Stereochemistry and Deuterium Isotope Effects in Camphor Hydroxylation by the Cytochrome $P450_{cam}$ Monoxygenase System†

Michael H. Gelb, David C. Heimbrook, Pentti Mälkönen,‡ and Stephen G. Sligar*

ABSTRACT: Bacterial cytochrome $P450_{cam}$ catalyzes the hydroxylation of camphor to yield 5-*exo*-hydroxycamphor in vivo and in a reconstituted system with oxygen, pyridine nucleotide, flavoprotein dehydrogenase, and putidaredoxin. Product is also formed when the ferric form of the hemoprotein is mixed with the exogenous oxidants iodosobenzene, *m*-chloroperbenzoic acid, and hydrogen peroxide. In this paper we show that when the $P450_{cam}$-dependent hydroxylation reactions are studied with camphor analogues containing deuterium at either the 5-exo or 5-endo position, a very small intermolecular isotope on the overall reaction velocity is observed and a significant intramolecular isotope effect is documented. We suggest the existence of an intermediate substrate–carbon radical and demonstrate that abstraction can occur from either the exo or endo position at carbon 5 on the camphor skeleton, with the oxygen stereospecifically added to only the *Re* face to give 5-*exo*-hydroxycamphor as the unique product. Using these substrates, we observed nearly identical hydrogen/deuterium isotope ratios in the product alcohol for the pyridine nucleotide/atmospheric dioxygen as well as exogenous oxidant supported hydroxylations, suggesting that these reactions share a common hydrogen-abstracting species. The relatively small magnitude of the measured intramolecular isotope effect can be rationalized with a model involving a reversible hydrogen-abstraction step and/or the involvement of heavy-atom motion in the reaction coordinate.

The P450 cytochromes are a ubiquitous class of enzymes occurring in various forms in a wide variety of mammalian tissues and bacteria. The more commonly studied P450 mixed function monoxygenase systems can be conceptually grouped into two classes based on the observed substrate specificity and the intermediate proteins required to transfer the necessary reducing equivalents from reduced pyridine nucleotide (NADH or NADPH)[1] to the terminal cytochrome P450 component. The hepatic microsomal P450 system, for example, includes a cytochrome P450 reductase containing both FMN and FAD cofactors, each with distinct electron transfer functions (Vermilion et al., 1981; Iyanagi & Mason, 1973; Yasukochi & Masters, 1976), and hydroxylates a wide variety of substrates with varying regiospecificity and epimeric specificity. In contrast, the adrenal mitochondrial P450 monoxygenase system consists of a three-component electron transfer chain containing an FAD-dependent flavoprotein, an intermediate iron–sulfur protein, and cytochrome P450. Hydroxylations catalyzed by the adrenal mitochondrial systems exhibit a high degree of regioselectivity and epimeric selectivity (Hamberg et al., 1974). The camphor hydroxylase system isolated from the soil bacterium *Pseudomonas putida* (Gunsalus et al., 1974; Gunsalus & Wagner, 1978) displays striking similarity to the adrenal mitochondrial steroid 11β-monoxygenase. This bacterial monoxygenase system contains a three-component electron transfer chain of reductase, putidaredoxin, and cytochrome $P450_{cam}$ and also exhibits rigid regioselectivity and epimeric selectivity, hydroxylating camphor only at the 5-exo position (Figure 1). An important area of current interest in cytochrome P450 research is the elucidation of the chemistry of substrate hydroxylation with precise identification of the activated oxygen and/or substrate species responsible for the hydroxylation of unactivated alkanes. The demonstration that exogenous oxidants such as peracids (Nordblom et al., 1976; Sligar et al., 1976), peroxides (Rahimtula & O'Brien, 1974), and iodosobenzene (Lichtenberger et al., 1976; Gustafsson et al., 1979) can be utilized by cytochrome P450 in the hydroxylation of substrate in the absence of O_2 and reduced pyridine nucleotide significantly advanced our understanding of the hydroxylation reaction. Although it is tempting to assume that these exogenous oxidants support hydroxylation by generating an active oxygen species similar to that of the $NAD(P)H/O_2$ process, little direct evidence exists in the literature to support such a conclusion.

In this study, we investigate deuterium kinetic isotope effects occurring in cytochrome $P450_{cam}$ supported reactions. Using camphor derivatives specifically deuterated at either the exo or endo position of the oxidizable carbon, we have examined intermolecular and intramolecular isotope effects in both the $NADH/O_2$-dependent reconstituted system as well as the reaction mediated by various exogenous oxidants. The analysis of isotope effects in this "mitochondrial-type" P450 system is interesting due to the apparent lack of substrate positional interchange at the active site of the enzyme. The determination of isotope effects occurring with various oxidants allows us to compare the stereochemistry and nature of the hydroxylating species generated from each source and to probe the chemistry of substrate oxygenation in molecular terms.

Materials and Methods

Cytochrome P450, putidaredoxin reductase, and putidaredoxin were purified from frozen cell paste (ATCC 29607) essentially as reported by Gunsalus & Wagner (1978). Substrate-free P450 was prepared by passing substrate-bound enzyme over a small P2 column (Bio-Rad) equilibrated in 50 mM Tris-HCl, pH 7.4, followed by repeated dialysis against 50 mM potassium phosphate, pH 7.0. Gas chromatographic analysis of a chloroform extract of the substrate-free enzyme

† From the Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06511. *Received May 20, 1981.* This work was supported by National Institutes of Health Grant GM24976. S.G.S. is a recipient of Research Career Development Award AM00778. We acknowledge the support of the NSF Northeast Regional NMR Facility at Yale University, CDP-7916210.

‡ Present address: Department of Chemistry, University of Joensuu, SF-80101 Joensuu 10, Finland.

[1] Abbreviations: NADH, reduced nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; Tris, tris(hydroxymethyl)aminomethane; THF, tetrahydrofuran; NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid.

