pears to involve a bound form of partially oxidized water. The nature of the strong oxidant (high potential electron carrier) that removes electrons one at a time from the complex is not yet known; however, a chlorophyll monomer has been proposed[44] as a candidate for the primary donor of the reaction center of photosystem 2, and cytochrome *b*-559 in some special high-potential form may serve to mediate electrons between it and the water-splitting complex.

The pioneering mechanism proposed by Bessel Kok to account for the periodic oscillations in O_2 flash yields is still the best framework for seeking an understanding of this process. Most of what we have learned in the 10 years since it was first presented can be incorporated easily into his overall scheme. As more detailed information becomes available we can expect to approach a better understanding of the molecular basis of the process. This will clearly be of importance in designing chemical model systems for simulating the photosynthetic mechanism that utilizes sunlight, water, and carbon dioxide to provide us with our best cheap source of available energy.

This Account is dedicated to the memory of Dr. Bessel Kok who made enormously fruitful contributions to our present understanding of photosynthetic electron transport and especially to the subject of this paper. I am indebted to my colleagues whose work is described in the text of this article. Much of this work was supported by the Divisions of Basic Energy Sciences and Biomedical and Environmental Research of the U. S. Department of Energy (Contract W-7405-ENG-48) and by a grant from the National Science Foundation (PCM 76-05074).

(44) M. S. Davis, A. Forman, and J. Fajer, *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 4170–4174 (1979).

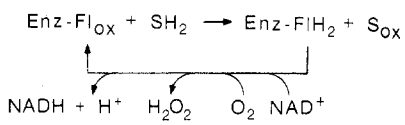
Mechanisms of Flavin Catalysis

THOMAS C. BRUICE

Department of Chemistry, University of California at Santa Barbara, Santa Barbara, California 93106

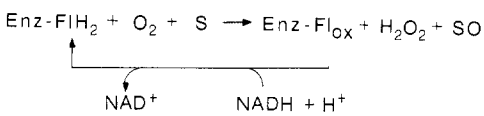
Received December 26, 1979

There are now recognized over 100 flavoproteins. Many contain more than one flavin, and others require the presence of Fe_nS_n cluster cores, exotic and fascinating inorganic cofactors of molybdenum, etc. (for examples, see ref 1). Flavoenzymes, for which flavin and substrate undergo a direct oxidation–reduction reaction, may, in the main, be placed in one of three categories: (i) the flavodoxins which are responsible for the transport of electrons by alternating between reduced and radical states; (ii) biological dehydrogenating agents (eq 1); and (iii) biological agents responsible for



(1)

the oxidation of substrate by the "activation" of molecular oxygen and transfer of one or two oxygen atoms from $^3O_2$ to the substrate (eq 2). We shall deal in this

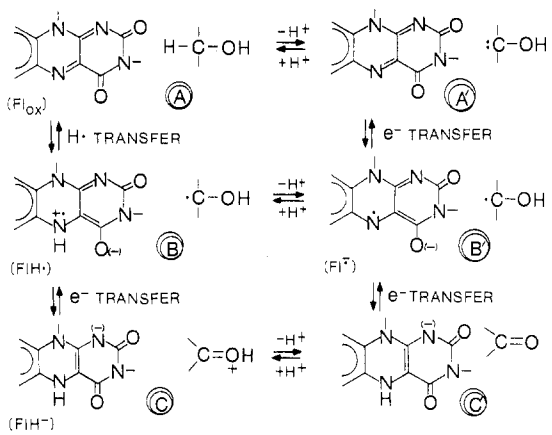


(2)

Account with the mechanisms by which non-enzyme-bound flavins enter into the forward reactions of eq 1 and 2. The mechanistic deductions derived from these model studies are extrapolated to the mechanisms of the flavoenzymes.

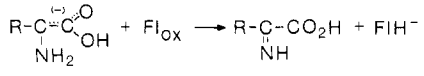
Thomas C. Bruice is Professor of Chemistry at the University of California, Santa Barbara. He attended the University of Southern California (B.S., 1950, Ph.D., 1954) and received postdoctoral training at UCLA. Prior to moving to Santa Barbara, he held faculty positions in biochemistry in the medical schools of Yale and Johns Hopkins Universities and in chemistry at Cornell University. He is a member of the National Academy of Sciences.

Scheme I

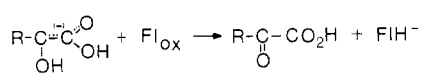


Dehydrogenation Reactions

Introduction of unsaturation α,β to a carboxyl group is performed by an important group of flavoenzymes. It has been proposed that the initial step is the ionization of the proton α to the carboxyl group followed by oxidation of the resultant carbanion (D- and L-amino acid oxidase (eq 3), lactic acid oxidase (eq 4), succinic



(3)

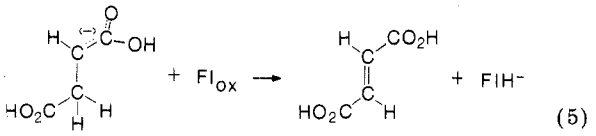


(4)

acid oxidase (eq 5), etc.). Evidence for the formation

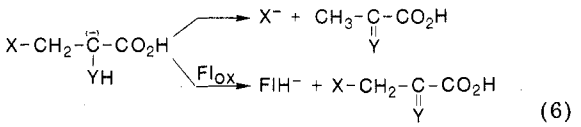
(1) S. P. Cramer, H. B. Gray, and K. V. Rajagopalan, *J. Am. Chem. Soc.*, **101**, 2772 (1979); T. D. Tullius, D. M. Kurtz, Jr., S. D. Conradson, and K. O. Hogson, *ibid.*, **101**, 2776 (1979).

