On the Mechanism of Flavin-Catalyzed Dehydrogenation α,β to an Acyl Function. The Mechanism of 1,5-Dihydroflavin Reduction of Maleimides

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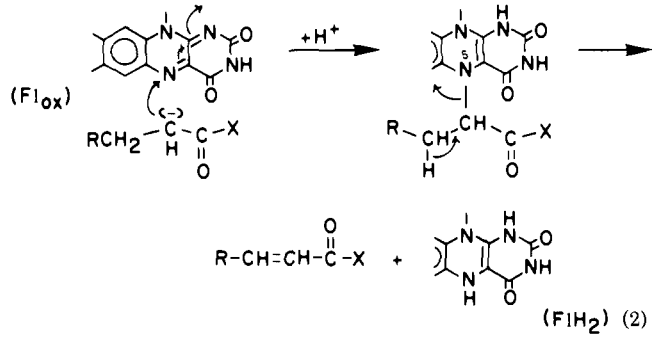
Abstract: The dihydroflavin reductions of maleimide (MI) and *N*-methylmaleimide (MMI) have been investigated using the water-soluble 1,5-dihydro-3-(3-sulfopropyl)lumiflavin (FlH_2). The reaction of the dihydroflavin with MI and MMI to yield oxidized flavin (Fl_{ox}) and succinimides is biphasic. The first phase involves general-acid-assisted nucleophilic attack of dihydroflavin anion (FlH^-) upon the maleimide to yield 4a-substituted 4a,5-dihydroflavin covalent adducts. The Brønsted α for the general-acid catalysis is −0.1 to −0.2. This observation would be expected for a Michael addition to FlH^- upon MMI to yield an unstable anionic 4a adduct which is trapped by proton transfer from general-acid species. The rate of decomposition of the intermediate to yield Fl_{ox} and succinimide is not measurably dependent upon the concentration of general-base species. The reaction was found to be first order in $[HO^-]$. A mechanism involving preequilibrium ionization of the N(5)–H followed by elimination of succinimide anion (specific-base catalysis) and formation of Fl_{ox} would require that the pK_a for dissociation of the aniline-like N(5)–H is less than 23 in order that the rate of elimination from the anion does not exceed that for the vibration of the C–C bond that is broken. It is concluded that the HO^--catalyzed elimination represents a concerted process with a Brønsted β approaching 1.0. From the results of this study and by involving the principal of microscopic reversibility the mechanism of N-methylsuccinimide anion oxidation by Fl_{ox} to yield maleimide and FlH^- can be constructed. The possible implications of this mechanism to the mechanism of such dehydrogenating flavoenzymes as succinic acid dehydrogenase, fumarate reductase, acyl-CoA dehydrogenase, and acyl-CoA oxidase are considered.

Introduction

A relatively unexplored aspect of flavin chemistry is the oxidative formation of C–C double bonds α,β to polar unsaturated substituents (as in eq 1) and the retroreductive process. Among

$$RCH_2CH_2C(=O)X + Fl_{ox} \rightarrow RCH{=}CH(=O)X + FlH_2 \quad (1)$$

flavoenzymes responsible for the catalysis of such reactions there may be cited succinic acid dehydrogenase of the metabolically central tricarboxylic acid cycle (and related bacterial fumarate reductase)[1] and the acyl-CoA dehydrogenases and oxidases of fatty acid catabolism. The mechanisms of these enzymes have in common the ionization of an α-proton of substrate and subsequent oxidation of the resultant carbanion by flavin cofactor.[2] What remains to be clarified is the precise mechanism by which a pair of electrons and a proton are transferred from substrate carbanion to flavin cofactor. Mechanisms that must be considered are (a) addition of the carbanion intermediate to the N(5)-position of the flavin to provide a covalent intermediate which by fragmentation yields reduced flavin and the α,β-unsaturated product (eq 2), (b) general-acid-catalyzed addition of the carbanion to the



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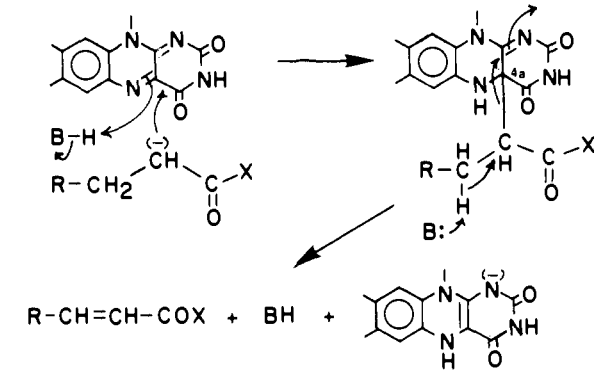
C(4a)-position of Fl_{ox} followed by general-base-catalyzed elimination of FlH_2 (eq 3), (c) initial le^- transfer from carbanion to flavin to provide a radical pair intermediate which could then collapse to the N(5) intermediate of eq 2 or undergo H· transfer as in eq 4, and (d) hydride transfer from carbanion to the N-(5)-position of Fl_{ox} (eq 5). Discussion of experimental evidence



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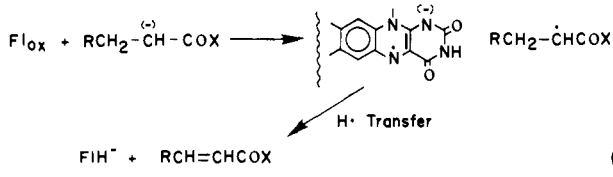

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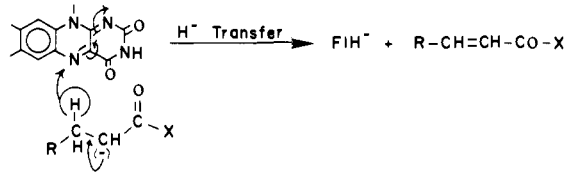

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for and against N(5) and C(4a) adducts as intermediates in various other flavin oxidative mechanisms has been presented.[3,4]

It has recently been shown that 5-carba-5-deazaflavin mononucleotide acts as a hydride acceptor in C–C double bond formation in dehydrogenation reactions catalyzed by the general acyl-CoA dehydrogenase of pig kidney (eq 6).[5] Also, hydride

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(2) Unden, G.; Kroger, A. *Eur. J. Biochem.* **1981**, *120*, 577–584.
(3) Chan, T. W.; Bruice, T. C. *Biochemistry*, **1978**, *17*, 4784–4793.
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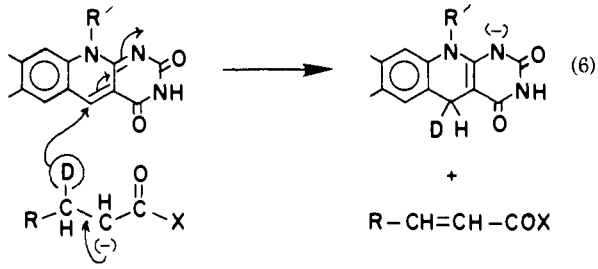

