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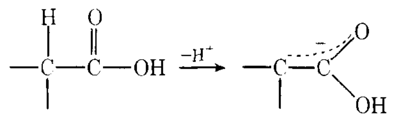
# Importance of $C^{4a}$- and $N^5$-Covalent Adducts in the Flavin Oxidation of Carbanions[†]

T. W. Chan and Thomas C. Bruice*

ABSTRACT: The reaction of nitroalkanes, furoin, and phenacyl alcohol with 7- and 8-cyano-3,10-dimethylisoalloxazine (7-$CNFl_{ox}$, 8-$CNFl_{ox}$), 5-ethyl-3-methyllumiflavinium ion ($Fl_{ox}{}^+Et$), and 2,6-dichloroindophenol (DCI) have been studied. Nitromethane anion forms an adduct on reaction with $Fl_{ox}{}^+Et$. The condensation reaction has been shown (nuclear magnetic resonance) to occur through addition of the methylene carbon of the nitromethane anion to the 4a position of $Fl_{ox}{}^+Et$. This adduct undergoes spontaneous dissociation to its components in acid. It does not undergo conversion in base to reduced flavin (1,5-FlEtH), $NO_2{}^-$, and $CH_2O$. For this reason one may conclude that *nitroalkane anion oxidation by flavin does not involve the intermediacy of a 4a-covalent intermediate*. Oxidation of nitroalkane anion through formation of an $N^5$-flavin adduct is discussed in terms of the peculiar requirements of the substrate when compared with carbanions derived from normal flavoenzyme substrates. It is concluded that the mechanism of nitroalkane oxidation by flavin must bear no relationship to the flavin oxidation of normal substrates. Furoin and phenacyl alcohol (compounds containing the -CH(OH)—CO- functionality) are oxidized by 8-$CNFl_{ox}$, 7-$CNFl_{ox}$, DCI and by the $N^5$-blocked flavin $Fl_{ox}{}^+Et$. These reactions are found to be zero order in oxidant at the concentrations of oxidants ($\sim 1.0 \times 10^{-5}$ M) and reductant ($\sim 10^{-3}$ to $10^{-4}$ M) employed. This finding, and that of an $^1H/^2H$ kinetic isotope effect of 3.5, as well as certain equalities of the determined rate constants establish the rate-determining step in all these oxidations to be carbanion formation. That the $N^5$-blocked flavin ($Fl_{ox}{}^+Et$) serves just as well as an oxidant as do substituted and unsubstituted isoalloxazines provides evidence that the *flavin oxidation of carbanions of general structure* (*-$C^-$(OH)—CO-*) *does not require the formation of an intermediate $N^5$-flavin adduct.* This conclusion when taken with the fact that the $N^5$-flavin adduct formed on reaction of dihydroflavin with -C(=O)—CO- is not an intermediate in the reduction of -C(=O)—CO- to -CH(OH)—CO- by dihydroflavin shows that $N^5$ adducts do not arise as intermediates in these oxidations. Remaining mechanisms (4a addition and radical pair formation) are discussed. 4a addition of carbanions to $Fl_{ox}{}^+Et$ is shown to be subject to considerable steric strain by the instability of the nitroethane adduct and the inability to observe the formation of the 2-nitropropane adduct.

The carbon acid substrates which are oxidized by flavoenzymes are divisible into two groups dependent upon the acidity of the C-H bond which ultimately gives up its bonding electrons to the flavin cofactor. In the first group, the $pK_a$ of the C-H function is so great that oxidation cannot involve the transfer of electrons from a derived carbanion (glucose, NADH, etc.). The second grouping consists of such substrate types as carbonyl compounds, carboxylic acids ($\alpha$-amino, $\alpha$-hydroxy, and others) or esters which may undergo $\alpha$-C-H bond dissociation to provide a resonantly delocalized carbanion as the species which undergoes oxidation. (Presumably the carboxylic acid substrates are bound to the enzyme in the undissociated form allowing resonance stabilization of the $\alpha$-carbanion (eq 1).)



$$-\overset{H}{\overset{|}{\underset{|}{C}}}-\overset{O}{\overset{\|}{C}}-OH \xrightarrow{-H^+} -\underset{|}{C}\overset{-}{\cdots}C{\overset{O}{\diagup}\atop\underset{OH}{\diagdown}} \qquad (1)$$

The evidence for the formation of intermediate carbanion species in these enzymatic oxidations derives from studies of competitive oxidation and halide elimination and others (succinic acid dehydrogenase, see Tober et al., 1970; D-amino acid oxidase, see Cheung & Walsh, 1976; lactic acid oxidase, see Walsh et al., 1973; Ghisla & Massey, 1977). The nonenzymatic (flavin) oxidation of carbanion forming carbon acids has been a topic of investigation in this laboratory (see Discussion section).