0001-4842/80/0113-0256$01.00/0 © 1980 American Chemical Society



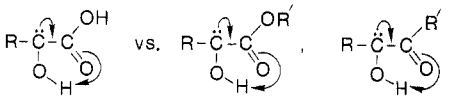
(5)

of carbanion intermediates derives from studies of β-halogenated substrates in which case halogen elimination is competitive (eq 6) with the oxidation of substrate



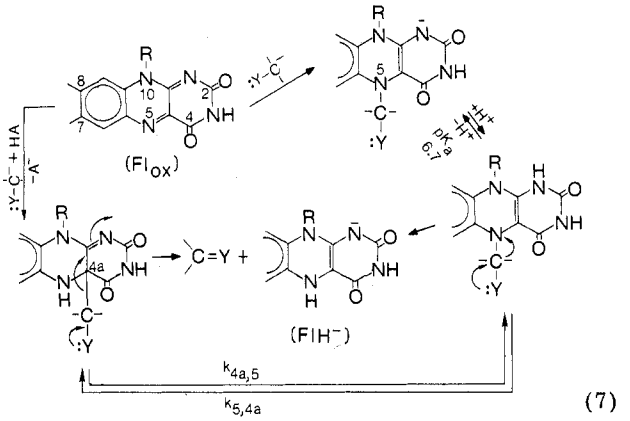
(6)

and reduction of flavoenzyme.[2-5] The formation of carbanion intermediate (eq 3–5) would require the delocalization of the electron pair to the undissociated carboxyl group. For investigations of nonenzymatic flavin oxidations, the corresponding carboxylic acid esters[6] and α-ketols may be employed. In the oxidation



of a number of such substrates by flavins, the reductant has been shown to be the carbanion. Involvement of a carbanion is established by the zero-order dependence of the disappearance of Fl_{ox} upon its concentration when experimental conditions are such that the observed rate constant is identical with the rate constant for carbanion formation (as determined by the rates of racemization, isomerization, and C–H/C–D isotope effects).[7-11] Oxidation of an intermediate carbanion is not the only mechanism available for flavin oxidation of α-substituted carboxylic acids. For instance, lactate reduces flavin at alkaline pH while pyruvate is reduced to lactate by dihydroflavin at low pH in a general-acid-catalyzed reaction.[12] It is unreasonable to suppose that a carbanion is involved in these reactions. Alternate routes for one-electron redox processes involving flavin and carbonyl compounds are provided in Scheme I. According to the scheme, carbanion oxidation by Fl_{ox} would follow A′ → B′ → C′ while the oxidation of lactate to pyruvate would proceed from A to B with concerted general-base-catalyzed one-electron transfer to convert B to C′. The latter mechanism is not easily distinguishable from general-base-catalyzed hydride transfer. In the oxidation of carbanion, the transfer of the electron pair to flavin may involve radical (Scheme I) and/or covalent intermediates.

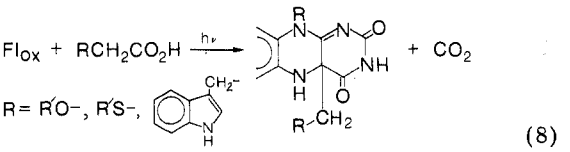
The postulation of mechanisms involving N^5 or C^{4a} covalent intermediates (eq 7) in flavoenzyme-catalyzed



(7)

dehydrogenation reactions has been supported by (1) the interpretation of photochemical decarboxylative alkylation reactions;[13] (2) the formation of N^5 adducts in the reaction of flavoenzymes with nitroalkane anions;[14] (3) the spectral observation of covalent species in the flavoenzyme oxidation of a substrate analogue;[15] and (4) nucleophilic substitution upon the flavin cofactor in the reaction of flavoenzymes with suicide inhibitors. Arguments based upon the observations of (1) to (4) will now be considered. Suicide inhibitors characteristically exhibit many oxidative turnovers with flavoenzymes without detectable intermediate formation prior to the suicide event.[16] There is presently no means to determine if the suicide product (SP) is formed from an adduct (A) of Fl_{ox} and inhibitor (SI) formed along the reaction path for oxidation of SI (i.e., Fl_{ox} + SI → A → FlH_2 + S_0 and A → SP) or if SP is formed in a reaction which is off the oxidative reaction path (i.e., Fl_{ox} + SI → FlH_2 + S_0 and Fl_{ox} + SI → SP or FlH_2 + S_0 → SP).

The mechanism for the photolytic decarboxylative alkylation reactions of eq 8 has been proposed to involve

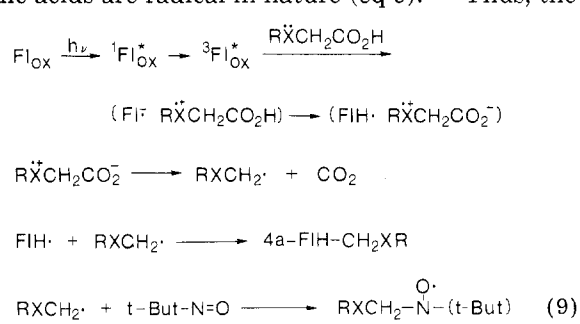


(8)

triplet flavin ($^3FL_{ox}$*) sensitization of the α-hetero acetic acid accompanied by its decarboxylation and the addition of the resultant carbanion to the 4a position of Fl_{ox}.[13] The assumption that photocatalytic decarboxylation yields carbanion which condenses with Fl_{ox} has led, in turn, to the supposition that these photolytic reactions are models for the covalent addition of carbanion to flavin.[17] Decarboxylations of α-amino-, α-thio-, or α-phenoxyacetic acids accompany their quenching of the photogenerated triplet states of benzophenone, quinones, and various organic dyes. The radical nature of these photoreactions has been estab-

(2) C. L. Tober, P. Nicholls, and J. D. Brodie, *Arch. Biochem. Biophys.*, **138**, 506 (1970).
(3) Y. F. Cheung and C. Walsh, *Biochemistry*, **15**, 2432 (1976).
(4) C. T. Walsh, O. Lockridge, V. Massey, and R. H. Abeles, *J. Biol. Chem.*, **248**, 7049 (1973).
(5) S. Ghisla and V. Massey, *J. Biol. Chem.*, **252**, 6729 (1977).
(6) R. G. Pearson and R. L. Dillon, *J. Am. Chem. Soc.*, **75**, 2439 (1953).
(7) L. Main, G. J. Kasperek, and T. C. Bruice, *Biochemistry*, **11**, 3991 (1972).
(8) S. Shinkai, T. Kunitake, and T. C. Bruice, *J. Am. Chem. Soc.*, **96**, 7140 (1974).
(9) T. C. Bruice and J. P. Taulane, *J. Am. Chem. Soc.*, **98**, 7769 (1976).
(10) I. Yokoe and T. C. Bruice, *J. Am. Chem. Soc.*, **97**, 450 (1975).
(11) T. W. Chan and T. C. Bruice, *Biochemistry*, **17**, 4784 (1978).
(12) R. F. Williams and T. C. Bruice, *J. Am. Chem. Soc.*, **98**, 7752 (1976).
(13) F. Hemmerich, *Prog. Chem. Org. Nat. Prod.*, **33**, 451 (1976).
(14) (a) D. J. T. Porter and H. J. Bright, *J. Biol. Chem.*, **252**, 4361 (1977); (b) D. J. T. Porter, J. G. Voet, and H. J. Bright, *ibid.*, **248**, 4400 (1973).
(15) (a) S. Ghisla and V. Massey in "Mechanisms of Oxidizing Enzymes", T. P. Singer and R. Ondarza, Eds., Elsevier, Amsterdam, 1978, p 55. (b) V. Massey, S. Ghisla, and K. Kieschke, *J. Biol. Chem.*, **255**, 2796 (1980).
(16) C. Walsh, *Acc. Chem. Res.*, **13**, 148 (1980).
(17) (a) P. Hemmerich, V. Massey, and G. Weber, *Nature (London)*, **213**, 728 (1967); (b) W. H. Walker, P. Hemmerich, and V. Massey, *Eur. J. Biochem.*, **13**, 258 (1970); (c) W. Haas and P. Hemmerich, *Z. Naturforsch. B*, 1035 (1972); (d) W. R. Knappe and P. Hemmerich, *Justus Liebigs Ann. Chem.*, 2037 (1976).

