Dioxygen Transfer from 4a-Hydroperoxyflavin Anion. 4. Dioxygen Transfer to Phenolate Anion as a Means of Aromatic Hydroxylation

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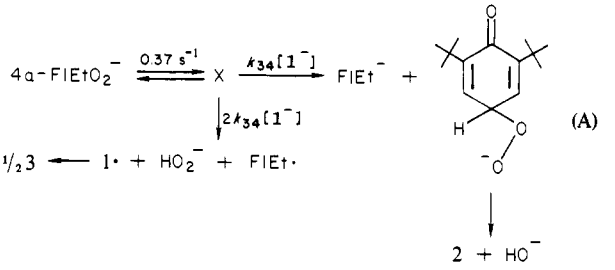
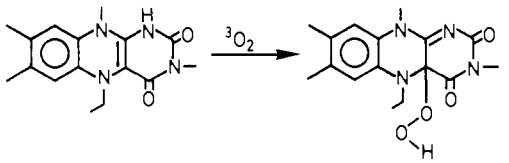
Abstract: Potassium 2,6-di-*tert*-butylphenolate (1^-) in the presence of N^5-ethyl-4a-hydroperoxy-3-methyllumiflavin anion (4a-FlEtOO$^-$) yields (30 °C, absolute *t*-BuOH; anaerobic) 2,6-di-*tert*-butylbenzoquinone (**2**), 4,4′-dihydroxy-3,3′,5,5′-tetra-*tert*-butylbiphenyl (**4**), and 3,3′,5,5′-tetra-*tert*-butyl-4,4′-diphenoquinone (**5**). The 4a-FlEtOO$^-$ is converted in turn to N^5-ethyl-3-methyllumiflavin radical (FlEt·) and 1,5-dihydro-N^5-ethyl-3-methyllumiflavin anion (FlEt$^-$). Kinetic and product studies establish the sequence of eq A to be competent. The product **4** is proposed to arise by rearrangement of 3,3′,5,5′-tetra-



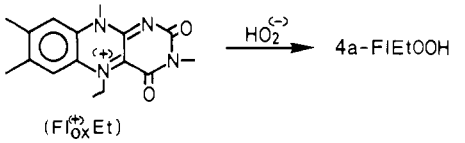
(A)

tert-butyl-[1,1′-bi-2,5-cyclohexadiene]-4,4′-dione (**3**), which is known to be a product of dimerization of **1**. In separate experiments, the rearrangement **3** → **4** has been shown to occur (absolute *t*-BuOH) spontaneously ($k = 1.3 \times 10^{-4}$ s^{-1}) and to be catalyzed by *t*-BuO$^-$K$^+$ ($k = 3.5 \times 10^3$ M^{-1} s^{-1}). The transfer of the peroxy substituent from 4a-FlEtOO$^-$ to 1^-, yielding the quinone **2** and FlEt$^-$, undoubtedly occurs via the cyclohexadienone peroxide anion (as shown). This is supported by the finding that the rate constant for conversion of 4a-FlEtOO$^-$ to reactive intermediate (**X**) is the same (0.37 s^{-1}) as found previously for peroxidation of other ambident nucleophiles by 4a-FlEtOO$^-$ (i.e., $0.37_5 \pm 0.01_6$ s^{-1}). Of possible relevance to the mechanism of dioxygen transfer are the findings that 1^- undergoes $1e^-$ oxidation by FlEt· ($k = 2.45 \times 10^4$ M^{-1} s^{-1}) (products FlEt$^-$ + $^1/_2$**4**), a feature shown by other ambident nucleophilic substrates, and that the second-order rate constant for reaction of 1^- with $^3O_2$ ($k \simeq 0.9$ M^{-1} s^{-1}) is too small for this to be of importance in the formation of **2**. It is pointed out that, since dioxygen transfer from 4a-FlEtOO$^-$ to 1^- yields reduced flavin (FlEt$^-$) and a quinone (**2**) and since FlEt$^-$ reduces quinones to hydroquinones, the dioxygen-transfer mechanism could serve as a means of hydroxylation of phenols.

In previous studies from this laboratory, it has been shown that (i) 4a-hydroperoxyflavins (as 4a-FlEt–O–OH) are formed on reaction of reduced flavin (FlEtH) or flavinium cation (Fl_{ox}^+Et) with $^3O_2$ and HO_2^-, respectively (eq 1 and 2),[1-5] (ii) the 4a-

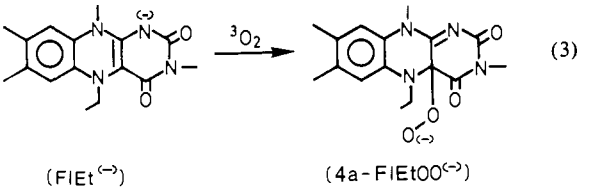


(1)



(2)

hydroperoxyflavin anion (4a-FlEtOO$^-$) is formed upon reaction of reduced flavin anion (FlEt$^-$) with $^3O_2$,[6] by reaction of $O_2^{-\cdot}$ with the flavin radical FlEt·[7] or by base-catalyzed ionization of 4a-FlEtOOH[6] (eq 3–5), (iii) 4a-FlEtOOH serves as an efficient



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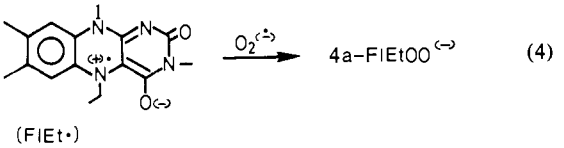

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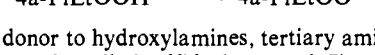

(5)

monooxygen donor to hydroxylamines, tertiary amines, and secondary amines and to alkyl sulfide (e.g., eq 6, 7) to yield *N*- and *S*-oxides along with the pseudobase 4a-FlEtOH as initial products;[8,9] and (iv) 4a-FlEtOO$^-$ is reduced by ambident nucleophiles

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(9) Ball, S.; Bruice, T. C. *J. Am. Chem. Soc.* **1980**, *102*, 6498.