An understanding of the mechanism of such flavoenzymes as the amino acid oxidases and lactic acid oxidase, etc. is obtained only when it is understood just how the electron pair of the appropriate carbanions are transferred to oxidized flavin. One may envision the formation of covalent intermediates [at either the 4a position (I and II) or $N^5$ position (III and IV)] or caged radical pairs as intermediates in these oxidations.

In the present study we have included the $N^5$-blocked 5-ethyl-3-methyllumiflavin ($Fl_{ox}{}^+Et$)[1] as oxidant and nitroal-

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[1] Abbreviations used: $Fl_{ox}{}^+Et$, 5-ethyl-3-methyllumiflavinium; DCI, 2,6-dichloroindophenol; 7-$CNFl_{ox}$ and 8-$CNFl_{ox}$, 7- and 8-cyano-3,10-dimethylisoalloxazine, respectively; Mops, morpholinopropanesulfonic acid.

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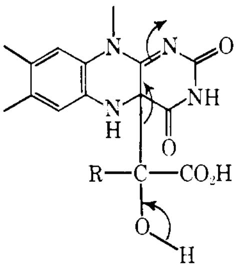

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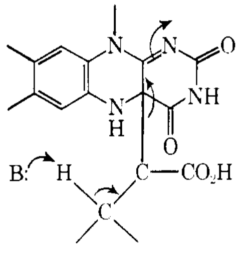

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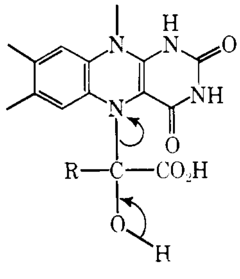

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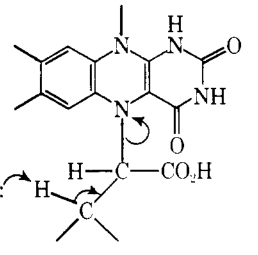
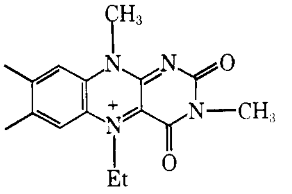


IV

kanes, furoin and phenacyl alcohol as substrate. By inclusion of $Fl_{ox}{}^+Et$ we have been able to draw some definite conclusions concerning the mechanism of flavin oxidation of carbanions.



$Fl_{ox}{}^+Et$

## Experimental Section

*Materials.* 5-Ethyl-3-methyl-1,5-dihydrolumiflavin (FlEtH) and 5-ethyl-3-methyllumiflavin perchlorate ($Fl_{ox}{}^+Et\text{-}ClO_4{}^-$) were synthesized and purified in this laboratory by published procedures (Ghisla et al., 1973). 7-Cyano-3,10-dimethylisoalloxazine ($7\text{-}CNFl_{ox}$) and 8-cyano-3,10-dimethylisoalloxazine ($8\text{-}CNFl_{ox}$) were synthesized in a previous study (Bruice et al., 1977).

Furoin (Aldrich) was purified by chromatography on silica gel using chloroform as eluent and recrystallized from ethanol, mp 138.5–139.5 °C (lit. 138–139 °C, Huntress, 1941). α-Deuteriofuroin (furoin-α-*d*) was prepared by dissolving furoin in acetonitrile followed by addition of an equal volume of 0.001 M sodium deuteroxide under anaerobic conditions. After 15 min the solution was acidified with a slight excess of DCl in $D_2O$. Furoin-α-*d* was precipitated by bubbling nitrogen through the solution for about 10 min and collected by filtration after cooling the suspension of product in an ice–water bath. The furoin-α-*d* so obtained was recrystallized from ethanol, mp 138.5–139.5 °C.

