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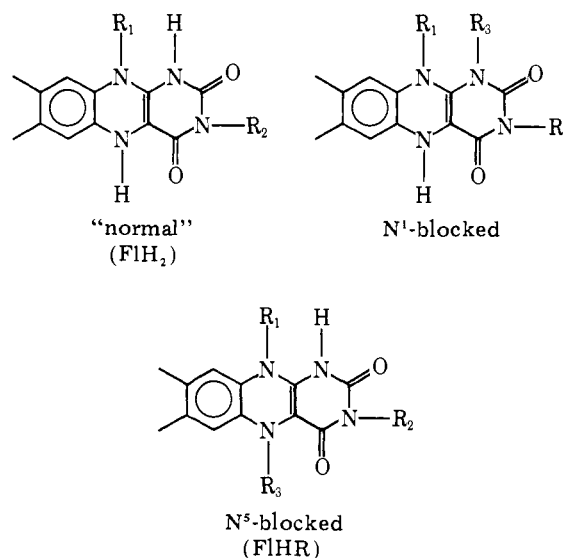
Reaction of ³O₂ with Dihydroflavins. 1. N^{3,5}-Dimethyl-1,5-dihydroflumiflavin and 1,5-Dihydroisalloxazines

C. Kemal,^{1a} T. W. Chan,^{1b} and T. C. Bruice*

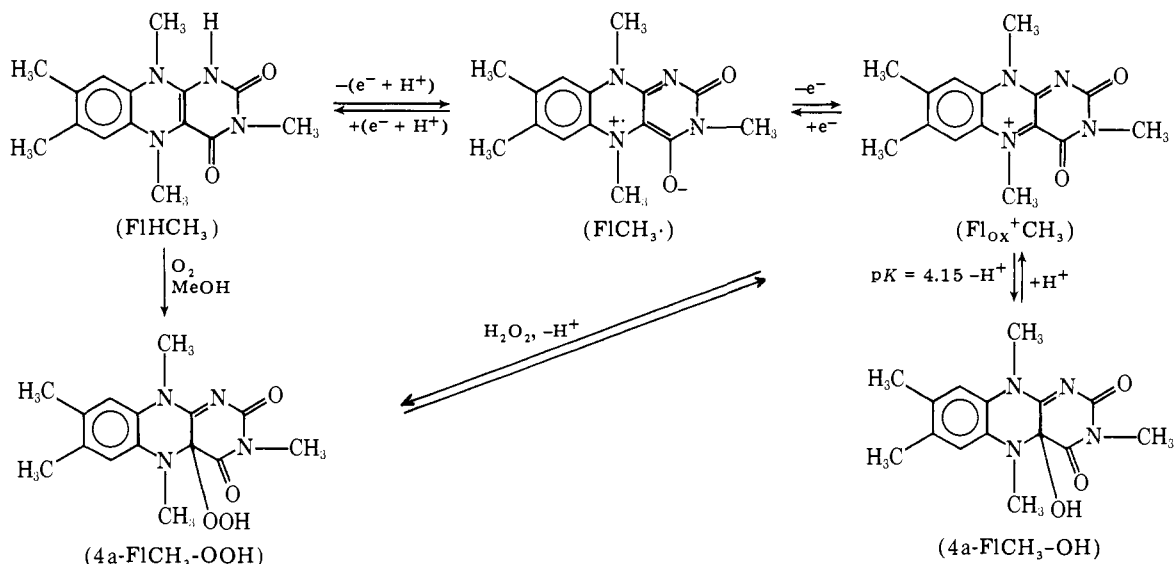
Contribution from the Department of Chemistry, University of California at Santa Barbara, Santa Barbara, California 93106. Received April 11, 1977

Abstract: The reactions of oxygen with N^{3,5}-dimethyl-1,5-dihydroflumiflavin (FIHCH₃) and a number of 1,5-dihydroisalloxazines have been investigated and mechanisms suggested which are based on kinetic measurements, product ratios, and computed potentials. The rate constants for the pertinent reactions which must be considered in the oxidation of FIHCH₃ in methanol (30 °C) are provided in eq 11–18. These include the formation of a 4a-hydroperoxyflavin (4a-FICH₃-OOH), its reaction with FIHCH₃ and FICH₃[•], and ionization to the oxidized flavin (Fl_{ox}⁺CH₃). The autocatalytic role of 4a-FICH₃-OOH and the importance of each reaction in the overall ³O₂ oxidation of FIHCH₃ have been established. Previous proposals of mechanism have involved homolytic scission of the C_{4a}-OOH bond. No evidence for this process could be discerned. In place, the 4a-FICH₃-OOH species is shown to undergo elimination of hydrogen peroxide to yield Fl_{ox}⁺CH₃ (both in methanol and water solvents). The autoxidation of FIHCH₃ in water (30 °C) provides a complex dependence of both rate and products on pH. Arguments are presented which point to the operation of the reactions of eq 26, 27, and 30 above pH 8, the reactions of eq 26 and 27 between pH 7 and 8, and the reactions of eq 26–29 below pH 7 (Scheme V). Autocatalytic oxidation of 1,5-dihydroisalloxazines (FIH₂) are discussed in terms of the reactions of Scheme VI. It is pointed out that the second-order rate constants for reaction of O₂ with FIH₂ and with FIHCH₃ are comparable (i.e., 250 vs. 220 M⁻¹ s⁻¹ at pH ~6.5) and that the greater lability of FIH₂ to O₂, when compared to FIHCH₃, is due to autocatalysis and in particular to the much greater rate of reaction of FI⁻ with O₂ when compared to FICH₃[•]. The time course for Fl_{ox} production has been shown to be reproducibly simulated when employing rate data from this and other studies. Lastly, feasibility of the intermediacy of the [FIH_T-O₂⁻] intimate radical pair in the oxidation of singlet FIH₂ by triplet O₂ has been established by comparison of the standard free energy of formation of FIH_T[•] + O₂⁻ (standard states 1 M, p[O₂] = 1 atm, 30 °C, pH 7.0) to the value of ΔG[‡] for the second-order reaction of oxygen with 1,5-dihydroflavin.

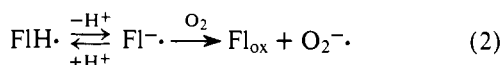
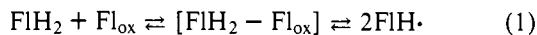
The not yet understood phenomenon of flavoenzyme mediated hydroxylation reactions² has prompted a number of investigations of the nonenzymic reduction of molecular oxygen by reduced flavins^{3–11} and has inspired much speculation concerning the mechanisms of both enzymic and model reactions.^{2,12–15} A large number of reduced flavins have been employed as models; these can be divided into three classes: (1) "normal" (FIH₂); (2) N¹-blocked; and (3) N⁵-blocked. Of these, the first two have received the most attention.^{3–8} Studies on the autoxidation of N¹-blocked reduced flavins by Mager and co-workers⁶ have emphasized the ability of this system to hydroxylate phenylalanine (by HO[•] generated from 1e⁻ reduction of a flavin hydroperoxide intermediate and/or H₂O₂). Müller and co-workers,⁷ on the other hand, have reported spectral evidence for the formation of a flavin hydroperoxide upon reaction of an N¹-blocked reduced flavin with oxygen in organic solvents.¹⁶ The most detailed kinetic investigations have employed "normal" reduced flavins such as 1,5-dihydroflavin mononucleotide (FMNH₂) and 1,5-dihydrotetraacetylriboflavin (TARFH₂). Working solely with FMNH₂,



Scheme I



Gibson and Hastings⁴ were first to discover the autocatalytic nature of the reaction and identified oxidized flavin as an autocatalytic agent. Massey, Palmer, and Ballou,⁵ who employed TARFH₂ as the principal substrate, later found out that in addition to oxidized flavin, O₂^{•-} was an autocatalytic agent. These two findings, taken together with the known high reactivity of flavin radical anion (FI⁻) with O₂,¹⁷⁻¹⁹ established the reaction sequence of eq 1-3 as a major part of "normal" reduced flavin autoxidation⁵ (see Discussion). The reaction sequence of eq 1-3 is, however, of little or no relevance to the action of flavoenzyme hydroxylases.



What is of concern is the primary reaction of (enzyme bound) FIH₂ with O₂ which has been shown (or proposed as the case may be) to yield an (enzyme bound) flavin hydroperoxide.^{2,20,21} The "side reactions" of eq 1-3, in model reactions, simply obscure the direct reaction of FIH₂ with O₂. In a model study, it is therefore desirable to eliminate all or at least some of these side reactions in order to obtain more information about the biologically important step, i.e., the interaction of FIH₂ with O₂. N⁵-Blocked reduced flavins possess desirable properties in this respect providing that the N⁵-blocking group is not labile in any one of three redox states of flavin (i.e., reduced, radical, and fully oxidized states). If the N⁵-blocking group is stable, then the reaction of flavin radical with O₂ (eq 2) is considerably slowed down. This is a consequence of preventing the ionization of the flavin radical to a radical anion, a species that is extremely reactive with O₂.¹⁷⁻¹⁹ The suppression of the reaction of eq 2 will of course make the reaction of eq 3 of less significance by lowering the concentration of O₂^{•-} in solution.

N^{3,5}-Dimethyl-1,5-dihydroflavin (FIHCH₃) employed by us in a number of studies^{11,22-24} is a satisfactory N⁵-blocked model (Scheme I). Though the comproportionation reaction of eq 1 is very rapid with N⁵-methylflavins (or any other known model), the reaction of eq 2 (and consequently the reaction of eq 3) would be expected to be suppressed owing to the stability of the N⁵-methyl blocking group in all three redox states of the flavin.²⁵ Preliminary work from this laboratory¹¹ has afforded spectral evidence for the formation of 4a-hydroperoxy-3,5-dimethyl-1,5-dihydroflavin (4a-FICH₃-OOH) on reaction of FIHCH₃ with O₂ in methanol solvent. In addition, we have

synthesized and characterized 4a-FIC₂H₅-OOH¹¹ and have shown the 4a-hydroperoxyflavin to react with aldehydes to yield mixed peroxides of general structure 4a-FIROO-CH(OH)R.²⁶ The latter decompose in a chemiluminescent reaction²⁶ mimicking the bioluminescent reaction of bacterial luciferase.²¹

We communicate herein the kinetics and product ratios for the reaction of ³O₂ with N^{3,5}-dimethyl-1,5-dihydroflavin and several 1,5-dihydroisalloxazines.

Experimental Section

Materials. 3,5-Dimethyl-,²⁷ 3-methyl-5-trideuteriomethyl-²³ and 3-methyl-5-ethyl-²⁷ 1,5-dihydroflavins (FIHCH₃, FIHCD₃, and FIHC₂H₅, respectively) and their oxidized forms²⁷ were prepared according to literature procedures. The synthesis of 5-alkyl-4a-hydroperoxy-3-methylflavins has been reported elsewhere.¹¹ 3-Methylflavin (VI) was prepared according to the N-oxide method.²⁸ Thin layer chromatography (TLC) on silica established the compound to be pure, mp 297 °C dec (lit.²⁸ mp 298-301 °C dec). Lumiflavin (IV) was prepared according to the method of Hemmerich et al.²⁹ The product was homogeneous by TLC, mp 324-325 °C dec (lit.²⁹ mp 322 °C dec). Lumiflavin-3-acetic acid (V) was obtained by derivatizing lumiflavin.³⁰ 10-(2',6'-dimethylphenyl)-3-methylisalloxazine (III) was from a previous study.³¹ The synthetic scheme for the preparation of 8-cyano-3,10-dimethylisalloxazine (I) and 7-cyano-3,10-dimethylisalloxazine (II) will be published in a separate manuscript dealing with the properties of these two flavins.³²

Superoxide dismutase (SOD) was isolated from human red blood cells following a procedure slightly modified from the one established by McCord and Fridovich for bovine superoxide dismutase³³ and purified by chromatography on a DEAE-cellulose (1.5 × 25 cm) column employing a linear KH₂PO₄ gradient at 4 °C. The fractions containing superoxide dismutase center at 0.05 M KH₂PO₄ as indicated by OD₂₈₀, copper test,³⁴ and cytochrome c/xanthine oxidase assay.^{33,35} Fractions were frozen and stored at -10 °C.

Xanthine oxidase (grade I from buttermilk, 5.6 μ/mL measured activity), cytochrome c (type III from horse heart), xanthine, and hypoxanthine were obtained from Sigma. 2,2'-Biquinoline was obtained from Aldrich. Thioxane (Aldrich) and methanol were distilled prior to use. Buffer salts and all other materials were analytical reagent grade and used without purification.

Apparatus. Kinetic measurements were made on a Cary 16, Cary 118C, or a Durrum-Gibson Model 13001 stopped-flow spectrophotometer. The latter instrument was enclosed in a glovebox under a nitrogen atmosphere. Spectra were recorded on a Cary 118C spectrophotometer. pH measurements were taken using a Radiometer Model 26 pH meter equipped with a standardized Model EA-125 Metrohm or GK-2302 C Radiometer electrode at 30 °C. A PDP 11/03 microprocessor based system was used in computer simulations.³⁶

Kinetic Measurements. All kinetic measurements reported in this paper were carried out at 30 ± 0.2 °C.

The reaction between FIHCH₃ and O₂ was followed by observing the appearance of *N*(3,5)-dimethylmonohydroxylumiflavin (FICH₃[•]) at 585 (in water) and 620 nm (in methanol). The following procedure was used for reactions carried out in water. A portion of an anaerobic stock solution of FICH₃[•] (3×10^{-3} to 2×10^{-2} M in 0.01 M KOH) was diluted to 25–50 mL with an anaerobic buffer solution (0.1 M, $\mu = 1.0$) containing EDTA (ca. 3×10^{-4} M). Buffer solutions of different O₂ concentrations were prepared separately by bubbling O₂-N₂ mixtures for 1 h at 30 °C. In appropriate experiments, 3-methylumiflavin or superoxide dismutase were added to the buffer solution before being saturated with the O₂-N₂ mixture. For the reactions carried out in methanol, O₂ solutions were prepared in much the same manner as in water. Reduced flavin solutions were prepared, just prior to being used, by dissolving crystals of FIHCH₃ in argon-saturated methanol.

The oxidations of IH₂, IIH₂, IIH₂, and IVH₂ by O₂ were followed by monitoring the appearance of the oxidized forms of these flavins at 441, 426, 434, and 450 nm, respectively. Anaerobic stock solutions of I, II, and III were prepared using acetonitrile as solvent (2.3 – 3.5×10^{-3} M). Stock solutions of lumiflavin (IV) were either 5×10^{-4} M (DMF) or 6×10^{-5} M (pH 6.4, phosphate buffer). Aliquots of the stock solutions were diluted to final volumes of 25–50 mL with appropriate anaerobic phosphate buffers (pH 6.4, $\mu = 0.1$ to 0.2). All solutions to be reduced by photolysis contained a 100-fold excess of EDTA (over flavin). Oxygen-saturated solutions were obtained by bubbling pure oxygen through the buffer solution (with or without flavin) at 30 °C by means of a gas dispersion tube for 45–60 min. Addition of superoxide dismutase was made to the oxygen saturated solutions after transferral into the glovebox surrounding the stopped-flow spectrophotometer, but just prior to reaction initiation. The storage syringes of the stopped-flow spectrophotometer were filled by needle through the serum-capped bottles to ensure oxygen-free atmosphere. Once the storage syringes were placed in the spectrophotometer, the reduced flavin solution was prepared by photolysis using a high-intensity tensor lamp (the other syringe containing oxygenated solution was protected from light) for typical time periods of 45–60 min to ensure complete reduction. The pHs of solutions were measured before and after reaction. Runs varying by pH drifts of >0.05 were discarded.