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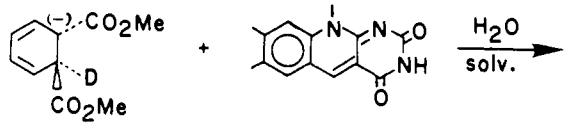
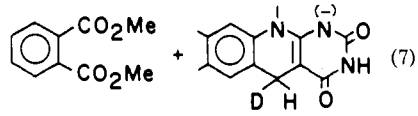
transfer has been shown to be involved in the oxidation of dimethyl *trans*-dihydrophthalate by 5-carba-5-deazalumiflavin (eq 7) in





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a model study.[6] Although these results, employing 5-carba-5-deazaflavins as oxidants, establish the feasibility of hydride (or equivalent le^-, H^+, le^-) transfer from carbanion to flavin, they do not establish that flavins carry out such oxidations by hydride-equivalent oxidation. The structure of the 5-carba-5-deazaflavins resemble nicotinamides as much as flavins and past experience has led to the suggestion that the 5-deazaflavins are nicotinamides in flavin clothing.[2,7]

By the principal of microscopic reversibility, the mechanism of C–C double-bond reduction by dihydroflavin to yield carbanion should be the same as that for C–C double-bond formation by oxidation of carbanion by flavin providing the experimental conditions for studying both processes are the same. The objective of this study has been to elucidate the detailed mechanism for the reduction of maleimides by dihydroflavin so that the mechanism of flavin oxidation of succinimides can be understood. The maleimides have been chosen as substrates since they readily undergo radical and nucleophilic addition[8] (allowing the formation of N(5)- and C(4a)-flavin adducts as in eq 2 and 3), undergo reduction by hydrides[9] (allowing the mechanism of eq 5), and support radical species on le^- reduction[10] as do fumarates[11] (as would be required of the mechanism of eq 4). We have previously shown that the low-potential dihydroflavin analogue 3,7,10-trimethyl-(1*H*,3*H*,5*H*,7*H*,9*H*,10*H*)-pyrimido[5,4-*g*]pteridine-2,4,6,8-tetraonedianion readily reduces *N*-methylmaleimide and diethyl fumarate at pH 7.[12]

Experimental Section

Instruments. All kinetic measurements were carried out on either a Cary 118 spectrophotometer or Perkin-Elmer Lambda-3 instrument in which the cell holder had been maintained at 30 ± 0.2 °C by circulating thermostated water. Fast reaction kinetics were followed on a Durham-Gibson stopped-flow spectrophotometer housed in a nitrogen glovebox and equipped with Biomation Model 805 wave form recorder. pH measurements were carried out on a Radiometer Model M26 pH meter equipped with a Radiometer GK2402C glass–calomel combination electrode. GC analyses were carried out on a Varian 3700 series gas chromatograph (5% OV-17 on chromosorb 80-100W-HP, 6 ft column) using a Varian CDS101 electronic integrator for peak area determinations. The GC/MS analysis was carried out on a HP 5992A instrument (fused silica column, OV101; 0.5-μm film, 25 m long/0.3 μm o.d.; inlet pressure 8 psi). The rate constants were calculated on a HP 9825A desk top calculator equipped with a digitizer HP 9864A and plotter HP 9862A utilizing a program written in this laboratory.

Materials. *N*-Methylmaleimide was obtained from Sigma Chemical Co. and purified by recrystallization (3 times) from ether. The recrystallized sample had a melting point of 93–95 °C (lit.[13] 94–96 °C) and appeared as a single peak on GC. Maleimide was obtained from Aldrich Chemical Co. and used as such. 3-(3-Sulfopropyl)lumiflavin (Fl_{ox}) was prepared according to published procedures.[14] 1,5-Dihydro-5-ethyllumiflavin was prepared from 3-methylumiflavin[15] by reductive alkylation[16] and 1,5-dihydro-3-methyl-5-carba-5-deazalumiflavin was obtained as described previously.[17] *N*-Methylsuccinimide was prepared by the catalytic hydrogenation of *N*-methylmaleimide, mp 64 °C (lit.[13] 66 °C). The inorganic salts used in the preparation of buffer solutions were of analytical reagent grade obtained from Mallinkrodt and were used as such. The buffer solutions were prepared using doubly glass distilled water. Buffers employed were HCl (pH 1.0), phosphate (pH 2–3 and pH 6–8), chloroacetate (pH 2.5–3.5), formate (pH 3–4), and acetate (pH 4–5.5). The ionic strength was adjusted to 1.0 with KCl.

Kinetic Measurements. Stock solutions of Fl_{ox} and *N*-methylmaleimide (MMI) and maleimide (MI) were prepared in 1 M KCl solution. Typically 0.4 mL of Fl_{ox} (final concentration 5×10^{-5} M), 0.1 mL of EDTA (prepared in the buffer of that pH, final concentration 2×10^{-3} mM), and 4.1 mL of buffer were taken in the lower part of the Thunberg cuvette and 0.4 mL of MMI (final concentration (1×10^{-3})–(1×10^{-2}) M) was placed in the top port of the Thunberg cuvette. The solutions were deoxygenated by bubbling argon gas (filtered through an oxiclear filter and then humidified) for 30 min. The Thunberg was then closed and the flavin in the bottom port was photoreduced using a 300-W bulb in a chamber cooled by circulating water. The Thunberg cuvette was then thermostated in the spectrophotometer for 15–20 min. The solutions were then mixed by gently tipping in the contents of the top port of the Thunberg cuvette into the bottom port. The reaction kinetics were followed by observing the change in absorbance as a function of time. In "adduct-formation" studies the increase in absorbance was followed at 368 nm. In "adduct-decomposition" studies, the increase in absorbance at 443 nm was followed as a function of time. A similar procedure was adopted for buffer dilution studies for which the concentration was varied from 0.033 to 0.33 M with $\mu = 1.0$ with KCl.

For studies on the stopped-flow instrument, solutions of Fl_{ox} (in appropriate buffer, final concentration = 2×10^{-5} M) containing EDTA (in buffer, final concentration 1×10^{-3} M) and MMI (in 1 M KCl, concentration (1×10^{-3})–(5×10^{-3}) M) were deoxygenated by bubbling argon through the solutions in degassing bottles for 2 h. The Fl_{ox} solution was then photoreduced and taken inside the nitrogen box where the stopped-flow instrument was located.