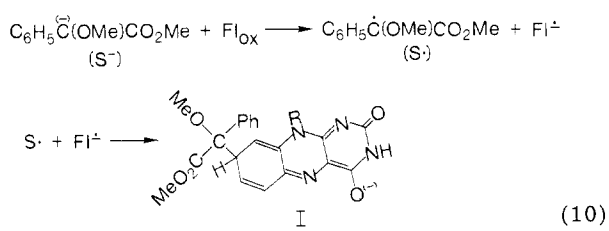
lished by a variety of methods including spin trapping, CIDNP, and product studies.[18] We have recently shown (by laser flash photolysis, spin trapping of radical intermediates, and kinetic inhibition of product formation by spin trap) that the decarboxylations (eq 8) which accompanies the quenching of triplet state flavin ($^3Fl_{ox}^*$) by these same α-heteroatom-substituted carboxylic acids are radical in nature (eq 9).[19] Thus, the



(9)

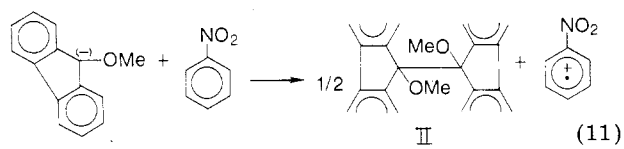
photocatalyzed decarboxylative coupling reactions of eq 8 are not examples of carbanion addition to Fl_{ox} and they cannot be employed to support a covalent mechanism for flavoenzyme catalysis.

The radical nature of carbanion oxidation by ground-state flavin has been established by radical trapping. Thus, methyl α-hydroxyphenylacetate carbanion [$C_6H_5C(-)(OH)CO_2Me$] is smoothly oxidized to methyl α-ketophenylacetate [$C_6H_5COCO_2Me$] by Fl_{ox} while the reaction of Fl_{ox} with methyl α-methoxyphenylacetate yields a product (I) which is formed by the coupling of flavin and substrate radicals (eq 10). In



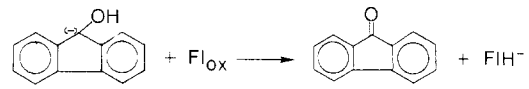
(10)

the presence of the radical trapping agents $^3O_2$ and 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy (>N–Ȯ) the yield of these coupling products is greatly reduced.[20] One-electron transfer from a carbanion to the electron-deficient aromatic Fl_{ox} with concomitant formation of radical coupling products is not unexpected. The oxidation of 9-methoxyfluorene anion by nitrobenzene serves as a useful example (eq 11).[21] While 9-



(11)

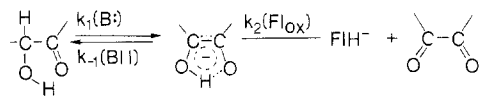
hydroxyfluorene anion is oxidized to 9-fluorenone by Fl_{ox} (eq 12), 9-methoxyfluorene anion undergoes a one-electron oxidation by Fl_{ox} to yield both the dimers



(12)

formed by the coupling of 9-methoxyfluorene radical with itself (i.e., II or eq 11) and with $Fl^{-}\cdot$. In the presence of the radical traps O_2 or >N–O· the products are reduced flavin and fluorenone.[22] The formation of 9-fluorenone from 9-methoxyfluorene anion by one-electron oxidation with Fl_{ox} in the presence of O_2 is completely analogous to the formation of 9-fluorenone upon radical oxidation of 9-methoxyfluorene anion with nitro aromatics in the presence of O_2.[21] Thus, evidence is rather overwhelming for the formation of radical intermediates in the Fl_{ox} oxidation of carbanions stabilized by resonance. Since C^{4a} and N^5 flavin adducts do not normally undergo homolytic cleavage, the source of these radicals cannot be found in the homolytic dissociation of a covalent compound obtained by nucleophilic addition of carbanions to Fl_{ox} (as in eq 7). *One must assume that the rate of one-electron transfer from substrate carbanion to Fl_{ox} exceeds that for nucleophilic addition of carbanion upon Fl_{ox}.* Covalent adducts may form by coupling of the intermediate flavin and substrate radicals. This may occur in competition with the transfer of the second electron to complete the redox reaction. Adduct formation by this means, in enzymatic reactions, is most likely to occur with modified or unusual substrate molecules. Covalent adducts are to be expected when the transfer of the second electron generates an unstable carbonium ion or a carbonium ion which can only undergo nucleophilic addition.