The reactions of protonated 1,5-dihydroxylumiflavin-3-acetic acid (VH₃⁺) and its aminium cation radical (VH₂^{•+}) with oxygen were studied as follows: under a nitrogen atmosphere there was placed in the lower portion of a Thunberg cuvette 1.6 mL of a solution 10^{-3} M in EDTA and 1 M in KCl and 0.5 mL of a methanolic solution 2.24×10^{-4} M in Fl_{ox} (V). After deoxygenation with vanadous ion scrubbed argon, the oxidized flavin was photoreduced. The spectrum of the reduced solution was recorded and under a nitrogen atmosphere 2.5 mL of 10 M HCl (the latter was prepared by deoxygenation with freeze-thaw vacuum techniques and saturated with vanadous ion scrubbed argon) was added to the cuvette. The spectrum was once again recorded and again under a nitrogen atmosphere 1 mL of O₂-saturated H₂O was added to provide a solution 2.44×10^{-5} M in VH₂, 5.44 M in HCl ($H_0 = -1.89$). Formation of VH₂^{•+} was followed at 485 nm and its disappearance at 485 or 383 nm (λ_{\max} of VH₂^{•+} and protonated V, respectively).

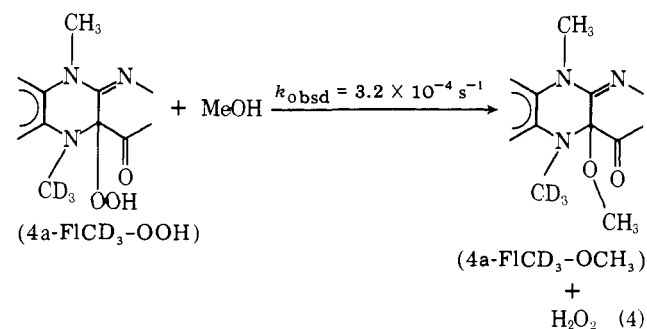
Determination of 4a-Hydroperoxy-3,5-dimethylumiflavin (4a-FICH₃-OOH) in Methanol.¹¹ The reaction of FIHCH₃ with O₂ (in MeOH) was carried out in a Thunberg cuvette. Then a weighed amount of NaI was added to the reaction solution after the oxidation of FIHCH₃ was complete (i.e., a few seconds after the concentration of FICH₃[•] reaches a maximum). Any 4a-FICH₃-OOH present as product is reduced by I⁻ ($k_{\text{obsd}} = 1.1 \text{ s}^{-1}$ when $[I^-] = 0.12 \text{ M}$) to yield I₂ and 4a-hydroxy-3,5-dimethylumiflavin (4a-FICH₃-OH). At the concentrations of I⁻ used (0.1–0.5 M), all of I₂ produced is as I₃⁻. The concentration of I₃⁻ ($= [4a\text{-FICH}_3\text{-OOH}]$) was determined spectrophotometrically at 349 nm ($\epsilon_{349} 2.29 \pm 0.03 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) after correcting for flavin absorbance at this wavelength. (Since $\epsilon_{349} \approx 6800 \text{ M}^{-1} \text{ cm}^{-1}$ for both 4a-FICH₃-OOH and 4a-FICH₃-OH, the correction simply requires subtraction of initial OD₃₄₉ from that observed after the addition of NaI.)

Results

Oxidation of 3,5-dimethyl-1,5-dihydroxylumiflavin (FIHCH₃) by O₂ in methanol was studied as a function of $[FIHCH_3]$ and

$[O_2]$. The condition, $[O_2] > [FIHCH_3]$, was maintained in all of the experiments. The progress of the reaction was monitored at 620 nm up to the point of maximal formation of the 1e⁻ oxidation product of FIHCH₃, i.e., FICH₃[•]. The appearance of FICH₃[•] is accompanied by a lag phase. (The disappearance of FICH₃[•] due to reaction with O₂ was found to be much slower; the subject will be taken up later.) FICH₃[•] was not the sole product of the reaction and its yield (determined spectrophotometrically, $\epsilon_{620} \approx 4500 \text{ M}^{-1} \text{ cm}^{-1}$) was found to vary as a function of the concentrations of both the reduced flavin and oxygen.

The dependence of the percent yield of FICH₃[•] on $[FIHCH_3]$ ($[O_2] = 1.0 \times 10^{-3} \text{ M}$) is shown in Figure 1. Inspection of Figure 1 reveals that the yield of the radical product increases as $[FIHCH_3]$ is increased. This dependence is not linear, however, and the maximum yield possible appears to be ca. 50% when the limit, $[O_2] \approx [FIHCH_3]$, is reached. In contrast, the dependence of percent yield of FICH₃[•] on $[O_2]$ is in the reverse direction, i.e., as $[O_2]$ is increased ($[FIHCH_3] = \text{constant}$), the percent yield of FICH₃[•] decreases. For example, increasing oxygen concentration from 1.0×10^{-3} to $3.7 \times 10^{-3} \text{ M}$ ($[FIHCH_3] = 2.7 \times 10^{-4} \text{ M}$) decreases the percent yield of FICH₃[•] produced from 42 to 24%. These observations are most useful in deciphering the mechanism of O₂ oxidation of FIHCH₃ (see Discussion). They also clearly indicate conditions that must be chosen in order to detect other flavin product(s) of the reaction. Thus the dependence of the percent yield of FICH₃[•] on $[FIHCH_3]$ and $[O_2]$ dictates that when the ratio $[O_2]/[FIHCH_3]$ is maximized, the yield of FICH₃[•] should approach zero; hence, the yield of the other flavin product(s) should be maximal. Indeed, the oxidation of $2 \times 10^{-5} \text{ M}$ FIHCH₃ in O₂-saturated methanol ($[O_2] \approx 7.5 \times 10^{-3} \text{ M}$) yields only ca. 10% of FICH₃[•]. The spectrum of the reaction mixture which possesses absorption peaks at 370 and 308 nm is almost identical with the spectrum of authentic 4a-hydroperoxy-3,5-dimethylumiflavin (4a-FICH₃-OOH, Figure 2) synthesized as previously described.¹¹ In addition, it was determined that a product of the O₂ oxidation of FIHCH₃ (1) oxidizes I⁻ to I₂ at exactly the same rate ($k_{\text{obsd}} = 2.3 \text{ s}^{-1}$ when $[I^-] = 0.2 \text{ M}$) as does authentic 4a-FICD₃-OOH; (2) forms the 4a-methoxy adduct (Figure 2) as does authentic 4a-FICD₃-OOH¹¹ (eq 4). These results unambiguously establish



that in addition to FICH₃[•], 4a-FICH₃-OOH is produced in the reaction of FIHCH₃ with O₂. The oxidation of I⁻ by the flavin hydroperoxide has previously been used to quantitate the active oxygen content of 4a-FIC₂H₅-OOH.¹¹ The same method (see Experimental Section) was used to determine the yield of 4a-FICH₃-OOH produced in the reaction FIHCH₃ + O₂. Thus, when $[FIHCH_3] = 2 \times 10^{-5} \text{ M}$ and $[O_2] \approx 7.5 \times 10^{-3} \text{ M}$, the yield of 4a-FICH₃-OOH was ca. 80%. On the other hand, when $[O_2]/[FIHCH_3] \approx 1.0$, no 4a-FICH₃-OOH could be detected.

Since 4a-FICH₃-OOH is the major final product when the ratio $[O_2]/[FIHCH_3]$ is large, and at best a minor product when $[O_2] \approx [FIHCH_3]$, it follows that it can be consumed during the O₂ oxidation of FIHCH₃. Based on the extensive

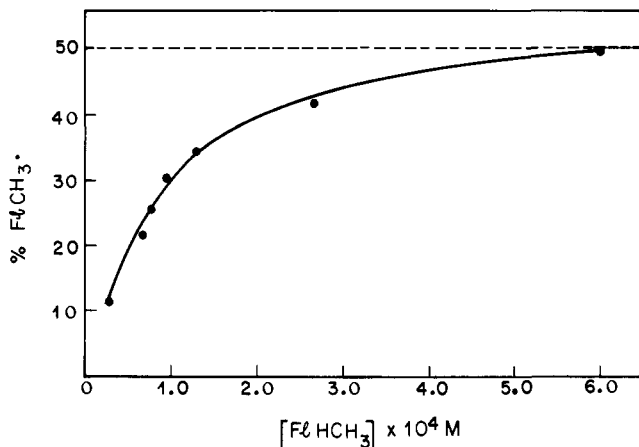
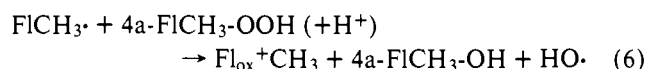
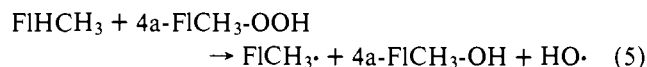
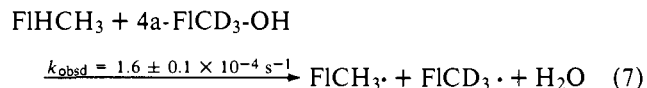


Figure 1. The observed percent yield of $\text{FIClH}_3\cdot$ on O_2 ($1 \times 10^{-3} \text{ M}$) oxidation of FlHCH_3 as a function of the initial concentration of FlHCH_3 (30°C , methanol solvent).

literature on hydroperoxide reactions,³⁷ one-electron reduction of $4\text{a-FlCH}_3\text{-OOH}$ by FlHCH_3 and $\text{FIClH}_3\cdot$ would be expected (eq 5 and 6).

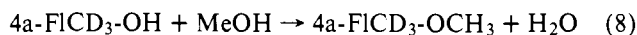


Indeed, in separate experiments both FlHCH_3 and $\text{FIClH}_3\cdot$ were found to be capable of reducing $4\text{a-FlCD}_3\text{-OOH}$. The formation of $\text{FIClH}_3\cdot$ from the reaction of FlHCH_3 and $4\text{a-FlCD}_3\text{-OOH}$ (10^{-5} and $1.0\text{--}2.5 \times 10^{-4} \text{ M}$, respectively) (eq 5) as well as the disappearance of $\text{FIClH}_3\cdot$ due to its reaction with $4\text{a-FlCD}_3\text{-OOH}$ (eq 6) was monitored at 620 nm . Formation of $\text{FIClH}_3\cdot$ obeyed first-order kinetics for at least 3 half-lives and a second-order rate constant of $5 \pm 0.5 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$ was calculated as an average of nine kinetic runs. The rate of the reaction of $\text{FIClH}_3\cdot$ with $4\text{a-FlCD}_3\text{-OOH}$ (eq 6) was estimated from the same experiments by taking the initial slope of the ensuing OD decrease at 620 nm . The second-order rate constant so obtained was $20 \pm 10 \text{ M}^{-1} \text{ s}^{-1}$. In the above experiments, the yield of $\text{FIClH}_3\cdot$ was $\sim 100\%$ (at the OD_{620} maximum) based on the limiting reagent (i.e., FlHCH_3). When the same reaction was studied using \geq twofold excess FlHCH_3 over $4\text{a-FlCD}_3\text{-OOH}$, the production of $\text{FIClH}_3\cdot$ was found to occur in two phases. The first phase ($k = 500 \text{ M}^{-1} \text{ s}^{-1}$) yielded one $\text{FIClH}_3\cdot$ per $4\text{a-FlCD}_3\text{-OOH}$ as before, while the second phase yielded two more flavin radicals per $4\text{a-FlCD}_3\text{-OOH}$. The observed rate constant for the second phase was $1.6 \pm 0.1 \times 10^{-4} \text{ s}^{-1}$. In a separate experiment, the reaction of $4\text{a-FlCD}_3\text{-OH}$ ($1.8 \times 10^{-5} \text{ M}$) with FlHCH_3 ($1.8 \times 10^{-4} \text{ M}$) also yielded the same rate constant (eq 7).



Hence it can be concluded that the reaction of FlHCH_3 with $4\text{a-FlCH}_3\text{-OOH}$ yields $4\text{a-FlCH}_3\text{-OH}$ as well as $\text{FIClH}_3\cdot$ (eq 5) (see Discussion).

In methanol, $4\text{a-FlCD}_3\text{-OH}$ was found to convert slowly to $4\text{a-FlCD}_3\text{-OCH}_3$ (eq 8).



Although the spectra of the two species are almost identical, small but sufficient absorbance changes at 420 nm allowed the determination of a rate constant, $1.6 \pm 0.1 \times 10^{-4} \text{ s}^{-1}$, for the

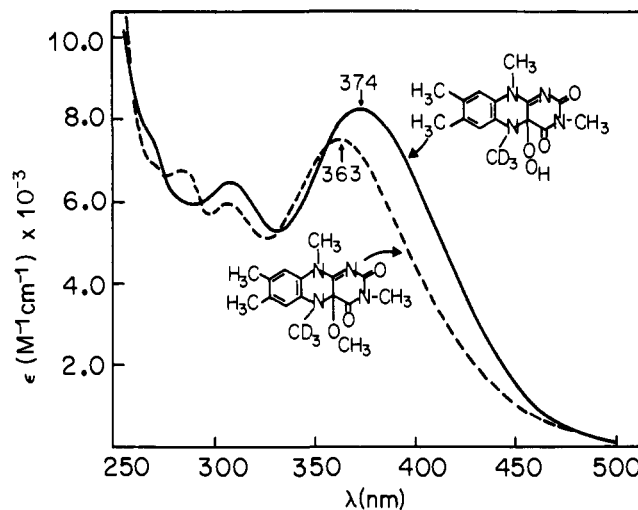
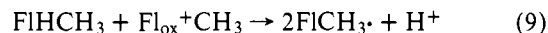


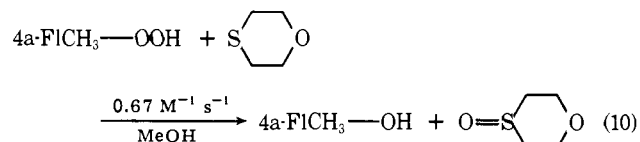
Figure 2. The spectra of $4\text{a-FlCD}_3\text{-OOH}$ and $4\text{a-FlCD}_3\text{-OCH}_3$ in methanol. The former yields the latter with an associated pseudo-first-order rate constant of $3.2 \times 10^{-4} \text{ s}^{-1}$ (30°C). The somewhat smaller absorbance of the methoxy pseudobase compared to that of $4\text{a-FlCD}_3\text{-OOH}$ could be due to a few percent of intramolecular oxidation of the isoalloxazine ring of the $4\text{a-FlCD}_3\text{-OOH}$ species which is in competition with the exchange reaction.

process. Since the reactions depicted in eq 7 and 8 proceed at the same rate, it can be concluded that the rate-determining step in both cases is the dissociation of HO^- from $4\text{a-FlCD}_3\text{-OH}$. The cation, $\text{Fl}_{\text{ox}}^+\text{CD}_3$, thus produced is trapped either by MeOH or FlHCH_3 . The second-order rate constant for H_2O attack on $\text{Fl}_{\text{ox}}^+\text{CH}_3$ was previously determined to be $1.2 \times 10^{-2} \text{ M}^{-1} \text{ s}^{-1}$.²³ Methanol would be expected to react with $\text{Fl}_{\text{ox}}^+\text{CH}_3$ at about the same rate. On the other hand, the comproportionation rate



is near diffusion controlled.²³ Therefore, in the presence of FlHCH_3 , $4\text{a-FlCH}_3\text{-OH}$ would be expected to react as in eq 7 rather than as in eq 8.