Spectral Identification of the Intermediate. In a typical experiment, 0.4 mL of Fl_{ox} (final concentration 1×10^{-4} M), 0.1 mL of EDTA (final concentration 2×10^{-3} M), and 4.1 mL buffer of appropriate pH (μ 1.0 and 0.33 M) were taken in the lower port of a Thunberg cuvette. MMI (0.4 mL, concentration $\sim 1 \times 10^{-4}$ M) was taken in the top port. The Thunberg was sealed with apeizon M grease, deoxygenated and the Fl_{ox} was photoreduced. The Thunberg was thermostated and the solutions were mixed. The spectra between 500 and 300 nm were repeatedly scanned. The spectra of the intermediate were obtained at pH 3.0, 4.0, 5.0, and 6.0. The spectrum of the intermediate in the reduction of MI by dihydroflavin (FlH_2) was similarly obtained at pH 5.0.

Product Determination. Fl_{ox} (13.2 mg, 0.035 mmol) and 1 g of EDTA (2.7 mmol) were dissolved in 50 mL of pH 7.0 phosphate buffer. The solution was deoxygenated and photoreduced. The solution of reduced flavin was then stirred with MMI (11.5 mg = 0.1 mmol) inside an oxygen-free nitrogen box for 5 h. The reaction solution was brought out and extracted with chloroform (5 × 40 mL). The chloroform extractions

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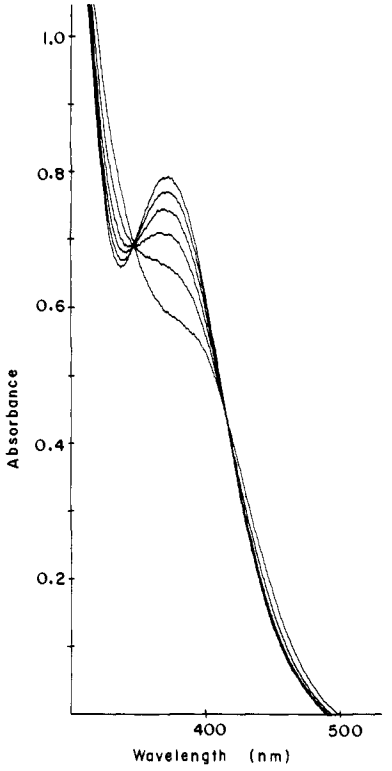
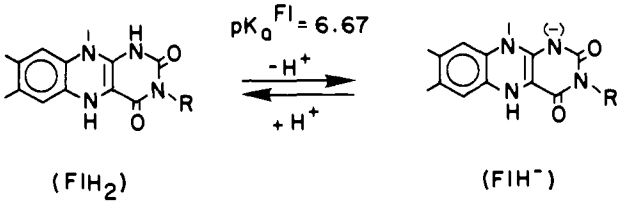


Figure 1. Repetitive scan of the reaction of FlH_2 (1×10^{-4} M) and MMI (1×10^{-4} M) to form the 4a adduct: pH 5.0 (acetate buffer), μ 1.0 with KCl and $T = 30 \pm 0.2$ °C. The time interval between the scans is 204 s. For clarity only the first few scans have been shown. Similar spectra were obtained at pH 3, 4, and 6 and also with MI (pH 5.0).

were pooled together, washed with water, dried, and evaporated. The yield (3.65 mg, 94% of theory) of *N*-methylsuccinimide (NMS) was determined by GC analysis of the residue by establishing a standard plot of concentration of NMS vs. peak area. The GC/MS analysis of a similarly obtained sample gave peaks corresponding to MMI [R_t = 3.7 min; *m/e* 111.9 (2.6%), 110.9 (43.7%), 83.1 (23.3%), 82.0 (27.3%), 53.9 (100%)] and NMS [R_t = 6.2 min; *m/e* 113.9 (4.8%), 113.0 (73.3%), 58.0 (23.2%), 57 (21.7%), 56 (100.0%), 55 (29.4%)] comparable to the authentic samples of MMI [R_t = 3.9 min; *m/e* 111.9 (3.01%), 110.9 (44.4%), 83.1 (24.8%), 82.0 (27.0%), 53.9 (100.0%), 53.1 (30.7%)] and NMS [R_t = 6.5 min; *m/e* 113.9 (6.3%), 113.0 (78.0%), 58.0 (21.8%), 57 (22.4%), 56 (100%), 55 (31.8%)]. No peak corresponding to any other product was detected under these conditions.

Results

The reduction of *N*-methylmaleimide by 1,5-dihydro-3-(3-sulfopropyl)lumiflavin (FlH_2) at pH 7.0 provides *N*-methylsuccinimide (Experimental Section) in 95% yield. The reduction of *N*-methylmaleimide (MMI) and maleimide (MI) by reduced flavin was followed under the pseudo-first-order conditions of [MI] or [MMI] >> $[FlH_2]_T$ (=$[FlH_2]$ + $[FlH^-]$) (eq 8). The hydrolysis of MMI and MI in the pH range studied[18] is slow compared to the rates observed in the present study.



(8)

The reaction of MMI and MI with dihydroflavin involves the rapid formation of spectrally observed intermediates followed by a much slower formation of oxidized flavin (Fl_{ox}). In the following account there is first presented the results pertaining to the kinetics of formation and identification of intermediate adduct followed by a description of kinetic studies dealing with the decomposition

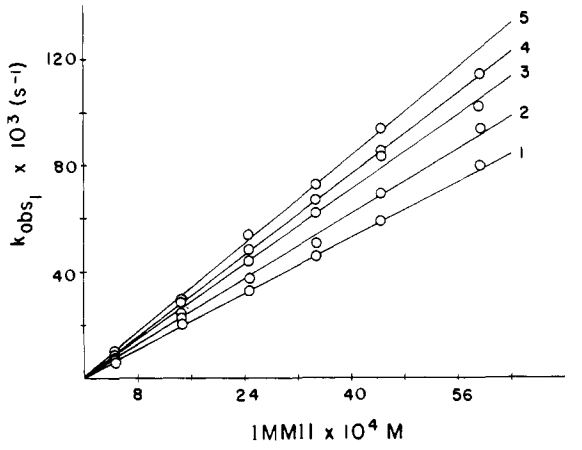


Figure 2. Plots of the first-order rate constants (k_{obsd1}) as a function of [MMI] at pH 5.5 (μ 1.0 and $T = 30 \pm 0.2$ °C). 1, 2, 3, 4, and 5 refer to total acetate/acetic acid concentrations of 0.033, 0.099, 0.165, 0.231, and 0.33 M, respectively. The slopes give the apparent second-order rate constants, k_2'. Similar plots were obtained at other buffer concentrations and pH's. Note that all the lines pass through the origin.

