containing organophosphonate, *E. coli* from the glycerol freeze were spread on control plates containing all of the growth medium components minus the organophosphonate.

Growth in liquid suspension entailed transfer of a single colony into a sterile test tube containing 5 mL of growth medium. The growth medium was identical with that used for growths on solid media with the exception that the agarose was omitted. Test tubes were shaken at 37 °C for 12–18 h or until a cloudy suspension developed. An inoculant (1.5 mL) was subsequently transferred from the test tube growth to a flask (145 mL total volume) containing 50 mL of growth solution. All containers, transfers, and solutions were sterile. The final growth flask was then sealed. After the flask was shaken for 12–18 h, a sample of the headspace of the sealed flask was withdrawn with a gas-tight syringe and analyzed by gas chromatography.

Quantitation of Inorganic Phosphate and Hydrocarbon Formed during *E. coli* Degradation of Methylphosphonates.[23] Methane produced during a growth was quantitated via the appropriate response factor relative to an ethane internal standard. Cells from growths in the sealed flasks were harvested by centrifugation (10000 g for 10 min) at 0 °C. After removal of the supernatant, the cells were twice washed with salt solution (trizma·HCl (64 mM), ammonium chloride (19 mM), and sodium chloride (8 mM)) and harvested by centrifugation (10000 g for 10 min) at 0 °C. The pellet was resuspended in 5 mL of salt solution and a portion (0.1 mL) transferred to each of three borosilicate test tubes. A range of concentrations of inorganic phosphate standards were added to separate test tubes. To each of the tubes containing *E. coli* cells and inorganic phosphate standards was added 5.0 M sulfuric acid followed by heating at 150 °C for 3 h. After the tubes were cooled, hydrogen peroxide (30% aqueous solution) was introduced to each tube and heating once again begun at 150 °C. The tubes containing cell debris were removed as soon as the clear solution began to yellow while the inorganic phosphate standards continued to be heated for 1.5 h at 150 °C. Subsequently 1.8 mL of a solution of ammonium molybdate (1.8 mM) was added to all of the tubes followed by 0.12 mL of Fiske and Subbarow reducer (obtained from Sigma). All of the tubes were then heated at 100 °C and cooled to room temperature, and the optical density of each tube measured at 812 nm. The amount of inorganic phosphate in the digested cell solutions was quantitated by comparison of optical density at 812 nm to the calibration curve derived from the inorganic phosphate standards.

Acknowledgment. Research was supported by the National Institutes of Health (GM36558-01) and the Department of the Army (Contract DAAG29-29-K-0242). The assistance of M. H. Schmidt with the electrochemical analysis was particularly helpful.

The Separation of the Intramolecular Isotope Effect for the Cytochrome P-450 Catalyzed Hydroxylation of *n*-Octane into Its Primary and Secondary Components

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Abstract: The intramolecular isotope effect for the cytochrome P-450b ω-hydroxylation of [1,1,1-$^2H_3$]-*n*-octane was separated into its primary and secondary components by the method of Hanzlik (Hanzlik et al. *J. Am. Chem. Soc.* **1985**, *107*, 7164). The primary isotope effect was found to lie between 7.3 and 7.9 while the secondary isotope effect was found to lie between 1.09 and 1.14. These data are consistent with a highly symmetrical transition state with 15% of the observed isotope effect being due to secondary isotope effects. Although the system was found to depart from the rule of the geometric mean, the phenomenon could not be attributed to tunneling.

Recently, we reported that the intrinsic isotope effect associated with the cytochrome P-450 catalyzed hydroxylation of the methyl group in *n*-octane was 9.8.[1] The large magnitude of this isotope effect and the even larger values reported by others[2,3] suggest that tunneling may contribute significantly to the reaction rate. For example, on the basis of Bell's analysis, the maximum allowable semiclassical isotope effect for cleavage of a carbon hydrogen vs. carbon deuterium bond would be 9 at 37°.[4] Unfortunately, the isotope effects associated with cytochrome P-450 aliphatic hydroxylation have invariably been reported as a combination of primary and secondary effects thereby precluding any assessment of the role tunneling might play in these reactions. We now report the separation of primary and secondary isotope effects, by the method of Hanzlik et al.,[5] for the aliphatic hydroxylation of *n*-octane. These data are consistent with a highly symmetrical transition state and indicate that up to 15% of the observed isotope effect is due to secondary isotope effects.