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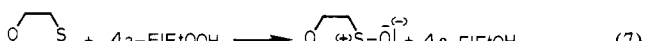
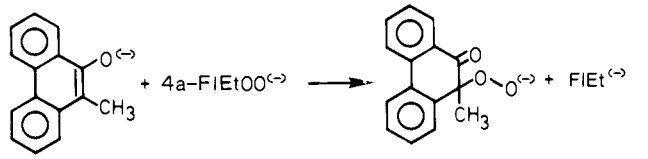


(7)

with accompanying peroxidation of the nucleophile (e.g., eq 8, 9).[6,10,11] The reactions of the type represented by eq 6 and 7 were



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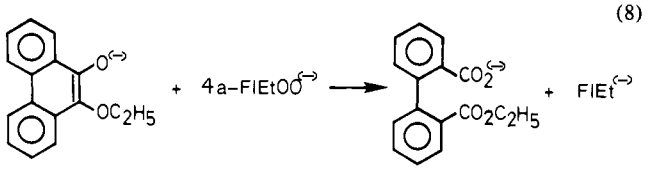
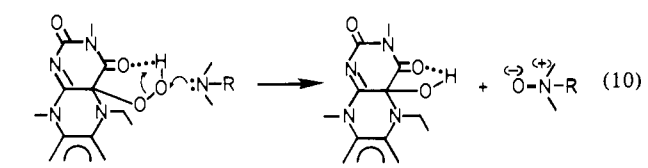


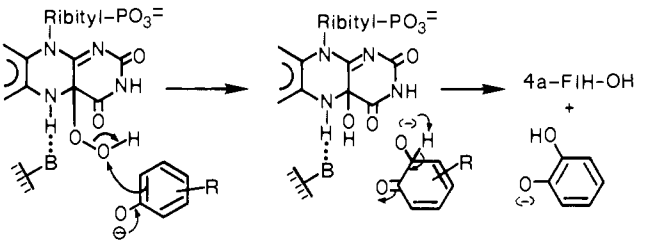
(9)

found to be strictly first order in the concentrations of the two reactants and to be quantitative.[9] The same reaction is, with little doubt, involved in the monoxygenation of nitrogen bases and organic sulfides by flavoenzyme monoxygenase.[12] The reaction involves a displacement of the terminal peroxide oxygen by nucleophile (eq 10). The ratios of the relative second-order rate



(10)

constants for sulfoxide formation from thioxane when comparing *t*-Bu–OOH:4a-FlEt–OOH:*m*-chloroperbenzoic acid are $\sim 6 \times 10^{-6}$:1:$7.5 \times 10^{2+}$.[13] Thus, the 4a-hydroperoxyflavins are incomparably better monooxygen donors than are alkyl hydroperoxides but are not comparable to percarboxylic acids. For this reason 4a-FlEtOOH does not epoxidize aromatic hydrocarbons, and there are now known flavoenzyme mixed-function oxidases which are capable of doing so. However, a number of flavoenzyme mixed-function oxidases hydroxylate phenolate substrates to provide catechols (e.g., salicylate hydroxylase,[14] *p*-hydroxybenzoate hydroxylase,[15] melilotate hydroxylase[16]). In these instances it would be reasonable to assume a priori that hydroxylation occurred by a nucleophilic displacement by phenolate anion upon 4a-hydroperoxyflavin (eq 11) much as seen in the reactions of eq 6 and 7 (i.e., eq 10). Hamilton assumed that the displacement of eq 11 would not occur because alkyl hydroperoxides possess little monooxygen-donating potential. (However, we now known, loc. cit., that 4a-hydroperoxyflavins possess much greater monooxygen-donating potential than do alkyl peroxides.) He suggested that the 4a-hydroperoxyflavin was converted to the carbonyl oxide of eq 12 (or vinylogous ozonide isomer) which then underwent



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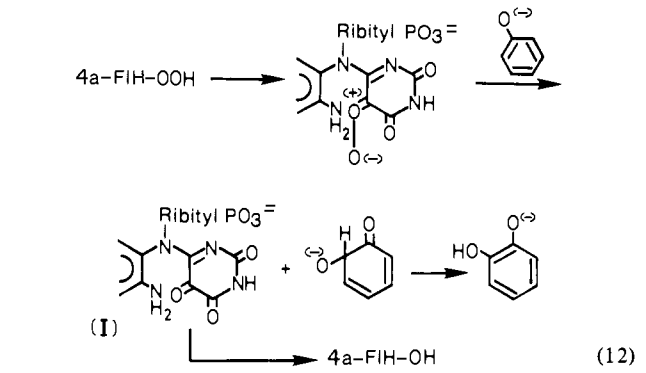

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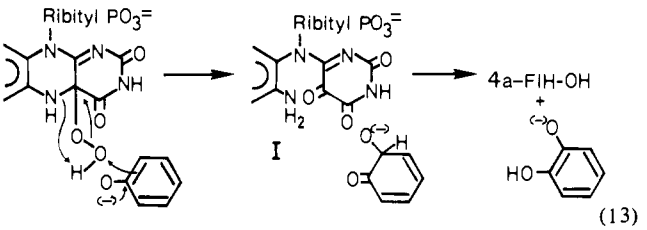
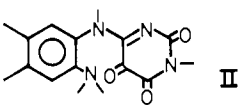
nucleophilic displacement to yield I.[17] A nucleophilic displacement by phenolate ion upon 4a-FlH–OOH to produce I was subsequently suggested by Entsch, Ballou, and Massey (eq 13) to



(13)

account for their observations with *p*-hydroxybenzoate hydroxylase.[15b] These workers identified an intermediate spectrally to which they assigned the structure I. This purported observation of I marks the only evidence offered for the existence of I. The unequivocal synthesis of II, which is a close analogue of I, and the establishment that the spectrum of II is not similar to I apparently remove the single experimental support for the existence of I.[18]



II

There is the possibility that phenolate hydroxylation occurs via (i) direct nucleophilic attack of the phenolate anion upon enzyme-bound 4a-FlHOOH (as depicted in eq 11) or (ii) dioxygen transfer from 4a-FlEtOO$^-$ or enzyme-bound 4a-FlHOO$^-$ to phenolate anion (as in eq 8) followed by expulsion of HO$^-$ from the intermediate peroxide and reduction of quinone by dihydroflavin anion (eq 14).[6] The experimental establishment of phenolate anion attack upon 4a-FlEt–O–OH is difficult because of the acidity of the peroxide and the necessity to generate phenolate anion in the presence of undissociated flavin hydroperoxide. The present study establishes the feasibility of phenolate hydroxylation by the sequence of eq 14.