The dependence of the yield of $\text{FIClH}_3\cdot$ on oxygen and reduced flavin concentrations suggests that a considerable amount of the radical is produced as a result of the reaction of $4\text{a-FlCH}_3\text{-OOH}$ with FlHCH_3 (eq 5). A means to assess the importance of the reaction of eq 5 would be obtained if the oxidation of FlHCH_3 was carried out in the presence of a selective flavin hydroperoxide trapping agent. Thioxane has been shown²⁶ to react with $4\text{a-FlCD}_3\text{-OOH}$ as in eq 10. Since this reaction (eq 10) competes with the reaction of eq 5, inclusion



of thioxane in the reaction mixture would be expected to lower the yield of $\text{FIClH}_3\cdot$.³⁸ This was found to be the case. The percent yield of $\text{FIClH}_3\cdot$ on O_2 ($1.0 \times 10^{-3} \text{ M}$) oxidation of FlHCH_3 ($2.6 \times 10^{-5} \text{ M}$) dropped from $\sim 15\%$ to $\sim 3\%$ on inclusion of 1.05 M thioxane (Figure 3).

The time courses of the oxidation of FlHCH_3 ($7.6 \times 10^{-5} \text{ M}$, $[\text{O}_2] = 1.0 \times 10^{-3} \text{ M}$) in the presence and absence of Fl_{ox} (3-methylumiflavin (VI), $2.2 \times 10^{-4} \text{ M}$) were found to be quite different. In the presence of Fl_{ox} , the formation of $\text{FIClH}_3\cdot$ was no longer associated with a lag phase and the rate of its formation was faster (Figure 4). In addition, Fl_{ox} seemed to affect the yield of $\text{FIClH}_3\cdot$ (Figure 4). Thus, the yield of $\text{FIClH}_3\cdot$ was 26% in the absence of Fl_{ox} and 19% in its presence. Since

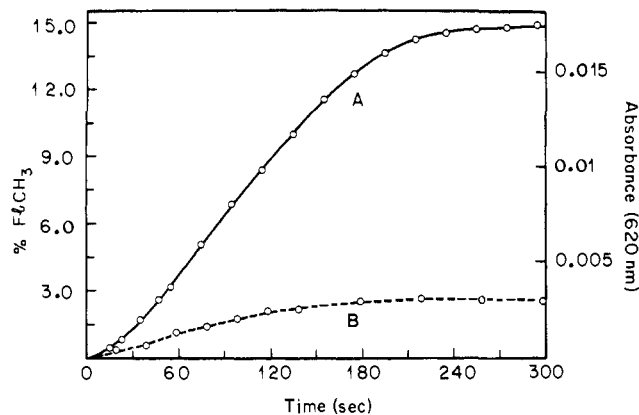


Figure 3. (A) The time course of O_2 (1.0×10^{-3} M) oxidation of $FIHCH_3$ (2.6×10^{-5} M) ($30^\circ C$, methanol solvent). (B) Same as A except 1.05 M thioxane is present. Left-hand axis provides the percent yield of $FICH_3\cdot$ (based on $[FIHCH_3]$).

the concentrations of $FICH_3\cdot$ were calculated using its extinction coefficient at 620 nm (≈ 4500 $M^{-1} cm^{-1}$), the possibility of an $Fl_{ox}-FICH_3\cdot$ complex possessing a lower ϵ_{620} was assessed. In the presence and absence of 3×10^{-4} M Fl_{ox} , the absorption of $FICH_3\cdot$ (10^{-5} M) was found to be the same. It can therefore be concluded that $FIHCH_3$ autoxidation yields less radical if the reaction is carried out in the presence of Fl_{ox} . It has been suggested that Fl_{ox} catalyzes the breakdown of flavin hydroperoxides.¹⁰ To check this possibility, the conversion of $4a-FICD_3-OOH$ (10^{-4} M) to $4a-FICD_3-OCH_3$ was studied in the presence of 10^{-4} M Fl_{ox} . No catalysis was detected. In addition, Fl_{ox} (3×10^{-4} M) was found to have no effect on the reaction of $FIHCH_3$ (10^{-5} M) with $4a-FICH_3-OOH$ (2.5×10^{-4} M).

The oxidation of $FICH_3\cdot$ by O_2 (in methanol) was studied at only one oxygen concentration ($[O_2] = 1.3 \times 10^{-3}$ M, $[FICH_3\cdot] \approx 10^{-5}$ M). The disappearance of $FICH_3\cdot$ (monitored at 620 nm) was found to obey excellent first-order kinetics to >4 half-lives. A second-order rate constant of ca. 0.4 $M^{-1} s^{-1}$ was calculated by dividing k_{obsd} by $[O_2]$ (average of three runs). When the reaction was over, the observed spectrum was that of $4a-FICH_3-OCH_3$ (λ_{ma} 363 and 308 nm, Figure 2).

The oxidation of $FIHCH_3$ by O_2 in water was investigated under the pseudo-first-order conditions of $[O_2] > [FIHCH_3]$ and as a function of $[O_2]$ and pH (4.85–10.5). As in methanol, the reaction was followed up to maximal formation of $FICH_3\cdot$ (λ_{max} 585 nm). In contrast to the $\leq 50\%$ yields of $FICH_3\cdot$ observed in methanol, the yield of $FICH_3\cdot$ in water was found to be $\sim 100\%$ below pH 8. Above pH 8, however, the yield of $FICH_3\cdot$ was found to decrease (Table I), and at pH 10.5, the observed yield of $FICH_3\cdot$ on reaction of 1.1×10^{-5} M $FIHCH_3$ with 4.16×10^{-4} M oxygen was only $\sim 15\%$. As shown in Table I, at pH 10.5, the yield of $FICH_3\cdot$ increased to $\sim 40\%$ upon increasing $[FIHCH_3]$ to 3.85×10^{-5} M while holding $[O_2]$ constant. In another experiment ($[FIHCH_3] = 3.85 \times 10^{-5}$ M, $[O_2] = 4.16 \times 10^{-4}$ M), the solution was acidified (to pH ~ 0) as soon as the maximal formation of $FICH_3\cdot$ had occurred. From the spectrum of the acidified mixture, $\sim 55\%$ yield of $Fl_{ox}^+CH_3$ was calculated (ϵ_{550} of $Fl_{ox}^+CH_3$ 7950 $M^{-1} cm^{-1}$,²³ ϵ_{550} of $FIHCH_3^+$ ~ 3000 $M^{-1} cm^{-1}$). The cation $Fl_{ox}^+CH_3$ arises on acidification of $4a-FICH_3-OH$.²³ This result suggests that at pH 10.5, the reaction of $FIHCH_3$ with O_2 yields $4a-FICH_3-OH$ as well as $FICH_3\cdot$. The dependence of the percent yield of $FICH_3\cdot$ on $[FIHCH_3]$ at pH 10.5 is similar to the results obtained in methanol (Figure 1). In contrast to the findings in methanol, the yield of $FICH_3\cdot$ exhibited no dependence on initial $FIHCH_3$ concentration (3.8×10^{-6} to 4.35×10^{-5} M) below pH 8 (Table I).

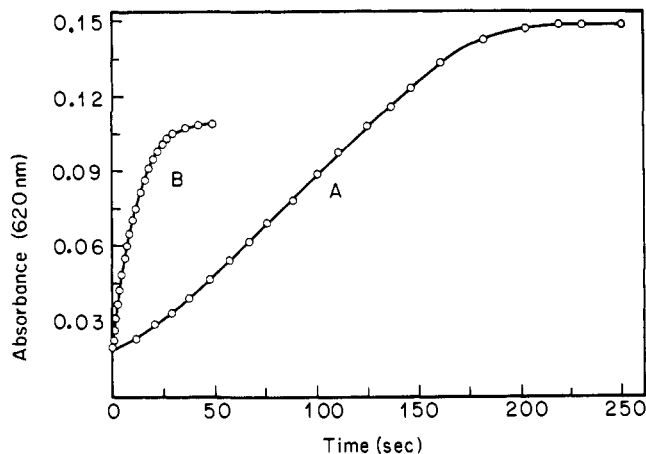


Figure 4. Absorbance vs. time plots for the reaction of $FIHCH_3$ (7.6×10^{-5} M) with O_2 (1.0×10^{-3} M) in the presence (B) and absence (A) of 2.2×10^{-4} M Fl_{ox} ($30^\circ C$, methanol solvent).

Table I. Summary of Rate Constants and Product Yields for the Reaction of $FIHCH_3$ with O_2 (4.16×10^{-4} M) in Aqueous Solvent ($30^\circ C$)

pH ^a	$[FIHCH_3]$	$k_{obsd}/[O_2]$, $M^{-1} s^{-1}$ ^b	% yield of $FICH_3\cdot$ ^c
4.85	6.2×10^{-6}	4.9×10	100
4.95	4.35×10^{-5}	5.8×10	100
5.38	6.7×10^{-6}	6.4×10	100
5.78	4.35×10^{-5}	1.76×10^2	100
6.03	3.8×10^{-6}	1.36×10^2	100
6.47	7.9×10^{-6}	2.55×10^2	100
6.57	4.35×10^{-5}	4.40×10^2	100
7.0	3.87×10^{-5}	6.00×10^2	100
7.63	4.35×10^{-5}	1.13×10^3	100
8.0	1.64×10^{-5}	1.03×10^3	100
8.03	4.35×10^{-5}	1.23×10^3	100
8.7	1.1×10^{-5}	1.25×10^3	85
10.5	1.1×10^{-5}	$\sim 1.3 \times 10^3$	15
10.5	3.85×10^{-5}	$\sim 1.3 \times 10^3$	40

^a All buffer concentrations were 0.1 M ($\mu = 1.0$ with KCl). Buffers used were acetate ($< pH$ 5), 2-(*N*-morpholino)ethanesulfonate (pH 5.38–6.47), morpholinopropanesulfonate (pH 6.57–8.0), Tris (pH 8–8.7), and carbonate (pH 10.5). ^b Rate constants for reactions carried out below pH 7 were obtained from initial slopes; k_{obsd} at pH 7 and above was obtained from first-order plots. ^c Based on the initial concentration of $FIHCH_3$ and determined spectrophotometrically at 585 nm.

Comparison of the time course of $FIHCH_3$ oxidation at six pHs between 4.85 and 8 (Figure 5A) indicates that as the pH is lowered to below 8, the rate of the reaction slows down. Inspection of Figure 5A also reveals that at pH 7–8 the reaction obeys the first-order rate law and that lowering the pH to below 7 is accompanied by increasing deviations from this law. The absorbance vs. time curves of these reactions (pH 4.85–6.5) do not show a lag phase; however, it is clear from the semi-logarithmic plots of Figure 5A that, as the reaction proceeds, the rate of $FICH_3\cdot$ production is speeded up. In Figure 5B, the time course of the O_2 oxidation of $FIHCH_3$ at pH 4.85 is compared to that obtained in the presence of 2×10^{-5} M Fl_{ox} (VI). Inspection of Figure 5B reveals that in the presence of Fl_{ox} , the rate of $FICH_3\cdot$ appearance is faster. In methanol, Fl_{ox} was found to affect radical production in a similar way (see above). However, in the latter solvent, there was an additional effect of lowering the yield of $FICH_3\cdot$ (Figure 4). In contrast, in water, Fl_{ox} was found not to affect the yield of $FICH_3\cdot$ which was $\sim 100\%$ both in the presence and absence of Fl_{ox} . In Figure 6 there is presented a plot of the pseudo-first-order rate con-

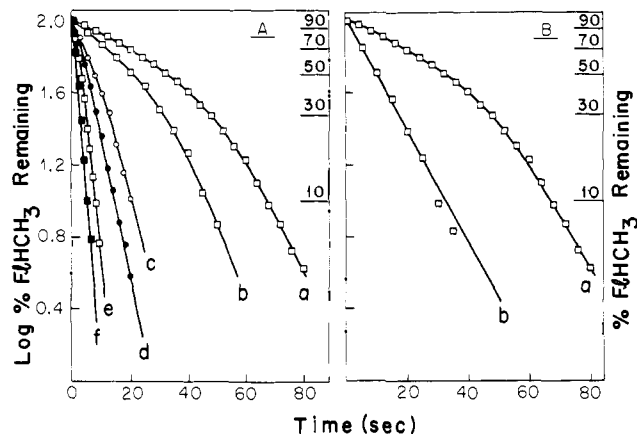


Figure 5. Time dependence of the reaction of FIHCH₃ with O₂ in water (30 °C, $\mu = 0.1$). (A) [FIHCH₃] = 8×10^{-6} to 1.5×10^{-5} M, [O₂] = 5.5×10^{-4} M at pH 4.85 in 0.1 M acetate (a), pH 5.38 in 0.1 M acetate (b), pH 6.08 in 0.1 M MES (c), pH 6.5 in 0.1 M MES (d), pH 7.0 in 0.1 M MOPS (e), and pH 8.0 in 0.1 M MOPS (f). (B) Reaction at pH 4.85 in 0.1 M acetate with [FIHCH₃] = 1.5×10^{-5} M (a), and [FIHCH₃] = 8×10^{-6} M with [Fl_{ox}] = 2×10^{-5} M (b).

stant (k_{obsd}) for the O₂ oxidation of FIHCH₃ (at pH 7) vs. the percent O₂ in O₂-N₂ gas mixtures employed in reaction solutions. From Figure 6 it is seen that k_{obsd} is a linear function of the partial pressure of O₂. One may conclude, therefore, that at pH 7.0, the oxidation of FIHCH₃ is first order in dihydroflavin and first order in oxygen. The apparent second-order rate constant from the slope of the line of Figure 6 is $\sim 600 \text{ M}^{-1} \text{ s}^{-1}$. This value must be divided by 2 to obtain the true second-order rate constant (at pH 7) for the reaction of O₂ with FIHCH₃ (see Discussion). At pHs 7.0 and 4.85, the OD₅₈₅ vs. time curves for the reaction of FIHCH₃ ($\sim 10^{-5}$ M) with O₂ (4.16×10^{-4} M) were found to be identical in the presence and absence of 5×10^{-7} M superoxide dismutase (SOD). This finding implies that little or no O₂^{•-} is formed on oxidation of FIHCH₃ to FICH₃[•] (see Discussion). In contrast, the rate of O₂ oxidation of FIH₂ (to Fl_{ox}) is retarded significantly by SOD⁵ (see below).

In methanol, as shown, the rate-determining step in the reaction of 4a-FICH₃-OH with FIHCH₃ (to yield two FICH₃[•]) is the dissociation of 4a-FICH₃-OH to Fl_{ox}⁺CH₃. In water the same mechanism would be expected to hold. In order to confirm this expectation, the reaction of 10^{-5} M FIHCH₃ with 10^{-5} M 4a-FICD₃-OH was studied (pH 6.5, 0.1 M phosphate, $\mu = 1.0$). Despite the fact that the two species were equimolar, the production of (FICH₃[•] + FICD₃[•]) was found to obey the first-order rate law ($k_{\text{obsd}} = 8 \times 10^{-3} \text{ s}^{-1}$). Thus, as in methanol, the rate-determining step of the reaction was the ionization of 4a-FICD₃-OH to Fl_{ox}⁺CD₃, which then reacted rapidly with FIHCH₃ to yield the observed product. In a previous paper from this laboratory,²³ the spontaneous and specific acid catalyzed rates of the ionization of 4a-FICH₃-OH were reported to be $\sim 10^{-2} \text{ s}^{-1}$ and $9.4 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$, respectively. The former rate, which was determined at pH 10, is in close agreement with the rate obtained at pH 6.5 in this study. Thus, it can be concluded that between pH ~ 6 and 10, the rate of ionization of 4a-FICH₃-OH is ca. $9 \times 10^{-3} \text{ s}^{-1}$.