Furil (Eastman) was recrystallized from ethanol, mp 165.5–166.5 °C (lit. 165 °C, Huntress, 1941). Phenacyl alcohol (Eastman) was recrystallized from ethanol, mp 85–86 °C (lit. 85.5–86 °C, Wiberg & Wesley, 1965). 2,6-Dichloroindophenol (DCI) was assayed with Mohr's salt (Lorenz & Arnold, 1938). Formaldehyde-2,4-dinitrophenylhydrazone was synthesized according to the method of Shriner, using concentrated hydrochloric acid instead of sulfuric acid. It was recrystallized from ethanol, mp 165–166 °C (lit. 165 °C, Shriner & Fuson, 1948). Acetaldoxime was purchased from Aldrich. Nitromethane (Aldrich), nitroethane (MC/B), and 2-nitropropane (Aldrich) were distilled before use. All other materials were obtained from commercial sources.

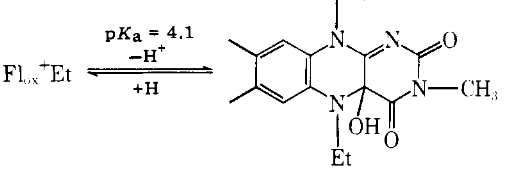
## Apparatus and Methods

*NMR spectra* were recorded on Varian T60 or XL100 FT spectrometers. *Kinetic measurements* were made on Cary 15 or 118 spectrophotometers. Reaction solutions ($\mu = 1$) were prepared in Thunberg cuvettes in a glove box under a nitrogen atmosphere. Reaction components were mixed after bubbling with vanadous ion scrubbed argon for 40 min. Zero-order disappearances of substrates ($S_{ox}$) or zero-order appearance of FlEt· were determined from the slopes of absorbance ($A$) vs. time plots making use of the relationship $d[A]/dt = \epsilon d[S_{ox}]/dt$, where $\epsilon$ is the extinction coefficient of $S_{ox}$.

*Preparation of Nitromethane Adduct to $Fl_{ox}{}^+Et$.* $Fl_{ox}{}^+Et$ (ca. 10 mg) was dissolved in a minimum of water which was diluted with 5 mL of phosphate buffer (0.2 M, pH 7.4, $\mu = 1$ with KCl). To the buffered solution was then added 200 μL of an 1 M aqueous solution of nitromethane. After a period of 15 min the reaction solution was lyophilized and the solid residue dissolved in $CDCl_3$ and filtered: NMR ($CDCl_3$, $Me_4Si$) 0.9 (t, 3 H), 2.29 (d, 6 H), 3.09 (q, 2 H), 3.42 (s, 2 H), 3.70 (s, 3 H), 4.34 (d, 1 H), 4.82 (d, 1 H), 7.01 (d, 2 H) (Figure 1).

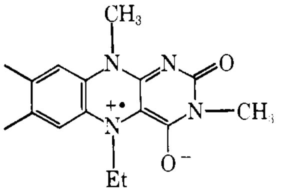
## Results

In aqueous solution $Fl_{ox}{}^+Et$ is in equilibrium with its pseudobase ($\lambda_{max} = 348$ nm, p$K = 4.1$) (Ghisla et al., 1973; Kemal & Bruice, 1976).

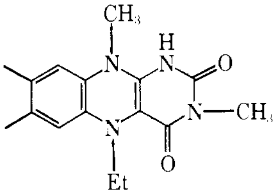


4a-FlEtOH (2)

Nevertheless, covalent addition of nitromethane to $Fl_{ox}{}^+Et$ occurs in aqueous solution at pH 7.0 (see Experimental Section). The formation of adduct is accompanied by the appearance of an absorbance maximum at 327 nm and the formation of about 5% of FlEt· (585 nm).