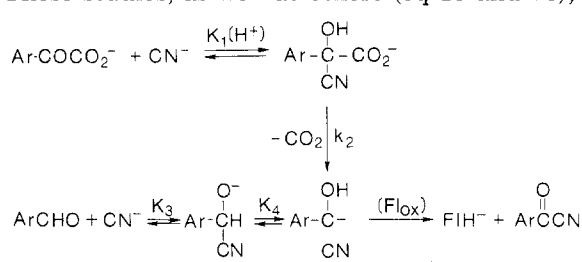
The oxidation of the α-ketols benzoin and furoin (K) by Fl_{ox} follow the kinetic sequence of eq 13.[8,9]



(13)

$$v = \frac{k_1k_2(B)(K)(Fl_{ox})}{k_{-1}(BH) + k_2(Fl_{ox})} \quad (14)$$

Steady-state assumption in intermediate enediolate species provides the expression of eq 14. At high [Fl_{ox}] ($\simeq 10^{-5}$ M) the constant $k_{obsd} = k_1[B][K]$ and the oxidation is zero order in [Fl_{ox}] (i.e., general-base-catalyzed carbanion formation is rate determining). The replacement of Fl_{ox} by N^5-ethyllumiflavin (Fl_{ox}^+Et) does not influence the rate of substrate oxidation[11] although the N^5 position of the flavin is blocked to adduct formation. Thus, formation of an N^5 adduct is not required in the facile oxidation of these α-ketols by flavin. These studies, as well as others (eq 15 and 16),[23] es-



(15)

(16)

(18) P. R. Bowers, K. A. McLauchlan, and R. C. Sealy, *J. Chem. Soc., Perkin Trans. 2*, 915 (1976); M. Weinstein, K. A. Muskat, and J. Dokkin, *J. Chem. Soc., Chem. Commun.*, 68 (1975); D. R. G. Brimage, R. S. Davidson, and P. R. Steiner, *J. Chem. Soc., Perkin Trans. 1*, 526 (1973); R. S. Davidson and P. R. Steiner, *J. Chem. Soc., Perkin Trans. 2*, 1357 (1972).

(19) M. Novak, A. Miller, T. C. Bruice, and G. Tollin, *J. Am. Chem. Soc.*, **102**, 1465 (1980).

(20) M. Novak and T. C. Bruice, *J. Chem. Soc., Chem. Commun.*, 372 (1980).

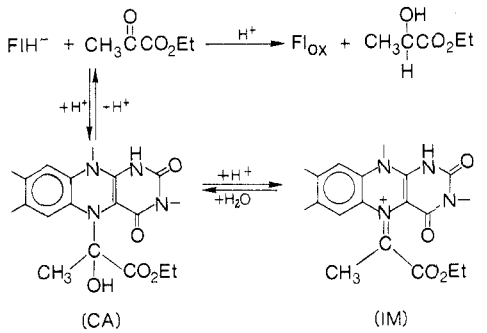
(21) R. D. Guthrie, D. P. Wesley, G. W. Pendygraft, and A. T. Young, *J. Am. Chem. Soc.*, **98**, 5870 (1976).

(22) M. Novak and T. C. Bruice, *J. Am. Chem. Soc.*, **99**, 8079 (1977).

(23) (a) S. Shinkai, T. Kamashita, and O. Manabe, *J. Chem. Soc., Chem. Commun.*, 301 (1979); S. Shinkai et al., *J. Am. Chem. Soc.*, **102**, in press; (b) S. Shinkai, T. Yamshita, Y. Kusano, and O. Manabe, *Chem. Lett.*, 1323 (1979).

tablish that oxidized flavins serve well as carbanion traps.

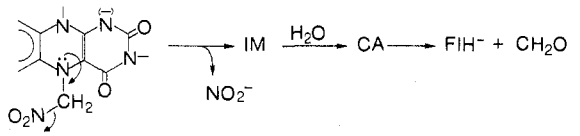
Other experiments show that when an N^5 adduct is formed it is not an intermediate in the oxidation reaction. In the acid-catalyzed reduction of pyruvic acid (and pyruvate, ethyl pyruvate, pyruvamide, and formaldehyde)[12,24,25] by FlH_2 and FlH^-, formation of N^5 adduct (i.e., carbinolamine, CA) is competitive with substrate reduction (eq 17)! Since CA is not an interme-



(17)

diate in this reduction process, *it cannot* (microscopic reversibility) be an intermediate in the reversal of the reaction (i.e., oxidation of lactic acid to pyruvic acid). Although interconversion of N^5 and C^{4a} flavin adducts occurs (eq 7), neither converts to oxidized flavin and reduced substrate.[26]

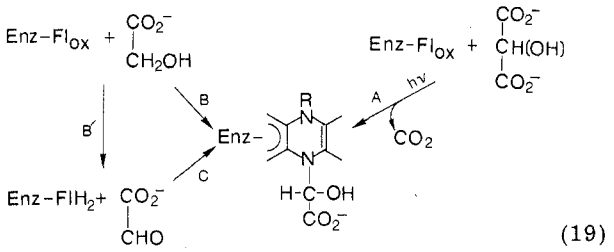
N^5 adducts have been observed during the oxidation of nitroalkane anions by glucose oxidase and D- and L-amino acid oxidase[14] and during the chiral oxidation of prochiral glycolic acid by lactate oxidase (for pertinent references, see ref 15). These reactions will now be considered. Formation of dihydroflavin by transfer of an electron pair from nitroalkane anion to Fl_{ox} cannot occur, regardless of the mechanism, because of the great instability of the resultant nitroalkane carbonium ion. For this reason, nitroalkane carbanions cannot undergo flavin oxidation by the same mechanism as the carbanions of α-amino and α-hydroxy acids. Adduct formation is all that can occur. However, this is not a facile reaction and, in model reactions, a highly electron-deficient flavin must be employed.[10] [The formation of the N^5 adduct may involve the collapse of an intermediate radical pair. Thus, reaction of nitroethane anion with glucose oxidase[14a] yields 65% of an N^5 adduct and 35% enzyme-bound flavin radical. Also, nitroalkane anions are known to enter into radical alkylation reactions.][27] Aside from dissociating to starting materials, the N^5 adduct may only react (as shown)[14] by the elimination of NO_2^- (eq 18). As previously discussed



(18)

(eq 17), formation of CA + IM from carbonyl compound and dihydroflavin is a dead-end equilibrium and does not provide oxidized flavin and alcohol product. Therefore, nitromethane anion may be considered as a reagent for the synthesis of an N^5 adduct of enzyme-bound flavin cofactor. The effectiveness of the reagent is determined by the facts that both electrons of the carbanion cannot pass to flavin and nitrite ion is a reasonable leaving group.