Experimental Section

General Remarks. All melting points were measured on a Thomas Model 40 micro hot-stage apparatus and are uncorrected. ^{1}H NMR

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(15) (a) Husain, M.; Schopfer, L. M.; Massey, V. *Methods Enzymol.* **1978**, *53*, 543. (b) Entsch, B.; Ballou, D. P.; Massey, V. *J. Biol. Chem.* **1976**, *251*, 2550, 7369. (c) Ghisla, S.; Entsch, B.; Massey, V.; Husain, M. *Eur. J. Biochem.* **1977**, 76, 139. (d) Husain, M.; Massey, V. *J. Biol. Chem.* **1979**, *254*, 6657. (e) Entsch, B.; Husain, M.; Ballou, D. P.; Massey, V.; Walsch, C. *Ibid.* **1980**, *255*, 1420.
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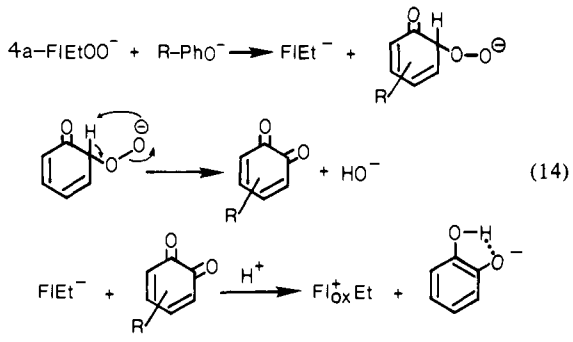
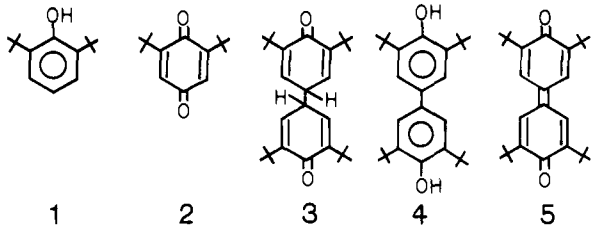


(14)

spectra were recorded on a Varian T-60 spectrometer using tetramethylsilane (Me_4Si) as internal reference. Chemical shifts are expressed as δ values (ppm downfield from Me_4Si). All spectrophotometric determinations were performed on a Cary 118 spectrophotometer, and rapid spectral changes were followed by a Durrum stopped-flow spectrophotometer under an O_2-free N_2 atmosphere. All kinetic studies were carried out at 30 ± 0.2 °C.

Materials. **2,6-Di-*tert*-butylphenol (1)** was purified by sublimation after two distillations. **2,6-Di-*tert*-butyl-*p*-benzoquinone (2)** was recrystallized from MeOH, yellow needles (mp 67–68 °C, ϵ_{255} (*t*-BuOH) 28 000 M^{-1} cm^{-1}). **3,3′,5,5′-Tetra-*tert*-butyl-[1,1′-bi-2,5-cyclohexadiene]-4,4′-dione (3)**, **4,4′-dihydroxy-3,3′,5,5′-tetra-*tert*-butyl-biphenyl (4)**, and **3,3′,5,5′-tetra-*tert*-butyl-4,4′-diphenoquinone (5)**, were prepared according to methods of Kharasch and Joshi:[19] **3**, pale yellow crystals, mp 150–151 °C, UV (*t*-BuOH) ϵ_{242} 15 000 M^{-1} cm^{-1}; **4**, pale yellow powder, mp 183–184 °C, UV (*t*-BuOH) ϵ_{267} 18 200 M^{-1} cm^{-1}, ^1H NMR ($CDCl_3$) δ 7.28 (4 H, s, Ar-H), 5.13 (2 H, s, OH), 1.49 ppm (36 H, s, *t*-Bu); **5**, red-brown needles, mp 246–247 °C, UV–vis (*t*-BuOH) ϵ_{258} 5700, ϵ_{270} 5800, ϵ_{420} 50 400 M^{-1} cm^{-1}, ^1H NMR ($CDCl_3$) δ 7.73 (4 H, s, olefinic proton), δ 1.40 (36 H, s, *t*-Bu). *tert*-Butyl alcohol was distilled from CaH_2 under N_2 and kept under a dry N_2 atmosphere. **N^5-Ethyl-4a-hydroperoxy-3-methyllumiflavin (4a-FlEtOOH)** was synthesized in this laboratory in 85–95% purity; ϵ_{370} (*t*-BuOH) 8000 M^{-1} cm^{-1}.[1,8]



Procedure for the Determination of Reaction Products. The reaction of 4a-FlEtOO⁻ with 2,6-di-*tert*-butylphenol anion (1^-) in anhydrous and O_2-free *tert*-BuOH solvent was carried out according to previously reported procedures.[6,10,11] Typical concentrations were as follows: [4a-FlEtOOH] = 2.0 × 10^{-4} M, [**1**] = 6.0 × 10^{-3} M, and [*t*-BuOK] = 6.1 × 10^{-3} M. The concentration of FlEt⁻ was measured by mixing a portion of the acidifed slution with a solution of 4-hydroxy-2,2,6,6-tetramethylpiperidinyl-1-oxy which is known to convert FlEtH to FlEt· (ϵ_{640} 5000 M^{-1} cm^{-1}).[20] The yields of products from substrate were determined by high-performance liquid chromatography (HPLC) employing standard curves prepared from authentic samples. The LC analysis was carried out with a Du Pont reverse-phase column (Zobax ODS 6.2 mm), using acetonitrile–water (90:10, v/v) and acetonitrile as solvents for **2**, **4**, and **5**, respectively, at a flow rate of 1.2 mL/min. The products were monitored at 255 nm (λ_{max} of **2**) and 420 nm (λ_{max} of **5**). The retention times of **1**, **2**, **4**, and **5** were 7.7, 9.1, 17.5, and 29.1 min, respectively.

Results

The products of the reaction of 4a-FlEtOO⁻ with 2,6-di-*tert*-butylphenol anion (1^-) (absolute *t*-BuOH and in the absence of O_2 (30 °C)) are FlEt·, FlEtH, **2**, **4**, and **5**. The percent yields of products based upon [4a-FlEtOO⁻] employed is presented in Table I. (For analytical methods see Experimental Section). The products **4** and **5** are known to arrive from **3** which, in turn, is formed from **1** via radical oxidation.[19] In *t*-BuOH, the conversion of **3** → **4** occurs by both spontaneous and base-catalyzed processes.