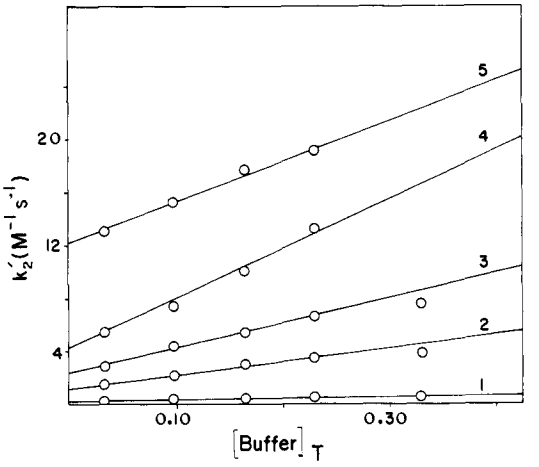


Figure 3. Plots of the apparent second-order rate constants (k_2' M^{-1} s^{-1}, obtained as slopes in Figure 2) against the total buffer concentrations. 1, 2, 3, 4, and 5 refer to pH values of 3.1, 4.08, 4.55, 4.97, and 5.58, respectively. Slopes give k_B and y intercept values give k_{lyate}.

of the intermediate to yield succinimides and oxidized flavin.

The kinetics of formation of the intermediate adduct of *N*-methylmaleimide and 1,5-dihydro-3-(3-sulfopropyl)lumiflavin were investigated in the pH range of 1 to 8. In Figure 1 there is presented repetitive spectral scans showing the formation of the adduct on reaction of dihydroflavin (10^{-4} M) with MMI (10^{-4} M) at pH 5. Between pH 6.0 and 3.0, the adduct has a λ_{max} of 368–370 nm, and its formation is associated with isosbestic points at ~420 and ~345 nm. The tight isosbestic points indicate the absence of accumulation of intermediates prior to the formation of the adduct. Adduct formation was followed in like manner with maleimide at pH 4.0–5.0. The spectrum of the maleimide adduct showed a λ_{max} at 365 nm, and its formation was noted to be associated with isosbestic points at 412 and 340 nm.

Formation of adduct was followed at 368 nm under the conditions of [MMI] >> $[FlH_2]_T$ and was found to follow the first-order rate law. The pseudo-first-order rate constants (k_{obsd1}) when plotted against [MMI] give straight lines passing through the origin. In Figure 2 are plotted k_{obsd1} vs. [MMI] at various buffer concentrations at pH 5.5. Similar linear plots were obtained at pH values between 3 and 8. The reaction is thus first order in $[FlH_2]_T$ and first order in [MMI]. The slopes of the lines provide apparent second-order rate constants (k_2'). Since the plots of k_{obsd1} vs. [MMI] pass through the origin, the reaction is not measurably reversible. In Figure 3 there is presented examples of plots of $[B_T]$, the total buffer concentration, vs. k_2' at constant pH values. Such plots were obtained at pH 2–8 and give straight

Table I. Values of k_B Determined as Best-Fit Slopes to Plots of Total Buffer Concentration [B_T] vs. k_{obsd}/[MMI] Compared To k_B Constants Generated from Eq 10 (i.e., $k_{B(calcd)}$)[a]

buffer acid (BH)	pH	k_B, M^{-2} s^{-1}	$k_{B(calcd)}$, M^{-1} s^{-1}	k_3, M^{-2} s^{-1}	pK_{app}	pK_B
H_3PO_4	2.03	4×10^{-1}	3.85×10^{-1}			
	2.51	5.45×10^{-1}	5.98×10^{-1}	1.66×10^4	2.09	2.12
	3.02	7.83×10^{-1}	7.39×10^{-1}			
$ClCH_2CO_2H$	2.52	3.5×10^{-1}	3.36×10^{-1}			
	2.96	5.3×10^{-1}	5.78×10^{-1}	3.78×10^3	2.81	2.85
	3.45	8.4×10^{-1}	8.02×10^{-1}			
HCOOH	3.1	1.18	1.12			
	3.45	1.85	2.0	2.79×10^3	3.67	3.75
	4.0	3.71	3.61			
CH_3COOH	4.08	10.16	8.91			
	4.55	18.91	21.4	2.07×10^3	4.99	4.75
	5.00	36.00	39.2			
	5.58	59.53	55.1			

[a] Included in the table are the calculated values of k_3 and a comparison of the kinetic and thermodynamic pK_{BH}'s ($pK_{BH(app)}$ vs. pK_{BH}).

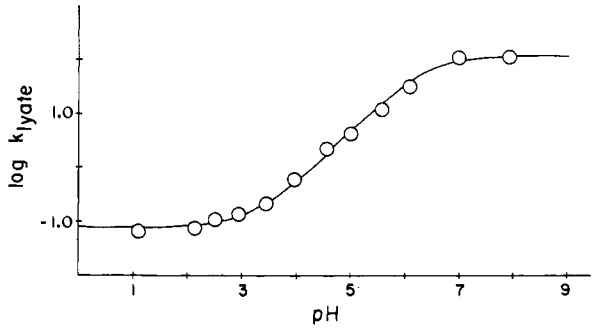
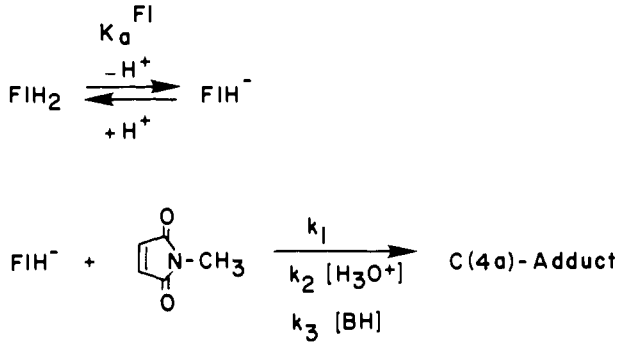


Figure 4. pH vs. log k_{lyate} profile. The circles represent the experimental points and the line was generated from the kinetic eq 9.

Scheme I



lines (slopes = k_B M^{-2} s^{-1}) with positive intercepts (k_{ly} M^{-1} s^{-1}). Thus, $k_2' = k_{ly} + k_B$. Buffers employed were HCl (pH 1.0), phosphate (pH 2–3 and 6–8), chloroacetate (pH 2.5–3.5), formate (pH 3–4), and acetate (pH 4–5.6).

