*-hydroxy	2.8 ± 0.9	95.9 ± 0.9	1.1 ± 0.2
norcamphor in D_2O	5-*exo*-hydroxy	0	1.93	98.07
	6-*exo*-hydroxy	0	1.59	98.43

[a] 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 $M^+ - 1$ was less than 6% of the M^+.

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*$_2$ was metabolized. The results are summarized in Table II. Several interesting features of norcamphor hydroxylation contrast the

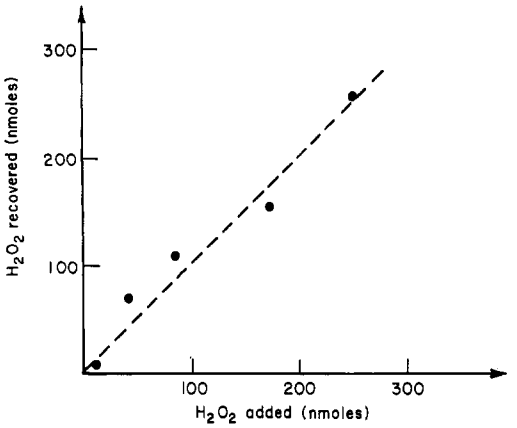

Figure 3. Recovery of hydrogen peroxide in the presence of norcamphor. The H_2O_2 recovered after addition of varying amounts of exogenous H_2O_2 was quantitated as described in the Experimental Section. The plotted values are corrected for the amount which is produced in the absence of added H_2O_2 (88 nmol). The dashed line represents the theoretical yield of 100% recovery.

stereochemical course observed with camphor.[5] 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.[5] 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$_{cam}$ 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 D_2O/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 D_2O 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 D_2O 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 O_2 consumptions were precisely quantitated along with formation of H_2O_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 H_2O_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.[2] In the presence of norcamphor, P-450$_{cam}$ 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 H_2O_2 produced, and, based on the 2:1 stoichiometry of excess NADH/excess 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$_{cam}$. 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

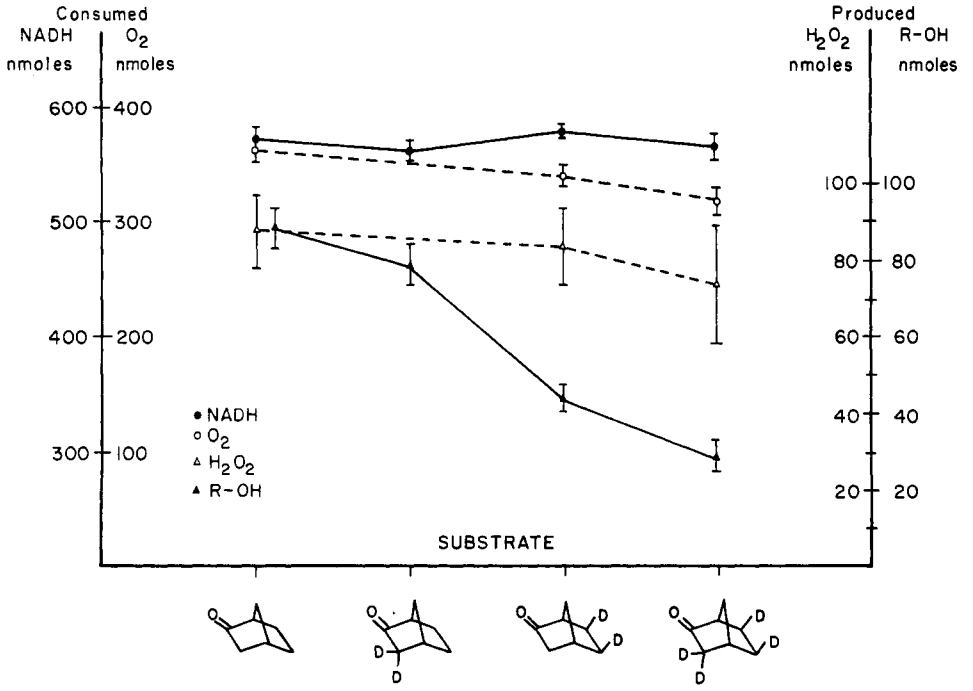

Figure 4. Deuterium isotope effects on the Stoichiometry of norcamphor metabolism.