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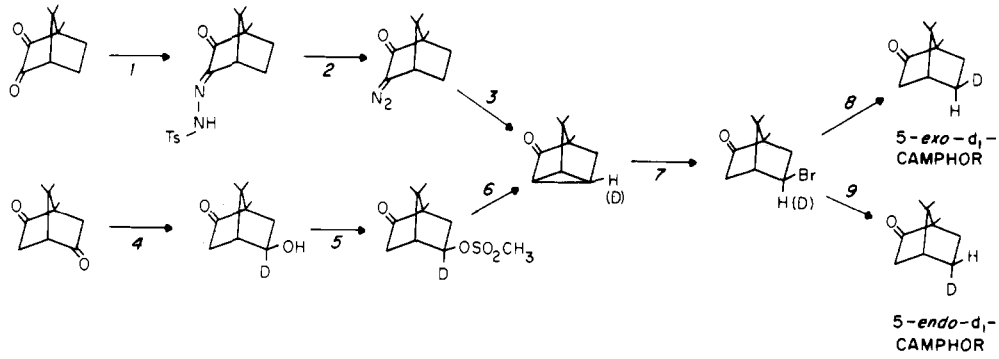
Scheme I: The Synthesis of Camphor-5-*exo*-d_1 and Camphor-5-*endo*-d_1 Using the Procedures Described under Materials and Methods[a]



[a] Reagents: (1) *p*-tosylhydrazine in methanol; (2) NaOH in water/petroleum ether; (3) copper-bronze in toluene; (4) lithium triethylborodeuteride in dry tetrahydrofuran; (5) methanesulfonyl chloride in dry dichloromethane containing a slight excess of triethylamine; (6) NaOH in water/dioxane; (7) anhydrous acetic acid saturated with HBr; (8) aluminum amalgam in dry tetrahydrofuran containing H_2O; (9) aluminum amalgam in tetrahydrofuran containing D_2O.

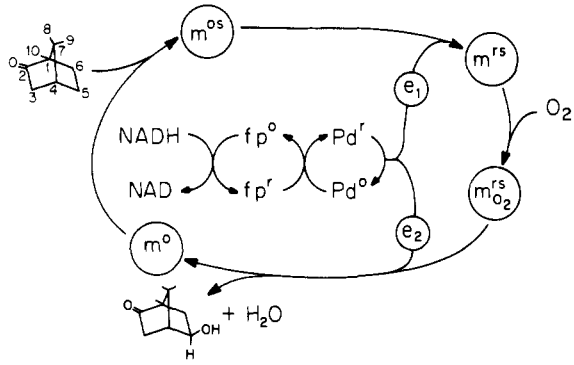


FIGURE 1: Cytochrome $P450_{cam}$ reaction cycle. The various stable intermediates of cytochrome $P450_{cam}$ are shown. Abbreviations: m^o, ferric cytochrome P450; m^{os}, the substrate-bound complex; m^{rs}, the ferrous substrate adduct; $m^{rs}_{O_2}$, the ferrous, oxygenated $P450_{cam}$ complex; Pd°, oxidized putidaredoxin; Pdr, one electron reduced putidaredoxin; fp, the flavoprotein NADH dehydrogenase–putidaredoxin reductase. The camphor skeleton has been numbered according to the conventional scheme for bicyclic systems. The diagram shows the structure of the D(+) isomer of camphor although the L(−) compound is also a substrate for the enzyme.

demonstrated that less than 1% of the cytochrome P450 was bound to camphor.

The synthesis of camphor-5-*endo*-d_1 was accomplished as illustrated in Scheme I. Attempts at the direct displacement of bromide from 5-*exo*-bromocamphor with lithium triethylborodeuteride, as suggested by the conversion of 2-*exo*-bromonorbornane to norbornane-2-*endo*-d_1 (Brown & Krishnamurthy, 1973), led to skeleton rearrangements. Instead, *l*-camphoroquinone was synthesized from *d*-camphor by oxidation with selenium dioxide as described by Lipp et al. (1967) and then treated with *p*-tosylhydrazine in methanol yielding the 2 adduct, followed by treatment in base to give 2-diazocamphor (Cava et al., 1958). Catalytic release of N_2 from the diazo compound affords *d*-pericylocamphanone, which when treated with HBr/acetic acid gives *d*-5-*exo*-bromocamphor (Bays et al., 1966). The bromocamphor was reduced to camphor by using a modification of the previously reported procedure of Joshi & Warnhoff (1972). Aluminum metal (20 mesh, Fisher Scientific Co.) was amalgamated by stirring 3 g in 20 mL of THF (freshly distilled over lithium aluminum hydride) containing 0.2 g of $HgCl_2$ under N_2 for 1–2 h. A solution of 0.56 g of *d*-5-*exo*-bromocamphor in 10 mL of dry THF was brought to reflux under N_2 and then 0.6 g of amalgam was added in one portion. Refluxing was continued for a few minutes and then 0.6 mL of D_2O (99.8% D) was added by syringe. The mixture was refluxed for 1 h under N_2 after which it was cooled, treated with anhydrous $MgSO_4$, and filtered by gravity. Flash evaporation of the solvent gave crude product, which was purified by preparative gas chromatography on a 3-ft DC-200 column at 175 °C. Mass spectral analysis of the purified camphor gave 98% d_1, 2% d_0, and 0% d_2. Proton NMR analysis at both 270 and 500 MHz indicated that 95% of the deuterium was in the 5-endo position. Thus, D_2O attacks the organometallic complex from the less hindered endo face of the camphor skeleton.[2]

The synthesis of camphor-5-*exo*-d_1 is also documented in Scheme I. *d*-5-Ketocamphor, obtained as a generous gift from Dr. I. C. Gunsalus or synthesized from the chromic acid oxidation of bornyl acetate (Mälkönen, 1964), was treated with 1.5 equiv of lithium triethylborodeuteride (Aldrich) in dry THF at 0 °C and stirred for 1 h at room temperature under N_2. Rigorously dry conditions were maintained throughout the reduction procedure. Gas chromatography and 270-MHz proton NMR demonstrated that 5-*exo*-hydroxycamphor-5-*endo*-d_1 was the major product formed (~80% of the total material) as expected from steric considerations. The alcohol was isolated by using an oxidative workup with alkaline hydrogen peroxide (Holder & Matturo, 1976) and converted to the mesylate by reaction with methanesulfonyl chloride in dry dichloromethane containing a slight excess of triethylamine (Crossland & Servis, 1970). The crude mesylate was converted directly to *d*-pericyclocamphanone-5-d_1 by stirring with 1 N NaOH in dioxane/water overnight at room temperature. Extraction with ether, drying, and flash evaporation gave the crude ketone, which was purified by vacuum sublimation at 50 °C (10 mmHg). The material was treated with HBr/acetic acid as described above to give *d*-5-*exo*-bromocamphor-5-*endo*-d_1, which was purified by vacuum sublimation at 50 °C (2 mmHg). Finally, reduction of the bromocamphor over aluminum amalgam in the presence of water (as described