Figure 4 gives the pH vs. log k_{ly} profile. The experimental points of Figure 4 have been fit to lines generated from eq 9. The

$$k_{ly} = \frac{K_a^{Fl}(k_1 + k_2 a_H)}{K_a^{Fl} + a_H} \quad (9)$$

constants employed for best fits are $k_1 = 1.20 \times 10^2$ M^{-1} s^{-1}, $k_2 = 1.94 \times 10^5$ M^{-1} s^{-1}, and $pK_a^{Fl} = 6.39$. The value of $pK_a^{Fl} = 6.39$ may be compared to the thermodynamic pK_a of 1,5-dihydro-3-(3-sulfopropyl)lumiflavin ($pK_a = 6.67$).[19] Values of k_B were fit to eq 10 to obtain the best value of k_3 for each buffer.

$$k_B = k_3 \left(\frac{K_a^{Fl}}{K_a^{Fl} + a_H} \right) \left(\frac{a_H}{K_a^{BH} + a_H} \right) \quad (10)$$

(19) Bruice, T. C.; Benkovic, S. J. "Bioorganic Mechanisms"; Benjamin: New York, 1966; Vol. II; Chapter I.

Scheme II

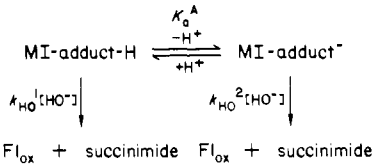


Table II. Pseudo-First-Order Rate Constants (s^{-1}) for Decomposition (30 ± 0.2 °C, Acetate Buffer at 0.33 M) of the 4a Adduct of MMI ([MMI] = 5.5×10^{-3} M) with FlH_2 ([FlH_2] = 4.7×10^{-5} M) under Anaerobic and Aerobic Conditions

pH	aerobic	anaerobic
4.0	5.62×10^{-6}	3.94×10^{-6}
4.97	3.66×10^{-5}	3.55×10^{-5}
6.04	3.30×10^{-4}	3.45×10^{-4}

In Table I are presented the experimental values of k_B along with those values generated from eq 10 which provide the constants k_3. Equations 9 and 10 may be derived from Scheme I.

The decomposition of the adducts, formed from reaction of the 1,5-dihydroflavin with maleimide (MI) and *N*-methylmaleimide (MMI), was followed spectrophotometrically by following Fl_{ox} formation (increase in A_{443} with time). Although MMI and MI were present in reaction solutions in great excess over adducts, the rate constants for decomposition of the adducts were independent of the concentrations of MMI and MI. The pseudo-first-order rate constants (k_{obsd2}) for decomposition of the adducts were found to be independent of buffer concentrations at given pH values. For example, in five kinetic runs at pH 7.0, employing phosphate buffer concentrations of 0.066–0.33 M, the values of k_{obsd2} averaged at $(2.9 \pm 0.2) \times 10^{-3}$ s^{-1} for decomposition of the MMI adduct, while k_{obsd2} averaged at $(1.36 \pm 0.35) \times 10^{-3}$ s^{-1} for decomposition of the adduct with MI. The log k_{obsd2} vs. pH profile for decomposition of MMI and MI adducts are provided in Figure 5. Examination of Figure 5 shows that, for MMI, log k_{obsd2} is a linear function of pH between pH 4 and 9. The slope of the line is 1.0. The slope of the plot and lack of buffer catalysis shows that the decomposition of the MMI adduct is apparent specific-base catalyzed ($k_{obsd2} = k_{HO}K_w/a_H$). Employing the autoprotolysis constant $pK_w = 13.833$ at 30 °C, the value of the second-order rate constant for reaction of adduct with HO^- (k_{HO}) is calculated as 2.27×10^4 M^{-1} s^{-1}. The plot of log k_{obsd2} for MI is parallel to that for MMI to pH 7, becoming independent of acidity at high pH. The line used to fit the log k_{obsd2} points to the profile for the MI adduct (Figure 5) was generated from eq 11 employing the constants $pK_a^A = 8.2$, $k_{HO}^1 = 8.0 \times 10^3$ M^{-1}

$$k_{obsd} = \frac{K_w}{a_H} \left(\frac{k_{HO}^1 a_H + k_{HO}^2 K_a^A}{K_a^A + a_H} \right) \quad (11)$$

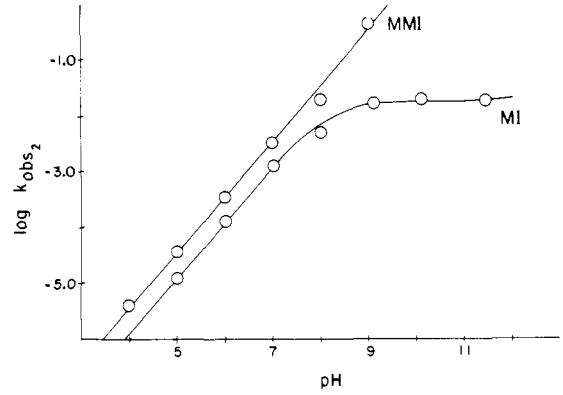


Figure 5. pH vs. log k_{obsd2} for the decomposition reaction of the 4a adducts of FlH_2 and MMI and FlH_2 and MI. k_{obsd2} values are the pseudo-first-order rate constants for the appearance of Fl_{ox} followed as a function of time at 443 nm. The circles represent the experimental points. The line for MMI was generated for the kinetic equation $k_{obsd2} = k_{HO}K_w/a_H$ and that for MI from eq 11.

s^{-1}, and $k_{HO}^2 = 1.7 \times 10^{-1}$ M^{-1} s^{-1}. It should be noted that the value used for k_{HO}^2 represents a maximum. Fitting of the points to the equation wherein $k_{HO}^2 = 0$ provides a plot which is, within experimental error, as good as that shown in Figure 5. Equation 11 is derivable from Scheme II. Adducts of MMI and MI when generated at pH 3.5 and pH jumped to pH 6.0 decomposed at the same rates as when generated at pH 6.0. Table II gives the rate constants for decomposition of the adduct of MMI under aerobic and anaerobic conditions.