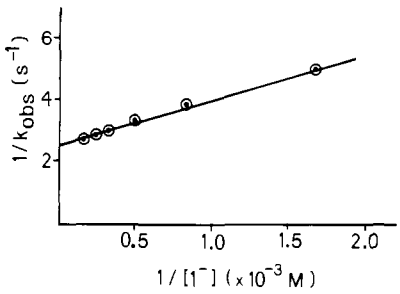
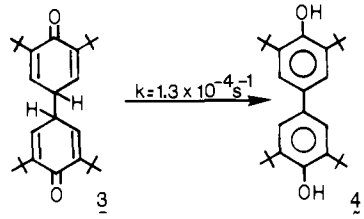


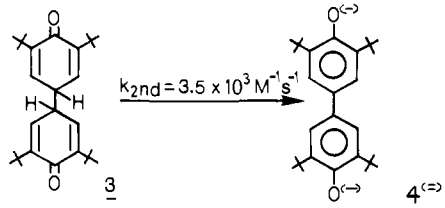
Figure 1. Plot of the reciprocal of the concentration of the anion of **1** vs. the reciprocal of the pseudo-first-order rate constant (k_{obsd}) for disappearance of 4a-FlEtOO⁻ (30 °C, degassed and anhydrous *t*-BuOH).

The disappearance of **3** (240 nm) and appearance of **4** (265 nm), in absolute *tert*-butyl alcohol (N_2 atmosphere), are associated with an isosbestic point at 255 nm. The reaction was found to be first order in substrate (eq 15) and to provide **4** in 94% yield (4% of



(15)

5 was also obtained). In *t*-BOH containing **3** [5 × 10^{-5} M] and *t*-BuO^-K^+ [2.5 × 10^{-3} M], the formation of 4^{2-} (k_{obsd} = 8.8 s^{-1}) occurred much more rapidly (eq 16). The % yield of 4^{2-}, based on **3**, was again 94% (5% of **5** was also obtained). The small yields of **5** most likely arise via oxidation of **4** during the product analysis.



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Kinetics for the reaction of 1^- with 4a-FlEtOO⁻ were followed by stopped-flow spectrometry under an N_2 atmosphere by monitoring the disappearance of 4a-FlEtOO⁻ at 370 nm. In practice, a solution of 4a-FlEtOOH (1.08 × 10^{-4} M in *t*-BuOH) was mixed on the stopped-flow bench with *t*-BuOH solutions containing 4.6 × 10^{-2} M *t*-BuO^-K^+ and **1** (0.6 × 10^{-3} to 6.0 × 10^{-3} M). All reaction were found to obey the first-order rate law to at least 3 half-lives. Plots of k_{obsd} vs. $[\mathbf{1}]_{initial}$ were found to approach a maximum in k_{obsd}. This observation is in accord with the reaction scheme of eq 17. From eq 17, there follows eq 18. In Figure

$$\text{4a-FlEt-OO}^- \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \times \xrightarrow{k_3[\mathbf{1}^-]} \text{products} \qquad (17)$$

1, there is plotted $1/k_{obsd}$ vs. $1/[\mathbf{1}^-]$. From the intercept, there is obtained $1/k_1$ (k_1 = 0.39 s^{-1}) and from slope/intercept, the value of k_2/k_3 (6.0 × 10^{-4} M).

$$1/k_{obsd} = 1/k_1 + k_2/k_1k_3[\mathbf{1}^-] \qquad (18)$$

The reduction of FlEt· by 1^- in *tert*-butyl alcohol was followed at the λ_{max} of the radical (640 nm) under an inert atmosphere of nitrogen. The N^5-ethylflavin radical was prepared by mixing equal volumes of *tert*-butyl alcohol solutions 1 × 10^{-4} M in oxidized (Fl_{ox}^+Et) and reduced N^5-ethyl-3,10-dimethylflavin (FlEtH). The comproportionation reaction of eq 19 has previously been shown

$$Fl_{ox}^+Et + FlEtH \rightarrow 2FlEt\cdot \qquad (19)$$

to proceed to completion and the radical species is stable.[21] The FlEt· solution was mixed on the stopped-flow bench with solutions

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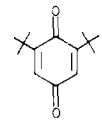
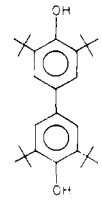
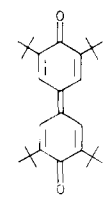
Table I. Reaction of 4a-FlEtO$_2$H with 2,6-Di-*tert*-butylphenol Anion in *t*-BuOH

FlEtO$_2$H (90%) [10^{-4} M]	 [10^{-3} M]	*t*-BuOK [10^{-3} M]	products, % FlEt·	FlEtH	 2	 4	 5
2.17	6.12	6.00	54	25	20	22	9
1.96	6.12	6.00	54	31	21	23	8
2.29	6.12	6.00	59	24	21	21	8
		av yield:	56%	26%	21%	22%	9%

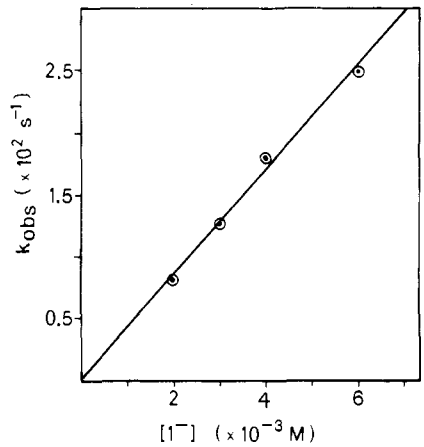
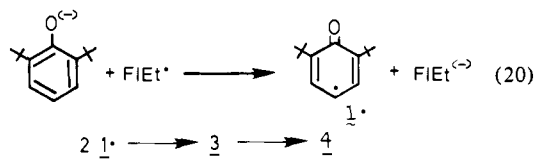


Figure 2. Plot of the pseudo-first-order rate constant for one electron oxidation of the anion of **1** by flavin radical (FlEt·) vs. the concentration of the anion of **1**.