[2] It has been previously reported that the reduction of 5-*exo*-bromocamphor to camphor using aluminum amalgam proceeds with retention of configuration (Joshi & Warnhoff, 1972), as judged by the partial loss of coupling in the proton NMR spectrum in the 3-exo region (δ 2.3). In our hands, the reduction proceeds with almost complete inversion of configuration. This conclusion is based on precise integration of the 5-exo region of the spectrum (δ 1.94) using 270- and 500-MHz proton NMR. Computer simulation demonstrates the danger in assigning label stereochemistry by coupling arguments alone. The complete high-resolution NMR analysis of camphor at 270 MHz yields the following chemical shift values (δ_{Me_4Si}) and couplings (Hz): 3x (3-exo proton), 2.33; 3n, 1.83; 4, 2.07; 5x, 1.94; 5n, 1.32; 6x, 1.66; 6n, 1.39; J_{3x-3n}, −18.64; J_{3x-4}, 4.50; J_{3x-5x}, 3.40; J_{3x-6x}, 0.70; J_{4-5x}, 4.50; J_{4-5n}, 0.10; J_{4-6x}, 0.50; J_{5x-5n}, −12.54; J_{5x-6x}, 11.70; J_{5x-6n}, 4.88; J_{5n-6x}, 3.68; J_{5n-6n}, 9.07; J_{6x-6n}, −13.43. All other couplings are less than 0.1 Hz.

above) gave the desired *d*-camphor-5-*exo*-d_1. The crude material was purified by high-performance liquid chromatography with octadecylsilane on 5-μm silica and eluted with 80% methanol/20% water. Mass spectral analysis of the pure camphor gave 96% d_1, 4% d_0, and 0% d_2, and NMR analysis showed 90% of the deuterium to be in the 5-exo position.

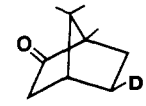
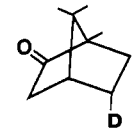
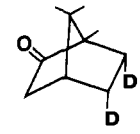
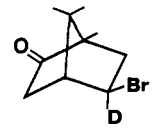
l-Dehydrocamphor was synthesized in seven steps from *d*-bornyl acetate as previously described (Mälkönen, 1964). The ketone was reduced with excess lithium aluminum hydride in dry ether to give a mixture of dehydroborneol and dehydroisoborneol. The alcohols (50 mg) were deuterated for 1 h at room temperature in 20 mL of *n*-pentane containing 100 mg of 10% Pd on carbon (Aldrich) in a Parr hydrogenator charged to 16 psi with 99% D_2.[3] The solvent was refluxed for 2–3 h with concentrated sulfuric acid prior to use to remove all traces of unsaturated hydrocarbons. The deuterated material was oxidized back to camphor with Jones reagent in acetone (Eisenbraun, 1965). Mass spectral analysis gave 94% d_2, 5% d_1, and 1% d_0. NMR showed that 95% of the 5-endo and 6-endo sites were deuterated.

Iodosobenzene was synthesized from iodobenzene via the dichloride as described previously (Lucas & Kennedy, 1955) and was shown by iodometry to be greater than 95% pure at the time of use. Hydrogen peroxide and *m*-chloroperbenzoic acid (*m*-CPBA) were obtained from standard suppliers in the highest available purity. 5-*exo*-Bromocamphor and 5-*exo*-bromocamphor-5-*endo*-d_1 were obtained as intermediates in the synthesis of camphor-5-*endo*-d_1 and camphor-5-*exo*-d_1, respectively, as described above; see Scheme I.

NADH-driven enzymatic turnovers were conducted under saturating conditions in 1 mL of 100 mM potassium phosphate buffer, pH 7.0, containing 1.5 μM substrate-free cytochrome P450, 3.3 μM putidaredoxin, 2.5 μM putidaredoxin reductase, and 300 μM substrate. Hydroxylations were initiated by the addition of NADH (200 nmol) (Sigma Chemical Co.) and followed by the decrease in absorbance at 340 nm, using an extinction coefficient of 6.2 mM^{-1} cm^{-1}. Enzymatic turnovers with exogenous oxidants were carried out in 1 mL of potassium phosphate buffer, pH 7.0, containing 20 nmol of substrate-free P450, and 200 μM substrate. The amounts of exogenous oxidants used were 10 μL of 100 mM methanolic iodosobenzene, 10 μL of 100 mM methanolic *m*-CPBA, or 10 μL of 0.5 M aqueous hydrogen peroxide. The iodosobenzene and *m*-CPBA reactions were worked up after 1 h (see below) while the hydrogen peroxide dependent reaction was left to stand overnight. Under these conditions more than five turnover reactions per enzyme were obtained. The use of higher oxidant concentrations is counterproductive due to the increasing rate of oxidant-dependent enzyme destruction (Heimbrook & Sligar, 1981; Sligar et al., 1976). After the reactions, excess *m*-CPBA and iodosobenzene were quenched by the addition of a small amount of sodium bisulfite. Product analysis consisted of extracting the solution 3 times with 1-mL portions of chloroform, followed by concentration to a small volume with a stream of dry air and addition of anhydrous sodium sulfate to ensure dryness. The extracts were analyzed by combined gas chromatography–mass spectrometry on a Hewlett-Packard 5985 or 5992B mass spectrometer with a 3-ft OV-101 column (Supelco) or a 6-ft SE-30 column (Supelco). Isotope ratios were determined from the integration of ion counts in the selective ion monitoring mode (see Results).

[3] Catalytic deuteration of pure dehydrocamphor under conditions identical with those described under Materials and Methods for dehydroborneol gave a mixture of isomers (approximately 70% endo and 30% exo) with respect to the position of deuterium, with the results in this case sensitive to the amount of catalyst and choice of solvent.

Table I: Intermolecular Isotope Effects in Camphor Hydroxylation

substrate	V_{max}^H/V_{max}^D [a]	substrate	V_{max}^H/V_{max}^D [a]
	1.23 ± 0.05		1.16 ± 0.05
	1.13 ± 0.05		1.15 ± 0.05

[a] The normalized NADH oxidation rate refers to the observed reaction velocity with nondeuterated substrate divided by the velocity with deuterated substrate under identical conditions.