Table III. Stoichiometry of Norcamphor Metabolism

substrate	catalase	consumed NADH, nmol	consumed O_2, nmol	produced H_2O_2, nmol	produced ROH, nmol	produced "extra H_2O",[a] nmol	$\frac{NADH - [H_2O_2 + ROH]}{O_2 - [H_2O_2 + ROH]}$
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[b]	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		
d-camphor	−	313 ± 8	290 ± 8	20 ± 12	290 ± 2	0	
	+	318 ± 3	280 ± 3		286 ± 9		

[a] Determined from samples not containing catalase. [b] 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$_{cam}$ 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.[11,12] 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,[12] 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.[13] 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 $[FeO]^{3+}$ 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 surprosing 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_2O rather than H_2O_2 or organic alcohol. Although the kinetics of H_2O_2, 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$_{cam}$ 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[a]

[a] 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$_{cam}$ 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_2O/nmol of P-450$_{cam}$/min or about 33% of the total NADH consumption rate. Typical rates of NADPH consumption by various hepatic isozymes are slower than this value,[14,15] as inferred from rates of product formation, so oxidase-derived water production is correspondingly quite slow compared to the P-450$_{cam}$ system. It is especially interesting that the mononuclear metal center of cytochrome P-450$_{cam}$, with no active-site acid/base groups,[11,12] possesses the ability to reduce molecular oxygen to water at a relatively high rate.

Deuteriation of the norcamphor skeleton has novel and profound effects on the stoichiometry of this system in addition to the isotope-dependent alteration in regiospecificity. It is unlikely that substrate deuteriation would have any appreciable effects on the kinetics or thermodynamics of substrate binding, electron transfer, or O_2 binding, although each of these is expected to be different from the corresponding parameters in the presence of camphor. Indeed, others have observed that deuterium substitution does not lead to an increase in the level of H_2O_2 production or in the steady-state levels of oxy–P-450 intermediates.[7] 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.[16] 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.[2] Our data indicate that the species undergoing two-electron reduction is sensitive to deuterium substitution of substrate and suggests that the $[FeO]^{3+}$ 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

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the absence of an easily hydroxylatable substrate, would not be a desirable situation due to the potential for destructive oxidative reactions. The fact that an electron-transfer pathway remains open in the complete reconstituted system allows for rapid quenching of an active intermediate in the absence of an acceptable substrate. This is consistent with the observation that multiple norcamphor turnovers do not result in any detectable levels of P-420 or of inactive enzyme. The decrease in product obtained with increasing amounts of deuteriation is not due to peroxide-mediated or $[FeO]^{3+}$-dependent heme oxidation. This is further demonstrated by the fact that NADH is consumed to the same degree for each of the deuteriated substrates. Furthermore, a second addition of NADH, after completion of the reaction, results in continued NADH and O_2 consumption at normal rates.

Due to complexity of the multiple pathways available for the flux of reducing equivalents to form organic products, peroxide and water, it is difficult to calculate an intrinsic isotope effect for norcamphor hydroxylation. However, some kinetic information is available if a minimal kinetic scheme is analyzed (Scheme I). By comparing the oxygenase/oxidase ratio for undeuteriated and deuteriated substrate (III), one can obtain the lower limit for an intrinsic isotope effect which would be accurate if all three hydroxynorcamphors were obtained from a single $[FeO]^{3+}$–substrate complex with no kinetic barrier to reorientation. In this simple model, the relative amount of organic product and oxidase-derived water for these two substrates is given by $[k_H/k_W]/[k_D/k_W]$, where k_H and k_D represent the rate constants for hydrogen or deuterium abstraction and k_W represents the rate constant for reduction of the intermediate to water. This second rate, k_W, whould be insensitive to isotopic substitution, and if insensitive to norcamphor orientation, would be equivalent for the two substrates. The ratio of oxygenase/oxidase activity, as measured by the ratio of total organic alcohol production to total water formation, should offer a measure of the intrinsic isotope effect k_H/k_D. This value is 3.78 for the data presented herein. Intrinsic isotope effects for P-450-mediated hydroxylations have been estimated by several methods with values ranging from 7.5 to 19.[6,8,17] These large isotope effects are expected for a radical mechanism involving hydrogen abstraction. The relatively small value obtained by this simplified kinetic scheme would suggest that a high degree of masking is operational and that reorientation of norcamphor occurs on the same time scale as, or faster than, reduction of the iron–oxo intermediate.

Several observations support the conclusion that H_2O_2 is not an intermediate in the four-electron reduction of O_2 in the P-450$_{cam}$ system. Exogenously added H_2O_2 had no effect on the rate or amount of NADH and O_2 consumption with any of the norcamphor analogues utilized. Furthermore, it is unlikely that the hydroxylation was actually mediated by H_2O_2 since exogenously added hydrogen peroxide or catalase had no effect on the amount of hydroxynorcamphor obtained. It is possible that an enzyme-bound H_2O_2 is generated followed by rapid peroxidase-type formation of a higher valent Compound I type intermediate. Such a fate for H_2O_2 would not be detected by our assay. If this type of mechanism were operative then these results still suggest that this intermediate was a substrate for further two-electron reduction to form water.