Experimental Section

Materials. *N,O*-Bis(trimethylsilyl)trifluoroacetamide and acetonitrile were obtained from Pierce Chemical Co., diethyl ether was obtained from J. T. Baker, and pentane was obtained from Burdick and Jackson. All biochemicals were obtained from Sigma Chemical Co.

Instrumentation. Gas chromatography was performed with an HP 5840A gas chromatograph modified for use with a J&W DB-5 or DB-1 capillary column. Difference spectra were recorded on an HP 8451A UV spectrophotometer. GC/MS analysis of the product alcohols was performed on a VG 7070H mass spectrometer in the selected ion recording mode, interfaced to a HP-5710A GC fitted with a J&W DB-5 fused silica capillary column. The derivatized metabolities were cold trapped at 40 °C, and then the temperature was ramped at 20 deg/min to 90 °C followed by isothermal elution. Mass spectral parameters were as follows: dwell, 5 ms; ionizing voltage, ca. 70 eV; source temperature 200–205 °C. The deuterium incorporation in each substrate was determined by bleeding each compound into the source of the mass spectrometer at a steady rate through the reference inlet and monitoring the ion current of the various isotopically substituted species with selected ion recording of the molecular ion. The mass spectrometric parameters for the substrates were the same as those for the analysis of the product alcohols except the dwell time was increased to 50 ms. The measured intensity of each ion monitored was corrected for the natural isotopic abundance of ^2H, ^{13}C, ^{14}C, ^{18}O, ^{29}Si.

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Table I. The Observed (Combined Primary (P) and Secondary (S)) Isotope Effects Associated with the Oxidation of Selectively Deuteriated Octanes

	isotope effect[a]	
	microsomes[b]	P-450b
[1,1,1-$^2H_3$]octane (d_3)	7.85 ± 0.76	9.45 ± 0.15
[1,1,8,8-$^2H_4$]octane (d_4)	3.20 ± 0.03	3.67 ± 0.02
[1,8-$^2H_2$]octane (d_2)	11.64 ± 0.43	12.71 ± 0.68

[a] The values reported are the means ± the standard deviation derived by propagation of the errors of at least five incubations. [b] The isotope effects that are reported for the microsomal hydroxylation represent an average of the isotope effects for different isozymes and thus interpretation of these data is ambiguous; however, since a large body of data have been collected from microsomal preparations the data have been included.

Dimethylsuberic Acid. Diazomethane, ca. 3 g, in ether was generated from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide, and added to suberic acid (0.028 mol) dissolved in 100 mL of diethyl ether. The ether was evaporated and the resulting dimethyl ester (5.75 g) used without further purification.

[1,1,8,8-$^2H_4$]Octane. Dimethylsuberic acid (0.2 mol) was dissolved in anhydrous tetrahydrofuran (ca. 50 mL) and added dropwise to 800 mg of lithium aluminum deuteride suspended in tetrahydrofuran. The reaction was stirred for 24 h and then terminated with 0.8 mL water, 1.6 mL of 15% NaOH, and finally 1.6 mL of water. The mixture was filtered, dried with sodium sulfate, and evaporated to yield 2.56 g of [1,1,8,8-$^2H_4$]-1,8-octanediol. The diol was dissolved in dichloromethane containing triethylamine (10 mL) and tosyl chloride (0.04 mol). The solution was stirred at 4 °C for 20 h and washed with water and saturated sodium bicarbonate solution. The organic phase was dried and evaporated and the residue washed with methanol. Yield 6.1 g, mp 69–71 °C. The solid (9 mmol) was dissolved in diethyl ether and added dropwise to a stirred suspension of lithium aluminum hydride (600 mg) in ether. The reaction was terminated by the addition of H_2O (0.6 mL), 15% NaOH (1.2 mL), and H_2O (1.2 mL). The mixture was filtered and the ether distilled until ca. 5 mL remained. The residual solution was applied to a silica gel column (60 Å, 230–240 mesh) and eluted with pentane. The resulting octane was greater than 99.5% pure and had a deuterium enrichment of 96.50 ± 0.01% [1,1,8,8-$^2H_4$]octane, 2.43% [1,1,8-$^2H_3$]octane, 0.55% [1,8-$^2H_2$]octane and 0.52% [1-^2H]octane.