Single turnover reactions were carried out as previously described (Lipscomb et al., 1976) in 1.35 mL of 100 mM potassium phosphate, pH 7.0, containing 7.4 mM EDTA, 7.4 μM proflavin (Aldrich Chemical Co.), 6.2 μM substrate-free P450, and a saturating concentration of substrate (200 μM). The solution was degassed in a Thunberg tube and irradiated for several minutes with a heat-filtered 150-W xenon lamp. The closed tube was equilibrated in a 10 °C water bath for 5 min and was opened to the atmosphere, and the contents were quickly transferred to a thermostated cuvette containing 0.066 nmol of putidaredoxin. Decay of oxyferrous cytochrome P450 generated by this procedure was followed by the absorbance decrease at 418 nm. The initial velocity for the decay was calculated from the first 15 s of the reaction.

Results

Site-Specific Hydroxylation of Camphor by Cytochrome P450$_{cam}$. Cytochrome $P450_{cam}$ catalyzes the hydroxylation of camphor to 5-*exo*-hydroxycamphor as the first step in the microbial degradation of this monoterpene. Gas chromatographic analysis of the product from either the $NADH/O_2$ or exogenous oxidant supported reactions demonstrates that no 5-*endo*-hydroxycamphor is formed, even when the enzymatic reaction is carried out with camphor deuterated at the 5-exo position. The gas chromatographic retention time of an authentic sample of the endo alcohol is substantially longer than that of the exo isomer and would be easily detectable by this technique if it were formed in the reaction. Thus, the addition of the hydroxyl group to the camphor skeleton occurs in a highly specific fashion such that only the exo or 5*R* geometrical isomer is formed.

Intermolecular Deuterium Isotope Effects. An intermolecular deuterium isotope effect refers to a decrease in the overall reaction velocity when a deuterated substrate is substituted for the normal, nondeuterated material. However, enzymatic reaction cycles often contain multiple steps, which can lead to a partial or complete masking of such an isotope effect. Table I presents the NADH oxidation rates for the enzymatic turnover of the deuterated camphor derivatives with the reconstituted enzyme system under the conditions described under Materials and Methods. The NADH oxidation reaction is rigorously linear during the time necessary to completely exhaust the supply of reducing equivalents, thus validating these conditions for initial velocity determinations. Also included in Table I are the NADH oxidation rates observed for the turnover of deuterated and nondeuterated 5-*exo*-bromo-

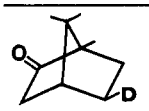
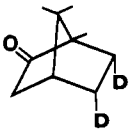
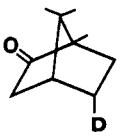
camphor. In a previous report (Gould et al., 1981), we demonstrated that 5-*exo*-bromocamphor is converted solely to 5-ketocamphor by this cytochrome P450 monoxygenase, presumably as a result of 5-endo hydrogen abstraction followed by oxygen addition to give the *gem*-halohydrin which readily loses hydrogen bromide to form the 5-ketone. As can be seen from Table I, a small but statistically significant difference exists between the NADH oxidation rates of deuterated (camphor-d_1) and nondeuterated (camphor-d_0) substrates (k_H/k_D = 1.1–1.2). In addition, although the NADH oxidation rate with bromocamphor is slower than with camphor, there is also a small difference in the rates when deuterated bromocamphor is compared to the nondeuterated analogue. These data are consistent with a partially masked intermolecular isotope effect on the maximal velocity of camphor hydroxylation.

To investigate this point further, we measured the rate of breakdown of the ferrous-oxygenated form of cytochrome $P450_{cam}$ in the presence of either deuterated or nondeuterated 5-*exo*-bromocamphor. With this analogue, only one position at the 5 carbon is open for hydrogen abstraction (Gould et al., 1981), and hence possible intermolecular isotope effects should be maximized. Ferrous-oxygenated cytochrome P450 has been previously characterized as an isolatable intermediate in the camphor hydroxylation cycle, which interacts with the iron–sulfur protein putidaredoxin to form a catalytically competent dienzyme complex (Lipscomb et al., 1976). Electron transfer and subsequent decay of this complex can be followed by monitoring the decrease in the Soret absorbance at 418 nm with the concomitant regeneration of ferric $P450_{cam}$ and the production of hydroxycamphor (Materials and Methods). The values for the initial velocities for the breakdown of ferrous-oxygenated $P450_{cam}$ in the presence of saturating amounts of either deuterated (5-*endo*-d_1) bromocamphor or nondeuterated bromocamphor are 0.18 and 0.19 μM/s, respectively. This small difference in oxy P450 decay rate observed when the deuterated substrate is compared to nondeuterated material parallels the intermolecular isotope effects seen in NADH oxidation measurements. These results demonstrate that the input of the first electron and the binding of O_2 to P450 are not responsible for the partial masking of the intermolecular isotope effect.

In order to probe the chemical intermediates in camphor hydroxylation by cytochrome $P450_{cam}$, we studied the reactions of ferric cytochrome P450 with *m*-CPBA, hydrogen peroxide, and iodosobenzene. For multiple turnover investigations using these artificial oxidants, intermolecular isotope effects were measured by examining the deuterium content of the remaining substrate pool after 10–20% of a mixture containing equal concentrations of nondeuterated and deuterated (5-*exo*-d_1) substrates were enzymatically converted to product. An intermolecular isotope effect in these competitive studies would be apparent from an increase in the fraction of deuterated camphor remaining in the substrate pool as a result of the slower flux of this material into product. Under all conditions studied, no change in the substrate deuterium content was observed (data not shown). This competitive measurement, which probes the lumped parameters V_{max}/K_m (Northrop, 1977), indicates no effect on this ratio by single deuteration of the substrate.

Intramolecular Deuterium Isotope Effects. An expressed intramolecular deuterium isotope effect in this hydroxylase system would result from the preferential abstraction of a hydrogen atom from camphor containing both hydrogen and deuterium at the 5 position. As stated in the previous section, only a very small intermolecular isotope effect was observed. An intramolecular isotope effect, however, is masked by different factors and could thus be significantly larger (Northrop, 1975).