As will be made clear (see Discussion), a knowledge of the rate of dissociation of 4a-FICH₃-OOH to Fl_{ox}⁺CH₃ and HO₂⁻ is very useful in judging the possible intermediacy of this species in the autoxidation of FIHCH₃. Attempts were made to obtain this rate constant by employing a procedure similar to that used in methanol, i.e., absorbance at 420 nm was monitored for the expected OD decrease on conversion of 4a-FICD₃-OOH to 4a-FICD₃-OH (see Figure 2). Conversion of 4a-FICD₃-OOH to 4a-FICD₃-OH occurred in the 15-s time

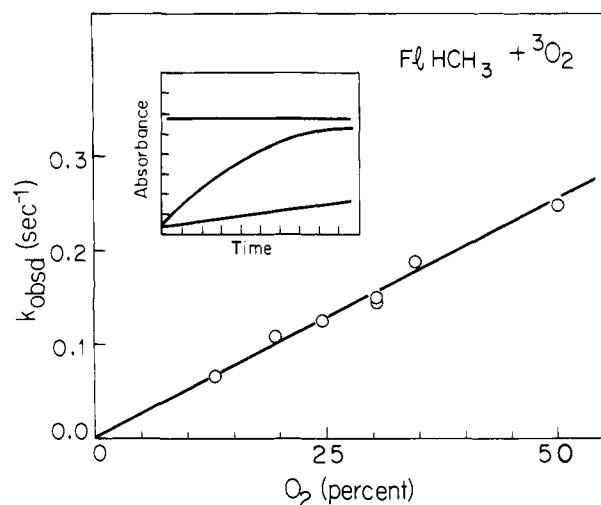


Figure 6. Plot of the pseudo-first-order rate constant (k_{obsd}) for the O₂ oxidation of FIHCH₃ vs. percent O₂ in N₂-O₂ mixtures employed to saturate solutions at 30 °C. Initial concentration of the dihydroflavin was ca. 8×10^{-6} M (pH 7.0 with 0.1 M MOPS buffer, $\mu = 1.0$ with KCl, solvent H₂O). The insert represents the absorbance vs. time trace for reaction of FIHCH₃ with oxygen (4.16×10^{-4} M). The time units for the lower trace and middle trace are 0.1 and 1 s per division, respectively. There is noted to be no lag period. The same reaction carried out in the presence of ca. 5×10^{-7} M superoxide dismutase produced a similar trace.

of mixing a dioxane solution of 4a-FICD₃-OOH with an aqueous 0.1 M phosphate buffer [20–30% dioxane (v/v), final pH ~ 7]. The species in solution was determined spectrophotometrically to be 4a-FICD₃-OH. From these results a minimum value of $\sim 0.2 \text{ s}^{-1}$ can be estimated for the spontaneous ionization rate of 4a-FICH₃-OOH at neutral pH. It has been suggested that flavin hydroperoxide yields flavin and superoxide radicals upon homolytic cleavage⁵ (especially at pHs greater than 8).^{10b,15} However, no FICD₃[•] is formed upon dissolving 4a-FICD₃-OOH in H₂O (pH ~ 7). Also, crystals of 4a-FICD₃-OOH when dissolved in pH 9.5 bicarbonate buffer (0.1 M, anaerobic conditions) did not give rise to the formation of FICD₃[•]; instead, 4a-FICD₃-OOH was converted to 4a-FICD₃-OH (λ_{max} 355, 307 nm) which was then hydrolyzed²³ (note also that the yield of FICH₃[•] from the reaction of FIHCH₃ with O₂ decreases with increasing pH; see above).

Oxidation of FICH₃[•] by O₂ in Water. The rate of this reaction was estimated from the initial slope of the absorbance decrease at 585 nm following the formation of FICH₃[•] from the reaction of FIHCH₃ with O₂. Between pH 4.85 and ~ 7 , the second-order rate constant so obtained was ca. $8 \text{ M}^{-1} \text{ s}^{-1}$. In these experiments, the disappearance of FICH₃[•] was expectedly found not to obey first-order kinetics. This is due to the fact that the flavin product from the reaction of FICH₃[•] with O₂, i.e., Fl_{ox}⁺CH₃, is solvolyzed to yield FICH₃[•] in a general-base-catalyzed reaction (see Scheme III of ref 23). With this knowledge and the knowledge that the rate of solvolysis of Fl_{ox}⁺CD₃ is $>$ tenfold slower than that for Fl_{ox}⁺CH₃,²³ it was possible to manipulate the conditions and thus avoid complicating secondary reactions. Thus, the reaction of FICD₃[•] (8×10^{-6} M) with O₂ (4.16×10^{-4} M) in 0.001 M MOPS buffer (pH 6.56, $\mu = 1.0$) was found to obey first-order kinetics. The second-order rate constant calculated from this experiment was $7.9 \text{ M}^{-1} \text{ s}^{-1}$, which agrees well with the rate calculated from initial slopes of FICH₃[•] disappearance.

Oxidation of 1,5-Dihydroflumiflavin (IVH₂) by O₂ (pH 6.4). The time course of the reaction of FIH₂ (2.4×10^{-6} M) with oxygen (5.5×10^{-4} M) is significantly altered when the reaction is carried out in the presence of Fl_{ox} (IV) (Figure 7). Thus the extent of the lag phase (observed in the absence of

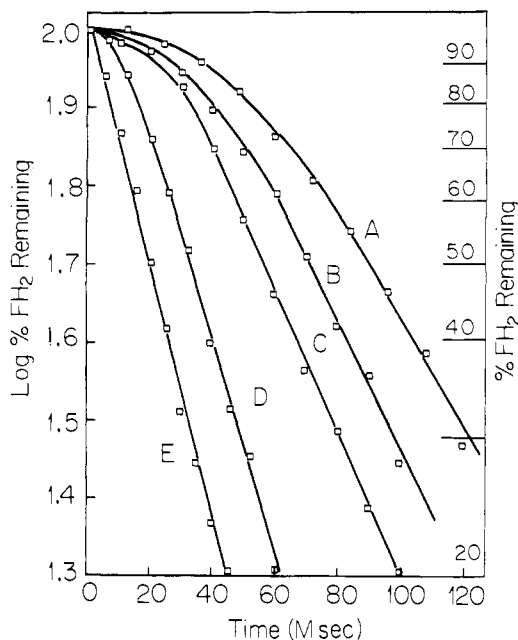
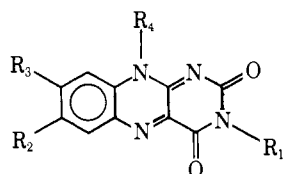


Figure 7. The effect of added Fl_{ox} (IV) on the time dependence of the reaction of FlH_2 (IVH_2 , 2.4×10^{-6} M) with oxygen (5.5×10^{-4} M). The concentration of Fl_{ox} was varied from 2.75×10^{-6} (curve A) to 10^{-5} M (curve E) (30°C , $\mu = 0.1$ with KCl, buffer 0.05 M phosphate).

added Fl_{ox}) is found to decrease when Fl_{ox} is present from the start and at the highest initial Fl_{ox} concentration (10^{-5} M), there is essentially no lag phase (Figure 7E). Also, one may note the increase in the rate of Fl_{ox} appearance following the lag phase when $[\text{Fl}_{\text{ox}}]_{\text{initial}}$ is increased (Figure 7). In agreement with previous studies,⁵ superoxide dismutase (SOD) was found to alter the time course of the reaction of FlH_2 with oxygen. The effects of SOD (10^{-7} M) are illustrated in Figures 8A-C. When the initial Fl_{ox} concentration was somewhat greater than $[\text{FlH}_2]$ (1.5×10^{-5} and 5×10^{-6} M, respectively), the initial rate observed in the absence of SOD was found to be identical with the overall rate observed in the presence of SOD (Figure 8A). The effects of SOD on the autoxidation of 2.24×10^{-6} M FlH_2 were determined in the presence of ~ 0 , 2.24×10^{-6} , and 9.4×10^{-6} M Fl_{ox} . When no Fl_{ox} was present initially, inclusion of SOD resulted in an increase of the lag phase and \sim three-fold decrease in the rate observed after the lag phase (see Figures 10a,b in the Discussion). When $[\text{Fl}_{\text{ox}}]_{\text{initial}}$ and $[\text{FlH}_2]$ were equimolar (2.24×10^{-6} M), the extent of the lag phase was slightly longer in the presence of SOD than that observed in its absence while the rate after the lag phase was again slower in the presence of SOD (Figure 8B). At the highest Fl_{ox} concentration (9.4×10^{-6} M) there was little or no lag phase present (both in the presence and absence of SOD) and the overall rate was slower (ca. fourfold) in the presence of SOD (Figure 8C).



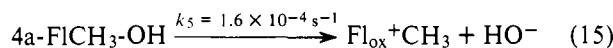
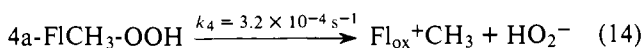
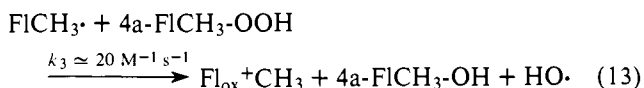
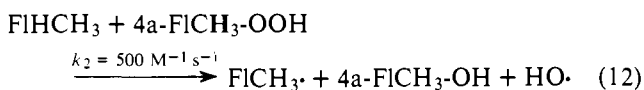
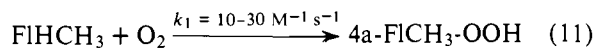
	R_1	R_2	R_3	R_4
I	CH_3	H	CN	CH_3
II	CH_3	CN	H	CH_3
III	CH_3	H	H	2',6'-dimethylphenyl
IV	H	CH_3	CH_3	CH_3
V	CH_2CO_2^-	CH_3	CH_3	CH_3
VI	CH_3	CH_3	CH_3	CH_3

Oxidation of 1,5-Dihydroflavins IH_2 - IIIH_2 by O_2 . Results are shown in Figure 8D-F for the oxidation of IH_2 , IIH_2 , and IIIH_2 carried out with and without SOD and in the presence of Fl_{ox} concentrations that suppress the lag phase. Inspection of Figure 8D-F reveals that the autoxidation of these flavins does not follow first-order kinetics. As observed with IVH_2 , the reaction is slower in the presence of SOD; however, at about 50% completion the observed rate of Fl_{ox} production slows down further. (Both in the presence and absence of SOD the autoxidations of IH_2 - IIIH_2 are not first order, but in Figures 8D-F, curvature of the first-order plots is demonstrated only for the reactions carried out in the presence of SOD.) Gibson and Hastings⁴ reported that in the oxidation of FMNH_2 the appearance of FMN , following the lag phase, was not first order while Massey, Palmer, and Ballou⁵ have reported that for the oxidation of 1,5-dihydrotetraacetylriboflavin the appearance of Fl_{ox} , at completion of the lag phase, is first order. Examination of Figure 8 reveals that the order may or may not appear to be first order in Fl_{ox} . From what follows (Discussion) it is obvious that there is no reason to anticipate first-order appearance of Fl_{ox} .

O_2 Oxidation of 1,5-Dihydroflavin-3-acetic Acid (VH_2) in the H_0 Region. In strong acid 1,5-dihydroflavins become protonated ($\text{FlH}_2 + \text{H}^+ \rightleftharpoons \text{FlH}_3^+$). The reaction of FlH_3^+ (VH_3^+ , $\text{pK}_a = 0.8$) (5.4 M HCl , $H_0 = -1.89$) with O_2 was found to be biphasic. Repetitive scanning of the reaction solution showed the initial buildup of the red flavin radical cation ($\text{VH}_2^{\cdot+}$) with λ_{max} at 485 nm and with an isosbestic point at 332 nm. The second-order rate constant for reaction of VH_3^+ with O_2 to provide $\text{VH}_2^{\cdot+}$ was determined to be ~ 4 $\text{M}^{-1} \text{s}^{-1}$. Plots of ϵ/OD vs. time for the disappearance of $\text{VH}_2^{\cdot+}$ ($\epsilon_{485} 1.1 \times 10^4 \text{ M}^{-1} \text{cm}^{-1}$) were found to be linear. Thus under aerobic conditions, the disappearance of the radical cation (isosbestic points at 437 and 357 nm) is second order in this species ($k \approx 5.6 \times 10^{-2} \text{ M}^{-1} \text{s}^{-1}$). The disappearance of the neutral radical ($\text{FlH}\cdot$) from aerobic solution has previously been shown to be second order in $\text{FlH}\cdot$ ($k_{\text{rate}} < 3 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$) while the radical anion $\text{Fl}^{\cdot-}$ reacts with oxygen with a second-order rate constant of $\sim 3 \times 10^8 \text{ M}^{-1} \text{s}^{-1}$.¹⁷⁻¹⁹ Moorthy and Hayon³⁹ have found that the radical cations generated from pyrazine, pyrimidine, and quinoxaline are relatively stable toward O_2 while the radical anions react with O_2 with second-order rate constants $> 10^8 \text{ M}^{-1} \text{s}^{-1}$.

Discussion

Reaction of O_2 with FlHCH_3 in Methanol. Prerequisite to an understanding of the O_2 oxidation of 1,5-dihydroflavins is an elucidation of the reactions of a number of flavin species with each other as well as with O_2 and $\text{O}_2^{\cdot-}$. An appreciation of this may be gained by a cursory examination of eq 11-18 which summarize the reactions (and rate constants determined in this study) occurring on O_2 oxidation of FlHCH_3 in methanol solvent.



