Using eq A8 and A9 (and their analogous forms for more heavily labeled species) and substituting for f_1 and f_2, we obtain:

$$3(A + B)\,(\mathrm{d}f_p/\mathrm{d}t) = (k_{1c}A + k_{2c}B)f_w - (k_{1c}Af_1 + k_{2c}Bf_2) \quad \text{(A11)}$$

This equation becomes identical with eq 21 of Fordham and Wang[5]

$$3(A + B)\,(\mathrm{d}f_p/\mathrm{d}t) = (k_{1c}A + k_{2c}B)(f_w - f_p) \quad \text{(A12)}$$

only if $f_1 \approx f_2 \approx f_p$. These equalities only hold under two circumstances, *either if*

$$k_{c1}/k_{c2} = [A/B]_{eq}$$

which states that the cyclic diester partitions to **1** and **2** in the same ratio as the equilibrium proportions of **1** and **2**, *or if*

$$k_{12}, k_{21} \gg k_{1c}, k_{2c}$$

which states that **1** and **2** equilibrate much more rapidly than any wash-in of solvent label occurs via c. Since neither of these limiting conditions holds, the equation of Fordham and Wang (A12) cannot be used. The rate constants were therefore evaluated as follows.

The term in large square brackets in eq A7 is known from the slope of Figure 1. Since we know K_{eq} and θ from independent direct measurement, we can express k_{12} in terms of k_{1c}. Now with use of eq A5, k_{21} and k_{2c} are determined. We therefore vary k_{1c} (and in accord, k_{12}, k_{21}, and k_{2c}) and, using a simple computer program, derive a best fit to the plot of f_p vs. time (Figure 2). The solid line plotted in Figure 2 is the "best-fit" line obtained with this procedure, and the kinetic constants listed in Table I are derived from this fit. For the sensitivity of the experimental data to the value of k_{1c} to be illustrated, Figure 2 contains the predicted lines if k_{1c} is 25% larger than the best-fit value (upper dashed line) or 25% smaller than the best-fit value (lower dashed line).

Aliphatic Hydroxylation by Cytochrome P-450. Evidence for Rapid Hydrolysis of an Intermediate Iron–Nitrene Complex

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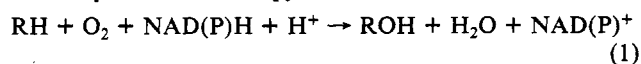
Abstract: Studies with synthetic metalloporphyrin-based hydrocarbon oxidation systems have suggested that the cytochrome P-450 class of heme-containing enzymes may be able to transfer a nitrene equivalent to a hydrocarbon substrate in direct analogy to their currently known ability to transfer an oxene equivalent. Exposure of cyclohexane to the nitrenoid reagent (*N*-(*p*-toluenesulfonyl)imino)phenyliodinane (**1**) in the presence of a purified cytochrome P-450 isozyme (P-450_{LM2}) did not result in any amidation of the hydrocarbon. Instead, cyclohexane was efficiently hydroxylated to cyclohexanol. Hydroxylation was also observed with two other hydrocarbons, methylcyclohexane and *p*-xylene. This reaction is a true catalytic activity of the P-450. Anaerobic and isotope-labeling studies demonstrated that the oxygen in cyclohexanol derived from the water of the reaction medium. These results are interpreted by a scheme whereby the nitrenoid reagent generates a transient iron(V)–nitrene complex which is rapidly hydrolyzed in the aqueous environment to the corresponding iron(V)–oxo complex, which then hydroxylates the substrate.

The cytochromes P-450 are a class of heme-containing enzymes able to catalyze the introduction of a single oxygen atom into an unactivated substrate from atmospheric dioxygen. During this process the other oxygen atom from dioxygen is reduced to water at the expense of reduced pyridine nucleotide.[1]



$$RH + O_2 + NAD(P)H + H^+ \rightarrow ROH + H_2O + NAD(P)^+ \quad (1)$$

This monooxygenation reaction is formally an oxene transfer and is suggestive of sequestration of a single reactive oxygen atom at the enzyme's catalytic site as, for instance, a high-valent iron–oxo species ($Fe^V{=}O$). Unfortunately, characterization of the P-450 reactive oxygen intermediate has been hindered by the lack of a stable, readily observable intermediate as is available in the peroxidase series (i.e., compound I).[2] Thus, the only information that has been gleaned about the P-450 intermediate is the result of indirect inferences from studies of special substrates, artifical oxidants, and synthetic models. A number of the model systems have employed iodosylarenes as oxygen donors with striking success.[3] Indeed, the P-450 enzymes themselves may use iodosylbenzene to supplant dioxygen and NADH in aliphatic hydroxylation.[4] Recently, Groves[5] and Breslow[6] have revealed metalloporphyrin-based catalytic systems capable of transferring a nitrene equivalent to aliphatic and olefinic hydrocarbons. We now report the results of similar experiments using an isolated P-450 enzyme and (*N*-(*p*-toluenesulfonyl)imino)phenyliodinane (**1**), one of the nitrene sources used by Breslow.