Table II: Mass Spectrometry Analysis of the Product Alcohol Deuterium Content

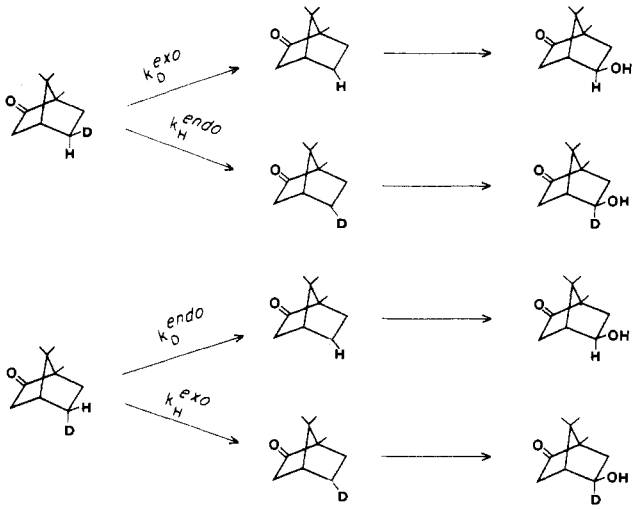
substrate	oxidant	alcohol-d_1/ alcohol-d_0 [a]	av
	$NADH/O_2$	1.15, 1.20	1.18
	iodosobenzene	1.47, 1.50	1.48
	m-CPBA	1.58, 1.69	1.64
	H_2O_2	1.19, 1.21	1.20
	$NADH/O_2$	4.40, 4.62	4.51
	iodosobenzene	3.77, 3.75	3.76
	m-CPBA	4.03, 3.88	3.96
	H_2O_2	3.85, 3.70	3.78
	$NADH/O_2$	4.25, 4.52	4.39
	iodosobenzene	4.72, 4.65	4.69
	m-CPBA	4.22, 3.99	4.11
	H_2O_2	3.97, 3.90	3.94

[a] The double entries are the results of independent experimental trials.

The intramolecular isotope effects in camphor hydroxylation were determined by measuring the deuterium content of the 5-*exo*-hydroxycamphor product of the reaction with combined gas chromatography–mass spectrometry (Materials and Methods). The mass ion intensity was used for all calculations since this ion is easily detected, being approximately 10% of the base peak, and provides unambiguous determination of the product deuterium content. Table II presents the results of several experiments in which the deuterated camphor derivatives were enzymatically hydroxylated with either $NADH/O_2$ in the reconstituted enzyme system or one of three external oxidants: iodosobenzene, *m*-CPBA, or hydrogen peroxide (Materials and Methods). Table II clearly shows that in all cases a significant amount of both deuterium and non-deuterium-containing 5-*exo*-hydroxycamphor is formed when deuterium is substituted at either the 5-exo or the 5-endo position on the camphor skeleton. Jones oxidation of the product alcohol to 5-ketocamphor removed all of the deuterium, confirming that the heavy isotope was exclusively in the 5-endo position and that no migration to the 6 carbon occurred (data not shown). These results strongly indicate the existence of a planar intermediate, either radical or ionic, as previously documented with hepatic cytochrome P450 (Groves et al., 1978). The nearly identical hydrogen/deuterium isotope ratio in the product alcohol resulting from hydroxylation supported by the various exogenous oxidants and $NADH/O_2$ suggests similar hydrogen-abstracting species in all cases.

The simplest kinetic scheme for describing intramolecular isotope effects and the intrinsic geometrical preference of the enzyme for exo vs. endo abstraction is presented in Scheme II, where the putative hydrogen abstraction step is considered to be irreversible. The intramolecular isotope effects for the abstraction of the exo and endo hydrogens can then be estimated as follows. k_H^{exo} and k_D^{exo} are defined as the rates for exo abstraction of hydrogen or deuterium, respectively, while k_H^{endo} and k_D^{endo} are the corresponding values for endo abstraction. The intrinsic isotope effect for exo abstraction I_{exo} is thus $I_{exo} = k_H^{exo}/k_D^{exo}$, that for endo abstraction is $I_{endo} = k_H^{endo}/k_D^{endo}$, and the inherent partitioning or selection of the enzyme for exo or endo abstraction, given hydrogen at both

Scheme II: Intramolecular Isotope Effects Occurring in Camphor Hydroxylation by the Cytochrome $P450_{cam}$ Monoxygenase[a]



[a] Included is a carbon-centered free radical at the 5 position of camphor. In this scheme, the hydroxylation cycle begins with the hydrogen-abstraction step and partitions into paths involving either exo or endo abstraction.

positions, is $G = k_H^{exo}/k_H^{endo}$. Measurement of the fraction of 5-*exo*-alcohol containing deuterium using camphor-5-*exo*-d_1 as substrate defines the ratio $A = k_H^{endo}/k_D^{exo}$. The same measurement of product deuterium content with camphor-5-*endo*-d_1 as substrate yields $B = k_H^{exo}/k_D^{endo}$. The nonzero values of A due to the presence of deuterium in the 5-*exo*-alcohol product, Table II, unambiguously show that the $P450_{cam}$ cytochrome may abstract the endo hydrogen with oxygen addition occurring stereospecifically from the exo face.

The isotope effects for exo and endo abstraction, I_{exo} and I_{endo}, can thus be expressed in terms of the experimental parameters A, B, and the constant G as

$$I_{exo} = \frac{k_H^{exo}}{k_D^{exo}} = \left(\frac{k_H^{endo}}{k_D^{exo}}\right)\left(\frac{k_H^{exo}}{k_H^{endo}}\right) = AG$$

$$I_{endo} = \frac{k_H^{endo}}{k_D^{endo}} = \left(\frac{k_H^{exo}}{k_D^{endo}}\right)\left(\frac{k_H^{endo}}{k_H^{exo}}\right) = B/G$$

Since using the two deuterium-labeled compounds yields two experimentally determined numbers, A and B, and as there are three unknowns, I_{exo}, I_{endo}, and G, we chose to write the isotope effects as a function of the geometrical selection effect G and to examine various solutions to these equations under Discussion.