It is worth noting that the experiments reported herein utilized racemic norcamphors. It is possible that the enantiomers of this substrate are processed with different kinetic parameters and that the oxygenase/oxidase ratio may be enantiomer dependent. Although these various alternatives are remotely feasible, they do not affect the proposal that an oxidase activity exists in the P-450$_{cam}$ isozyme and that this activity involves reduction of $[FeO]^{3+}$ via NADH and the associated electron-transport chain.

Finally, several questions are raised by the observation that norcamphor turnover elicits a high degree of oxidase activity. The structure of norcamphor would be expected to result in an active site complex with two features significantly different from that formed with camphor. Firstly, on the basis of the percent ferric high-spin complex (45%) obtained with saturating norcamphor, it would be expected that a significant amount of water remained at the active site in the presence of this substrate.[12,13] Hydration of the $[FeO]^{3+}$ species may allow for, and even be required for, reduction to H_2O, since protons would be required to completely balance the oxidase stoichiometry. Thus, the relative rate for the reduction of $[FeO]^{3+}$ may be a function of the degree of hydration of this intermediate. Secondly, one can imagine that there is an increase in the amount of substrate motion for the complex formed with norcamphor as compared with the complex obtained with camphor. Such an increase in substrate dynamics could decrease the effective residence time that any substrate hydrogen spends in the correct geometry for efficient abstraction, thus resulting in a larger fraction of reducing equivalents being channeled to water production without a change in the actual rate of this reaction. At present it is not clear whether norcamphor binding allows for a faster rate of $[FeO]^{3+}$ reduction or simply unmasks this reaction by slowing down hydrogen abstraction due to increased substrate motion or unfavorable geometry.

Experimental Section

Enzymes. Cytochrome P-450$_{cam}$, putidaredoxin, and putidaredoxin reductase were purified by the method of Gunsalus and Wagner.[18] Substrate free P-450 was prepared immediately prior to use by passage over a Sephadex G-25 column equilibrated with 50 mM Tris buffer, pH 7.4. It was found to be absolutely essential to remove 2-mercaptoethanol from the stock putidaredoxin solution in order to obtain reproducible hydrogen peroxide assays. This was accomplished by passage over a Bio-gel P-4 column. Catalase was purchased from Sigma Chemical Co.

Chemicals. Norcamphor and camphor were purchased from Aldrich Chemical Co. and used without further purification.

Deuteriated Norcamphors. The 5,6-*exo*,*exo*-norcamphor-*5,6-d*$_2$ (I) was prepared by previously established methods.[19] Briefly, this was achieved by catalytic deuteriation of 5-norbene-2-ol (Aldrich) followed by a modified Jones oxidation.[20] Identity of the product was confirmed by GC and mass spectral comparison to commercially available norcamphor: mass spectrum 97.7% d_2, 1.5% d_1, 0.8% d_0; GC indicated >98% purity.

Norcamphor-*3,3-d*$_2$ (II) was also prepared by established procedures.[21] Norcamphor was heated to 45 °C in dioxane/D_2O, 60:40, where the D_2O was 40% NaOD. After 12 h the mixture was extracted and purified by silica column chromatography (ether:petroleum ether 1:2): mass spectrum 38.1% d_2, 58.5% d_1, 3.3% d_0. The d_1 species is expected to be >95% exo D.[21] GC analysis indicated this preparation was >97% norcamphor.

5,6-*exo*,*exo*-Norcamphor-*3,3,5,6-d*$_4$ (III) was prepared by subjecting norcamphor-*5,6-d*$_2$ to solvent exchange at the 3-position as described;[21] mass spectrum 34.0% d_4, 51.0% d_3, 14.8% d_2. This compound was >98% pure based on GC.

Hydroxylated Norcamphors. 5-*exo*-Hydroxynorcamphor was prepared from the 2,5-*exo*,*exo*-diol which was obtained by base saponification of the 2,5-diformate in 40% KOH.[22] This was followed by titration with dichromate solution.[23] The keto alcohol obtained could be oxidized to the 2,5-diketone by further addition of dichromate. ^1H and ^{13}C NMR of this compound clearly showed four resonances, thus demonstrating complete absence of the 2,6-diketone.

5-*endo*-Hydroxynorcamphor was obtained from the 2,5-diketone by titration with several reducing agents including lithium tri-*tert*-butoxyaluminohydride and L-Selectride (Aldrich).[24] The species obtained could readily be converted back to the diketone.