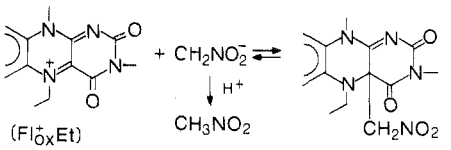
The formation of an N^5 adduct of the alternate substrate glycolic acid with lactate oxidase may be carried out in three different ways (eq 19).[15a] The mechanism



(19)

of the photocatalytic reaction of path A has already been considered, and it is radical in nature. The kinetics for the nonphotolytic reactions do not allow a choice between the alternatives of CA formation along the reaction coordinant for the catalytic reaction (path B followed by the reverse of C) or the formation of CA in a dead-end equilibrium from reduced flavin cofactor and glyoxylate products (B′ followed by C) prior to glyoxalate release (eq 19).[15a,b]

The possibility of C^{4a} addition of carbanion (eq 7) in the enzymatic reactions must also be considered. From nitroalkane anion and N^5-ethyllumiflavin there can actually be synthesized a 4a adduct which is completely stable in base, reverting to starting materials with specific acid catalysis.[11] In the addition of nitroalkane anions to Fl_{ox}^+Et, the rate of reaction with nitromethane anion is stopped flow, while adduct formation with nitroethane anion is much slower and there is no detectable reaction with 2-nitropropane anion (eq 20).[11]

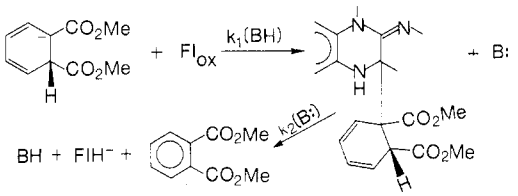


(20)

4a-Addition would appear to be under considerable steric constraint. Stuardt–Briegleb models of the 4a adducts of benzoin and furoin anion with Fl_{ox}^+Et cannot be constructed. Nevertheless, both furoin and benzoin anion are readily oxidized by Fl_{ox}^+Et.

The kinetics of the oxidation of carbanions by Fl_{ox} do not support the intermediacy of 4a adducts. The kinetic expressions of eq 13 and 14 are general for carbon acid oxidation by Fl_{ox}. Oxidation of carbanion by Fl_{ox} is not subject to acid–base catalysis though 4a addition to Fl_{ox} is a general-acid-catalyzed reaction.[28] If dehydrogenation by Fl_{ox}, resulting in carbon–carbon double bond formation, should involve 4a addition of intermediate carbanion, then this step should be general acid catalyzed and the ensuing elimination reaction should be general base catalyzed (eq 21). Regardless of whether the 4a addition or the elimination step were rate determining, the reaction would require general catalysis. However, aromatization of dimethyl *trans*-1,2-dihydrophthalate occurs in a noncatalyzed reaction

(24) S. Shinkai and T. C. Bruice, *J. Am. Chem. Soc.*, **95**, 7526 (1973).
(25) R. F. Williams, S. Shinkai, and T. C. Bruice, *J. Am. Chem. Soc.*, **99**, 921 (1977).
(26) D. Clerin and T. C. Bruice, *J. Am. Chem. Soc.*, **96**, 5571 (1974).
(27) G. A. Russell and W. C. Danen, *J. Am. Chem. Soc.*, **88**, 5663 (1966); N. Kornblum, R. E. Michel, and R. C. Kerber, *J. Am. Chem. Soc.*, **88**, 5660, 5662 (1966).
(28) T. C. Bruice, L. Hevesi, and S. Shinkai, *Biochemistry*, **12**, 2083 (1973); L. Hevesi and T. C. Bruice, *ibid.*, **12**, 290 (1973).

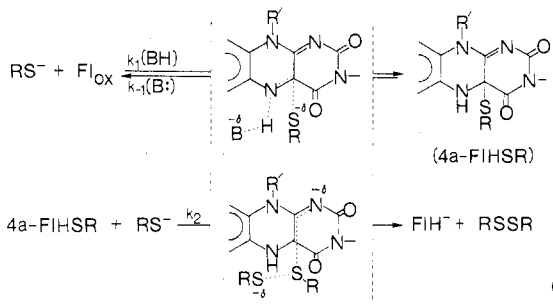


(21)

within a complex of the substrate-derived carbanion and Fl_{ox}.[7]

Thiol Oxidation

In contrast to carbanion oxidation, oxidation of thiol anion by Fl_{ox} involves general acid catalysis of the formation of an intermediate 4a-thiol adduct (eq 22).[10,29]



(22)

Spectral evidence for a 4a-thiol adduct has been reported for lipoamide dehydrogenase.[30] The enhanced nucleophilicity of RS^- toward carbon, the instability of RS^+, and the involvement of nucleophilic displacement by RS^- upon the adduct differentiate mercaptan oxidation and carbon acid dehydrogenation reactions.

Oxygen Activation by Dihydroflavins

Reduced flavomonooxygenases react with $^3O_2$ to form a labile enzyme-bound dihydroflavin oxygen compound (FlH_2O_2; λ_{max} 370–380 nm).[31] The rates of reaction of $^3O_2$ with reduced flavomonooxygenases and free dihydroflavin are generally comparable. Although kinetic investigations of the reaction of dihydroflavin with oxygen have provided a general understanding of the autocatalytic nature of the reaction,[32] the species FlH_2O_2 is not seen and purported evidence supporting its presence may be questioned.[33] Thus, after about 1% conversion of dihydroflavin to oxidized flavin, the remaining dihydroflavin is consumed in reactions which involve one-electron transfer from flavin radical anion to oxygen ($k_3 = 3 \times 10^8\ M^{-1}\ s^{-1}$)[34] and to superoxide[32c,d] (eq 23).

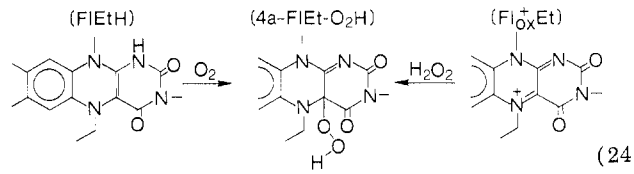
$$FlH_2 + {}^3O_2 \rightarrow Fl_{ox} + H_2O_2$$

$$Fl_{ox} + FlH_2 \rightleftarrows 2FlH\cdot$$

$$FlH\cdot \underset{+H^+}{\overset{-H^+}{\rightleftharpoons}} Fl^-\cdot \xrightarrow{k_3[O_2]} Fl_{ox} + O_2^-\cdot \qquad pK_a = 8.4$$

$$FlH_2 + O_2^-\cdot\ (+H^+) \rightarrow FlH\cdot + H_2O_2 \qquad (23)$$

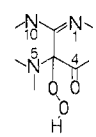
4a-(Hydroperoxy)flavins (whose spectra are identical with or very similar to the spectrum of the initially formed monooxygenase-bound dihydroflavin oxygen compound) are obtained on oxygenation of reduced N^5-alkylflavins (FlRH) or upon reaction of H_2O_2 with N^5-alkylflavinium cations ($Fl_{ox}{}^+R$)[32e,35] (eq 24). The