The above analysis for I_{exo} and I_{endo} in terms of A, B, and G assumes both complete isomeric purity (exo vs. endo deuterium substitution) and label purity (total deuterium vs. total hydrogen) for the 5-exo and 5-endo deuterated substrates. This is an excellent approximation considering the documented purity for these compounds as determined by high-resolution NMR and mass spectrometry (Materials and Methods). In order to be completely rigorous, however, we can correct for the small effects due to incomplete labeling as follows. The two substrates, predominantly 5-*exo*-deuterio and 5-*endo*-deuterio, are considered to actually have respective fractions of 5-*exo*-d_1, d_0, and 5-*endo*-d_1 components of a–c and d–f, respectively. From Materials and Methods these values are determined to be $a = 0.84$, $b = 0.04$, $c = 0.10$, $d = 0.05$, $e = 0.02$, and $f = 0.93$. Considering the various pathways to product formation and using straightforward algebra, it can be shown that the isotope effects I_{exo} and I_{endo} are now given

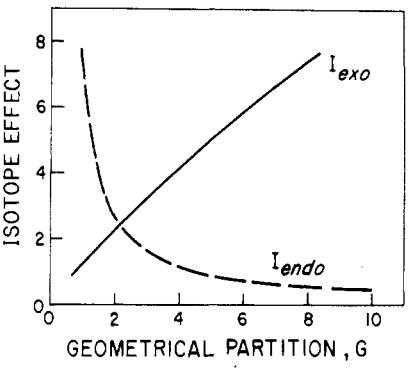


FIGURE 2: Intramolecular isotope effects for exo, I_{exo}, and endo, I_{endo}, abstraction at the 5 carbon of the camphor skeleton as a function of G, the geometrical selection for exo vs. endo abstraction. See text for a discussion of the relationship between I_{exo}, I_{endo}, and G and the experimentally observed isotope ratios into the product alcohol using the two specifically deuterated substrates camphor-5-*exo*-d_1 and camphor-5-*endo*-d_1.

in terms of the experimental parameters (A, B, G) and the weighting factors (a–f) by

$$A = \frac{ak_H^{endo} + ck_H^{exo}}{ak_D^{exo} + b(k_H^{exo} + k_H^{endo}) + ck_D^{endo}}$$

$$B = \frac{dk_H^{endo} + fk_H^{exo}}{dk_D^{exo} + e(k_H^{exo} + k_H^{endo}) + fk_D^{endo}}$$

$$I_{exo} = \frac{ABG(af - dc)}{(pfB - cqA)}$$

$$I_{endo} = \frac{AB(cd - fa)}{(pdB - aqA)}$$

where for notational simplicity we have written $p = a + cG - Ab(1 + G)$ and $q = d + fG - Be(1 + G)$. If $a = f = 1$ and $b = c = d = e = 0$, these results reduce to the simplified expressions presented earlier, $I_{exo} = AG$ and $I_{endo} = B/G$.

The exo and endo isotope effects derived from the d_1/d_0 partitioning data can thus be plotted as a function of G as exemplified in Figure 2 for the pyridine nucleotide/dioxygen dependent reaction of the complete reconstituted system. If the isotope effects for endo and exo hydrogen abstraction were the same, as would be the case for symmetric, identical transition states (Melander & Saunders, 1980), the intersection of the two curves would define the geometrical selection effect operating in this system. However, if a single species is responsible for both exo and endo abstraction, it is unlikely that geometrical factors would allow identical transition states and hence identical isotope effects in both cases (see Discussion) and one must consider other limiting solutions, such as when G becomes large.

Discussion

The three observations presented in this manuscript are (1) a small observed intermolecular isotope effect on the cytochrome $P450_{cam}$ dependent camphor hydroxylation, (2) the existence and magnitude of observed intramolecular isotope effects, and (3) the loss of stereochemistry during substrate hydroxylation. These will be discussed sequentially, with final comments on the importance and implications of the nearly identical results observed with the $NADH/O_2$ reconstituted system and the exogenous oxidant supported oxygenations.

The hydroxylation of camphor by the reconstituted cytochrome P450 monoxygenase system is a multistep process, including electron transfer from NADH to the cytochrome via the two intermediate redox proteins and all of the chemical reactions involved in the actual scission of the oxygen–oxygen

bond of O_2 and hydroxylation of substrate. Therefore, intermolecular deuterium isotope effects in this system might be expected to show considerable steady-state suppression (Northrop, 1977). The substantial masking of the intermolecular isotope effect observed in the studies reported herein is a result of such suppression although this is insufficient information to conclude that hydrogen abstraction is not rate limiting (Northrop, 1981). It is apparent, however, that the transfer of the first electron into the cytochrome and O_2 binding are not responsible for this masking since no difference in intermolecular isotope effect is observed in single turnover experiments starting from the one electron reduced, ferrous, oxygenated hemoprotein. The absence of intermolecular isotope effects in the hydroxylation reactions supported by *m*-CPBA, iodosobenzene, and hydrogen peroxide was determined by a competitive method that documents isotope effects on V_{max}/K_m rather that just V_{max} as was determined in the noncompetitive studies with the NADH/O_2 reconstituted system.

The second point to be addressed is the existence and relatively small magnitude of the observed intramolecular isotope effects. Previous studies in liver microsomal P450 systems have shown that intermolecular isotope effects are usually small whereas a large range of intramolecular isotope effects are observed. For example, hepatic cytochrome P450 catalyzed N-demethylation reactions are characterized by a small intramolecular isotope effect of 1.6–2.0 (Miwa et al., 1980). Other studies of P450-dependent alkane hydroxylation reactions yield large values of isotope effects (Groves et al., 1978; Hjelmeland et al., 1977). The reasons for this variability in intramolecular isotope effects are somewhat uncertain, although they may easily result from differences in the relative rate at which the two symmetrical but isotopically distinct regions of the substrate interchange at the enzyme's active site as compared to the subsequent oxygen addition and product formation (Miwa et al., 1980).