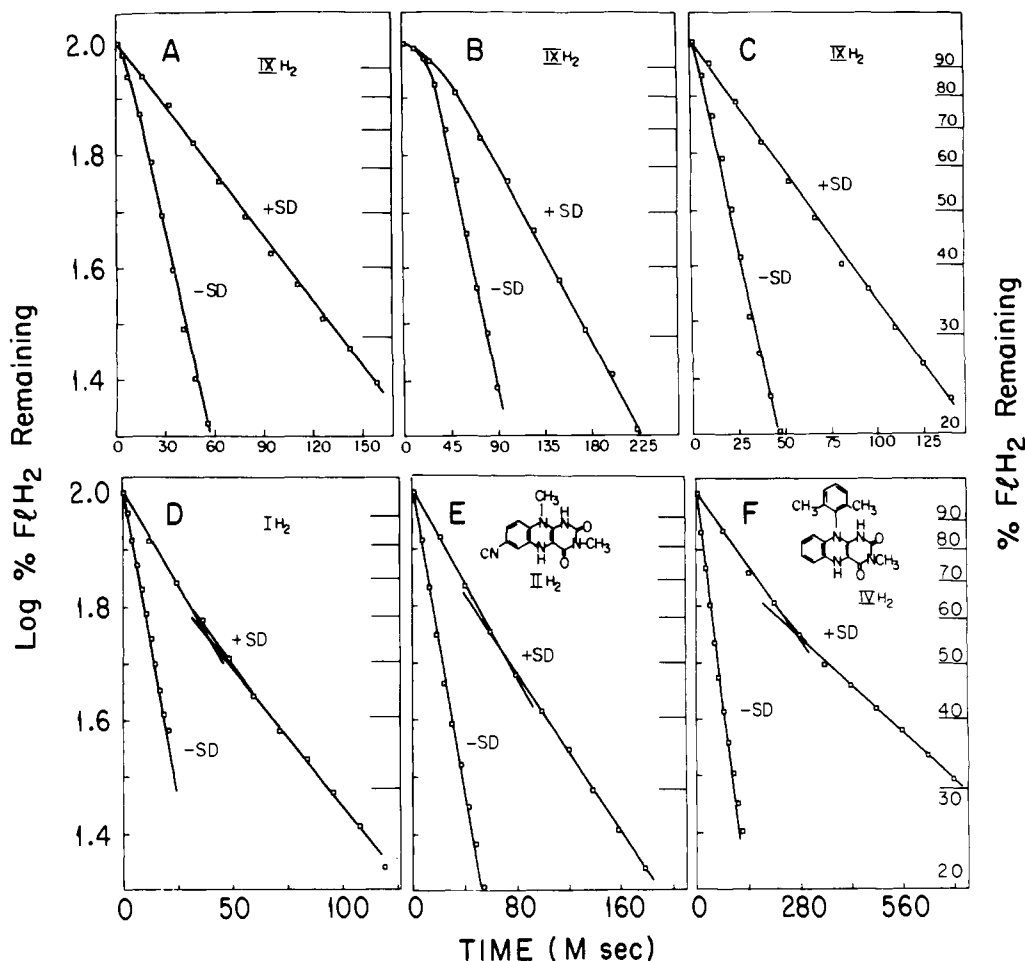
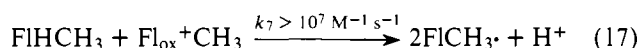
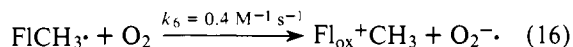


Figure 8. Time dependence of the reaction of dihydroflavins with oxygen (5.5×10^{-4} M) in the presence and absence of superoxide dismutase (pH 6.4). Initial concentrations of FIH_2 and FI_{ox} were A, 5×10^{-6} and 1.5×10^{-5} M; B, 2.24×10^{-6} and 2.24×10^{-6} M; C, 2.24×10^{-6} and 9.4×10^{-6} M; D, 1.9×10^{-5} and 5.3×10^{-5} M; E, 2.3×10^{-5} and 4.8×10^{-5} M; F, 1.5×10^{-5} and 4.4×10^{-5} M, respectively. Superoxide dismutase when present was at 10^{-7} M.



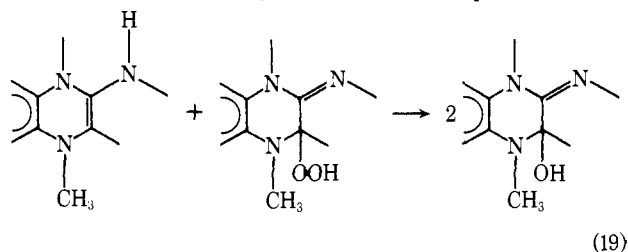
Each of the rate constants and stoichiometries (of flavin species) for six (eq 12–17) of these reactions were determined independently (see Results). The rate constant for 4a- $FIHCH_3$ -OOH formation (eq 11) was estimated from the initial slope of the time course of the autoxidation of $FIHCH_3$. Contribution to the overall reaction from the reaction of O_2^- with $FIHCH_3$ (eq 18) can only be minor because of the slow rate of O_2 oxidation of $FIHCH_3$ (eq 16) which (presumably) yields O_2^- . Of these eight reactions, five (eq 11–13, 17, 18) are irreversible. The reactions of eq 14–16 are effectively irreversible because the $FI_{ox} + CH_3$ product reacts very rapidly with $FIHCH_3$ (eq 17). Once all of $FIHCH_3$ has been consumed, $FI_{ox} + CH_3$ reacts with the solvent to yield 4a- $FIHCH_3$ -OCH₃ (see Results).

Both spectral and kinetic evidence establish that a 4a-hydroperoxyflavin (4a- $FIHCH_3$ -OOH, Scheme I, eq 11) is formed on O_2 oxidation of $FIHCH_3$. Thus, a product of the reaction possesses a spectrum ($\lambda_{max} \sim 370$ nm) very similar to that of authentic 4a- $FIHCH_3$ -OOH ($\lambda_{max} \sim 370$ nm, Figure 2) and it oxidizes I^- to I_2 at exactly the same rate as does authentic 4a- $FIHCH_3$ -OOH (see Results). The evidence for the participation of 4a- $FIHCH_3$ -OOH as an autocatalytic agent (eq 12)

in the reaction of $FIHCH_3$ with O_2 is threefold. (1) Trapping of 4a- $FIHCH_3$ -OOH with thioxane (eq 10) results in significantly lesser yields of $FIHCH_3$ (Figure 3). This means that (in the absence of thioxane) much of the observed radical product arises from the reaction of eq 12. (2) As O_2 concentration is increased ($[FIHCH_3] = \text{constant}$) the yield of $FIHCH_3$ decreases while the yield of 4a- $FIHCH_3$ -OOH increases. These results are consistent with eq 11 and 12 which indicate respectively that the velocity of 4a- $FIHCH_3$ -OOH formation is $[O_2]$ dependent while that of its disappearance (with radical formation) is not. (3) As $[FIHCH_3]$ is increased ($[O_2] = \text{constant}$) the yield of $FIHCH_3$ increases (Figure 1) while the yield of 4a- $FIHCH_3$ -OOH decreases. These results also confirm predictions based on the reactions of eq 11 and 12. As the concentration of $FIHCH_3$ approaches that of O_2 , the percent yield of $FIHCH_3$ levels at ca. 50% (Figure 1). This observation is in accord with the trapping (eq 12) of all formed 4a- $FIHCH_3$ -OOH (eq 11) by $FIHCH_3$, i.e., eq 11 and 12 are the most important steps in determining the appearance of $FIHCH_3$ in methanol.

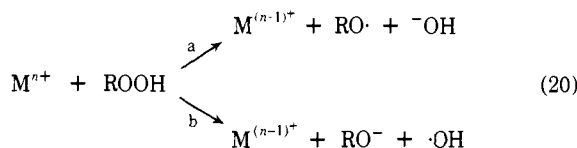
Working with 1,3-dimethyl-1,5-dihydroflumiflavin, Mager and co-workers⁶ were first to recognize that reduced flavin and flavin radical can reduce a flavin hydroperoxide as in eq 12 and 13, i.e., via one-electron transfer. This conclusion was based on the extent of O_2 consumption (50% based on reduced flavin concentration) and the fact that H_2O_2 was absent as a product.^{6b} These results, and the finding that the oxidation of reduced flavin could be coupled to hydroxylation of aromatic compounds (by $HO\cdot$),^{6d} strongly suggested reactions similar to those in eq 11–13. A priori, three possible mechanisms can

be written for the reaction of FlHCH_3 with $4a\text{-FlCH}_3\text{-OOH}$. The first would involve nucleophilic attack of FlHCH_3 upon the terminal peroxy oxygen of $4a\text{-FlCH}_3\text{-OOH}$ yielding two $4a\text{-FlCH}_3\text{-OH}$ species (eq 19). The second requires dissocia-

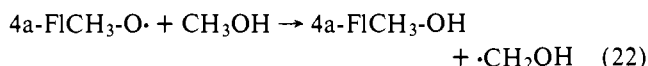
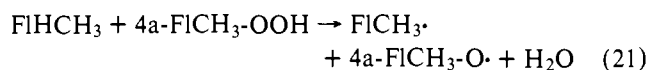


tion of HO_2^- from $4a\text{-FlCH}_3\text{-OOH}$ (eq 14) and reaction of the resultant $\text{Fl}_{\text{ox}}^+\text{CH}_3$ with FlHCH_3 (eq 17) yielding two $\text{FlCH}_3\cdot$ but no $4a\text{-FlCH}_3\text{-OH}$. The third possible mechanism involves $1e^-$ reduction of $4a\text{-FlCH}_3\text{-OOH}$ by FlHCH_3 to yield one $\text{FlCH}_3\cdot$ and one $4a\text{-FlCH}_3\text{-OH}$. These three possible reaction paths can be distinguished by the products actually formed. Since experimentally it was shown that $\text{FlCH}_3\cdot$ and $4a\text{-FlCH}_3\text{-OH}$ are formed in a 1:1 ratio (see Results), the correct mechanism must be that which involves $1e^-$ reduction of $4a\text{-FlCH}_3\text{-OOH}$ by FlHCH_3 . A combination of the first and second mechanism (eq 19 plus eq 14 followed by eq 17) which would give the same product ratio is not possible because of the slow dissociation rates of $4a\text{-FlCH}_3\text{-OOH}$ and $4a\text{-FlCH}_3\text{-OH}$ (eq 14 and 15, respectively). However, a minor contribution from the reactions of eq 14 and 17 is quite likely [in H_2O , where the ionization of $4a\text{-FlCH}_3\text{-OOH}$ occurs faster, this pathway may actually predominate (see below)].

It is interesting to note that FlHCH_3 and $\text{FlCH}_3\cdot$ behave analogously to a reducing transition metal (eq 20). The type



of homolytic cleavage of the O-O bond of an organic hydroperoxide (i.e., 20a vs. 20b) depends on the nature of the R group.³⁷ Therefore, the formation of $4a\text{-FlCH}_3\text{-OH}$ on reaction of FlHCH_3 with $4a\text{-FlCH}_3\text{-OOH}$ could also be explained by eq 21 and 22

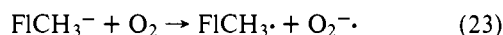


(instead of eq 12). The available data do not allow us to distinguish between the two possibilities.⁴⁰ The same considerations also apply to the reaction of $\text{FlCH}_3\cdot$ with $4a\text{-FlCH}_3\text{-OOH}$ (eq 13). The possible reactions of $\text{HO}\cdot$ (eq 12, 13) and/or $\cdot\text{CH}_2\text{OH}$ (eq 22) with flavin species have not been considered owing to lack of information.

It can be estimated that in the reaction of FlHCH_3 (2.6×10^{-5} M) with O_2 ($\sim 1.0 \times 10^{-3}$ M) in the presence of thioxane (1.05 M), >99% of $4a\text{-FlCH}_3\text{-OOH}$ intermediate is trapped by thioxane, i.e., the $\text{FlCH}_3\cdot$ producing reaction of eq 12 is inoperative. Yet, even under these conditions, ca. 3% $\text{FlCH}_3\cdot$ is obtained (Figure 3). However, the main source of this small amount of radical is most likely due to the slow reaction of $4a\text{-FlCH}_3\text{-OH}$ (product of thioxane reaction of eq 10) with FlHCH_3 which occurs in two steps (eq 15 and 17). Autocatalysis by $\text{FlCH}_3\cdot$ (eq 16 followed by eq 17 and 18) also contributes about 1% to the final yield of $\text{FlCH}_3\cdot$.

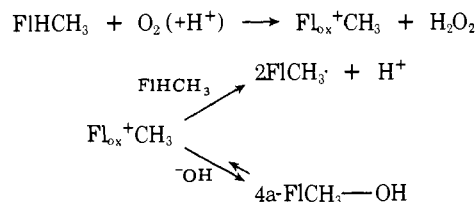
Reaction of O_2 with FlHCH_3 in Water. Autoxidation of FlHCH_3 is pH dependent and it is convenient to divide the

discussion of mechanism according to the observations made in the three pH regions: pH 4.8–7, 7–8, and 8–10.5. Between pH 4.8 and 8 the sole product is $\text{FlCH}_3\cdot$; however, the kinetics differ in the pH regions of 4.8–7 and 7–8. At pH 4.8 the reaction is slower than at higher pH and the kinetic behavior is complex. As the pH is increased from 4.8 to 7 the rate increases and the reaction gradually becomes first order (Figure 5). On increasing pH from 8 to 10.5, the rate does not change substantially but the yield of $\text{FlCH}_3\cdot$ decreases. For example, at pH 10.5 the reaction of $\text{FlCH}_3\cdot$ (1.1×10^{-5} M) with O_2 (4.16×10^{-4} M) yields only $\sim 15\%$ $\text{FlCH}_3\cdot$. Although the stability of $\text{FlCH}_3\cdot$ is going from neutral pH to pH 10.5 decreases, its rate of production still exceeds its rate of disappearance by ~ 50 -fold. Therefore, this drastic change in the observed yield of $\text{FlCH}_3\cdot$ cannot be attributed to its instability at pH 10.5. It must therefore be concluded that in the main ($\geq 85\%$) $\text{FlCH}_3\cdot$ produced in the reaction of $\text{FlCH}_3\cdot$ with O_2 does not arise from a simple one-electron transfer as in eq 23. (The radical pair $[\text{FlCH}_3\cdot \text{O}_2^-]$ may exist as an intermediate (see latter discussion) but if so it collapses to $4a\text{-FlCH}_3\text{-OOH}$.)



The results imply that there is a precursor of $\text{FlCH}_3\cdot$ which is susceptible to trapping by HO^- . The most logical candidate for the precursor is fully oxidized flavin, $\text{Fl}_{\text{ox}}^+\text{CH}_3$, which can either comproportionate with $\text{FlCH}_3\cdot$ to yield $\text{FlCH}_3\cdot$ or react with HO^- to yield $4a\text{-FlCH}_3\text{-OH}$. The rate of HO^- attack on $\text{Fl}_{\text{ox}}^+\text{CH}_3$ to form $4a\text{-FlCH}_3\text{-OH}$ is $5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$.²³ The comproportionation rate between $\text{Fl}_{\text{ox}}^+\text{CH}_3$ and $\text{FlCH}_3\cdot$ is even faster²³ ($\sim 10^9 \text{ M}^{-1} \text{ s}^{-1}$). It follows that HO^- and $\text{FlCH}_3\cdot$ would begin to compete with each other in reacting with $\text{Fl}_{\text{ox}}^+\text{CH}_3$ once the concentration of HO^- becomes comparable to that of $\text{FlCH}_3\cdot$. If $[\text{FlCH}_3\cdot] \approx 10^{-5}$ M then the reaction of HO^- with $\text{Fl}_{\text{ox}}^+\text{CH}_3$ should become important at ca. pH 9. In other words, at a given concentration of reduced flavin and oxygen, the yield of radical should decrease as the pH is increased. This was found to be the case (Table I). A scheme which is consistent with the data obtained between pH 8 and 10.5 is given in Scheme II.

Scheme II

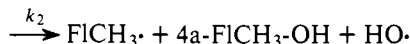
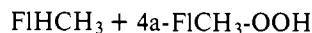
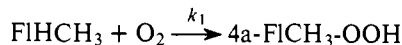


In Scheme II, $4a\text{-FlCH}_3\text{-OOH}$ is most likely the precursor to $\text{Fl}_{\text{ox}}^+\text{CH}_3$ and H_2O_2 (it must be recalled that $4a\text{-FlCH}_3\text{-OOH}$ is the direct product of reaction of FlHCH_3 with O_2 in methanol). The observation of a small lag phase (lasting ca. 2% of the total time course of the reaction) at pH 8.7 may be indicative of the intermediacy of $4a\text{-FlCH}_3\text{-OOH}$ (see below). Further evidence in support of Scheme II was obtained by showing that at pH 10.5 the percent yield of $\text{FlCH}_3\cdot$ increases from 15% to $\sim 40\%$ upon increasing the initial concentration of $\text{FlCH}_3\cdot$ from 1.1×10^{-5} M to 3.85×10^{-5} M (compare the $[\text{FlHCH}_3]$ dependent and independent disposition of $\text{Fl}_{\text{ox}}^+\text{CH}_3$ in Scheme II) and that $\sim 55\%$ of product in the latter case is indeed $4a\text{-FlCH}_3\text{-OH}$ (see Results).⁴¹ In addition, the fact that superoxide dismutase does not alter the time course of $\text{FlCH}_3\cdot$ oxidation (pH 7) finds ready explanation in Scheme II which indicates that no O_2^- is formed.