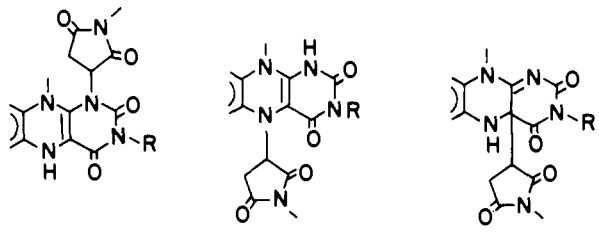
No reaction was observed when *N*(5)-ethyl-1,5-dihydrolumiflavin (7×10^{-5} M) was allowed to react with MMI (1×10^{-4} M) in pH 5 buffer for a period of 15 h. Admittance of O_2 provided the flavin semiquinone (λ_{max} 580, 503, 355, and 325 nm). Also, 1,5-dihydro-5-carba-5-deazalumiflavin (5.11×10^{-5} M) exhibited no reactivity with MMI (2.83×10^{-3} M) in aqueous solution at pH 8.0 when observed by spectral scanning for 48 h under anaerobic conditions.

Discussion

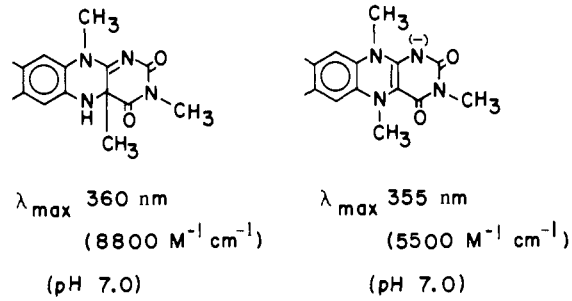
The reduction of maleimide (MI) and *N*-methylmaleimide (MMI) to yield, respectively, succinimide and *N*-methylsuccinimide plus oxidized flavin involves the rapid formation of an intermediate species (eq 12). This finding supports mechanisms

reduced flavin + maleimide → covalent adduct → oxidized flavin + succinimide (12)

that involve either nucleophilic attack of reduced flavin ($FlH_2 \rightleftharpoons FlH^- + H^+$) upon the MMI and MI substrates or le^- transfer from FlH^- to the substrates to form radical pairs which then collapse to provide the required intermediates (see Introduction). Both flavin and maleimide radical species are sufficiently stable to allow such a radical mechanism.[4,10] Plausible adduct structures would involve addition to the dihydroflavin moiety at N(1), N(5), or C(4a). It is not possible to clearly differentiate flavin N(5) and

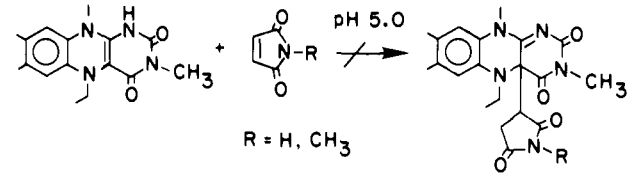


C(4a) adducts from λ_{max} values[20] (MMI adduct λ_{max} 368 nm (ϵ 4920 M^{-1} cm^{-1}), MI adduct λ_{max} 365 nm (ϵ 5450 M^{-1} cm^{-1})).



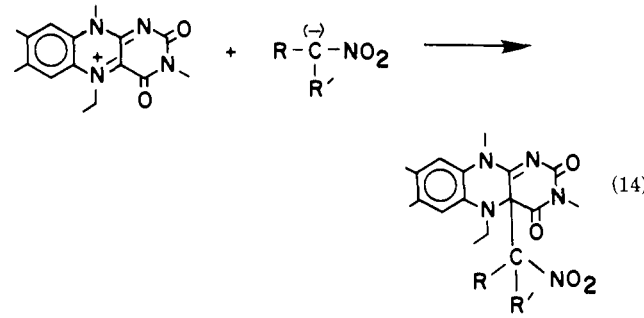
Adduct formation at N(1) or N(5) leaves the 1,5-dihydroisoalloxazine ring intact and for this reason these types of adducts are rapidly air oxidized. For example, the rate of reoxidation of FlH_2 is greater than the rate of reoxidation of N(5) adduct ($t_{1/2}$ = 30–60 s), which is greater than the rate of reoxidation of C(4a) adduct ($t_{1/2} = 4 \times 10^5$ s).[21] 4a-Flavin adducts are devoid of the 1,5-dihydroisoalloxazine structure and are resistant to O_2 oxidation in the dark. The covalent adducts of dihydroflavin with both maleimide and *N*-methylmaleimide are unreactive to oxygen. Additional support for 4a adducts comes from the pH dependence of the kinetics of their solvolysis (Results). No inflection in the log k_{obsd} vs. pH profile (Figure 5) is seen in the vicinity of the pK_a (~7) for dissociation of the N(1) proton for a N(5) adduct. It can be safely concluded from our results that the intermediates are indeed C(4a)-flavin adducts. The formation of a C(4a) adduct on reaction of dihydroflavin with a maleimide amounts to the Michael addition of an enamine. Ample precedence for such reactions exists in the literature and Michael addition of enamines upon MI and MMI have been reported.[22]

The inability of 5-ethyl-1,5-dihydrolumiflavin to react with MMI and MI must be due to steric crowding in what would be the C(4a) adducts (eq 13). The lack of bulk tolerance for 4a



(13)

adduct formation accompanying N(5)-alkyl substitution of flavin has been previously observed by Chan and Bruice.[3] A good example involves C–C bond formation in the addition of nitroalkane anions to the 4a-position of *N*(5)-ethyllumiflavin cation (eq 14). The addition of nitromethane anion is very rapid, and



(14)

the resultant 4a adduct is quite stable in basic solution; the 4a adduct formed from nitroethane anion is unstable while 2-nitropropane anion does not react with flavinium cation. Inspection of molecular models shows that 5-ethylflavin 4a adducts of 2-nitropropane and succinimide exhibit similar steric compression.

The kinetics of formation of 4a adduct on reacting 1,5-dihydroflavin with MMI are in accord with the reactions of Scheme I (Results) from which eq 15 may be derived. However, the term

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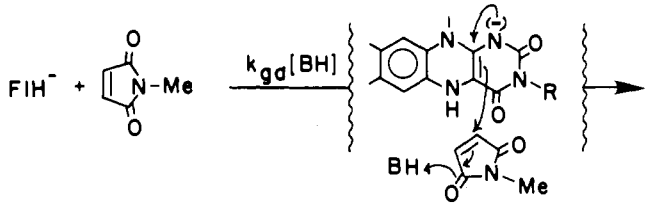
(22) Flavin, M.; Slaughter, C. *J. Biol. Chem.* **1969**, *244*, 1434–1444.