Experimental Section

Analytical Procedures. Infrared spectra were recorded on a Perkin-Elmer 735 instrument with potassium bromide pellets with use of the 1601-cm^{-1} band of polystyrene for calibration. Proton NMR spectra were determined on a Varian EM-360 instrument at 60 MHz, referenced

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to tetramethylsilane as internal standard. Gas chromatography was performed on a Varian 3700 Chromatograph equipped with a flame ionization detector. The columns used were the following: column A, 3% OV-17 on Supelcoport (80/100), 6 ft × $^1/_8$ in.; column B, 10% Carbowax 20M on Supelcoport (80/100), 6 ft × $^1/_8$ in.; column C, 0.2% Carbowax 1500 on Carbopack C (80/100), 5 ft × $^1/_8$ in. Integration of gas-chromatographic peaks was handled by a Hewlett-Packard 3990A Integrator with use of the internal standard technique for absolute quantitation. Gas chromatography–mass spectrometry (GC-MS) was performed on a Hewlett-Packard 5992 instrument with use of electron-impact ionization at an energy of 70 eV. Ultraviolet–visible spectra were determined on a Varian-Cary 219 spectrophotometer. Melting points were measured on a Thomas-Hoover melting point apparatus. Iodometry was performed according to a standard procedure.[7] Elemental analyses were performed by Micro-Tech Laboratories, Inc., Skokie, IL.

Materials. The homogeneous enzyme cytochrome P-450$_{LM2}$ was isolated from livers of phenobarbital-treated rabbits as described previously.[8] The phospholipid, dilauroylglyceryl-3-phosphorylcholine, was purchased from Sigma Chemical Co. and used as a sonicated suspension in water. The tosylamides, *p*-toluenesulfonamide, *N*-cyclohexyl-*p*-toluenesulfonamide, and *N*-phenyl-*p*-toluenesulfonamide were prepared by the Schotten-Baumann reaction of *p*-toluenesulfonyl chloride with the corresponding amine. Other organic chemicals were obtained from Aldrich Chemical Co. Stohler Isotope Chemicals was the source of the 99% ^{18}O water. BASF catalyst was obtained from Ace Glass Co. Iodosylbenzene was prepared from diacetoxyiodobenzene by a literature procedure.[9]

(*N*-(*p*-Toluenesulfonyl)imino)phenyliodinane (1). The procedure described by Yamada et al. was followed.[10] Diacetoxyiodobenzene (3.2 g, 10 mmol) was added to a solution of KOH (1.4 g, 25 mmol) and *p*-toluenesulfonamide (1.7 g, 10 mmol) in methanol (40 mL) at 8 °C. A yellow color developed within 1 min. The cooling bath was removed, and the solution was allowed to warm to room temperature as it was stirred for 3 h. Water (45 mL) was added, and the solution was refrigerated overnight at 0 °C. The precipitate was collected by filtration and air dried to yield 2.9 g of a light-yellow powder. Iodometry indicated 56% purity. The powder was dissolved in methanol (60 mL), a small residue was removed by filtration, and water (60 mL) was added. This solution was refrigerated at −20 °C overnight. Filtration and air drying of the precipitate afforded **1** as a finely divided yellow powder (2.0 g, 52% yield). This material extensively decomposed over the range 90–120 °C. Iodometry indicated this material to be 99.3% pure as $C_{13}H_{12}INO_2S$. Anal. ($C_{13}H_{12}INO_2S$) C, H, N. Reduction of the powder with excess aqueous sodium bisulfite afforded 1 mol of iodobenzene and 1 mol of *p*-toluenesulfonamide per mol of **1** as the only products, as determined by gas chromatography on column A. Infrared spectrum: 3067 (w), 1593 (w), 1571 (w), 1468 (w), 1436 (w), 1232 (m), 1209 (s), 1123 (s), 1087 (s), 933 (s), 811 (m), 728 (w), and 651 (m) cm^{-1}. NMR spectrum (in CD_3OD): 2.45 (singlet, 2.6 H), 7.2–8.4 ppm (11 line multiplet, largest peak at 7.78 ppm, 9.0 H). UV-vis spectrum: (in MeOH) λ_{max} 222 (ϵ 109 000 M^{-1} cm^{-1}), 400 (end absorption, ϵ 0.97 M^{-1} cm^{-1}; in 25% H_2O–75% MeOH) λ_{max} 225 (ϵ = 89 000 M^{-1} cm^{-1}), 400 nm (end absorption, ϵ 1.5 M^{-1} cm^{-1}).