(24)

kinetics for reaction of FlEtH with O_2 and the disposition of 4a-$FlEtO_2H$ (CH_3OH solvent) have been investigated in some detail.[32e] A major pathway of decomposition in methanol involves dissociation to yield $Fl_{ox}{}^+Et$ plus hydrogen peroxide and the reaction of this species with solvent to provide 4a-FlEtOMe. Solvents of choice for investigations of the chemistry of 4a-(alkylperoxy)- and 4a-(hydroperoxy)flavins are anhydrous and oxygen-free *tert*-butyl alcohol and dimethylformamide. Dissociation of the peroxides is minimal in these solvents. A major mode of decomposition of both 4a-(alkylperoxy)- and 4a-(hydroperoxy)flavins provides 10a-spirohydantoins.[36]

Flavoenzyme monooxygenases are the gentle oxygen addition or insertion reagents of biochemistry (hydroxylation of electron-rich aromatic rings, N-oxidation of amines, etc.)[37] A number of mechanisms whereby 4a-$FlEtO_2H$ is converted into more powerful oxidants (oxygen guns)[38] have been proposed.[38–41] The premise that 4a-FlHOOH, as an organic hydroperoxide, should not be expected to behave as a hydroxylating agent at the active site of a flavin monooxygenase may be questioned. The O–O bond of 4a-$FlHO_2H$ is inductively polarized by the electronegativity of the N^1, N^5, and N^{10} and $>C^4{=}O$ members of the isoalloxazine ring. This



electronegativity of the 4a position is responsible for the

(29) E. L. Loechler and T. C. Hollocher, *J. Am. Chem. Soc.*, **97**, 3235 (1975). J. M. Sayer, P. Conlon, J. Hupp, J. Fancher, R. Belanger, and E. J. White, *J. Am. Chem. Soc.*, **101**, 1890 (1979).

(30) C. Thorpe and C. H. Williams, Jr., *J. Biol. Chem.*, **251**, 7726 (1976).

(31) (a) T. Spector and V. Massey, *J. Biol. Chem.*, **247**, 5632 (1972); B. Entsch, D. P. Ballou, and V. Massey, *ibid.*, **251**, 2550 (1976); (b) S. Strickland and V. Massey, *ibid.*, **248**, 2953 (1973); (c) J. W. Hastings, C. Balny, C. Le Peuch, and P. Douzou, *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 3468 (1973).

(32) (a) G. H. Gibson and J. W. Hastings, *Biochem. J.*, **83**, 368 (1962); (b) H. Gutfreund, *ibid.*, **74**, 17p (1960); (c) V. Massey, G. Palmer, and D. Ballou in "Flavins and Flavoproteins", H. Kamin, Ed., University Park Press, Baltimore, MD, 1971, p 349; (d) V. Massey, G. Palmer, and D. Ballou in "Oxidases and Related Redox Systems", Vol. I, J. E. King, H. S. Mason, and M. Morrison, Eds., University Park Press, Baltimore, MD, 1973, p 25; (e) C. Kemal, T. W. Chan, and T. C. Bruice, *J. Am. Chem. Soc.*, **99**, 7272 (1977); (f) V. Favaudon, *Eur. J. Biochem.*, **78**, 293 (1977).

(33) T. C. Bruice, in ISOX III, the Third International Symposium on Oxidases and Related Oxidative Reduction Systems, Albany, NY, 1979.

(34) S. P. Vaish and G. Tollin, *Bioenergetics*, **2**, 61 (1971); M. Farraggi, P. Hemmerich, and I. Pecht, *FEBS Lett.*, **51**, 47 (1975).

(35) C. Kemal and T. C. Bruice, *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 995 (1976).

(36) M. Iwata, T. C. Bruice, H. L. Carrell, and J. Glusker, *J. Am. Chem. Soc.*, **102**, 5036 (1980).

(37) (a) J. W. Gorrod, "Biological Oxidation of Nitrogen", Elsevier/North Holland, Amsterdam, 1978; (b) B. S. S. Masters and D. M. Ziegler, *Arch. Biochem. Biophys.*, **145**, 358 (1971); (c) F. F. Kadlubar, E. M. McKee, and D. M. Ziegler, *ibid.*, **156**, 46 (1973).

(38) P. Hemmerich, *Prog. Chem. Nat. Prod.*, **33**, 451 (1976).

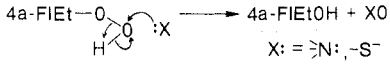
(39) G. A. Hamilton, *Prog. Bioorg. Chem.*, **1**, 83 (1971).

(40) H. W. Orf and D. Dolphin, *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 2646 (1974).

(41) G. I. Dmitrienko, V. Snieckus, and T. Viswanatha, *Bioorg. Chem.*, **6**, 421 (1977).

large rate constants (and low pK_a values) for pseudobase (4a-FlEtOH) formation from Fl_{ox}^+Et.[42]

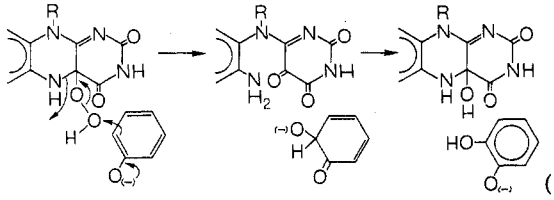
4a-(Hydroperoxy)flavins are very effective agents for N-oxidation of secondary, tertiary, and hydroxyl amines (and for the S-oxidation of alkyl sulfides) to produce the same N-oxidation products formed by hepatic flavoprotein microsomal oxidase.[31] In both protic and aprotic solvents, the reactions are first order in both 4a-$FlEtO_2H$ and substrate to provide 4a-FlEtOH and *N*-oxide (or *S*-oxide) in near theoretical yields (eq 25).[43,44] The second-order rate constant for S-oxidation



(25)

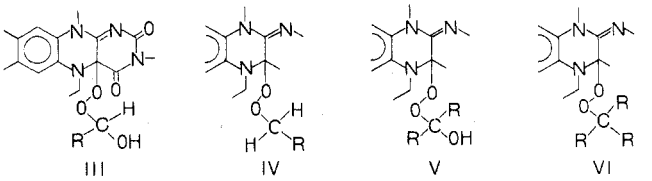
and N-oxidations by 4a-$FlEtO_2H$ exceeds those obtained with *t*-BuOOH and H_2O_2 by 10^4 to 10^5.