$$d[adduct]/dt = (k_1 + k_2[H_3O^+] + k_3[BH])[FlH^-][MMI] \quad (15)$$

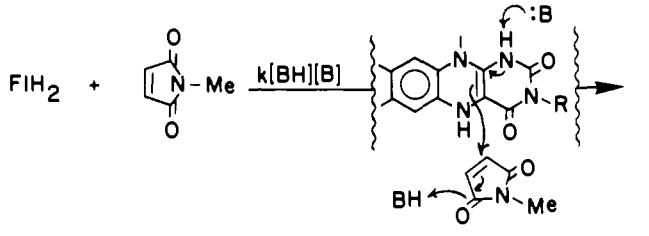
$k_2[H_3O^+][FlH^-][MMI]$ is kinetically equivalent to $k_2K_a^{Fl}[H_2O][FlH_2][MMI]/K_a^{H_3O^+}$, while $k_3[BH][FlH^-][MMI]$ is kinetically equal to $k_3K_a^{Fl}[B][FlH_2][MMI]/K_a^{BH}$. Thus, the mechanism of adduct formation may be explained by use of either or both of two kinetically equivalent mechanisms. The first (Scheme I) requires the reaction of FlH^- with MMI to be a general-acid-catalyzed process, and the second would require general-base catalysis of the reaction of undissociated FlH_2 with MMI. The general-acid catalysis may represent one of two situations. The general acids H_3O^+, BH (=H_3PO_4, $ClCH_2CO_2H$, HCO_2H, CH_3CO_2H), and H_2O assist the nucleophilic attack of FlH^- upon MMI either by donating a proton to the developing negative charge on the maleimide moiety in a concerted reaction (eq 16) or by protonation of anionic intermediate (as in eq 17).



4a-Adduct (16)
+
B:

$$FlH^- + MMI \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} adduct^- \xrightarrow{k_b[BH]} adduct\text{-}H + B \quad (17)$$

The kinetically equivalent general-base mechanism involves removal of a proton from the N(1)-position of FlH_2 by H_2O, $H_2PO_4^-$, $ClCH_2CO_2^-$, HCO_2^-, $CH_3CO_2^-$, or HO^- in concert with the latter's addition to MMI. This mechanism can be rejected on the basis that it would be nonsensical at pH values much above the pK_a of FlH_2 (6.72). Also, this mechanism might be anticipated to require protonation of adduct anion, yet a kinetic term second order in buffer (eq 18) was not detected.



4a-Adduct
+
B: + BH
(18)

Equation 15 must represent general-acid catalysis of FlH^- addition to MMI. In Figure 6 there are plotted the logs of the general-acid rate constants of Scheme I (k_3, $k_1/[H_2O]$, and k_2) vs. the pK_a of the acid catalysts BH, H_2O, and H_3O^+, respectively. In the plot the value of k_3 for H_3PO_4 and k_2 have been divided by three and the constant $k_1/[H_2O]$ by two in order to correct for the number of equivalent protons that may be transferred. The slope of the correlation line of Figure 6 (i.e., α) equals −0.2 with H_3O^+ exhibiting a positive deviation of 2-fold and H_2O a negative deviation of 8-fold (if the points for H_3O^+ and H_2O are ignored $\alpha = -0.1$). The small value of α supports a mechanism wherein proton transfer to the negative charge on the maleimide moiety has not proceeded far. The mechanism may be best represented as involving addition of FlH^- to MMI to provide an unstable adduct which undergoe rate-determining protonation by H_3O^+, H_2O, and the buffer acids BH (eq 17).

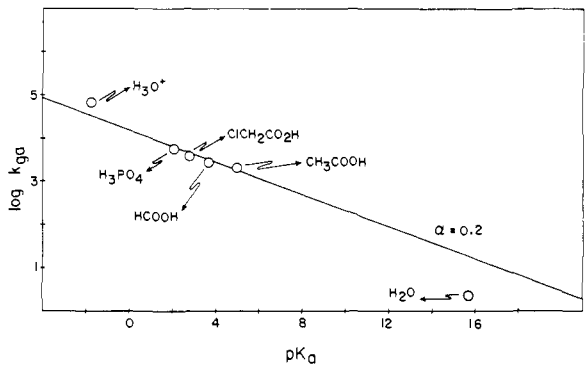
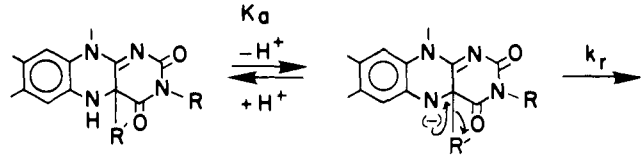


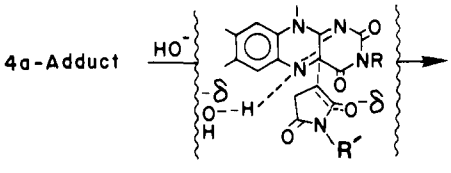
Figure 6. Brønsted plot of pK_a vs. log k_{ga}. k_{ga} refers to values of k_3 for the general acids H_3PO_4, $ClCH_2COOH$, and CH_3CO_2H, k_1 for H_2O, and k_2 for H_3O^+. The values of k_3 (H_3PO_4), k_1, and k_2 have been statistically corrected before including in the plot (see text).

The rates of fragmentation of the 4a adducts of flavin with MMI and MI are not appreciably influenced by buffer concentration at given constant pH values. The fragmentations are catalyzed by HO^-. From the second-order rate constant for HO^- catalysis of the decomposition of the 4a adduct with MMI (2.35×10^4 M^{-1} s^{-1}) it can be calculated that the pK_a for dissociation of the N(5) proton would have to be less than 19 in order that HO^- catalysis could represent a preionization (specific base, eq 19) mechanism.



Fl_{ox} + R'
(19)

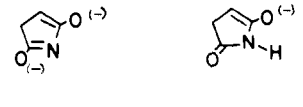
Values of pK_a above 19 would require k_r to exceed 10^9 M^{-1} s^{-1} (a pK_a of 23 would require k_r to equal the rate constant for a single-bond vibration). The pK_a for dissociation of an aniline N–H bond to yield an anion is much greater than 19. It is reasonable to conclude that the elimination is general-base catalyzed with a Brønsted coefficient approaching 1.0, in which case catalysis by bases weaker than HO^- would be difficult to detect. The eliminative fragmentation of the 4a adducts would then be associated with a transition state in which ionization of the N(5)–H is quite advanced (eq 20). Inspection of the *minmal* relative



$$H_2O + Fl_{ox} + \text{(pyrrolidinone anion)} \quad (20)$$

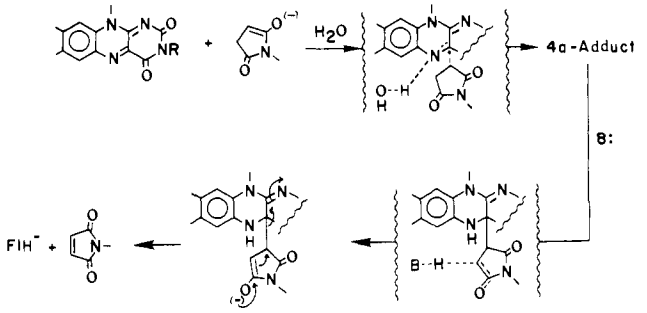
R'	Me	H	electron pair
k_{HO}(rel)	1×10^5	5×10^4	1

second-order rate constants of eq 20 shows that the possession of a negative charge on the group that departs decreases the rate of departure minimally ~50000-fold. If C–C bond breaking were far advanced in the transition state then the rate differential should be approximated by the difference in the free energy contents of the dianion (A) and monoanion (B) of sucinimide. At 30 °C