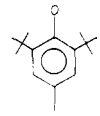
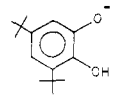
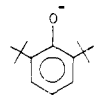
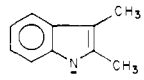
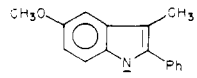
of **1**$^-$ (4×10^{-3} to 1.2×10^{-2} M) prepared in *tert*-butyl alcohol containing potassium *tert*-butoxide. The disappearance of FlEt· followed the first-order rate law to completion. The second-order rate constant for the reduction of FlEt· to FlEt$^-$ by **1**$^-$ was then obtained as the slope (4.25×10^4 M^{-1} s^{-1}) of a plot of k_{obsd} vs. [**1**$^-$] (Figure 2). The products of the reactions of **1**$^-$ with FlEt· were shown to be **1**· and FlEt$^-$ as follows. Under an inert nitrogen atmosphere, a solution of FlEt· (1.61×10^{-4} M) in *tert*-butyl alcohol was mixed with a *tert*-butyl alcohol solution of **1** (6×10^{-3} M) containing potassium *tert*-butoxide (6.1×10^{-3} M). The flavin radical disappeared immediately. After about 10 min, the reaction solution was acidified with a few drops of oxygen-free acetic acid. An aliquote was then withdrawn and the FlEt$^-$ formed during the reaction reoxidized to FlEt· with nitroxide radical (Experimental Section). The concentration of FlEt· was determined spectrally. In this manner, it could be shown that the sequence of **1**$^-$ reduction of FlEt· to FlEt$^-$ and reoxidation to FlEt· by nitroxide occurs in 98% yield. By use of high-pressure liquid chromatography, it could be established that **4** is formed from **1**$^-$ in 37% yield based upon FlEt· employed (eq 20). The formation of **2** and **5** could not be detected by HPLC nor by uv/vis spectrophotometry.



(20)

The reaction of oxygen with 1$^-$ occurs at a rate sufficiently slow to allow the disappearance of **1**$^-$ to be followed by repetitive scanning with a recording spectrophotometer. For this purpose, a solution of **1**$^-$ (9×10^{-4} M) was prepared in *tert*-butyl alcohol as before. This was then mixed with 9-fold volumes of air-saturated (30 °C) *tert*-butyl alcohol ([O_2] = 3.15×10^{-3} M). The disappearance of **1**$^-$ (306 nm) was accompanied by the appearance of **2** (255 nm) and the reaction exhibited an isosbestic point at 274 nm. The reaction followed the first-order rate law to at least 3 half-lives. High-pressure liquid chromatography established the formation of **2** in 72% and **5** in 6% yield based upon [**1**$^-$] employed. From the pseudo-first-order rate constant obtained (2.5×10^{-3} s^{-1}) and the oxygen concentration, the second order-rate constant for reaction of **1**$^-$ with O_2 is ~0.90 M^{-1} s^{-1}.

Table II. Derived Rate Constants for the Dioxygen Transfer from 4a-FlEtOO$^-$ to Various Substrates

substrate	k_1, s^{-1}	k_2/k_3, M	ref
	0.36	2.2×10^{-4}	6, 10
	0.37	2.8×10^{-4}	10
	0.39	6.0×10^{-4}	this work
	0.33	8.3×10^{-3}	11
	0.37	1.3×10^{-2}	11

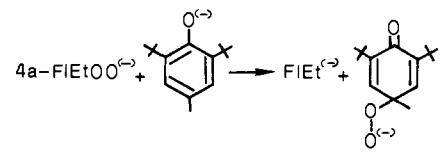
Discussion

The reaction of the anion of 2,6-di-*tert*-butylphenol (**1**$^-$) with the anion of N^5-ethyl-4a-hydroperoxy-3-methyllumiflavin (4a-FlEtOO$^-$) occurs by the trapping of a thermodynamically unstable intermediate (X) formed reversibly from 4a-FlEtOO$^-$ (eq 17). This very same reaction sequence has been observed on reaction of 4a-FlEtOO$^-$ with other ambident nucleophiles. The rate constant for formation of X ($k_1 = 0.37_5 \pm 0.01_6$ s^{-1}) is independent of the nature of the substrate and, as expected from eq 17, the partition coefficient k_2/k_3 is substrate dependent (Table II).

Evidence has been presented previously[10,11] that X does not represent the solvent-separated flavin and oxygen species.

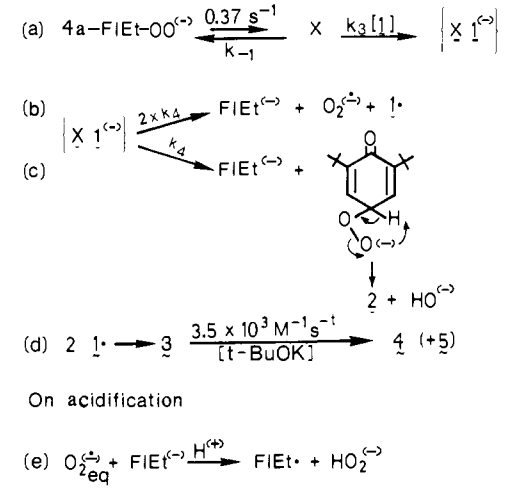
$$\text{FlEt}\cdot\|O_2^-\cdot \qquad \text{FlEt}^-\|^3O_2 \qquad \text{FlEt}^-\|^1O_2$$

Thus, the radical formed by one-electron oxidation of 2,6-di-*tert*-butyl-4-methylphenolate does not couple with O_2^-· to yield the observed hydroperoxide product of eq 21; the singlet oxygen



(21)

Scheme I



traps, 2,5-dimethylfuran and 2,3-dimethyl-2-butene, do not react with X, and the reaction of the various substrates (Table II) with triplet oxygen is too slow to allow oxygen to be an intermediate in the oxygen transfer reaction. In the instance of the reaction of $\mathbf{1}^-$ with 4a-FlEtOO$^-$, we find again that X cannot be FlEt$^-$‖$^3O_2$. In the present study, the second-order rate constant for reaction of $^3O_2$ with $\mathbf{1}^-$ has been determined to be $\sim 0.90\ M^{-1}\ s^{-1}$. If $\mathbf{1}^-$ reacted with $^3O_2$ produced from 4a-FlEtOO$^-$, eq 22 and 23 would

$$\text{4a-FlEtO}_2^- \underset{k_2\ s^{-1}}{\overset{0.37}{\rightleftharpoons}} \text{FlEt}^- + {}^3O_2$$

$$^3O_2 + \mathbf{1}^- \xrightarrow{k_3,\ M^{-1}\ s^{-1}} \text{products} \qquad (22)$$