FlEt·



1,5-FlEtH

Reduction of $Fl_{ox}{}^+Et$ to 1,5-FlEtH does not occur during the reaction. This can be shown by the fact that addition of nitroxide (2,2,6,6-tetramethylpiperidinol-1-oxy) at completion of reaction under argon does not give rise to FlEt· (Chan & Bruice, 1977). The addition of nitromethane anion was found to be reversible. At pH values well below the p$K_a$ of nitromethane (pH 1–3), the adduct reverts to its components ($Fl_{ox}{}^+Et$, $\lambda_{max} = 545$ nm) at a first-order rate constant ($k_{obsd} = 2.3\text{–}3.5 \times 10^{-3}\ s^{-1}$) which is independent of the concentration of buffers ([phosphate] = 0.2 M, pH 2.08; [chloroacetatel] = 0.05–0.2 M, pH 2.9) and hydrogen ion concentration ([HCl] = 0.03–1.0 M). Nitroethane also reacts with $Fl_{ox}{}^+Et$ under conditions similar to those employed with nitromethane, but on acidification $Fl_{ox}{}^+Et$ reappeared on the stopped-flow time scale. This reaction was not studied further. 2-Nitropropane does not react, under the conditions employed, with $Fl_{ox}{}^+Et$ to any significant extent. These observations

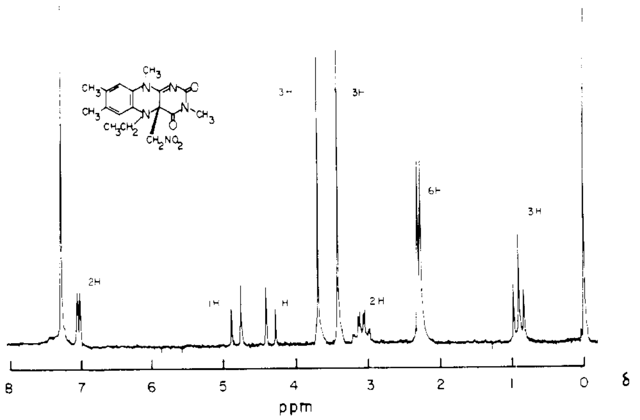


FIGURE 1: The 100-MHz $^1$H NMR spectrum for $Fl_{ox}{}^+Et$-nitromethane adduct in $CDCl_3$ at 23 °C.

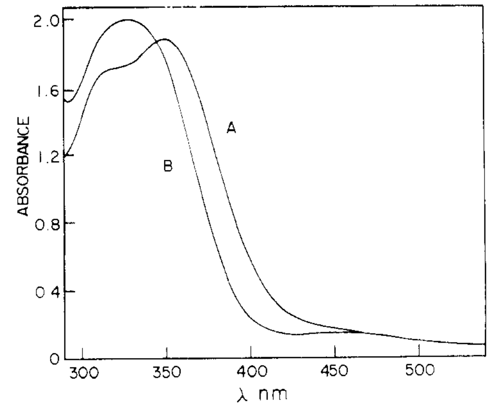
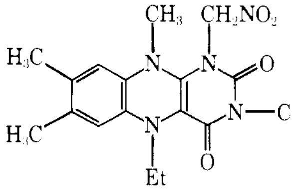


FIGURE 2: Spectrum of (A) FlEtOH ($2 \times 10^{-4}$ M) in phosphate buffer (0.18 M, pH 7.0, $\mu = 1$ with KCl) at 30 °C and (B) $Fl_{ox}{}^+Et$-nitromethane adduct ($2 \times 10^{-4}$ M) in the presence of excess nitromethane ($6 \times 10^{-3}$ M) in the same buffer solution.

suggest a considerable steric destabilization of the adduct on substitution of the nitromethane hydrogens with methyl groups (i.e., 4a addition is subject to considerable steric hindrance). The adduct of nitromethane and $Fl_{ox}{}^+Et$ has no propensity to go on to reduced flavin at pH 6.4–12. Acidification of a solution of the adduct results in its spontaneous dissociation (see above).