A B

Scheme III



a rate constant ratio of 50 000 equals 27 kJ M^{-1}. Although we cannot point with any certainty at 50 000 difference in the thermodynamic stability of A and B in H_2O, a minimal value of 27 kJM^{-1} is reasonable. It is fair to assume, however, that departure of the leaving succinimide anion has not proceeded far in the critical transition state. This conclusion is based on the observation that general-acid assistance to leaving group departure in the HO^--catalyzed fragmentation is not observed between pH 4 and 9. This feature would have been evident in a dependence of the rate upon the concentration of buffer base species (eq 21).

$$v = k[HO^-][HB][\text{adduct}] = (kK_w/K_a^B)[B][\text{adduct}] \quad (21)$$

The mechanism of flavin oxidation of *N*-methylsuccinimide to *N*-methylmaleimide may be deduced from this study to involve the addition of succinimide anion to the 4a-position of Fl_{ox} concerted with proton transfer from water to the N(5)-position of Fl_{ox}. In the transition state, C–C bond formation is well developed while proton transfer is early. Decomposition of the 4a adduct to provide reduced flavin and maleimide involves step-wise general-base-catalyzed removal of an α proton of the succinimide carbon acid moiety of the 4a adduct followed by elimination of dihydroflavin anion and formation of the maleimide. In the general-base-catalyzed carbon acid ionization step, C–H bond breaking is far advanced in the transition state (Scheme III).

The hydride reducing agent, 1,5-dihydro-5-carba-5-deazaflavin ($dFlH^-$) does not reduce MMI even though the redox potential for $dFlH^-$ is 120 mV more negative than that for FlH^-.[23] It is clear that hydride transfer is not favored over 4a adduct formation in the redox reaction of flavin/dihydroflavin with maleimide/succinimide. Also, the formation of N(5) adduct on reaction of FlH^- with MMI and MI does not appear to occur.

The biochemical significance of the present results are of importance. Certainly, one cannot extrapolate mechanistic findings that deal with the dihydroflavin/flavin–maleimide/succinimide redox couples in model studies to dihydroflavin/flavin–fumarate/succinate (or dihydroflavin/flavin–α,β-unsaturated acyl CoA/acyl CoA) redox couples in an enzymatic reaction. One can only say that for the former the mechanism is known to involve a 4a adduct intermediate and that this should not be ignored in the consideration of mechanism for the latter.

Before concluding the discussion it is appropriate to consider what apparently amounts to the only model study of a flavin oxidation of a carbanion that leads to C–C double bond formation.

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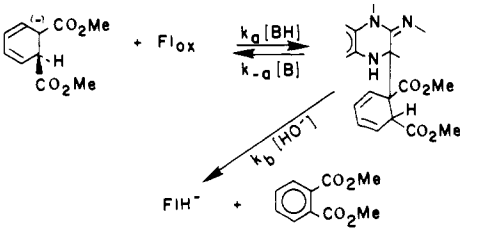
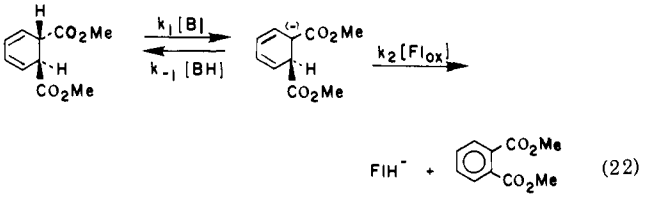
Scheme IV



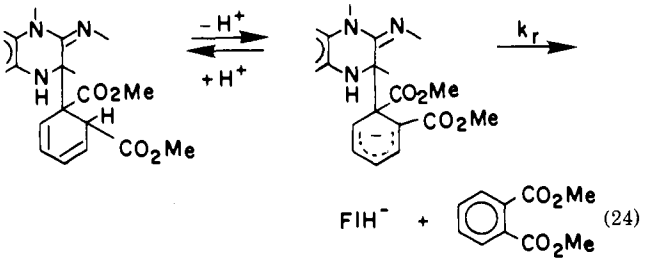
The oxidation of dimethyl *trans*-dihydrophthalate by Fl_{ox} has been shown to follow the kinetics depicted in eq 22.[24] The reaction



of carbanion with Fl_{ox} does not exhibit a dependence upon general-acid catalysis (i.e., $k_2[Fl_{ox}][\text{carbanion}]$). Such a situation would be in effect if carbanion oxidation involved the sequence of Scheme IV providing the 4a adduct was at steady state and $k_{-a}[B] >> k_b[HO^-]$, viz:[25]

$$v_{C^{-}\rightarrow C_{ox}} = \frac{k_a k_b[BH][HO^-][Fl_{ox}][\text{carbanion}]}{k_{-a}[B] + k_b[HO^-]} = \frac{k_a k_b K_w}{K_a^{BH} k_{-a}}[Fl_{ox}][\text{carbanion}] \quad (23)$$

This situation would require the ionization of the 4a adduct to be either specific-base catalyzed (eq 24) or general-base catalyzed



with the Brønsted constant β approaching 1.0. The latter possibility has been considered by us to be quite unlikely,[3,25] but in the light of the results of the present study it must be reconsidered. Inspection of Scheme III shows that decomposition of the 4a adduct of flavin and maleimide is associated with a general-base-catalyzed formation of 4a adduct anion and that the transition state for formation of this anion has a structure in which proton transfer to general base is far advanced (i.e., β approaches 1.0). Thus, the mechanism of Scheme III may apply to the flavin oxidation of the carbanion of dimethyl dihydrophthalate, providing the formation of the 4a adduct is sterically allowed.

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

Registry No. MI, 541-59-3; MMI, 930-88-1; FlH_2, 91385-12-5; Fl_{ox}, 36469-13-3; *N*-methylsuccinimide anion, 91385-13-6.

(24) Main, L.; Kasperek, G. J.; Bruice, T. C. *Biochemistry* **1972**, *11*, 3991–4000.

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