$$k_{obsd} = \frac{0.37[\mathbf{1}^-]}{k_2/k_3 + [\mathbf{1}^-]} \qquad (23)$$

$$k_{obsd} = \frac{0.37[\mathbf{1}^-]}{6 \times 10^{-4} + [\mathbf{1}^-]} \qquad (24)$$

pertain. Employing the determined value of k_2/k_3, eq 23 becomes eq 24. At saturation in $\mathbf{1}^-$, the concentration of $\mathbf{1}^-$ (i.e., $[\mathbf{1}^-] = 10k_2/k_3 = 6 \times 10^{-3}$ M) will exceed the steady-state concentration of $^3O_2$ and the product $k_3[\mathbf{1}^-]$ $(=0.9 \times 6 \times 10^{-3}\ s^{-1}) = 5.4 \times 10^{-3}\ s^{-1}$ should be the observed pseudo-first-order rate constant for reaction of **1** with $^3O_2$. Since $k_3[\mathbf{1}^-]$ at saturation is ~ 70 times smaller than the limiting rate constant ($0.37\ s^{-1}$), X cannot represent solvent separated FlEt$^-$‖$^3O_2$.

It is obvious that X must represent a compound derived from 4a-FlEtOO$^-$ or a complex of a flavin species and an oxygen species. Two compounds are formed from $\mathbf{1}^-$ in its reaction with X. These are the *p*-quinone **2** and the dimer **3**. Formation of **3** is expected to occur via radical (i.e., **1**·) coupling reaction. The reaction sequence of Scheme I suffices to explain the % yields of **2**, **3**, FlEt·, and FlEt$^-$ (Table I) if it is assumed that the titer of 4a-FlEtO$_2$H is at 85% at the time of rapid mixing of reagent solutions (solid 4a-FlEtOOH used was 90% pure). In Scheme I, {X **1**$^-$} need not represent one species. It could represent either the composition of transition states or of intermediates. Reaction b may be accounted for by eq 25–27. Equilibrium between FlEt$^-$ + O_2 ⇄

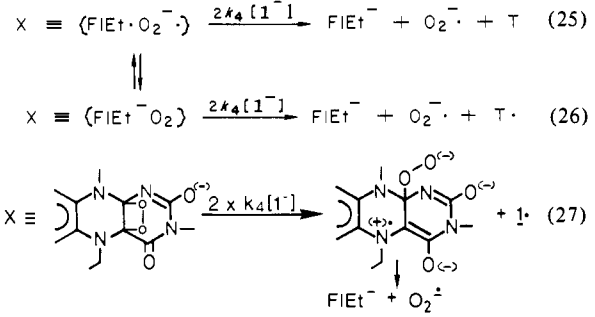
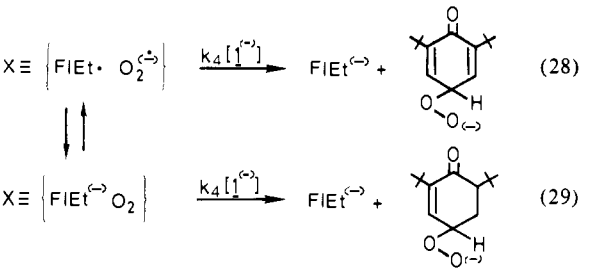


Table III. Second-order Rate Constants for the One-Electron Reduction of FlEt· by Various Anions

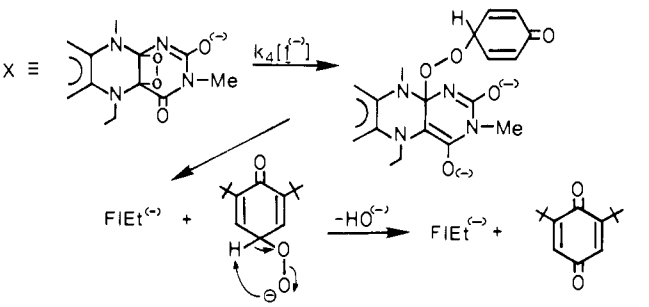
anion of	k, $M^{-1}\ s^{-1}$	ref
2,6-di-*tert*-butyl-*p*-methylphenol	$>10^7$	6
2,6-di-*tert*-butylphenol	4.25×10^4	this work
3,5-di-*tert*-butylcatechol	1.95×10^4	10
5-methoxy-3-methyl-2-phenylindole	2.60×10^3	11
10-ethoxy 9-phenanthrol	1.02×10^3	10
2,3-dimethylindole	2.03×10^2	11
10-methyl-9-phenanthrol	9.97	10

4a-FlEtO$_2^-$ has been established[6] as has the equilibrium between FlEt· + O_2^-· ⇄ 4a-FlEtO$_2^-$.[7] The rapid reduction of FlEt· by $\mathbf{1}^-$ to produce FlEt$^-$ + **1**· has been established in this study. To date, all ambident anions which react with X have been shown to reduce FlEt· (Table III). Formation of **1**· by reaction of $\mathbf{1}^-$ with O_2 is a well-known process.[19] Reaction c of Scheme I may be considered in terms of the mechanisms of eq 28–30.



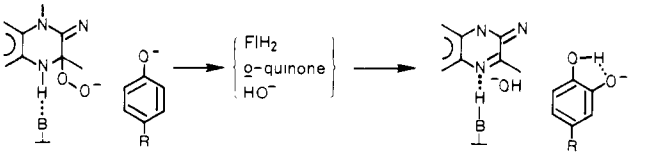
(28)

(29)



(30)

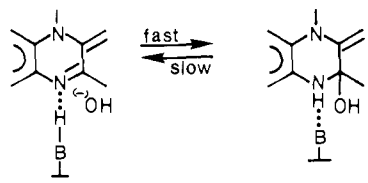
Dioxygen transfer may be of biochemical importance as a means of hydroxylating phenolate anions (see introductory section). Thus, dioxygen transfer from enzyme-bound 4a-FlEtO$_2^-$ to *p*-hydroxybenzoic acid dianion followed by elimination of HO$^-$ from the peroxidized substrate would provide HO$^-$, an *o*-quinone, plus reduced flavin at the active site (as in eq 14). Reduction of the *o*-quinone by the dihydroflavin would then provide at the active site HO$^-$, oxidized flavin, and a catechol. The reduction of quinones by dihydroflavins is rapid (eq 31).[22,23] For benzoquinone