Enzymatic Hydroxylations. Reaction mixtures contained P-450$_{LM2}$ (3–5 nmol), phospholipid (100–200 μg), one of the various substrates, potassium phosphate buffer (100 μmol, pH 7.4), and the oxidant (either **1**, iodosylbenzene, or cumene hydroperoxide, added last as a concentrated solution in MeOH) in a total volume of 1 mL of water. Solutions of the iodine-based oxidants were used within minutes of their preparation. In some control experiments, P-450$_{LM2}$ was omitted from the reaction mixture. In others, the enzyme was gently denatured to the P-420 form by careful heating at 60 °C for about 10 min. After being cooled to 25 °C, the reaction was initiated as usual. This gentle procedure avoided enzyme precipitation. Reactions were conducted at 25 °C in closed vials and were terminated after an appropriate reaction interval by injection of 0.3 mL of 2 M NaOH/0.1 M Na_2SO_3. A suitable aliquot of internal standard was injected and the reaction mixture was extracted twice with 1-mL portions of chloroform. After concentration of the combined extracts to about 0.1 mL under a stream of dry nitrogen, the sample was analyzed for products by gas chromatography. The product, GC column, and internal standard for each analysis were as follows: *N*-cyclohexyl-*p*-toluenesulfonamide, column A (220 °C), *N*-phenyl-*p*-toluenesulfonamide; cyclohexanol, column B (100 °C), *trans*-4-methylcyclohexanol; isomeric methylcyclohexanols, column C (140 °C), benzyl alcohol; *p*-methylbenzyl alcohol, column A (120 °C), benzyl alcohol

For the anaerobic experiments, the reaction vials were flushed for 20 min through a needle and septum with nitrogen which had been deoxygenated by passage over a column of reduced BASF catalyst maintained at 150 °C. The solution of **1** was also deoxygenated in this manner prior to injection to initiate the reaction. For the ^{18}O-label experiment, 0.2 mL of 99% $H_2{}^{18}O$ was added to the reaction mixture. The remainder of the water required to bring the volume to 1.0 mL was ordinary. These vials were also deoxyygenated prior to reaction initiation. The product of the ^{18}O-labeling experiment was analyzed for heavy oxygen content by GC-MS on column B (150 °C) with use of the selected ion monitoring mode (SIM). Areas were determined by computer integration of the intensity of the ions m/z 107,109,122, and 124 with respect to time at the *p*-methylbenzyl alcohol chromatographic peak. Isotope abundances were calculated from these areas.

Results and Discussion

The nitrenoid reagent chosen for study in these experiments is (*N*-(*p*-toluenesulfonyl)imino)phenyliodinane (**1**; p-$CH_3C_6H_4SO_2N{=}IC_6H_5$), the imino analogue of iodosylbenzene. This representation of **1** is convenient for the purposes of our discussion, but the solid substance may be polymeric in analogy to the iodosylarenes.[11] Hill has shown that the latter compounds "dissolve" in alcohol through an alcoholysis reaction to yield a soluble dialkoxyiodinane monomer (e.g., $PhI(OCH_3)_2$). The solubility properties of **1** are in accord with this idea. It is soluble to about 0.17 M in methanol; it is also soluble in dimethyl sulfoxide and dimethylformamide, moderately soluble in dimethoxyethane, and slightly soluble (ca. 0.01 M) in tetrahydrofuran. The solid reagent is essentially insoluble in water, diglyme, dioxane, ether, acetonitrile, acetone, dichloromethane, benzene, and pentane. Thus, dissolution of **1** requires a coordinating solvent, but **1** is too hydrophobic to be appreciably soluble in water and apparently is not solvolyzed to a monomer in nonnucleophilic solvents. However, the molecular nature of the species in methanol solution is not clear. If **1** were solvolyzed to dimethoxyphenyliodinane by methanol, as is iodosylbenzene,[11] then we would not expect to recover **1** efficiently from repeated methanol recrystallizations. Furthermore, **1** is much less soluble in methanol than is iodosylbenzene (0.17 vs. > 2.3 M).[11] If both **1** and iodosylbenzene react to form $PhI(OCH_3)_2$, then their apparent solubilities ought to be similar. Therefore, we suggest that **1** retains an I–N bond in methanol solution. However, rigorous solution structural studies will be needed to resolve this question.