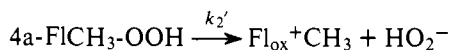
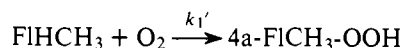
The observed rate constants of oxidation of FlHCH_3 at pH 7–8 (Table I), taken together with the quantitative yield of $\text{FlCH}_3\cdot$, impose a stringent constraint on the mechanism of the reaction. For example, if $4a\text{-FlCH}_3\text{-OOH}$ is an intermediate

it must be converted to $\text{FICH}_3\cdot$ within the experimentally determined time course of the reaction. The conversion of $4a\text{-FICH}_3\text{-OOH}$ to $\text{FICH}_3\cdot$ is not spontaneous (see Results) and would have to follow either of two paths (Schemes III and IV).

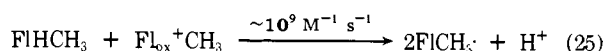
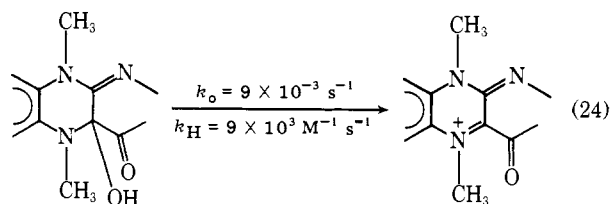
Scheme III



Scheme IV



As in methanol, direct reaction of FIHCH_3 with $4a\text{-FICH}_3\text{-OOH}$ (Scheme III) would be expected to yield one $\text{FICH}_3\cdot$ and one $4a\text{-FICH}_3\text{-OH}$ (see before). Therefore, the yield of $\text{FICH}_3\cdot$ by the mechanism of Scheme III would at best be 50% providing $k_2 \gg k_1$. The reaction of $4a\text{-FICH}_3\text{-OH}$ with FIHCH_3 also yields $\text{FICH}_3\cdot$. However, the rate of $\text{FICH}_3\cdot$ formation (eq 24, 25, where k_0 = rate constant for the spontaneous or



H_2O -catalyzed departure of HO^- and k_H = the rate constant for specific-acid-catalyzed departure of HO^-)²³ is governed by the rate of ionization of $4a\text{-FICH}_3\text{-OH}$ (eq 24) which, at pH 8, is ~ 50 -fold slower than the observed rate of O_2 oxidation of FIHCH_3 . It can therefore be concluded that no significant contribution to the yield of $\text{FICH}_3\cdot$ could arise from the reaction of $4a\text{-FICH}_3\text{-OH}$ and FIHCH_3 via the sequence of eq 24 and 25. Thus, Scheme III, even if it were to include eq 24 and 25, would not explain the data.⁴² Scheme IV, on the other hand, would be consistent with the observed rate law and yield of product only if the rate constant for dissociation of $4a\text{-FICH}_3\text{-OOH}$ to $\text{Fl}_{\text{ox}}^+\text{CH}_3 + \text{HO}_2^-$ (k_2') was $>$ tenfold greater than $k_1' [\text{O}_2]$. Unfortunately, a precise value for the dissociation of $4a\text{-FICH}_3\text{-OOH}$ could not be determined experimentally (see Results) and a minimum value of 0.2 s^{-1} was estimated. By computer simulation of the time course for $\text{FICH}_3\cdot$ formation using Scheme IV (see below) it could be shown that if k_2' was less than $\sim 5 \text{ s}^{-1}$, the production of $\text{FICH}_3\cdot$ at pH 8 would exhibit a lag phase. Since no lag phase was observed (pH 7–8), it can be concluded that if $4a\text{-FICH}_3\text{-OOH}$ is on the reaction pathway, it must ionize with an associated rate constant of $\geq 5 \text{ s}^{-1}$ in this pH range. A small lag phase was observed at pH 8.7. It is not surprising that the rate constant for dissociation of $4a\text{-FICH}_3\text{-OOH}$ would exceed that for $4a\text{-FICH}_3\text{-OH}$ (eq 24). Thus, Dixon and Bruice⁴³ found that even though the equilibrium constants for the formation HO^- and HOO^- adducts of malachite green were about the same; both the on and off rates for HOO^- were greater by 3–4 orders of magnitude.

Scheme V

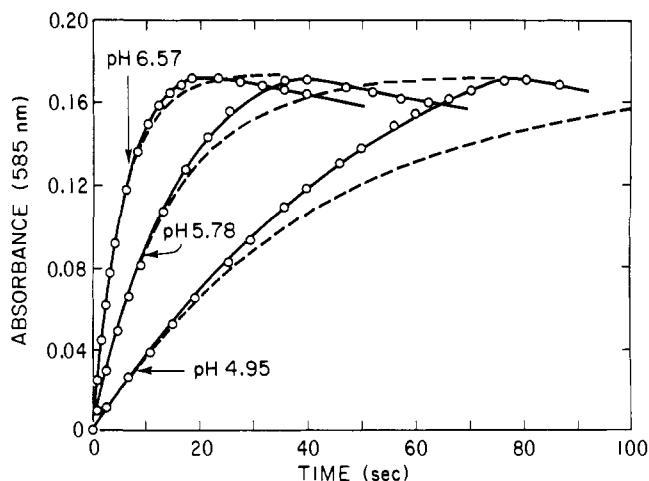
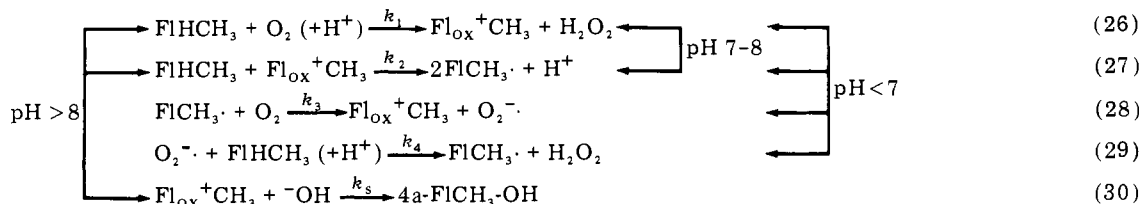
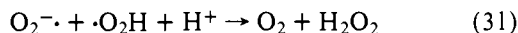


Figure 9. Computer simulation of the O_2 ($4.16 \times 10^{-4} \text{ M}$) oxidation of FIHCH_3 ($4.35 \times 10^{-5} \text{ M}$). The points were experimental; the solid and dashed lines were generated using eq 26–29 and 26–27 of Scheme V, respectively. The difference between solid and dashed lines (at each pH) represents the contribution to the overall reaction from the autocatalytic steps of eq 28, 29 [$k_1 = 29, 88, \text{ and } 220 \text{ M}^{-1} \text{ s}^{-1}$ at pH 4.95, 5.78, and 6.57, respectively; the same values of $k_2 (> 2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1})$, $k_3 (8 \text{ M}^{-1} \text{ s}^{-1})$, and $k_4 (> 2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1})$ were used at all three pHs; see Scheme V].

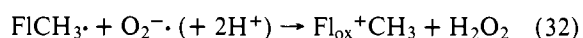
$\text{FICH}_3\text{-OOH}$ is on the reaction pathway, it must ionize with an associated rate constant of $\geq 5 \text{ s}^{-1}$ in this pH range. A small lag phase was observed at pH 8.7. It is not surprising that the rate constant for dissociation of $4a\text{-FICH}_3\text{-OOH}$ would exceed that for $4a\text{-FICH}_3\text{-OH}$ (eq 24). Thus, Dixon and Bruice⁴³ found that even though the equilibrium constants for the formation HO^- and HOO^- adducts of malachite green were about the same; both the on and off rates for HOO^- were greater by 3–4 orders of magnitude.

Since at pH 7–8 the rate of $\text{FICH}_3\cdot$ oxidation to FICH_3 is 100–150 times faster than the rate of the reaction of $\text{FICH}_3\cdot$ with O_2 , it was not essential to consider the latter reaction in following the appearance of $\text{FICH}_3\cdot$. However, upon lowering the pH to below 7 it becomes necessary to do so. The reason for this is that while the rate of the reaction of FIHCH_3 ($\text{pK}_a \approx 6.5$)²² with O_2 decreases upon lowering the pH to below 7, the rate of the reaction of $\text{FICH}_3\cdot$ (pK_a of $\text{FICH}_3\text{H}^+ = 2.65$)²³ with O_2 remains the same. The $\text{Fl}_{\text{ox}}^+\text{CH}_3$ and O_2^- produced on reaction of O_2 with $\text{FICH}_3\cdot$ react with FIHCH_3 to produce more radical. This autocatalysis increases the rate of oxidation of FIHCH_3 to $\text{FICH}_3\cdot$. With these considerations in mind, Scheme V is proposed to explain all the experimental observations which we have collected for the autoxidation of FIHCH_3 between pH 4.8 and 10.5 in water (in the overall reaction of eq 26, $4a\text{-FICH}_3\text{-OOH}$ may well be the precursor to $\text{Fl}_{\text{ox}}^+\text{CH}_3 + \text{H}_2\text{O}_2$). To test the scheme, kinetic runs between pH 4.8 and 7 were computer simulated. Three typical fits at pHs 4.95, 5.78, and 6.58 are shown in Figure 9. The solid and dashed lines of Figure 9 were generated using eq 26–29 and 26 and 27, respectively. As shown, the difference between the solid and dashed lines becomes more noticeable upon lowering pH, i.e., as the pH is lowered there is more and more contribution to $\text{FICH}_3\cdot$ production from the autocatalytic steps of

eq 28 and 29. The values of k_1 that were used in these computer simulations were obtained by dividing the observed second-order rate constants ($k_{\text{obsd}}/[\text{O}_2]$) of Table I by 2.⁴⁴ The value of k_3 was also experimental ($\sim 8 \text{ M}^{-1} \text{ s}^{-1}$), and k_2 and k_4 were set equal to $2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (the same solutions were obtained as long as k_2 and k_4 were greater than $10^6 \text{ M}^{-1} \text{ s}^{-1}$, i.e., their precise values are unimportant as long as k_1 and $k_3 \ll k_2$ and k_4). As mentioned previously, the actual value of k_2 is $\sim 10^9 \text{ M}^{-1} \text{ s}^{-1}$. The dismutation of superoxide radicals



was not included in Scheme V. This of course is equivalent to assuming that all of $\text{O}_2^{\cdot-}$ radicals produced from the reaction of $\text{FICH}_3\cdot$ with O_2 (eq 28) react with FIHCH_3 (eq 29). For this assumption to hold true, k_4 (eq 29) must be $> 10^7 \text{ M}^{-1} \text{ s}^{-1}$.⁴⁵ The possible reaction of $\text{FICH}_3\cdot$ with $\text{O}_2^{\cdot-}$ (eq 32) was also not included in Scheme V. If the rate constant for the reaction of eq 32 is fast enough to suppress dismutation of $\text{O}_2^{\cdot-}$ (eq 31), then the sum of eq 32 and 27 become identical with eq 29.

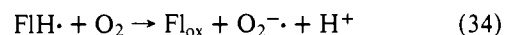
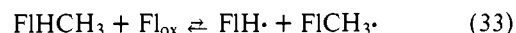


In any event, the reaction of eq 32 is not required in the computer simulation of the overall time course of the reaction of FIHCH_3 with O_2 .

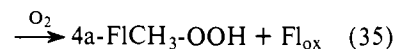
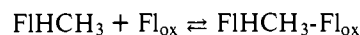
It has been shown (see Results) that inclusion of SOD does not alter the time course of FIHCH_3 autoxidation at pH 4.85. This may be due to the fact that during the reaction $\text{O}_2^{\cdot-}$ concentration is very low ($< 3 \times 10^{-8} \text{ M}$).⁴⁵ In any case, the contribution of the reaction of FIHCH_3 with $\text{O}_2^{\cdot-}$ to the autoxidation of FIHCH_3 must be a minor one. This can be shown by computer simulation of the time course of $\text{FICH}_3\cdot$ production and ignoring the reaction of FIHCH_3 with $\text{O}_2^{\cdot-}$ (eq 29, Scheme V). When this is done, a curve lying exactly at the mean of the solid (eq 26–29) and dashed (eq 26 and 27) lines of Figure 9 is obtained (this is true up to experimental OD maximum). At pH 4.95, the maximum deviation of the line generated from eq 26–28 from the experimental points is 7–8% of the total OD. As the pH is increased, the deviation is even smaller and at pH 6.57 is hardly noticeable.

Though certain aspects of the mechanism for reaction of $N(5)$ -alkyl-1,5-dihydroflavins with O_2 remain for investigation (i.e., complete study of the pH dependence for the reaction which includes studies of the buffer dependence, etc.), the present study has encompassed the major reaction steps and serves to correct many speculations and even fallacious conclusions in the literature. The only prior studies of the oxidation of $N(5)$ -alkyl-1,5-dihydroflavins have involved 3-benzyl-5-(2-thioly)-1,5-dihydrolumiflavin (VIIH_2)¹⁰ and 5-benzyl-1,5-dihydroflavin (VIIIH_2).⁹ The study with the latter simply established the aminium cation radical as a product. The examination of VIIIH_2 was meant to be more inclusive. With VIIIH_2 the 2-thioly blocking group is lost in the course of oxidation. This interjects additional complicating reaction steps (see introduction). Furthermore, several conclusions from this study are suspect. Thus, though general acid catalysis may be found at a latter date, the conclusions by Hemmerich et al. that the "M"-shaped pH-rate profile (change in rate maximally seven- to eightfold between pH 1 and 10) supports general acid catalysis of the oxidation is of course fallacious. The finding that Fl_{ox} does not appear to comproportionate with FIHR to yield $\text{FIR}\cdot$ and $\text{FIH}\cdot$ in the case of VIIIH_2 is supported by previous results from this laboratory²³ which established that $\text{Fl}_{\text{ox}} + \text{FICH}_3\cdot$ does not yield a measurable concentration of $\text{FICH}_3\cdot$. From the inability to detect $\text{FIR}\cdot$ on reaction of Fl_{ox} with FIHR , Hemmerich and associates¹⁰ concluded that Fl_{ox} acts upon a flavin hydroperoxide intermediate to provide autocatalysis. In this study we show that there is no catalytic decomposition of 4a- $\text{FICH}_3\text{-OOH}$ on addition of Fl_{ox} . The

reaction of FIHCH_3 with 4a- $\text{FICH}_3\text{-OOH}$ (in methanol) is also unaffected by Fl_{ox} (see Results). Therefore, the suggestion¹⁰ that the observed autocatalysis of the O_2 oxidation of FIH_2 by Fl_{ox} is due to the reaction of the latter with a flavin hydroperoxide intermediate cannot be correct. Yet, Fl_{ox} does catalyze the O_2 oxidation of FIHCH_3 both in methanol (Figure 4) and water (Figure 5B) despite the fact that no observable "comproportionation" of $\text{Fl}_{\text{ox}} + \text{FIHCH}_3$ is occurring. This catalytic effect of Fl_{ox} may be due to (1) the trapping (by O_2) of a spectrophotometrically undetectable low steady state concentration of $\text{FIH}\cdot$ obtained via a thermodynamically unfavorable reaction of Fl_{ox} and FIHCH_3 (eq 33, 34), and/or (2) the reaction of O_2 with a charge transfer complex of Fl_{ox} and FIHCH_3 (eq 35). The finding of lesser yields of $\text{FICH}_3\cdot$ in the presence of Fl_{ox} (in methanol solvent, Figure 4) would seem to suggest that the latter is correct, though contribution from the former path cannot be ruled out. Thus, the reaction sequence