[1,8-$^2H_2$]Octane. 1,8-Octanediol (0.027 mol) was treated with tosyl chloride (0.05 mol) and the ditosylate (0.01 mol) was reduced as per [1,1,8,8-$^2H_4$]octane except lithium aluminum deuteride was used instead of lithium aluminum hydride. The resulting [1,8-$^2H_2$]octane was purified, as per [1,1,8,8-$^2H_4$]octane, to greater than 97.5% by GC and had a deuterium enrichment of 97.65 ± 0.06% [1,8-$^2H_2$]octane, 1.69% [1-^2H]octane, and 0.66% octane.

[1,1,1-$^2H_3$]Octane. The synthesis of [1,1,1-$^2H_3$]octane has been described previously and was greater than 99.8% octane by GC with a deuterium enrichment of 96.94 ± 0.01% [1,1,1-$^2H_3$]octane, 2.24% [1-$^2H_2$]octane, and 0.82% [1-^2H]octane.

The methods used for the microsomal preparation, the P-450b preparation, the derivatization of metabolites, and the calculation of isotope effects were done as previously reported.[1]

Results and Discussion

The observed value of 9.45 that was obtained for the intramolecular isotope effect associated with the cytochrome P-450b catalyzed oxidation of [1,1,1-$^2H_3$]octane (d_3) is a combination of one primary (P) and two secondary (S) isotope effects, Table I. If we use the nomenclature of Hanzlik, the relationship between the observed isotope effect and its primary and secondary components can be expressed by eq 1. In this equation, hydroxylation

$$d_3 = k_H^{HH}/k_D^{DD} = PS^2 \quad (1)$$

at the protio end of the molecule is represented by k_H^{HH} and that at the deuterio end of the molecule by k_D^{DD}. The subscripts indicate the isotopic species involved in the primary bond breaking event and the superscripts indicate the isotopic species responsible for any secondary effects.

In an analogous manner the observed isotope effects for the substrates [1,1,8,8-$^2H_4$]octane (d_4) and [1,8-$^2H_2$]octane (d_2) can be expressed by eq 2 and 3, respectively. The 2 in the denominator of eq 2 and in the numerator of eq 3 accounts for the statistical probability of removing a proton vs. a deuterium in the two substrates. The secondary isotope effect associated with hydroxylation of octane can now be determined from the observed isotope effects for d_3 and d_4 or d_3 and d_2 as indicated by eq 4 and 5.

Table II. The Separated Primary and Secondary Isotope Effects Associated with the Oxidation of Octane

isotope effect[a]	microsomes		P-450b	
	d_3/d_4	d_3/d_2	d_3/d_4	d_3/d_2
primary	6.85 ± 0.30	6.49 ± 0.30	7.95 ± 0.07	7.27 ± 0.50
secondary	1.07 ± 0.05	1.10 ± 0.05	1.09 ± 0.01	1.14 ± 0.08

[a] The values reported are the means ± the standard deviation derived by propagation of the errors of at least five incubations.

$$d_4 = k_H^{DD}/2k_D^{HD} = P/2S \quad (2)$$

$$d_2 = 2(k_H^{DH}/k_D^{HH}) = 2P/S \quad (3)$$

$$d_3/d_4 = 2(k_H^{HH}/k_D^{DD})(k_D^{HD}/k_H^{DD}) = 2S^3 \quad (4)$$

$$d_3/d_2 = \tfrac{1}{2}(k_H^{HH}/k_D^{DD})(k_D^{HH}/k_H^{HD}) = S^3/2 \quad (5)$$

The primary and secondary isotope effects calculated from these equations are listed in Table II. The large primary (7.3–7.9) and the intermediate secondary isotope effects (1.09–1.14) are consistent with a highly symmetrical reaction coordinate.[6] It can be seen that the secondary isotope effects account for a large portion, ca. 15%, of the observed isotope effect. Thus, with use of the criteria of Bell, tunneling need not be invoked since the primary isotope effect has a value of less than 9.