Breslow was able to demonstrate transfer of the tosylnitrene equivalent from **1** to cyclohexane mediated by iron(II) tetraphenylporphyrin.[6a] Since cyclohexane is also a good substrate in the liver microsomal cytochrome P-450 enzyme system, we used it as the potential nitrene acceptor in this work as well. The expected reaction would then be:

$$p\text{-}CH_3C_6H_4SO_2N{=}IC_6H_5 + C_6H_{12} \rightarrow p\text{-}CH_3C_6H_4SO_2NH\text{-}C_6H_{11} + C_6H_5I \quad (2)$$

However, at concentrations of **1** ranging from 1 to 4 mM, the isolated isozyme we were using (P-450$_{LM2}$) displayed no detectable nitrene transferase activity toward cyclohexane. From the detection limit of the GC method employed, we can set an upper limit of 3×10^{-5} s^{-1} for the turnover number of P-450$_{LM2}$ in the catalysis of eq 2.

When **1** (0.5 mM) was mixed with P-450$_{LM2}$ in the absence of a hydrocarbon substrate, spectrophotometry indicated that **1** immediately bound to the enzyme's catalytic site (a strong Type I binding spectrum resulted).[12] We observed no spectrum which could be attributed to a high-valent iron intermediate. Subsequently, the heme absorption was bleached in a first-order process with $k_{obsd} = 1.5 \times 10^{-3}$ s^{-1} ($t_{1/2}$ = 7.7 min). Thus, **1** was apparently binding to the enzyme and oxidizing the heme but not in a manner

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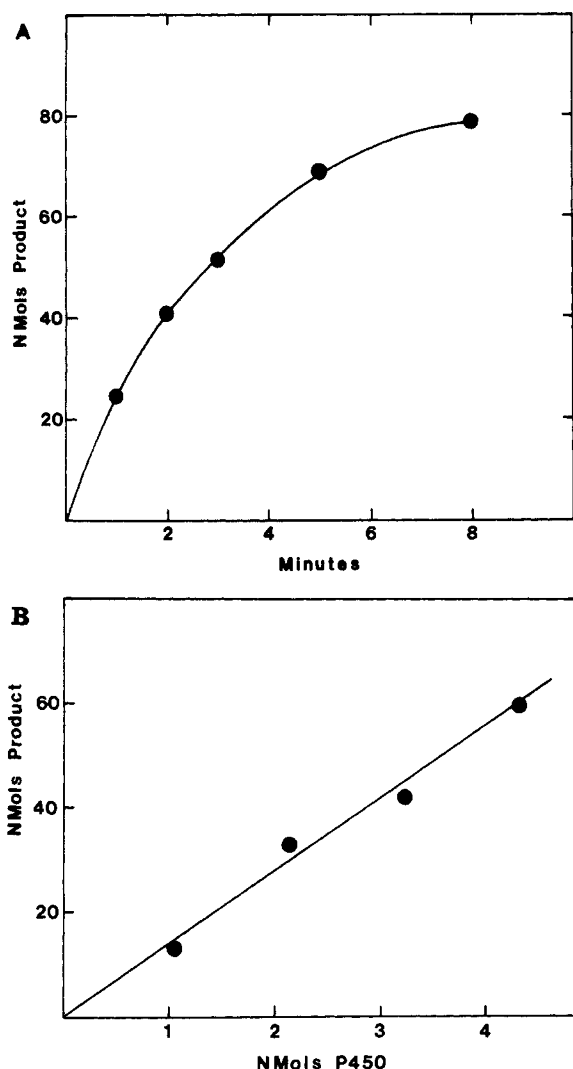

Figure 1. Hydroxylation of cyclohexane by **1** and cytochrome P-450. The concentration of cyclohexane was 10 mM. Other conditions were as in the Experimental Section. (A) Accumulation of cyclohexanol as a function of time. The concentration of $P\text{-}450_{LM2}$ was 3.6 μM and the concentration of **1** was 2 mM. (B) Total nmol of cyclohexanol accumulated after a 2-min reaction period as a function of P-450 concentration. The concentration of **1** was 4 mM.