The data presented in this paper unambiguously demonstrate that cytochrome $P450_{cam}$ can effect hydrogen abstraction from either the exo or endo position and yet yields only one stereospecific product (5-*exo*-hydroxycamphor). With these two pathways for hydrogen abstraction available, one might expect a large intramolecular isotope effect to be expressed. The experimental data reported herein (Figure 2) suggest relatively small values of the intramolecular isotope effects in the absence of excessively large geometrical selections (G) or substantial inverse secondary isotope effects. The minor intramolecular isotope values actually allowed for the carbon–hydrogen bond breaking step in the $P450_{cam}$ reaction cycle suggest either that the abstraction step is reversible, thereby masking the inherent effect of deuterium through a commitment to catalysis factor, or that the geometry of the reaction trajectory through the transition state is more complicated than the simplistic model cases (Melander & Saunders, 1980). Reversible hydrogen abstraction can be treated by defining functions that represent the flux of the substrate through a path involving either exo or endo abstraction using the formalism of net rate constants (Cleland, 1975). The four net rate constants (indicated by asterisked quantities) applicable in this case are

$$*k_H^{exo} = k_H^{exo}k_p/(k'_H{}^{exo} + k_p)$$

$$*k_D^{exo} = k_D^{exo}k_p/(k'_D{}^{exo} + k_p)$$

$$*k_H^{endo} = k_H^{endo}k_p/(k'_H{}^{endo} + k_p)$$

$$*k_D^{endo} = k_D^{endo}k_p/(k'_D{}^{endo} + k_p)$$

where the primed rate constants represent the reverse of the corresponding hydrogen abstraction steps. The rate k_p includes all steps subsequent to hydrogen abstraction and is considered to be the same for both exo and endo branches of the reaction path. The actual number of steps included in k_p is presently unknown but clearly includes the addition of the oxygen atom to the activated substrate to form the exo alcohol. With these definitions the experimentally determined values of A and B will reflect the reversibility of the hydrogen abstraction step:

$$A = \frac{*k_H^{endo}}{*k_D^{exo}} = \left(\frac{k_H^{endo}}{k_D^{exo}}\right)\left(\frac{k'_D{}^{exo} + k_p}{k'_H{}^{endo} + k_p}\right)$$

$$B = \frac{*k_H^{exo}}{*k_D^{endo}} = \left(\frac{k_H^{exo}}{k_D^{endo}}\right)\left(\frac{k'_D{}^{endo} + k_p}{k'_H{}^{exo} + k_p}\right)$$

A and B reduce to the previous definitions as the reverse reaction rates (primed quantities) become much smaller than the lumped catalytic rates, k_p. Alternatively, if the primed terms are very large compared to k_p, the reaction is highly reversible with a poor commitment to catalysis, and the expressions for A and B reduce to expressions of equilibrium constants:

$$A = \left(\frac{k_H^{endo}}{k'_H{}^{endo}}\right)\left(\frac{k'_D{}^{exo}}{k_D^{exo}}\right) = K_H^{endo}/K_D^{exo}$$

$$B = \left(\frac{k_H^{exo}}{k'_H{}^{exo}}\right)\left(\frac{k'_D{}^{endo}}{k_D^{endo}}\right) = K_H^{exo}/K_D^{endo}$$

With these modified definitions for A and B it is clear that the derived values for I_{exo}, I_{endo}, and G may be masked by the reversible step. Thus, the large isotope effects observed in microsomal systems might result from an effectively irreversible hydrogen abstraction as a result of much faster oxygen addition to the carbon-centered radical, whereas in the camphor hydroxylase system the oxygen-transfer step may be slower and therefore allow masking to occur. If the hydrogen abstraction step in the camphor hydroxylase system is indeed reversible, then one might predict a $P450_{cam}$-dependent racemization at the 5 carbon, although such an aspect of the reaction would complicate the analysis and render a unique solution in terms of individual rates unlikely.

The geometry of the transition state for the hydrogen abstraction step can also limit the value of the observed isotope effect (Kresge, 1977). For a colinear transition state of donor, hydrogen, and acceptor atoms, the intrinsic isotope effect depends on the position of the hydrogen atom in the transition state, being a maximum when the hydrogen is midway between the donor and acceptor. Hence, the isotope effect is directly dependent on the relative hydrogen atom affinities of donor and acceptor (Melander, 1960). Geometrical nonlinearity in the transition state can also decrease the intrinsic isotope effect (Melander & Saunders, 1980). The loss in stereochemistry during camphor hydroxylation suggests that the hydrogen abstraction species is situated between the exo and endo hydrogens, which would be expected to give rise to a nonlinear transition state and a small value for I_{exo}, I_{endo}, or both. The nature of the hydrogen-abstracting species with respect to its hydrogen atom affinity relative to the C–H bond energy of camphor, the nonlinearity of the transition state, and the reversibility of the actual abstraction step are all potentially important in determining the value of the isotope effect actually expressed. Unfortunately, since the identity of the abstracting species and its geometrical positioning are unknown

at present, no absolute value for these quantities can be determined. As a final comment on the masking of intramolecular isotope effects in this P450 system, we note that a substantial secondary isotope effect may be operating in the hydrogen abstraction event. Thus, heavy atom motion linked to carbon orbital rehybridization during hydrogen transfer could modulate the observed isotope effects by altering the activation energy for the deuterium-containing transition state relative to that for the hydrogen material (Cleland et al., 1977). Note, however, that to first order, all secondary isotope effects cancel in the determination of G, the geometrical partitioning factor, since both 5-*exo*-d_1 and 5-*endo*-d_1 substrates are used, with the experimentally determined quantities A and B appearing only as a ratio.

Finally, we wish to discuss the stereochemical course of cytochrome $P450_{cam}$ oxygenation and its implication for the mechanism of alkane hydroxylation. Critical to these discussions is the observation that all oxygenation reactions of $P450_{cam}$ display remarkably similar loss of absolute stereochemistry, Table II. A previous study on isotope effects in a purified microsomal P450 preparation (Groves et al., 1978) examined the effect of deuterium on the ratio of *exo*- vs. *endo*-norborneol production from *exo,exo,exo,exo*-2,3,5,6-tetradeuterionorbornane and concluded that the data were consistent with a large intrinsic isotope effect. In addition, some of the *exo*-norborneol produced in the enzymatic reaction retained all four deuterium atoms and must have originated by abstraction from the endo position of the substrate. Their data were consistent with the formation of a carbon-centered radical intermediate undergoing a partial loss of stereochemistry at the active site of the enzyme and argued against a carbonium ion intermediate, since such a species might be unable to capture oxygen in the endo position. The partial loss of stereochemistry in the microsomal P450 hydroxylation of norbornane was rationalized by assuming that the radical intermediate could move in the active site of the enzyme and could thus be captured on the opposite face from which hydrogen abstraction occurred. In contrast to hepatic P450, where more than one alcohol product is formed in the reaction, our data document a substantial loss of stereochemistry at carbon 5 of camphor since the product 5-exo-hydroxycamphor retains a substantial fraction of deuterium when formed from either camphor-5-*exo*-d_1, camphor-5-*endo*-d_2, or camphor-5-*endo*-d_1. Such a loss in stereochemistry could be rationalized by assuming positional motion of the substrate radical in the active site following hydrogen abstraction, although such a mechanism might be expected to allow the formation of a significant amounts of endo alcohol (not observed), since norbornyl-type radicals display only a modest exo selectivity in their capture when compared to the almost exclusive exo capture of norbornyl-type cations (Bartlett et al., 1970). Our data do not exclude the possibility of carbonium ion formation in the camphor system, since, unlike studies with norbornane in hepatic P450 preparations, no endo alcohol is produced. Thus, it is possible that either an endo or exo hydride is removed by the enzyme in a concerted fashion or by sequential hydrogen abstraction and electron transfer to generate a nonclassical carbonium ion that is always captured at carbon 5 in the exo configuration. However, such a mechanism seems unlikely since no iron-oxo species is known to formally abstract hydride anion, and cytochrome $P450_{cam}$ and hepatic microsomal P450 would be expected to proceed through the same mechanism.