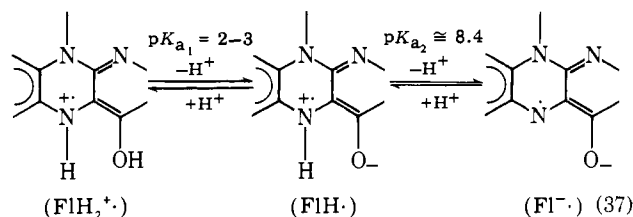
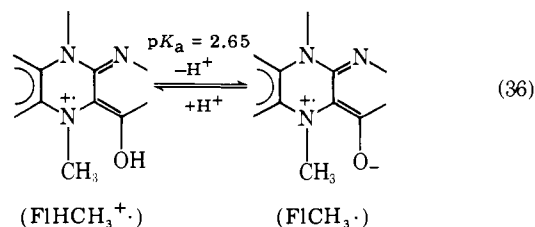


would be expected to result in quantitative yield of $\text{FICH}_3\cdot$ unless most of $\text{O}_2^{\cdot-}$ generated from the reaction of eq 34 reacted with $\text{FICH}_3\cdot$ to yield 4a- $\text{FICH}_3\text{-OOH}$. This may be considered unlikely because the reaction of FIHCH_3 with $\text{O}_2^{\cdot-}$ (which would yield $\text{FICH}_3\cdot$) would be expected to be as fast as (if not faster than) the reaction of $\text{FICH}_3\cdot$ with $\text{O}_2^{\cdot-}$. The second possibility, i.e., the reaction of a $\text{FIHCH}_3\text{-Fl}_{\text{ox}}$ charge transfer complex with O_2 , is evidently more reasonable because in this case the net effect of Fl_{ox} would be the catalysis of the rate of formation of 4a- $\text{FICH}_3\text{-OOH}$,



(assuming that 4a- $\text{FICH}_3\text{-OOH}$ formation from FIHCH_3 or $\text{FIHCH}_3\text{-Fl}_{\text{ox}}$ and O_2 is quantitative) without affecting the rate of the reaction of 4a- $\text{FICH}_3\text{-OOH}$ with FIHCH_3 which yields $\text{FICH}_3\cdot$ (see Results). Hence, as experimentally observed (Figure 4), the yield of $\text{FICH}_3\cdot$ in the presence of Fl_{ox} would be lower.

Comparison of the Chemistry of N^5 -Protio- and N^5 -Methylflavins. As will be shown below, our conclusions on the mechanism of the " $\text{FIHCH}_3 + \text{O}_2$ " reaction may be extrapolated to the " $\text{FIH}_2 + \text{O}_2$ " reaction. To do so, however, requires an understanding of the differences in the chemistry of the two reduced flavins (FIHCH_3 vs. FIH_2) and the corresponding radicals ($\text{FICH}_3\cdot$ vs. $\text{FIH}\cdot$) and oxidized forms ($\text{Fl}_{\text{ox}} + \text{CH}_3$ vs. Fl_{ox}). Two major differences between the two systems have been noted: (1) The radical $\text{FICH}_3\cdot$ does not possess an ionizable proton (eq 36) while $\text{FIH}\cdot$ does (eq 37). The flavin anion



radical, Fl^- , is extremely reactive with O_2 while FlH^{17-19} and FlCH_3 are not. (2) The disproportionation of FlH^\bullet to Fl_{ox} and FlH_2



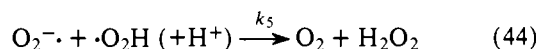
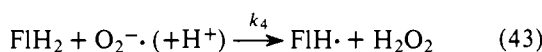
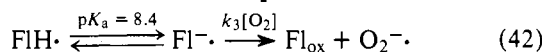
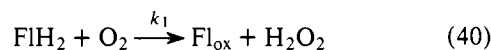
is rapid and thermodynamically favored. In contrast, FlCH_3 exhibits no discernible propensity to disproportionate to $\text{Fl}_{\text{ox}} + \text{CH}_3$ and FlHCH_3 .²³



The comparisons explain why FlCH_3 is the sole observed product of FlHCH_3 autoxidation (pH < 8, see before) while Fl_{ox} is the product of FlH_2 autoxidation.

Reaction of O_2 with 1,5-Dihydroflumiflavin (IVH_2). The results on the autoxidation of IVH_2 can be discussed most conveniently in terms of the sequence of reactions of Scheme VI.⁴⁷

Scheme VI



That a very significant portion of FlH_2 autoxidation occurs through the sequence of reactions of eq 41–43 has been recognized for some time.⁵ Thus, the observation that inclusion of Fl_{ox} speeds up the rate of FlH_2 consumption (Figure 7) finds explanation in the reaction sequence of eq 41–42.^{4,5} Suppression by SOD of the rate of FlH_2 disappearance (Figure 8) implies that the reaction of FlH_2 with $\text{O}_2^- \cdot$ (eq 43) is a very important step of the overall mechanism.⁵ Our finding that inclusion of SOD increases the lag phase for autoxidation of FlH_2 , when no or little Fl_{ox} is present initially (see below, Figures 10a,b), is a direct consequence of the suppression of the reaction of eq 43 by SOD. This is because under these conditions (i.e., no initial Fl_{ox} , SOD present) there is only one autocatalytic agent (i.e., Fl_{ox}) instead of two (i.e., Fl_{ox} and $\text{O}_2^- \cdot$), and as a consequence the time required for $d[\text{Fl}_{\text{ox}}]/dt$ term to reach a maximum is longer (i.e., the time required to reach the end of the lag phase on the experimental $[\text{Fl}_{\text{ox}}]$ vs. time plot is longer). In addition, the rate after the lag phase is slower in the presence of SOD (see below, Figures 10a,b) because of the suppression of the FlH_2 -consuming step of eq 43 (note that the reactions of eq 42 and 43—which are chain propagation steps—form a cycle). At the other extreme, i.e., when there is a large amount of Fl_{ox} present initially ($[\text{Fl}_{\text{ox}}] > [\text{FlH}_2]$) and SOD is absent, there is only one autocatalyst whose effect on the reaction has time dependence. This is $\text{O}_2^- \cdot$, the concentration of which must first build up (eq 41–42) before it can exert its autocatalytic effect (eq 43). The autocatalytic effect of Fl_{ox} , though present, has no time dependence because the concentration of Fl_{ox} does not change appreciably during the reaction (i.e., $[\text{Fl}_{\text{ox}}]_{\text{initial}} > [\text{FlH}_2]_{\text{initial}}$). Under these conditions then (i.e., excess Fl_{ox} , no SOD) there is only a small lag phase observed which is due to $\text{O}_2^- \cdot$ buildup. This small lag phase is of course eliminated by inclusion of SOD, i.e., the initial rate in the absence of dismutase closely approximates the overall rate found in the presence of dismutase⁵ (Figure 8A). Upon increasing $[\text{Fl}_{\text{ox}}]_{\text{initial}}$, this lag phase would become unobservable because it would be over during the

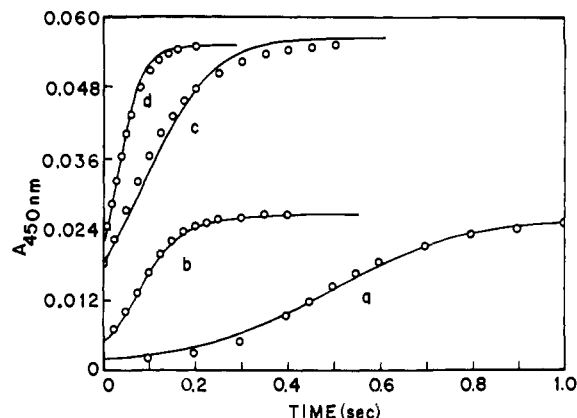


Figure 10. Computer simulation of the autoxidation ($[\text{O}_2] = 5.5 \times 10^{-4}$ M) of 1,5-dihydroflumiflavin (30 °C, pH 6.4): (a) $[\text{FlH}_2] = 2.24 \times 10^{-6}$ M, $[\text{Fl}_{\text{ox}}] = 2.4 \times 10^{-8}$ M, $[\text{SOD}] = 10^{-7}$ M; (b) $[\text{FlH}_2] = 1.96 \times 10^{-6}$ M, $[\text{Fl}_{\text{ox}}] = 3.16 \times 10^{-7}$ M, $[\text{SOD}] = 0$; (c) $[\text{FlH}_2] = 3.48 \times 10^{-6}$ M, $[\text{Fl}_{\text{ox}}] = 1.36 \times 10^{-6}$ M, $[\text{SOD}] = 10^{-7}$ M; and (d) $[\text{FlH}_2] = 3.08 \times 10^{-6}$ M, $[\text{Fl}_{\text{ox}}] = 1.65 \times 10^{-6}$ M, $[\text{SOD}] = 0$. The points were experimental; Schemes VI and VII were used in fitting the data obtained in absence (curves b and d) and presence (curves a and c) of SOD, respectively. The rate constants employed are given in Table 11.

mixing time of the stopped-flow spectrophotometer (Figure 8C).

The biologically most relevant process of Scheme VI is the direct reaction of FlH_2 with O_2 (see introduction). From the above considerations, it is obvious that information about the rate of this reaction (eq 40) can only be obtained from the initial slope of the time course of the reaction providing that the following three conditions are met: (1) no Fl_{ox} is present initially; (2) initial FlH_2 concentration is as low as experimentally feasible; and (3) SOD is present. The first two of these conditions assure that the autocatalytic effect of Fl_{ox} is minimized while the satisfaction of the third condition results in the elimination of the autocatalysis due to $\text{O}_2^- \cdot$. The time course of the reaction of 2.24×10^{-6} M FlH_2 (IVH_2) with 5.5×10^{-4} M O_2 [in the presence of $\sim 10^{-7}$ M SOD and in the absence of Fl_{ox} (IV), pH 6.4] is shown in Figure 10a (points are experimental, the line was computer simulated using Scheme VI, see below). From the initial slope of Figure 10a, the second-order rate constant for the reaction of IVH_2 with O_2 can be calculated and is found to be ca. $250 \text{ M}^{-1} \text{ s}^{-1}$ (pH 6.4). This is a rather interesting result because it indicates that the second-order rate constant for the reaction of FlH_2 with O_2 is about the same as that observed for the reaction of FlHCH_3 with O_2 ($220 \text{ M}^{-1} \text{ s}^{-1}$ at pH 6.57), i.e., the direct reaction of FlH_2 with O_2 appears to be much faster than the direct reaction FlHCH_3 with O_2 but it really is not! This “appearance” is due to the autocatalytic steps of eq 41–43 in general and to the reactivity of flavin radical anion with oxygen in particular. It may be noticed that (essentially) the same mechanism has been shown to apply to the autoxidation of both FlHCH_3 and FlH_2 (compare⁴⁸ Schemes V and VI). Our results with FlHCH_3 indicate that its reaction with O_2 is mainly an overall two-electron process yielding 4a- $\text{FlCH}_3\text{-OOH}$ (in methanol) and $\text{Fl}_{\text{ox}} + \text{CH}_3$ (in water, presumably through the intermediacy of 4a- $\text{FlCH}_3\text{-OOH}$). Though a flavin hydroperoxide has not been shown to be an intermediate in the reaction of FlH_2 with O_2 (eq 40), it is understood that it may be on the reaction path. If this is so, then the rate of the breakdown of the hydroperoxide to Fl_{ox} and H_2O_2 would have to be faster than its rate of formation.

Further support for Scheme VI was obtained by computer simulation of some of our results using this scheme. In the presence of SOD, Scheme VI reduces to Scheme VII.

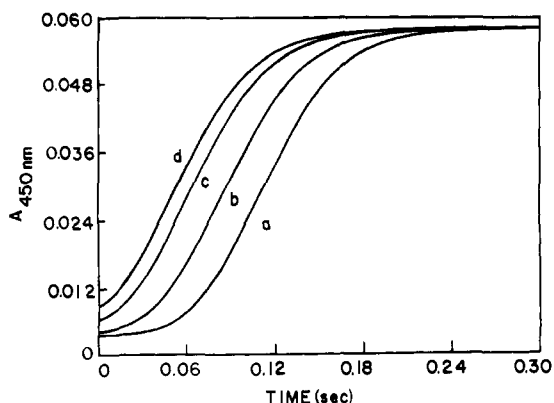


Figure 11. The time course of the (hypothetical) reaction of FlH_2 with O_2 ($5.5 \times 10^{-4} \text{ M}$) in the absence of SOD. The curves were computer generated using Scheme VI and the rate constants of Table II. Curve (a) is for autoxidation of $5 \times 10^{-6} \text{ M}$ FlH_2 in the absence of Fl_{ox} ; curves (b), (c), and (d) were obtained assuming 1, 5, and 10% reoxidation of FlH_2 prior to initiation of the reaction, respectively.

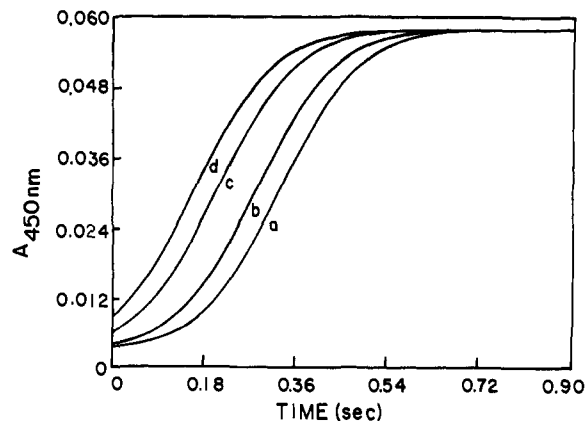


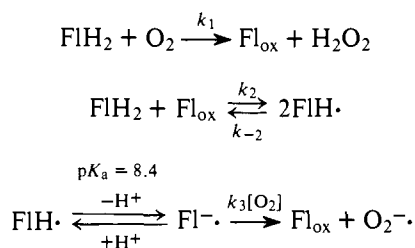
Figure 12. The time course of the (hypothetical) reaction of FlH_2 with O_2 ($5.5 \times 10^{-4} \text{ M}$) in the presence of SOD. The curves were computer generated using Scheme VII and the appropriate rate constants of Table II. Curve (a) is for the autoxidation of $5 \times 10^{-6} \text{ M}$ FlH_2 in the absence of Fl_{ox} , curves (b), (c), and (d) were obtained assuming 1, 5, and 10% reoxidation of FlH_2 prior to initiation of the reaction, respectively.