Table I. Reaction Requirements for Hydroxylation of Cyclohexane

reaction components	turnover no.,[a] min^{-1}
complete[b]	5.03 ± 0.37
P-450 omitted	0
P-450 denatured	0.37 ± 0.02
O_2 absent	4.85 ± 0.10

[a] Average ± deviation of duplicate determinations. [b] Concentrations of enzyme, cyclohexane, and **1** were 3.2 μM, 10 mM, and 4 mM, respectively. Reactions were carried out as described under the Experimental Section. Reaction periods were 2 min.

allowing nitrene transfer to cyclohexane.

Upon further examination of the reaction mixtures from the cyclohexane experiments we discovered a substantial amount of cyclohexanol had been produced. Figure 1A shows the time course for the accumulation of cyclohexanol in the reaction of **1** with cyclohexane as catalyzed by $P\text{-}450_{LM2}$. The reaction rate decreases with time (due to irreversible heme oxidation), the half-life for the decay being around 3 min under these conditions. The half-life is longer when lower concentrations of **1** are employed. That the enzyme is truly catalytic in this reaction is shown by the fact that 3.6 nmol of $P\text{-}450_{LM2}$ produced about 80 nmol of cyclohexanol in the time period covered by Figure 1A. The reaction course is approximately linear for the first 2 min, so

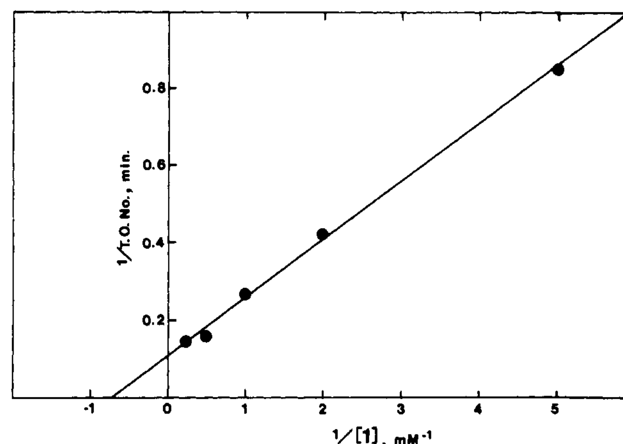

Figure 2. Lineweaver–Burk plot of the initial rate of cyclohexane hydroxylation as function of concentration of **1**. The concentration of $P\text{-}450_{LM2}$ was 3.6 μM. Other conditions were as in Figure 1B. The correlation coefficient of the linear regression line drawn is 0.999.

Table II. Mass Spectra of *p*-Methylbenzyl Alcohol[a]

oxidant	^{18}O content of water[b]	ratios of peaks (m/z)[c] 109/107	124/122	% incorporation of water oxygen
1	normal	0.0028	0.0053	
1	enriched	0.2534	0.2309	97
PhI=O	enriched	0.2423	0.2118	91
CHP[d]	enriched	0.0014	0.0021	0
$NADPH/O_2$[e]	enriched	0.0028	0.0044	0
none[f]	enriched	0.0041	0.0059	0
theory	enriched	0.0046	0.0054	0
theory	enriched	0.2520	0.2527	100

[a] Samples of product alcohol resulting from hydroxylation of *p*-xylene by P-450 and various oxidants. Oxidant concentrations were the following: **1**, 4 mM; PhI=O, 2 mM; CHP, 6 mM. The concentration of *p*-xylene was 10 mM. The reaction times were 5 min, except for $NADPH/O_2$ which was 1 h. Enzyme concentration was 3.2 μM. [b] Atom percent ^{18}O of water in the enzymatic reaction medium. Normal: 0.20%; enriched 20.0%. [c] Ion intensity peak areas were measured by the selected ion monitoring technique. The ratios of peak areas at different *m/z* values are compiled here. [d] Cumene hydroperoxide. [e] The normal enzyme system consisting of P-450, P-450 reductase, NADPH, and O_2 was employed. [f] A sample of *p*-methylbenzyl alcohol was incubated with enzyme but no oxidant for 5 min.

subsequent fixed time point rate measurements used a 2-min period. The initial rate is proportional to enzyme concentration, as shown in Figure 1B, as required if $P\text{-}450_{LM2}$ were catalytic.