Addition of nitromethane anion to $Fl_{ox}{}^+Et$ might be considered to occur at the 1 (V), 4a (VI), or 5 (VII) positions through either O addition $^-O—N^+(—O^-)=CH_2$ or C addition ($-CH_2—NO_2$). The NMR spectrum (Figure 1) of the nitromethane portion (δ 4.6) of the adduct is consistent with C addition. The peaks of vinyl protons resulting from O addition ($FlEt—O—N^+(—O^-)=CH_2$) should be at relatively low field (δ > 5). For comparison we have determined the chemical shifts of the vinyl protons of formaldehyde-2,6-dinitrophenylhydrazone and acetoxime to be ca δ 6.9–7.4. Structure III may be ruled out because the NMR peaks for the methylene protons of the $N^5$-ethyl group of the compound $N^5$-diethyl-3-methyl-1,5-dihydrolumiflavin are at δ 3.7 and 5.5 (Ghisla et al., 1973), whereas the doublets for the methylene



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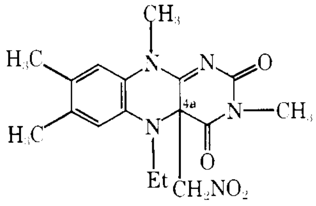
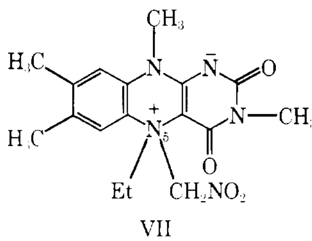


VI



VII

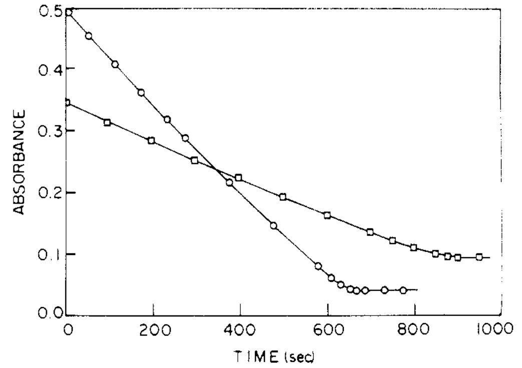


FIGURE 3: Plots of absorbance vs. time for the reaction of DCI ($2.4 \times 10^{-5}$ M, 603 nm, ○) with furoin ($7.96 \times 10^{-4}$ M) and $7\text{-}CNFl_{ox}$ ($2.60 \times 10^{-5}$ M, 430 nm, □) with furoin ($8.06 \times 10^{-4}$ M) at 30 °C in phosphate buffer (0.18 M, pH 7.0, $\mu = 1$ with KCl).

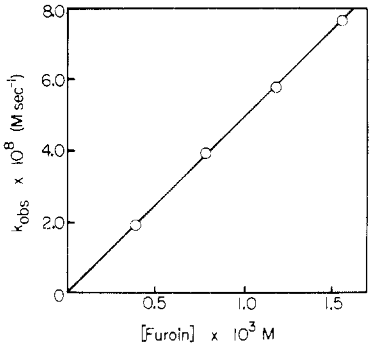


FIGURE 4: Plot of $k_{obsd}$ vs. [furoin] for the reaction of DCI ($2.40 \times 10^{-5}$ M) with furoin at 30 °C in phosphate buffer (0.18 M, pH 7.0, $\mu = 1$ with KCl).

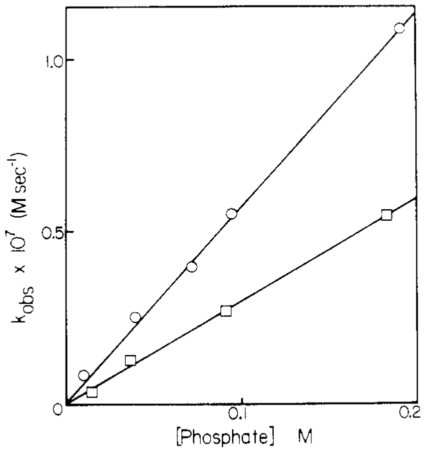


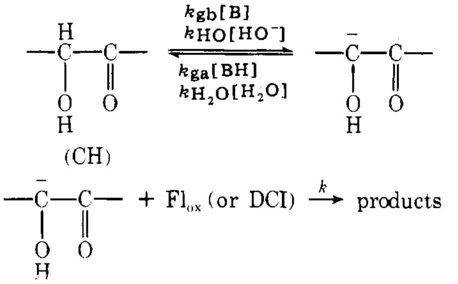
FIGURE 5: Plots of $k_{obsd}$ vs. [phosphate] for the reaction of DCI with furoin at 30 °C. (A) pH 7.4, [furoin] = $1.6 \times 10^{-3}$ M (○); and (B) pH 5.8, [furoin] = $4.55 \times 10^{-3}$ M (□).