Recently the interpretation of a secondary isotope effect has been clouded by the discovery that tunneling can increase its magnitude.[7–9] This has been reported for reactions with either a high or a low primary isotope effect in both chemical[8,9] and enzymatic systems.[10–12] Tunneling is assumed to be important for a given reaction if the kinetic isotope effect is greater than the equilibrium isotope effect.[7] This criteria is of little value if the equilibrium isotope effect cannot be measured directly, or approximated with fractionation factors. Since this is the case with reactions that form radical intermediates, such as is proposed for P-450 catalyzed oxidations, another method for assessing the importance of the role of tunneling in contributing to the magnitude of the secondary isotope effect is needed.

One approach to this problem is to use any departure from the rule of the geometric mean (RGM) as a measure of tunneling. If tunneling is significant a positive deviation would be expected.[5,7,9] For example, if one compares the data obtained for glutamate dehydrogenase, which has been shown to have a greater kinetic than equilibrium secondary isotope effect, the corresponding positive deviation associated with the RGM is between 13 and 14%.[10] As indicated by Hanzlik et al., any deviation from the RGM will be reflected by a deviation from the pure number 4 obtained by dividing eq 3 by eq 2. If eq 3 is divided by eq 2 the values obtained for microsomes (3.64 ± 0.14) and for P-450b (3.46 ± 0.19) deviate from the theoretical number of 4 in a *negative* direction. The direction of this deviation is not consistent with the generally accepted expectations for the effect of tunneling. No obvious explanation for this phenomenon is apparent.

In summary, the primary and secondary isotope effects for the cytochrome P-450 mediated oxidation of octane have been separated and determined. The primary isotope effect of 7.3–7.9 and the secondary isotope effect of 1.09–1.14 are consistent with a

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highly symmetrical reaction coordinate; however, the exact values are not known with certainty because of the departure from the rule of the geometric mean. Despite the symmetry of the reaction coordinate, no direct evidence for tunneling was obtained. However, the criteria used to assess the role of tunneling may be excessively rigid.[4] Further studies are needed to clarify the apparent partial failure of the RGM. It may be that the deviation results from an unrecognized source of error in the measurements or that it is in fact an unexplained but real physical phenomenon.

Acknowledgment. We are grateful to Ken Korzekwa for helpful discussions and Dr. Robert Hanzlik for providing us with a preprint of his toluene study. This work was supported in part by the National Institutes of Health (Grant GM 36922).

Communications to the Editor

Formation of Metallocycloimides from the Reaction of Isocyanates with a Neutral Transition-Metal Carbonyl

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Received April 10, 1986

We recently reported that the activation of CO_2 by η^1-C complexation to $[Fe(\eta\text{-}C_5H_5)(CO)_2]^-$ results in facile oxygen exchange between coordinated CO_2 and coordinated CO, and we suggested that these exchanges follow attack by a nucleophilic oxygen of the CO_2 ligand on a neighboring carbonyl ligand.[1] The transient metalloanhydride proposed is analogous to the metalloheterocycles which Fehlhammer has reported as major or minor products of the reactions of $[Fe(\eta\text{-}C_5H_5)(CO)_2]^-$ with heteroallenes such as carbodiimides,[2] isothiocyanates,[3] and ketenimines.[4] The facility of the oxide transfer in this anionic system suggested that metalloanhydrides and other heteroallene adducts might also be formed by carbonyl complexes which are neutral but electron rich,[5] and we now wish to report the syntheses of the first metallocycloimide complexes by addition of isocyanates to a neutral carbonyl complex.

Oxidative addition of methyl iodide[6] to $[W(\eta\text{-}C_5H_5)_2(CO)]$[7] (**1**) indicates that **1** is an exceptionally nucleophilic neutral carbonyl complex and a promising substrate for the addition of heteroallenes. Addition of methyl isocyanate (13.0 mL, 220 mmol) to an intensely green slurry of $[W(\eta\text{-}C_5H_5)_2(CO)]$ (0.648, g, 1.90 mmol) in pentane (60 mL) led over 1 h to formation of an orange solution and a bright orange precipitate. The supernatant was decanted off and the powder washed with pentane and vacuum dried to give 0.667 g (1.67 mmol ≡ 87%) of spectroscopically pure $[W(\eta\text{-}C_5H_5)_2[C(O)N(CH_3)C(O)]]$ (**2**). Analytically pure **2** was obtained as irregular needles (ca. 90% recovery) from acetone at −78 °C.[8]