Additional experiments established the requirements for the reaction and are listed in Table I. The formation of cyclohexanol was completely dependent on the presence of active $P\text{-}450_{LM2}$. When the enzyme was omitted or gently denatured, no cyclohexanol was produced. The small amount of cyclohexanol present in the denaturation experiments correlated well with the residual amount of native enzyme still present after gentle denaturation. On the other hand, when the reaction was carried out in the absence of molecular oxygen cyclohexanol production was unaffected, indicating that dioxygen cannot be the source of the oxygen atom in the cyclohexanol. The initial rate displayed saturation with respect to the concentration of **1**, as expected for an enzyme-catalyzed reaction. The dependence of the rate on the concentration of **1** is displayed in Figure 2 as a Lineweaver–Burk plot. From the *X* and *Y* intercepts of Figure 2 we calculate the enzymatic kinetic constants as K_m = 1.5 mM and V_{max} = 9.8 mol/mol of P-450/min.

Having established that the reaction is wholly dependent on enzyme and that the enzyme is catalytic in the process, we next sought to answer the question of the origin of the oxygen atom appearing in the product. Since, as shown above, atmospheric dioxygen was not the source, the only possible remaining source is solvent water. To confirm this we conducted a hydroxylation

Table III. Regioselectivity of Hydroxylation of Methylcyclohexane[a]

oxidants[c]	positional isomer distribution,[b] %				total product, nmol
	1	3c	4c	(3t + 4t)	
1	9.8 ± 0.3	55.7 ± 0.4	7.6 ± 0.1	26.9 ± 0.5	131 ± 9
PhI=O	9.7 ± 0.2	59.6 ± 0.8	8.1 ± 0.9	22.6 ± 0.9	139 ± 15
CHP	12.9 ± 0.5	60.8 ± 0.5	10.9 ± 0.5	15.0 ± 0.1	543 ± 79
$NADPH/O_2$[d]	11.4	48.7	6.7	33.3	

[a] Values are listed as the average ± average deviation ($n = 3$). [b] Abbreviations: c is cis, t is trans. [c] The concentration of methylcyclohexane was 10 mM. Other reaction conditions were as in Table II. [d] Reference 13.

reaction in water enriched in ^{18}O. For this experiment we selected *p*-xylene as the hydrocarbon substrate since the product, *p*-methylbenzyl alcohol, has favorable characteristics for the GC-MS analysis. The data are contained in Table II. When either the ion pair 109/107 or 124/122 is used, a substantial presence of ^{18}O was indicated. The conclusion is that the oxygen atom in *p*-methylbenzyl alcohol from hydroxylation of *p*-xylene by **1** is derived from water. Water incorporation was also seen with iodosylbenzene, but *not* with cumene hydroperoxide (CHP) or with $NADPH/O_2$. This marked difference in water exchangeability suggests a profound difference in the nature of the intermediates generated by the iodine-centered oxidants and those generated from CHP or from O_2.

Three possibilities for incorporation of solvent water into product alcohol from **1** are the following: (a) hydrolysis of **1** to iodosylbenzene during the reaction; (b) exchange of alcoholic oxygen for water oxygen during the exposure of the product to water; and (c) hydrolysis of an intermediate imino-iron complex prior to the oxidation of the hydrocarbon substrate. Possibility a may be ruled out since **1** was exposed to water and/or hydroxide for long periods at room temperature during its preparation. Therefore, if hydrolysis could have occurred during a 1-min enzyme incubation, it would already have occurred during preparation of **1**. The exception is that the hydrolysis might be enzyme catalyzed (see below). Possibility b was ruled out on the basis of a control experiment shown in Table II. Incubation of the product *p*-methylbenzyl alcohol with enzyme in ^{18}O-enriched water for 5 min resulted in no exchange of water and alcoholic oxygen atoms. Possibility c may then be the correct explanation of the incorporation of solvent water into the product and, in fact, is virtually required by the phenomenon of substrate hydroxylation in any event.

As a further probe of the nature of the species which transfers oxygen to the substrate, methylcyclohexane was chosen as an indicator of regioselectivity. Not counting enantiomers, there are eight possible positional isomers and stereoisomers of methylcyclohexanol. Of these eight isomers, our GC method using column C was able to resolve six. The other two isomers eluted as a single peak, making a total of seven GC peaks which could be determined. Three different oxidants were allowed to hydroxylate methylcyclohexane. Only four GC peaks were observed, corresponding to 1-, 3-*cis*-, 4-*cis*, and 3-*trans*- + 4-*trans*-methylcyclohexanol. The other possible isomers contributed only a few percent to the product mixture and were ignored. The absence of 2-hydroxylation probably reflects steric hindrance to hydrogen abstraction adjacent to the methyl group, while the methyl group itself is little attacked because it is primary. Table III lists the product distributions from the various oxidants. Literature data for hydroxylation of methylcyclohexane by the normal $NADPH/O_2$ enzyme system are also included for comparison. While small but statistically significant differences exist between the product distributions, the qualitative patterns are similar, suggesting similar enzyme-bound reactive-oxygen species among all the hydroxylating agents. In particular, the product distributions from **1** and from PhI=O are very close to one another. Such a result would be expected if both **1** and PhI=O generated an iron-oxo species (Fe^V=O) as the proximate oxidant of substrate.