Studies on the stereochemical course of microbial sterol hydroxylations have shown that in most cases the reaction proceeds with an almost complete retention of configuration (Bergstrom et al., 1958; Corey et al., 1958), although in one case the hydroxylation occurred with only 69% retention (Hayano et al., 1958). A recent study in the side chain cleavage reaction of adrenal mitochondrial cytochrome P450 (Byon & Gut, 1980) demonstrated that 22*R* hydroxylation of cholesterol, the first step in side chain cleavage to yield pregnenolone, retains 90% of a tritium label if 22(*S*)-[^{3}H]-cholesterol is used as substrate. As our study shows, it would be incorrect to conclude that the 22 hydroxylation of cholesterol occurs with complete retention of configuration since the tritium isotope effect must also be considered. Indeed, the analogous reaction in our work, the 5-exo hydroxylation of camphor-5-*endo*-d_1, shows an 80% retention of the deuterium label and yet represents a significant loss of absolute stereochemistry. Our results are consistent, however, with a model in which the *hydrogen-abstracting and oxygen-adding species are separate and distinct entities*, where the former can abstract hydrogen from either the exo or endo positions, while the latter is positioned over the exo face of the camphor skeleton such that the exo alcohol is always formed upon radical recombination. Such a model can also aid in interpreting the results observed in the microsomal P450 systems.

An additional important observation gleaned from this study concerns the similarity in observed intramolecular isotope effects occurring in the hydroxylation of camphor by either the reconstituted NADH/O_2-dependent system or the reactions of *m*-CPBA, iodosobenzene, or hydrogen peroxide with ferric cytochrome $P450_{cam}$. This suggests a mechanism for these reactions that involves a common hydrogen-abstracting agent. This species might be formally a perferryl intermediate, which could be generated from all three types of exogenous oxidants through heterolysis of an iron-bound peracid or peroxide or by direct transfer of an oxygen atom from iodosobenzene to iron (Lichtenberger et al., 1976). Such a mechanism for cytochrome P450 involving a perferryl intermediate has been previously suggested. In the "oxygen-rebound" mechanism (Groves & McClusky, 1976), the role of the iron-oxo species is 2-fold, first in the abstraction of a hydrogen atom from the substrate and second in the addition of oxygen to the carbon-centered substrate radical. This model seems, however, inconsistent with the loss of stereochemistry observed during $P450_{cam}$-catalyzed camphor hydroxylation. A suitable modification of this mechanism can involve generation of a radical center, X·,[4] at a site on the protein removed from the iron-oxo-porphyrin prosthetic group. Such a higher oxidation state of cytochrome $P450_{cam}$ might be similar to the structure "ES" in cytochrome *c* peroxidase (Yonetani et al., 1966; Poulos & Kraut, 1980) and could be generated by two conceptual pathways. Production of a $[(P)Fe^{5+}O^{2-}]^{3+}$ state by heterolysis of the oxygen–oxygen bond of O_2, ROOH, or RCO_3H bound to the heme iron could generate X· by hydrogen abstraction or electron transfer. Alternatively, homolysis of the peroxide bond could yield X· in a concerted process

[4] As the designation of the higher oxidation states of iron-porphyrin systems can become confusing, we suggest the following nomenclature. The porphyrin entity carries a formal charge of 4– (two propionic acid anions and two pyrrole nitrogen anions) and is designated P. Thus, the one electron oxidized porphyrin (π cation radical) is written as $P^{+}\cdot$. Iron is designated with its proper oxidation number and iron-bound oxygen with its formal charge. Thus, compound I in horseradish peroxidase is written as $[(P^{+}\cdot)Fe^{4+}O^{2-}]^{3+}$. The charge outside the brackets is written in this case as 3+ and represents the sum of the charges in the complex. Cytochrome *c* peroxidase, with one oxidation equivalent off the porphyrin ring, is written as $[(P)Fe^{4+}O^{2-}(X^{+}\cdot)]^{3+}$ where $X^{+}\cdot$ is the one electron oxidized species.

(Blake & Coon, 1981). The substrate radical intermediate is then envisioned to be generated via hydrogen transfer from either the exo or endo position of the substrate to X· with stereospecific radical recombination with the iron-bound oxidizing species giving the observed 5-exo alcohol product.

Summary

We have shown that cytochrome $P450_{cam}$ forms only 5-*exo*-hydroxycamphor from camphor deuterated at either the 5-exo or 5-endo positions in an oxygen-dependent reaction using pyridine nucleotide or in the reactions of ferric P450 with peracid, peroxide, or iodosobenzene as oxygen donors. All reactions occur with some loss of stereochemistry at the carbon undergoing oxidation and yield only a single product, 5-*exo*-hydroxycamphor. These results can be rationalized by assuming different identities for the hydrogen-abstracting and oxygen-donating species. Although a small intermolecular isotope effect was detected on the reaction velocity, a substantial intramolecular isotope effect was observed with all oxidants examined. This partially masked intramolecular isotope effect is consistent with a reversible hydrogen abstraction step and/or a highly nonsymmetric transition state. Furthermore, the similar isotope effects seen with the various oxidants included in this work suggest that a common intermediate is responsible for hydrogen abstraction in alkane hydroxylation by cytochrome $P450_{cam}$. The nature of the active oxygen and hydrogen-abstracting centers continues to receive extensive investigation in our laboratory.

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