(31)

reduction by FlH$_2$ or FlMeH, the second-order rate constant exceeds $10^8\ M^{-1}\ s^{-1}$. Those features, at the active site, which prevent dissociation of hydrogen peroxide from 4a-FlHO$_2$H to yield Fl$_{ox}$ might then provide stabilization for the 4a-pseudobase, viz. and loss of H_2O from the active site would then yield enzyme-bound oxidized flavin. Entsch, Ballou, and Massey[15] observe,

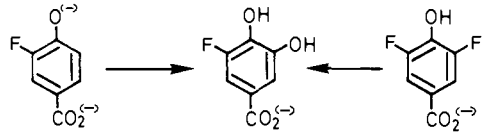
(22) Gibian, M. J.; Rynd, J. A. *Biochem. Biophys. Res. Commun.* **1969**, *34*, 594.
(23) Bruice, T. C.; Yano, Y. *J. Am. Chem. Soc.* **1975**, *97*, 5263.



(32)

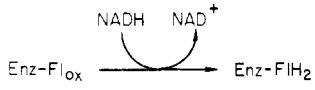
with *p*-hydroxybenzoate hydroxylase and certain alternate substrates, that enzyme-bound 4a-FlHOOH gives way to an intermediate (λ_{max} 395–420 nm ($\epsilon \simeq 15\,000$)) which then yields 4a-FlHOH. This, as yet, unidentified intermediate could be a complex of dihydroflavin and *o*-quinone.

Any purported mechanism for *p*-hydroxybenzoate monooxygenase must explain the fact that fluoride is substituted by a hydroxyl substituent in the enzymatic reaction.[24] Fluoride displacement was found to be kinetically less favorable than apparent "oxygen insertion between C and H" (eq 33), and when



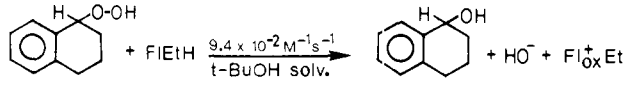
(33)

fluoride ion is displaced, the rate-determining step was found to be associated with the decomposition of enzyme-bound 4a-FlH-OOH. This means, of course, that intermediates do not accumulate and, therefore, they cannot be observed. What is known is that 2 equiv of NADH are required for enzymatic fluoride displacement, rather than the one NADH equivalent for "oxygen insertion" into normal substrates (eq 34). A reasonable mech-

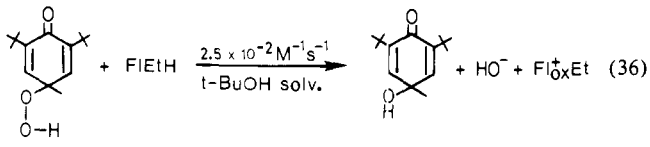


$$\text{Enz-FlH}_2 \xrightarrow{O_2,\ \text{substrate}} \text{Enz-Fl}_{ox} + \text{catechol} \qquad (34)$$

anism which involves i equiv of NADH may be written for the dioxygen-transfer reaction to displace fluoride ion by a hydroxyl substituent. The key step in the sequence of Scheme II is the reduction of the substrate-derived hydroperoxide by dihydroflavin. Precedence for this reaction exists in the literature[10] (see eq 35 and 36).



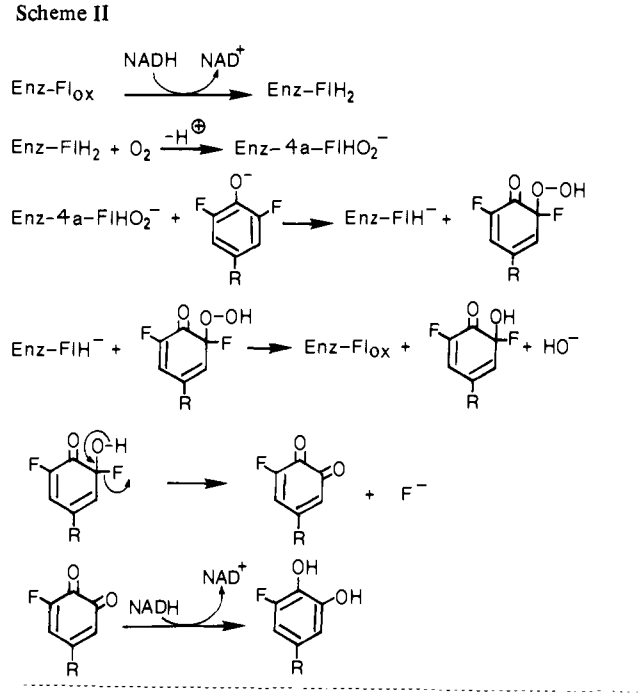
(35)

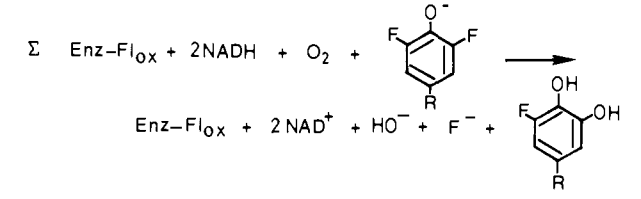


(36)

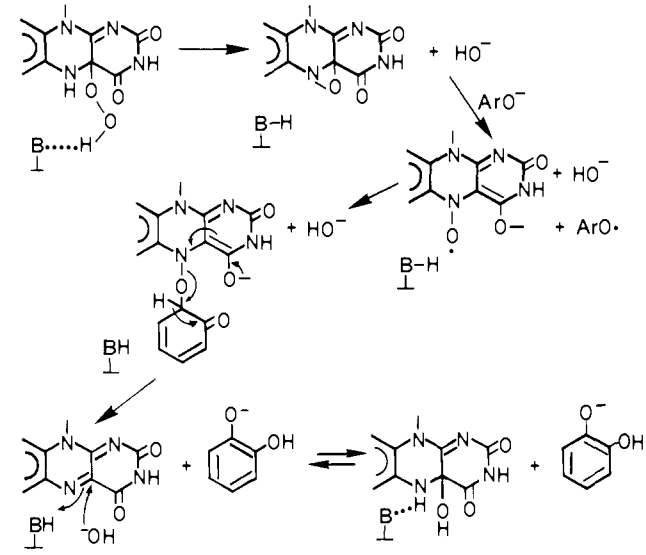
One other flavin species-dependent phenolate hydroxylation model has been established. Rastetter and colleagues[25] have shown that flavin N^5-oxide transfers an oxygen to the anion of **1**. Two moles of the N^5-oxide are consumed in the process of conversion of $\mathbf{1}^-$ to its *p*-quinone. Presumably, oxygen transfer from flavin N^5-oxide to $\mathbf{1}^-$ provides Fl_{ox} plus hydroquinone which is, in turn, oxidized by the second flavin N^5-oxide to yield quinone, EPR studies established the presence of a nitroxyl radical during the time course of the reaction. On the basis of the suggestion of these workers, the reaction sequence of Scheme III may be written for flavoenzyme monooxygenation of phenolate anion. In principal,