This idea is incorporated into Figure 3. We suggest that **1** reacts with ferric iron, extruding iodobenzene and generating a transient iron-nitrene complex. Attack of water on this complex is fast, such that hydrolysis to the iron-oxo species is complete

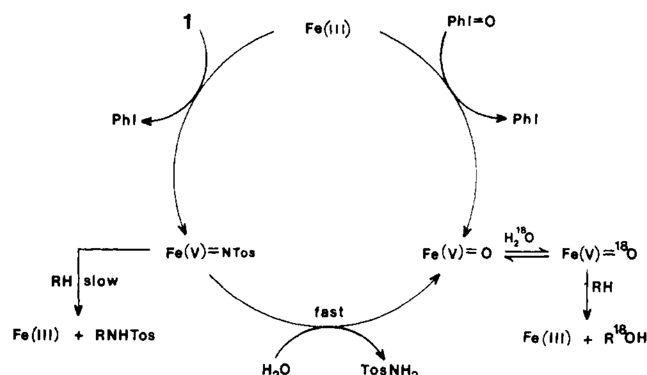

Figure 3. Possible mechanistic scheme to account for hydroxylation of hydrocarbons by **1** and cytochrome P-450.

before nitrene transfer to substrate (RH) can occur. Iodosylbenzene may directly convert ferric iron to the iron-oxo complex, which readily exchanges oxygen with solvent water regardless of its mode of formation. Such facile oxo-oxygen exchange with water has been previously unequivocally demonstrated in iron,[14] manganese,[15] and chromium[16] based metalloporophyrin P-450 model systems.

We have considered an alternative scheme in which P-450 catalyzes the hydrolysis of **1** to PhI=O, which then reacts with iron in the same manner as does exogenously added PhI=O. Of course, we do not know the direction of the thermodynamic equilibrium between **1** and PhI=O, so we cannot say if the hydrolysis is feasible. Furthermore, such a hydrolytic reaction would have to be extemely fast to convert the large excess of **1** in these reaction mixtures (4 mM **1** vs. 3 μM $P\text{-}450_{LM2}$) to PhI=O before **1** could react with iron. Since Figure 1A shows no lag we estimate the $k_{cat.}$ for the hypothetical enzymatic hydrolysis would have to be greater than 50 000 min^{-1}. However, the data in Table III show that **1** is just as efficient a hydroxylator as is PhI=O. Therefore, we believe the alternative scheme to be unlikely.

Finally, we observed that there was no incorporation of solvent water into product with CHP or with $NADPH/O_2$. Heimbrook and Sligar[4] previously observed this contrast between iodosylbenzene and other oxidants using bacterial cytochrome P-450. The reactions observed in this study, using **1** or iodosylbenzene, exhibit the behavior predicted on the basis of model iron porphyrin studies[14] for an iron-oxo intermediate, namely identical regioselectivity regardless of oxidant and water exchangeability. On the other hand, the peroxide-based oxidants, CHP and $NADPH/O_2$, never passed through an intermediate state that was able to exchange with water, suggesting the absence of an iron-oxo species with them. Whether this dichotomy results from two fundamentally different sorts of intermediates or merely differences in the kinetics of the respective hydroxylation events remains to be discovered. However, it is clear that we are not yet able to account for the total range of phenomena which may occur at the active sites of these interesting enzymes.

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Conclusion

(*N*-(*p*-Toluenesulfonyl)imino)phenyliodinane appears to react with the heme iron of cytochrome P-450 to form a transient iron(V)-nitrene complex. With the substrates studied here (cyclohexane, methylcyclohexane, *p*-xylene) the nitrene complex undergoes hydrolysis to the corresponding iron(V)-oxo complex before transer of the nitrene to a C-H bond of the substrate can occur. The iron-oxo complex is able to attack substrate, with hydroxylation being the net result. The synthetic metalloporphyrins upon which these enzymatic studies are based should be examined to determine if the nitrene hydrolysis is part of the normal chemistry of such species or if it represents a unique catalytic property of cytochrome P-450.