Table II. Summary of the Rate Constants Used in Computer Simulation of the Reactions of Schemes VI and VII

Rate constant	Value used, $\text{M}^{-1} \text{s}^{-1}$	Lit. value	Ref
k_1	250		
k_2	3.2×10^6		
k_{-2}	10^{10}	6.2×10^9	18
k_3	2.75×10^8	$2.5\text{--}3.0 \times 10^8$	18, 19
k_4	5×10^7		
k_5	3×10^6	$\sim 2 \times 10^6$	46
k_6	25^a	20–90	46

^a First-order rate constant (s^{-1}) for disappearance of $\text{O}_2^{\cdot-}$. (see text).

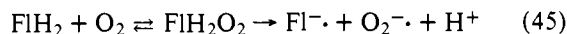
Scheme VII



Computer fits to data obtained by employing Scheme VII are shown in Figure 10 (curves a and c). The value of k_2 was chosen to provide the best computer fits; k_{-2} and k_3 were based on literature values (see Table II) and k_1 was estimated from initial slopes (see before). Computer simulation of two runs carried out in the absence of SOD were obtained using Scheme VI and are shown in Figure 10 (curves b and d). The values of k_1 , k_2 , k_{-2} , and k_3 employed were the same as those used for the other fits of Figure 10 in which SOD was present; the other rate constants which were used are given in Table II. It has been reported that the disappearance of $\text{O}_2^{\cdot-}$ from solution obeys mixed first- and second-order kinetics.⁴⁶ The first-order component was suggested to be due to impurities,⁴⁶ though special care did not remove this first-order component. The kinetic runs of Figures 10b and 10d were simulated using the reported rate constants for the disappearance of $\text{O}_2^{\cdot-}$ via the reactions first order (k_6) and second order (k_5 , eq 44) in this species (see Table II). However, fits as good as those shown in Figures 10b and 10d could also be obtained by leaving out k_6 [$\text{O}_2^{\cdot-}$] and using a rate constant ca. ten times larger than

reported for k_5 . There is some justification for this alternative.⁴⁹ We also had to leave out the reaction of an $\text{FlH}_2\text{--Fl}_{\text{ox}}$ charge transfer complex with O_2 owing to limitations in the computer simulation system used.⁴⁷ In view of these difficulties we conclude that the fits of Figure 10 which were obtained with one set of seven rate constants (most of which were determined separately by various people under various conditions) are as good as can be expected for such a complex series of reactions as shown in Scheme VI. In Figures 11 and 12, theoretical curves (obtained using Schemes VI and VII and the rate constants of Table II) are shown for reaction of $5 \times 10^{-6} \text{ M}$ FlH_2 with $5.5 \times 10^{-4} \text{ M}$ O_2 , in the absence and presence of SOD, respectively. It is interesting to note that the time course of the reaction is found to be significantly altered (both in the presence and absence of SOD) when only 1% of initial FlH_2 is assumed to be present as Fl_{ox} before the initiation of the reaction. As found experimentally, the lag phase in the presence of Fl_{ox} is shorter than that observed in its absence. The effects of 5 and 10% reoxidation of $[\text{FlH}_2]_{\text{initial}}$ prior to the initiation of the reaction are also shown (Figures 11, 12). Since only a percent of FlH_2 reoxidation prior to mixing FlH_2 and O_2 solutions (which is difficult to avoid experimentally) can alter the time course of the reaction, it is obvious that extreme care must be exercised in studying the kinetics of the $\text{FlH}_2 + \text{O}_2$ reaction. An interesting observation that may be made from the computations of Figures 11 and 12 is that the apparent rate of oxidation, following the lag phase, should always be the same providing that the total flavin concentration remains constant. This prediction finds verification in the results reported by Massey, Palmer, and Ballou^{5b} on the rates of oxidation of TARFH_2 and a mixture of TARFH_2 and TARF wherein $[\text{TARFH}_2] + [\text{TARF}]$ is constant.

From their study of the autoxidation of FlH_2 , Massey, Palmer, and Ballou⁵ concluded that the primary reaction of FlH_2 with O_2 occurs as in

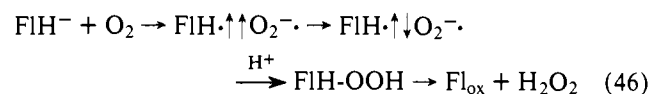


(compare to eq 40). The FlH_2O_2 compound (eq 45) was assumed to have either (or both) 4a-hydroperoxy and/or 10a-hydroperoxy structure and homolytically cleave to yield $\text{FlH}\cdot$ and $\text{O}_2^{\cdot-}$ (see also ref 10a and 15). It is important to realize, however, that demonstration of $\text{O}_2^{\cdot-}$ as a product of FlH_2 autoxidation⁵ does not necessarily mean that $\text{O}_2^{\cdot-}$ arises from homolytic cleavage of a flavin hydroperoxide or even from direct reaction of FlH_2 with O_2 (see Scheme VI for sources of $\text{O}_2^{\cdot-}$). Such a spontaneous homolytic cleavage of a C–O bond

(bond energy ~ 85 kcal/mol) should be highly unfavorable. If anything, the weaker O–O bond (~ 35 kcal/mol) should cleave and even that does not spontaneously occur at an appreciable rate.³⁷ To the authors' knowledge, no example of spontaneous homolytic cleavage of the C–O bond of a hydroperoxide exists in the literature. In any case, we find that 4a-FICH₃-OOH does not spontaneously (i.e., via homolytic cleavage of the C–O bond of 4a-FICH₃-OOH) yield FICH₃• and that it does not revert back to FIHCH₃ and O₂ (contrary to claims by Massey and co-workers^{5b} that the flavin hydroperoxide derived from FIH₂ and O₂ is in equilibrium with FIH₂ and O₂). Therefore, based on our findings on the chemistry of 4a-FICH₃-OOH, we suggest that the FIH₂O₂ compound of Massey and co-workers is not a covalent compound but perhaps a charge transfer complex.

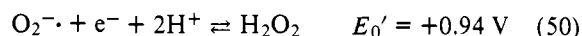
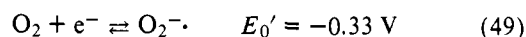
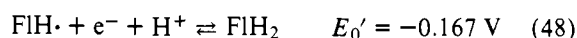
It has been reported^{5b} that the rate of FIH₂ autoxidation becomes slightly slower (by ca. three- to fourfold) upon increasing pH from 6.3 to 9.5. Since the time course of FIH₂ autoxidation is almost solely determined by the autocatalytic steps of eq 41–43 (Scheme VI) the cause of the rate drop must be due to a decrease in the rate of comproportionation (eq 41) and/or the rate of O₂^{•-} reaction with FIH₂ (eq 43) with increasing pH. In support of the latter is the finding by Massey and co-workers^{5b} that the observed yield of O₂^{•-} (from autoxidation of TARFH₂) increases with pH. This increase in O₂^{•-} yield (\sim fourfold in going from pH 8.4 to 10.6), though attributed to stability of O₂^{•-} at high pH,^{5b} is much greater than that which would be predicted from the reported⁴⁶ second-order rate constants for the dismutation of O₂^{•-} in this pH range. In any case it should be clear that the decrease in rate is not due to the direct reaction of FIH₂ with O₂ (Hemmerich and co-workers,¹⁰ on the other hand, have proposed that the decrease in rate is due to the direct reaction of FIH₂ with O₂).

The mechanism of direct reaction of 1,5-dihydroflavins with oxygen most likely involves the 4a-hydroperoxyflavin as an intermediate, as shown in the oxidation of FIHCH₃ in methanol (eq 11). Since ground state oxygen is a triplet and dihydroflavin a singlet, direct one-step reaction of the two to give a covalent compound is spin forbidden.¹³ Hence, the formation of the flavin hydroperoxide is most likely a multistage process. The change in multiplicity may be envisioned to occur through a 1,5-dihydroflavin–oxygen complex (eq 46).



Sufficient electrochemical data exist to allow the assessment of the allowability of the intermediacy of an [FIH• O₂^{•-}] species. This may be done by a comparison of ΔG^\ddagger for the oxidation of FIH_{2T} (= FIH⁻ + FIH₂) to the computed standard free energy (ΔG°) for the formation of flavin and superoxide radicals at a particular pH (7).

The relative free energies of FIH₂ + O₂, FIH• + O₂^{•-}, and Fl_{ox} + H₂O₂ may be computed by appropriate combinations of flavin⁵⁰ (eq 47, 48) and oxygen⁵¹ (eq 49, 50) half-cells⁵² ($\Delta G^\circ = -nF\Delta E_0'$) and are presented in Table III (pH 7, all species except O₂ 1 M, $p_{[\text{O}_2]} = 1$ atm).



From Table III it can be seen that the FIH• + O₂^{•-} products of 1e⁻ transfer from dihydroflavin to O₂ is of approximately 10 kcal/mol less free energy content than the critical transition

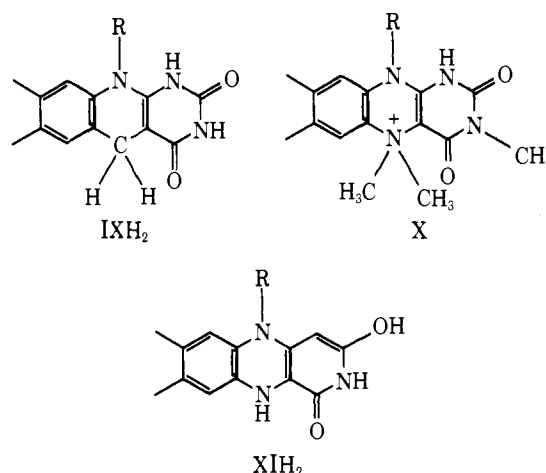
Table III. Calculated Relative Free Energies (G°) of Intermediates and Products Formed on Direct Reaction of O₂ with 1,5-Dihydroflavin (Standard States 1 M, $p_{[\text{O}_2]} = 1$ atm, 30 °C, pH 7.0)^a

Species	Relative G° , kcal/mol ^a
Transition state	14.4 (G^\ddagger) ^b
FIH _{2T} + O ₂ ^{•-}	3.8 ^c
FIH _{2T} + O ₂	0
(FIH _{2T} O ₂) _{complex} ^d	-1.1 ^e
Fl _{ox} + H ₂ O ₂	-23.2 ^c

^a Values were calculated relative to the free energy content of FIH_{2T} + O₂, which was taken as zero. ^b Calculated using the initial rate constant (k_r , pH 6.4) for reaction of O₂ with FIH₂ [$\Delta G^\ddagger = -RT \ln(k_r h/kT)$]. ^c Calculated using electrochemical data (see text). ^d Reported complex of FIH_{2T} with O₂ (see ref 5a). ^e Calculated from $\Delta G^\circ = -RT \ln K$. $K = 5 \times 10^3 \text{ M}^{-1}$ at pH 8.5 and 20 °C (see ref 5b). Saturated aqueous solution of O₂ is $1.27 \times 10^{-3} \text{ M}$ ($\mu = 0.1$, 20 °C). Thus, $K = 6.35 \text{ atm}^{-1}$. It is assumed that at pH 7 and 30 °C the value of K will be 6.35 atm^{-1} also.

state for reaction of FIH_{2T} with O₂ to produce Fl_{ox} + H₂O₂. One may conclude, therefore, that the sequence of eq 46 is energetically feasible. Perhaps a reasonable reaction coordinate diagram for the reaction of FIH_{2T} with O₂ would have as the rate-determining step the formation of the [FIH_{2T} O₂^{•-}] radical pair which would collapse to the hydroperoxyflavin at a rate exceeding that for radical pair dissociation. We have shown that the direct reaction of O₂ with FIHCH₃ does not yield FICH₃• + O₂^{•-}. The hydroperoxyflavin formed from [FIH_{2T} O₂^{•-}] would then rapidly dissociate to yield Fl_{ox} + H₂O₂ or dependent upon the isoalloxazine structure remain for some time in solution (as is the case with 4a-FICH₃-OOH in methanol solvent).

The intermediacy of a flavin radical–superoxide intimate ion pair would be in accord with the structural requirements for rapid reaction of 1,5-dihydroflavins with O₂. Thus, 5-deaza-1,5-dihydroflavins (IXH₂) react only slowly with O₂,⁵³ 3,5,5-trimethyl-1,5-dihydroflavin (X) is inert to O₂ even in 1.0 N NaOH or HCl⁵⁴ at 60 °C while 1-deaza-1,5-dihydroflavin (XIH₂) reacts rapidly with O₂.⁵⁵ These observations



establish the importance, to rapid oxidation, of the lone pair of electrons of N⁵ of 1,5-dihydroflavins (i.e., the integrity of the 1,4-dihydropyrazine structure). 1,4-Dihydropyrazines when planar are antiaromatic⁵⁶ and when bent exhibit lone-pair lone-pair Hoffman⁵⁷ orbital splitting. We have previously pointed out²² the importance of these features in the lowering of the free-energy barrier to 1e⁻ transfer reactions from dihydroflavins.⁵⁸

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References and Notes

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- (41) If the species that is produced in 55% yield at pH 10.5 (see Results) was 4a-FI_{ox}⁺-OOH, it too would yield FI_{ox}⁺CH₃ upon acidification. This possibility is considered unlikely because it would not explain why at pH 8 FI_{ox}⁺ is produced in 100% yield (see following discussion).
- (42) Scheme III would still be inconsistent with the results even if it is assumed that HO• reacts with FIHCH₃ to yield FI_{ox}⁺. If this reaction occurred, the maximum yield of FI_{ox}⁺ would be 75% and not 100%.
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- (45) From a knowledge of the rate of the reaction of FI_{ox}⁺ with O₂ (eq 28) and the rate of disproportionation of O₂^{-•} radicals at pH 4.85 (~10⁶ M⁻¹ s⁻¹),⁴⁶ it can be calculated that the maximum O₂^{-•} concentration possible (under the conditions of Figure 9) is ca. 2-3 × 10⁻⁸ M. It follows that if the rate constant for the reaction of FIHCH₃ (~10⁻⁵ M) with O₂^{-•} is >10⁷ M⁻¹ s⁻¹, then the bimolecular reaction of O₂^{-•} (~10⁻⁸ M) with itself (eq 31) will be totally suppressed. It is also quite likely that SOD is an ineffective scavenger of O₂^{-•} when in competition with FIHCH₃ at these low concentrations of O₂^{-•}. The values of K_m and k_{cat} for the reaction of SOD with O₂^{-•} are not known and the disappearance of O₂^{-•} (catalyzed by SOD) has been reported to obey second-order,^{5b} first-order [D. Klug, J. Rabani, and I. Fridovich, *J. Biol. Chem.*, **247**, 4839 (1972)], and complex 1d. Klug-Roth, I. Fridovich, and J. Rabani, *J. Am. Chem. Soc.*, **95**, 2786 (1973)] rate laws.
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- (48) Equation 30 of Scheme V is not included in Scheme VI because FI_{ox} is not known to add HO• as FI_{ox}⁺CH₃ does. Equation 45 of Scheme VI could have been included in Scheme V also; it was left out because, in contrast to the reaction of FIH₂ with O₂ which yields high concentrations of O₂^{-•},⁵ very low concentrations (<3 × 10⁻⁸ M) of O₂^{-•} are produced in the reaction of FIHCH₃ (<5 × 10⁻⁵ M) with O₂.⁴⁵ As a consequence, the second-order reaction of eq 45 cannot compete with the reaction of O₂^{-•} with FIHCH₃ (eq 29, Scheme V).
- (49) The values of k₅ (pH 8.4-10.5) that can be calculated for the disappearance of O₂^{-•} generated from the autoxidation of TARFH₂ (from the data of Massey et al., ref 5b) are 10-100 times larger than those reported by Rabani et al.⁴⁶
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