In the ring hydroxylation of the alternate substrate 2,4-dihydroxybenzoate (and others), the 4a-FMNH-OOH (λ_{max} 380 nm) bound to *p*-hydroxybenzoate hydroxylase is converted to a strongly absorbing intermediate (λ_{max} 390–420 nm) which then gives way to 4a-FMN-OH (λ_{max} 380–385 nm). Entch, Ballou, and Massey[45] propose a mechanism (eq 26) much akin to


(26)

the carbonyl oxide concept of Hamilton.[39] However, the spectra to be anticipated of the hypothetical intermediate are not known and the ring opening would not (as shown) be anticipated to provide a driving force for oxygen transfer. An alternative mechanism would involve a nucleophilic attack by the ambident phenoxide ion upon the terminal peroxide oxygen with proton transfer to yield the pseudobase (4a-FMNH-OH) directly (as in eq 25). The intermediate may represent a conformational perturbation of the spectra of the pseudobase product. The X-ray structure of *p*-hydroxybenzoate hydroxylase in a complex with its substrate reveals that cofactor and substrate are so aligned at the active site as to favor oxygen transfer from the 4a-(hydroperoxy)flavin moiety.[46]

Flavin hydroperoxide enters into the chemiluminescent oxidation of long-chain aldehyde substrates at the active site of the bacterial luciferase monooxidases.[47] Chemiluminescence (CL) accompanies the decomposition of the adduct of 4a-$FlEtO_2H$ with RCHO (III) or the adduct of an alkyl hydroperoxide with Fl_{ox}^+Et (IV), but CL is not observed to accompany the decomposition of the adducts of 4a-$FlEtO_2H$ with ketones (V) nor the adduct of *t*-BuOOH with Fl_{ox}^+Et (VI).[35,44,48] The minimal structure for CL appears to be 4a-$FlEtO_2C$-(H)<. Two excited species are formed on decompo-

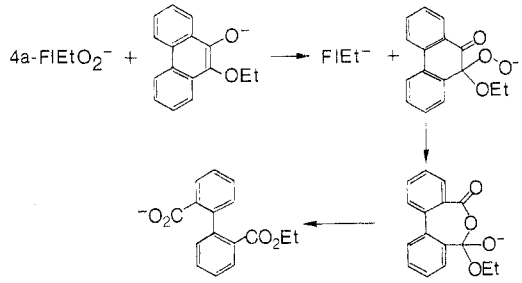


sition of III and IV.[49] In the absence of added fluorescer CL is due to the formation of Fl_{ox}^*. This reaction is characterized by a quantum yield (Φ) of ca. 10^{-4}. A second nonfluorescent excited species may be detected by including any number of fluorescers in the reaction solution (including Fl_{ox}). The observed value of Φ is dependent upon the fluorescence efficiency of the added fluorescer (for rhodamine B, $\Phi \simeq 10^{-2}$; for bacterial luciferase, $\Phi = 10^{-1}$). Regardless of the presence or absence of fluorescer, the substitution of the α-H by α-D results in an isotope effect of $\sim$2 upon the quantum yield (Φ^H/Φ^D), but there is no kinetic isotope effect upon the rates of disappearance of III or IV or upon $h\nu$ emission. This finding establishes that the formation of the chemiexcited singlet species occurs in minor reactions (not rate controlling for the disappearance of III or IV) and that scission of the C–H(D) bond contributes to the free-energy content of the critical transition state(s) for these minor reactions. Addition of fluorescer does not increase the first-order rate constant for disappearance of III or IV, nor is there any relationship between the one-electron transfer potential of fluorescer and Φ. For this reason, catalysis by chemically induced electron exchange luminescence[50] cannot be involved.

A number of mechanisms have been proposed for the mechanism of bacterial luciferase.[13,51] If(!) our biomimetic reaction possesses a mechanism similar to that for the enzymatic reactions, we are then allowed to conclude that the various proposals are incorrect because they require the presence of the α-HO group (compare III and IV), do not involve a mixed peroxide intermediate, or cannot be applied to an N^5-alkylflavin. We have proposed[48] a Russel fragmentation of III and IV which may account for the generation of the nonfluorescent excited species. The 4a-pseudobase of FMN would emit if generated at the active site of the enzyme.[52] Eberhard and Hastings[53] suggested that 4a-pseudobase may be the emitter in the enzyme reaction.

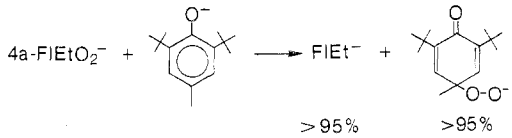
Just as 4a-$FlR\text{-}O_2H$ compounds provide biomimetic analogues to the flavomonooxygenase enzymes, the anions *4a-FlR-O_2^- behave as biomimetic flavodioxygenases* (eq 27).[28,54,55] The peroxide intermediate

(42) (a) S. Ghisla, U. Hartman, P. Hemmerich, and F. Müller, *Justus Liebigs Ann. Chem.*, 1388 (1973); (b) C. Kemal, T. C. Bruice, *J. Am. Chem. Soc.*, **98**, 3955 (1976).
(43) S. Ball and T. C. Bruice, *J. Am. Chem. Soc.*, **101**, 4017 (1979).
(44) C. Kemal, T. W. Chan, and T. C. Bruice, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 405 (1977).
(45) B. Entsch, D. P. Ballou, and V. Massey, *J. Biol. Chem.*, **251**, 2550 (1976).
(46) R. K. Wierenga, R. J. De Jong, K. H. Kalk, W. G. J. Hol, and J. Drenth, *J. Mol. Biol.*, **131**, 55 (1979).
(47) J. W. Hastings, *CRC Crit. Rev. Biochem.*, 163 (1978).
(48) C. Kemal and T. C. Bruice, *J. Am. Chem. Soc.*, **99**, 7064 (1977).
(49) P. Shepherd and T. C. Bruice, *J. Am. Chem. Soc.*, submitted for publication.
(50) B. G. Dixon and G. B. Schuster, *J. Am. Chem. Soc.*, **101**, 3116 (1979); J. Koo and G. B. Schuster, *ibid.*, **100**, 4496 (1978); S. P. Schmidt and G. B. Schuster, *ibid.*, **100**, 1966 (1978); J. P. Smith and G. B. Schuster, *ibid.*, **100**, 2564 (1978); J. Koo and G. B. Schuster, *ibid.*, **99**, 6107 (1977); G. B. Schuster, *Acc. Chem. Res.*, **12**, 366 (1979).
(51) R. E. Keay and G. A. Hamilton, *J. Am. Chem. Soc.*, **97**, 6875 (1975); F. McCapra and D. W. Hyseat, *Biochem. Biophys. Res. Commun.*, **52**, 298 (1973); F. McCapra and P. Leeson, *J. Chem. Soc., Chem. Commun.*, 1037 (1976); J. W. Lowe, L. L. Ingraham, J. Alspach, and R. Rasmussen, *Biochem. Biophys. Res. Commun.*, **73**, 465 (1976); J. W. Hastings, *ibid.*, **47**, 348 (1972).
(52) S. Ghisla, B. Entsch, V. Massey, and M. Husein, *Eur. J. Biochem.*, **76**, 139 (1977).