Acknowledgment. We thank Dr. Stephen G. Sligar for initially bringing to our attention the model work on iminoiodinane nitrenoid reagents. We thank Dr. John B. Schenkman for the use of the gas chromatograph-mass spectrometer in his laboratory. This work was supported by Research Grant GM28737 from the National Institutes of Health and by the University of Connecticut Research Foundation.

Registry No. 1, 55962-05-5; CHP, 80-15-9; PhI=O, 536-80-1; cytochrome P-450, 9035-51-2; cyclohexane, 110-82-7; methylcyclohexane, 108-87-2; *p*-xylene, 106-42-3.

Acid-Base Catalysis of the Elimination and Isomerization Reactions of Triose Phosphates

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Abstract: The nonenzymatic β-elimination, isomerization, and racemization reactions of L-glyceraldehyde 3-phosphate (LGAP) are through a common enediolate intermediate which partitions between leaving group expulsion, C-1 protonation, and C-2 protonation, respectively. The elimination reaction mechanisms of LGAP and dihydroxyacetone phosphate (DHAP) are Elcb because enolate intermediates have been identified in very closely related systems.[13,14] Strong general base catalysis of elimination demonstrates that the enediolate intermediate is formed essentially irreversibly by rate-determining substrate deprotonation. The pH rate profile for the elimination reaction of LGAP is first order in hydroxide at pH >10 due to direct substrate deprotonation by hydroxide, pH independent at pH 6-10 due to intramolecular deprotonation by the C-3 phosphate dianion, and first order in hydroxide at pH <6 due to a decrease in the reactive phosphate dianion form of the substrate. The Brønsted β values for buffer catalysis of DHAP and LGAP elimination by 3-substituted quinuclidines are 0.48 and 0.45, respectively. The solvent deuterium isotope effect on the second-order rate constant for quinuclidinone catalysis of DHAP elimination is 1.1. At >0.2 M concentrations of 3-oxo- and 3-hydroxy-substituted quinuclidine buffers there is curvature in the buffer catalysis plots for the elimination reaction of DHAP which is attributed to a change from rate determining substrate deprotonation to partially rate determining leaving group expulsion. The isomerization and racemization reactions of LGAP were followed by coupling the formation of DHAP and DGAP to enzymatic NADH oxidation under conditions where >90% of the product is from the elimination reaction. Uncatalyzed racemization is five times slower than uncatalyzed isomerization, and buffer-catalyzed racemization is estimated to be >20 times slower than buffer-catalyzed isomerization. The observed rate constant for LGAP isomerization is second order in total buffer concentration; the basic form of the buffer acts to increase the steady-state concentration of the enediolate, and the acidic form of the buffer increases the fractional partitioning of the enediolate to DHAP. Rate constant ratios k_{BH}/k_e and k_{-0}/k_e for partitioning of the enediolate between buffer-catalyzed or uncatalyzed protonation (k_{BH} or k_{-0}) and leaving group expulsion (k_e) were obtained from the slopes and the intercepts respectively of linear plots of the isomerization/elimination rate constant ratio against buffer concentration. The Brønsted α value for enediolate protonation at C-1 is 0.47. The $(k_{BH}/k_e)_{H_2O}/(k_{BD}/k_e)_{D_2O}$ ratio for quinuclidinonium catalysis in H_2O and D_2O is 3.2. The pH dependence plot of k_{BH}/k_e values shows pH-independent regions at pH <7 and >10, with a 100-fold greater limiting k_{BH}/k_e value at pH >10. The increased k_{BH}/k_e value at high pH is due to slower leaving group expulsion from the enediolate phosphate dianion compared to that of the enediolate phosphate monoanion. The nonenzymatic reactions of triose phosphates are compared with the enzymatic reactions catalyzed by triose phosphate isomerase and methylglyoxal synthase.

Triose phosphates undergo isomerization and elimination reactions in water,[1,2] and it has been proposed that these reactions are through the enediolate intermediate formed by substrate deprotonation (eq 1).[3] The reversible isomerization and the irreversible elimination reactions of dihydroxyacetone phosphate are catalyzed by the enzymes triose phosphate isomerase[4] and methylglyoxal synthase,[5] respectively.

In this paper we present a study of dihydroxyacetone phosphate (DHAP) and L-glyceraldehyde 3-phosphate (LGAP) isomerization and elimination in water. In contrast to past studies of this system[1-3] which report observed isomerization or elimination rate constants for single sets of reaction conditions, we have determined the uncatalyzed, hydroxide-catalyzed, and buffer-catalyzed components of these rate constants over a wide pH range.

This work was initiated primarily to obtain a good estimate of the rate acceleration for the triose phosphate isomerase catalyzed

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