The 7-hydroxynorcamphors, both syn and anti, were prepared by synthesis of the 2,7-diols[25] followed by oxidation with Pt/air (5% on charcoal) exactly as described.[26]

3-*exo*-Hydroxynorcamphor was prepared by $KMnO_4$ oxidation of norbornylene (Aldrich) to afford the 2,3-*exo*,*exo*-diol[27] followed by Pt/air oxidation.

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Enzyme Turnover. Reactions were performed in 50 mM Tris, pH 7.4, containing 1.1 μM P-450$_{cam}$, 1.79 μM putidaredoxin, 1.71 μM putidaredoxin reductase, 225 μM NADH, 20 mM KCl, 450 μM substrate, and, in the appropriate cases, 200 μg of catalase (2500 EU). Reactions were initiated with substrate in a final volume of 2.8 mL. Reactions were performed in a cuvette equipped with a Clark oxygen electrode, allowing NADH and O_2 consumption to be monitored simultaneously. NADH was quantitated by the change in absorbance at 340 nm, using an extinction coefficient of 6.22 mM^{-1} cm^{-1}. O_2 consumption was calculated by using a full scale deflection as a change in O_2 concentration of 213.8 μM. H_2O_2 was determined as the difference in O_2 consumption in the presence and absence of catalase.[28] After reaction was complete, a known amount of standard was added, either 2-*exo*-norborneol or 3-*endo*-bromocamphor. The mixture was extracted 3 times with an equal volume of $CHCl_3$. The organic extract was concentrated under a slow stream of N_2 and analyzed by GC on a 15-m megabore DB-17 column programmed at 70 °C for 4 min followed by temperature ramp to 200 °C at 7 °C/min. The amount of hydroxynorcamphor was determined by integration of peak areas with a HP 3390A reporting integrator.

Mass spectral data were obtained on VG 7070E mass spectrometer in line with an HP 5700 gas chromatograph equipped with a 30-m DB-5 capillary column. The mass spectrometer was interfaced with an 11250 VG multispec data system.

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Spectroscopic and Magnetic Studies of the Purple Acid Phosphatase from Bovine Spleen

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Abstract: The properties of the purple acid phosphatase from bovine spleen have been examined by optical, EPR, ^{57}Fe Mössbauer, and resonance Raman spectroscopy and by variable temperature magnetic susceptibility measurements. Anaerobic titrations monitored by optical and low-temperature EPR spectra show that conversion of the purple, enzymatically inactive form to the enzymatically active pink form is a one-electron process. The pink form exhibits a $g' = 1.77$ EPR spectrum due to a pH-dependent mixture of two rhombic species, with an apparent pK_a of ca. 4.4. The temperature dependence of the EPR signal of the pink form over the range 6-20 K is consistent with a weak antiferromagnetic coupling ($-2J = 11 \pm 2$ cm^{-1}) between an $S = 2$ Fe(II) and an $S = {}^5/_2$ Fe(III). Bulk magnetic susceptibility studies of a lyophilized sample of the purple, oxidized enzyme are consistent with the presence of a strongly antiferromagnetically coupled binuclear high-spin ferric system, with $-2J \geq 300$ cm^{-1}. Natural abundance ^{57}Fe Mössbauer spectra of the purple form confirm the coupled diferric site and indicate that the iron atoms are in relatively low symmetry environments. Resonance Raman spectra demonstrate the presence of tyrosyl phenolate ligands to iron in both the purple and pink forms, but experiments with $H_2{}^{18}O$ gave no direct evidence of a μ-oxo ligand. Integration of these data with previous spectroscopic results suggests the presence in the oxidized enzyme of a binuclear iron center bridged by a μ-oxo and possible additional ligands such as carboxylates. In the oxidized form, one ferric iron is coordinated by two tyrosinates, while the other iron is probably coordinated to a phosphate. In the reduced form, the phosphate is lost from the new ferrous site, the ferric site retains the tyrosinate ligands, and the putative bridging oxo group is probably protonated. Histidine imidazoles and other as yet unidentified ligands complete the coordination of the iron atoms.

The purple acid phosphatases[2] constitute an apparently diverse group of metalloenzymes involved in regulation of the levels of phosphate and phosphorylated metabolites in a wide range of organisms. Despite superficial spectral similarities (all exhibit an absorption band at 500-550 nm with $\epsilon \sim 2000$-4000 M^{-1} cm^{-1}), it now appears as if three distinct types of chromophore may be present in these enzymes. The purple acid phosphatases from plants are reported to contain either Mn(III) (the sweet potato enzyme[3]) or Fe(III) and Zn(II) (the kidney bean enzyme[4]). In contrast, those purple acid phosphatases obtained to date from mammalian sources, including porcine uterine fluid,[5] bovine spleen,[6] rat spleen,[7] and human spleen,[8] have been shown to contain two iron atoms. Several other mammalian enzymes[9-13] exhibit

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