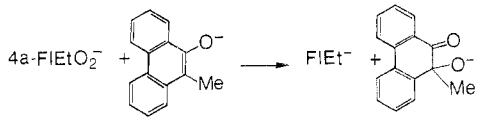
(27)

may be isolated from the reaction of eq 28. That



(28)

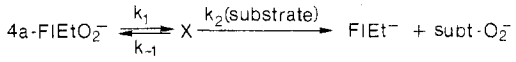
monoester of eq 27 is not formed from a substrate-derived dioxetane is shown by the fact that only tertiary alcohol is obtained in the reaction of eq 29. Since



(29)

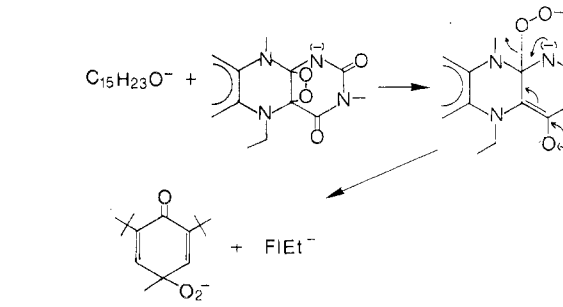
4a-flavin hydroperoxide is formed on reaction of oxygen with N^5-ethyldihydroflavin, these dioxygenation reactions amount to the catalysis, by dihydroflavin, of the reaction of molecular oxygen with substrate. Dioxygen transfer from 4a-FlEtO$_2^-$ to substituted indoles has also been studied.

The mechanism of dioxygen transfer is not completely understood. However, we do know that the kinetics are compatible with the sequence of eq 30.



(30)

Thus, the reaction exhibits saturation in substrate and k_1 is independent of the nature of the substrate. The intermediate (X) does not represent the completely dissociated species FlEt$^-$ + $^3O_2$ or FlEt$^-$ + $^1O_2$. These conclusions are based on the knowledge of the second-order rate constants for the reaction of $^3O_2$ with substrate and the inability of the singlet oxygen trap 2,5-dimethylfuran to act as a substrate. The intermediate does not likely represent FlEt· + O_2^-· since it has been reported that O_2^-· does not couple with ArO·.[56,57] Plausible mechanisms could include the intermediacy of 4a-10a dioxetane (eq 31), 4a-4 dioxetane, or a complex of FlEt$^-$ and an O_2 species. Work in this area of research is continuing.



(31)

Overview

Introduction of unsaturation α,β to a carboxyl group by a flavoenzyme is best explained by a two-step mechanism which involves α-C–H bond dissociation followed by enzyme-bound flavin oxidation of the resultant carbanion. Flavins and other isoalloxazine molecules serve per se as one-electron carbanion traps. Radical intermediates have been established in the photocatalytic decarboxylative couplings of α-heterosubstituted acetic acids with flavins. The proposals that these reactions are models for carbanion nucleophilic attack upon the isoalloxazine ring system appear to be unwarranted. The necessity for N^5-adduct formation in the oxidation of carbanions is rendered unlikely by the observation that N^5-blocked flavins serve admirably well as carbanion traps. 4a-Adduct formation in the oxidation of carbanions can be ruled out by kinetic arguments. One-electron transfer is not the sole mechanism available for the flavin oxidation of α-substituted carboxylic acids. These compounds can also be oxidized by hydride-equivalent transfer through a mechanism which may or may not involve radical intermediates. N^5-Adduct formation in these reactions is not along the reaction path for flavin catalysis. Thiol oxidation by flavins does involve 4a-addition of thiol anion to flavin.

N^5-Ethyl-4a-(hydroperoxy)-3-methyllumiflavin (4a-FlEtOOH) brings about the oxidation of secondary amines, tertiary amines, hydroxylamines, and organic sulfides in reactions first order in 4a-FlEtOOH and substrate to yield hydroxylamines, *N*-oxides, nitrones, and sulfones. The second-order rate constants exceed those for oxidation by hydrogen peroxide or alkyl hydroperoxide by 10^4- to 10^5-fold. These reactions serve to define the mechanism for the microsomal flavoenzyme monooxygenase responsible for N- and S-oxygenation. The monooxygenase reactions responsible for the chemiluminescent oxidation of aldehydes (bacterial luciferases) have also been modeled. The minimal structure for light production has been established, and it has been shown that two excited species are formed. The anion 4a-FlEt-O-O$^-$ transfers a dioxygen moiety to ambident anion substrates (S$^-$) to yield reduced flavin and SOO$^-$. Dioxygen transfer from 4a-FlEtOO$^-$ has its biochemical counterpart in the flavin dioxygenase enzymes.

I express my appreciation to the John Simon Guggenheim Memorial Foundation for its support.

(53) J. W. Hastings and K. H. Nealson, *Annu. Rev. Microbiol.*, **31**, 549, (1977).
(54) C. Kemal and T. C. Bruice, *J. Am. Chem. Soc.*, **101**, 1635 (1979).
(55) S. Muto and T. C. Bruice, *J. Am. Chem. Soc.*, **102**, 0000 (1980); work in progress.
(56) A. Nishinaga, T. Itahara, T. Shimizu, H. Tomita, K. Nishizawa, and T. Matsuura, *Photochem. Photobiol.*, **28**, 687 (1978).
(57) J. Wilshire and D. Sawyer, *Acc. Chem. Res.*, **12**, 105 (1979).