Scheme II

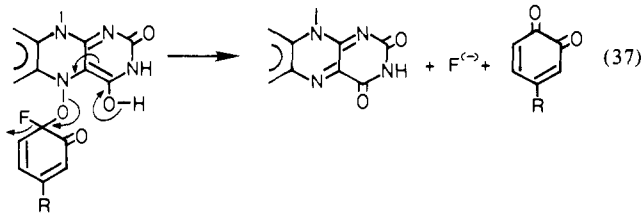




Scheme III



this mechanism could be applied to fluoro-substituted phenols (eq 37).



(37)

As a biomimic, the reaction of Rastetter is a very interesting one. However, two criticisms of the mechanism when applied to

(24) Husain, M.; Entsch, B.; Ballou, D. P.; Massey, V.; Chapman, P. J. *J. Biol. Chem.* **1980**, *255*, 4189.

(25) (a) Rastetter, W. H.; Gadeh, T. R.; Tone, J. R.; Frost, J. W. *J. Am. Chem. Soc.* **1979**, *101*, 2228. (b) Frost, J. W.; Rastetter, W. H. *Ibid.*, in press.

the enzymatic reaction, may be offered. (i) Though the N^5-oxide of the cofactor binds well to the active site of *p*-hydroxybenzoate hydroxylase, it does not transfer oxygen to substrate.[26] (ii) Monooxygenase-bound 4a-FlHOOH, in the absence of substrate, decomposes quantitatively by elimination of H_2O_2 with the formation of enzyme-bound oxidized flavin so that there is no evidence for the proposed intramolecular formation of a flavin oxaziridine.

(26) Massey, V.; Rastetter, W. H., private communication.

Acknowledgment. This study was supported by grants from the National Institutes of Health and the National Science Foundation.

Registry No. 1, 128-39-2; **1**$^-$·K^+, 24676-69-5; **2**, 719-22-2; **3**, 24457-07-6; **4**, 128-38-1; **5**, 2455-14-3; **4a**-FlEtOOH, 59587-26-7; **4a**-FlEtOO$^-$, 74976-70-8.

High-Pressure NMR Study of Dynamical Effects on Conformational Isomerization of Cyclohexane

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Received September 14, 1981

Abstract: The effect of temperature and pressure on the conformational inversion of cyclohexane in solution has been investigated with use of ^{1}H FT NMR spectroscopy. The solvents used in this study are methylcyclohexane-d_{14}, carbon disulfide, and acetone-d_6. It is found that the activation parameters as obtained from the temperature dependence of the rate constant are independent of solvent. However, the coalescence temperature in the methylcyclohexane-d_{14} solvent is 1.5 °C higher than in the other two solvents. It is observed that the ring inversion in cyclohexane is accelerated when pressure is increased, and this pressure dependence of the rate constant is nonlinear. In the lower viscosity solvents, acetone-d_6 and carbon disulfide, the rate constant shows a larger pressure dependence than in the more viscous methylcyclohexane-d_{14} solvent. The experimental data are interpreted with use of results of the stochastic models for isomerization reactions. In these models it is proposed that there are dynamical effects on isomerization because the reaction coordinate is coupled to the surrounding medium. We find that the observed activation volume is strongly pressure and solvent dependent as is the collisional contribution to the activation volume. Since the collision frequency reflecting the coupling of the reaction coordinate to the solvent mediums is proportional to solvent viscosity, the observed activation volume and the transmission coefficient κ correlate well with the solvent viscosity. In addition, the experimental data indicate a non-monotonic transition between the inertial (weak coupling) and diffusive (strong coupling) regimes of isomerization reactions. Our results represent the first experimental proof of the predictions of stochastic models for isomerization reactions in condensed phases.

I. Introduction

A large number of studies employing different NMR techniques have been devoted to the investigation of the temperature dependence of ring inversion of cyclohexane. This is not surprising since the cyclohexane moiety is of primary importance in the theory of conformational analysis. What is surprising, however, is that all these studies used a single solvent, carbon disulfide. Recently a limited pressure study (up to 2 kbar) of cyclohexane in a complex mixture of solvents has been performed by Lüdemann and co-workers.[1]

Our understanding of the ring-inversion process in cyclohexane is based upon these variable-temperature measurements and the results of force-field calculations. These calculations[2-12] have shown that the lowest energy path for ring inversion proceeds via a half-chair transition state with the twist-boat and its slightly higher energy pseudorotation partner the boat as intermediates. The barrier for isomerization, which is equal to the energy difference between the half-chair transition state and chair forms, depends on the parameters chosen for the calculation. The calculated value for the barrier height varies from 9.5 to 12.7 kcal/mol and is in good agreement with experiment. In order to describe the relatively complex potential energy surface of cyclohexane two degrees of freedom are necessary.[7,10,12] Since this potential corresponds to isolated molecules, direct comparison with experiment is not strictly correct and quantitative agreement should not be expected. Recently, it has been shown that even in the absence of electrostatic contributions the packing of solvent molecules can dramatically alter the gas-phase potential.[13-15] This would result in a possible dependence of the barrier height (ΔH^*) on the local structure of the solvent.

The application of stochastic models to isomerization reactions as proposed by Skinner and Wolynes[16] and Montgomery, Chan-

† Institute of Chemistry, College of General Education, Osaka University, Toyonaka, Osaka 560, Japan.

(1) H. D. Lüdemann, R. Rauchschwalbe, and E. Lang, *Angew. Chem.*, **87**, 340 (1977).
(2) J. B. Hendrickson, *J. Am. Chem. Soc.*, **83**, 4537 (1961).
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