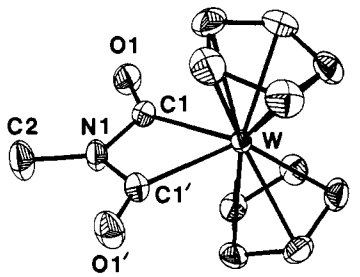

Figure 1. Molecular structure of $[W(\eta\text{-}C_5H_5)_2[C(O)N(CH_3)C(O)]]$ (50% probability ellipsoids). Primed atoms are symmetry generated by the plane through the W and N atoms perpendicular to the metallocycloimide plane. Selected bond lengths (Å) and angles (deg): W–C(1) = 2.195 (3), C(1)–O(1) = 1.213 (5), C(1)–N(1) = 1.393 (4), N(1)–C(2) = 1.451 (7); C(1)–W–C(1′) = 60.3 (2), W–C(1)–N(1) = 97.4 (2), C(1)–N(1)–C(1′) = 104.7 (4), C(1)–N(1)–C(2) = 127.5 (2), W–C(1)–O(1) = 139.4 (3).

The metallocycloimide structure of **2** (Figure 1) was established by an X-ray diffraction study of the orange cubes obtained by cooling a saturated acetone solution to 0 °C over a 4-h period.[9] The metallocycle core of the molecule is reminiscent of the structures of typical cyclic imides,[11] and the metal atom, the ring carbon atoms, and the nitrogen atom are essentially coplanar. The planar geometry of the nitrogen atom,[12] and the short nitrogen–ring carbon bond lengths of 1.393 (4) Å, indicates that the nitrogen lone pair is delocalized over the carbonyl carbons.

Formation of metallocycloimides from **1** is not restricted to methyl isocyanate, and a slurry of **1** (0.284 g, 0.83 mmol) in pentane (30 mL) reacted immediately with excess PhNCO (2.0 mL, 18.3 mmol) to give a pale orange powder. After 1 h the

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(8) ^{1}H NMR (acetone-d_6, 300.13 MHz) δ 4.95 (s, 10, $2C_5H_5$), 2.31 (s, 3, NCH_3); ^{13}C NMR (Me_2SO-d_6, gated decoupled, 75.47 Mhz) δ 176.2 (s, satellites J_{W-C} = 93.4 Hz, C=O), 86.3 (d, J = 183 Hz, C_5H_5), 22.8 (q, J = 138 Hz, CH_3); mass spectrum (parent ion, ^{184}W), m/e 399. Anal. Calcd for $C_{13}H_{13}NO_2W$: C, 39.12; H, 3.29; N, 3.51. Found (Schwarzkopf Laboratories, NY; sample dried at 10^{-4} mmHg for 24 h to remove occluded H_2O): C, 39.06; H, 3.37; N, 3.58.
(9) The diffraction study revealed the presence of one molecule of water of crystallization per molecule of **2** (IR confirmed the presence of H_2O in a bulk sample of the orange cubes and its absence from analytical samples). Crystal data: monoclinic space group $P2_1/m$, Z = 2, a = 7.659 (2) Å, b = 8.672 (2) Å, c = 9.656 (2) Å, β = 101.45 (2)°, d_{calcd} = 2.20 g/cm^3, μ = 97.1 cm^{-1}, λ(Mo Kα) = 0.710 69 Å. Of the 3158 reflections measured in the range $3° \le 2\vartheta \le 60°$ on a Nicolet R3 four-circle diffractometer, 1881 unique reflections with $I > 3\sigma$ were used in the structure solution (Patterson) and refinement (SHELXTL).[10] Least-squares refinement with a block-diagonalized matrix converged at R = 2.20% and R_w = 2.19%. The hydrogen atoms of the water molecule were not located, and all other hydrogen atoms were placed in calculated positions. The largest unassigned peak in the final difference map (0.88 e$^-$/Å$_3$) was located 0.91 Å from the oxygen atom of the water.
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(12) The nitrogen atom is only 0.041 Å above the plane defined by C(1), C(2), and C(1′).

0002-7863/87/1509-2173$01.50/0