TABLE I: Values of $k'_{obsd}$ (30 °C, $H_2O$, $\mu = 1$) for the Reaction of 7-$CNFl_{ox}$ ($6 \times 10^{-5}$ M), 8-$CNFl_{ox}$ ($6 \times 10^{-5}$ M), and DCI ($2.4 \times 10^{-5}$ M) with Furoin ($7.8 \times 10^{-4}$ to $3.13 \times 10^{-3}$ M) and Phenacyl Alcohol ($3.13 \times 10^{-3}$ to $1.56 \times 10^{-2}$ M).

| | $k'_{obsd}$ ($M^{-1}\ s^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | furoin, × $10^4$ | | | phenacyl alcohol, × $10^5$ | | |
| pH | 7-$CNFl_{ox}$ | 8-$CNFl_{ox}$ | DCI | 7-$CNFl_{ox}$ | 8-$CNFl_{ox}$ | DCI |
| 5.8 | | | 0.55 | | | |
| 6.02 | | | 0.86 | | | |
| 6.45 | 1.40 | 1.44 | 1.40 | | | 2.16 |
| 6.66 | | | 1.87 | | | |
| 6.85 | 2.44 | 2.30 | 2.31 | | | |
| 7.00 | 2.42 | 2.4 | 2.61 | | | |
| 7.40 | 3.32 | 3.45 | 3.99 | 4.01 | 4.30 | 4.64 |

protons ($N^5$-ethyl) of the adduct (Figure 1) are found at $\delta$ 3.1. The double doublets of the methylene protons ($-CH_2NO_2$) indicate that they are located next to an asymmetric center. This tends to rule out 1-$FlEtCH_2NO_2$ (V) in favor of 4a-$FlEtCH_2NO_2$ (VI). The UV-Vis spectrum of the adduct (Figure 2) is also consistent with the 4a adduct. The $\lambda_{max}$ of the adduct (327 nm) is about that anticipated for a bulky alkyl group at the 4a position of an isoalloxazine ring. Thus, the $\lambda_{max}$ of 4a,5-dihydrolumiflavin (Hemmerich et al., 1971) was found to be at 327 nm.

*Reactions of 8- and 7-Cyano-3,10-dimethylisoalloxazines (8-CNFl$_{ox}$ and 7-CNFl$_{ox}$, respectively), and 2,6-Dichloroindophenol (DCI) with Furoin and Phenacyl Alcohol (in 0.02 M to 0.2 M Phosphate Buffer).* The reduction of 8-$CNFl_{ox}$ (443 nm), 7-$CNFl_{ox}$ (430 nm), and DCI (600 nm) by furoin and phenacyl alcohol were found to be zero order in each of the three oxidants (Figure 3) and first order in carbon acid (Figure 4) and buffer (Figure 5). This finding suggests rate-determining carbanion (enediolate) formation by general-base catalysis (Bruice & Taulane, 1976) as shown in eq 3.



$$\mathrm{-CH(OH)-C(=O)-}\ \underset{k_{ga}[BH]\ \ k_{H_2O}[H_2O]}{\overset{k_{gb}[B]\ \ k_{HO}[HO^-]}{\rightleftharpoons}}\ \mathrm{-\bar{C}(OH)-C(=O)-}$$

$$\mathrm{-\bar{C}(OH)-C(=O)-} + Fl_{ox}\ (\text{or DCI}) \xrightarrow{k} \text{products} \tag{3}$$

The steady-state assumption in carbanion leads to eq 4.

$$\frac{-d[Fl_{ox}]}{dt} = \frac{(k_{gb}[B] + k_{HO}[HO^-])[CH][Fl_{ox}]}{(k_{ga}[BH] + k_{H_2O}[H_2O]) + k[Fl_{ox}]} \tag{4}$$

The observation of zero order dependence of the disappearance of the oxidants dictates that $k[Fl_{ox}] \gg k_{ga}[BH] + k_{H_2O}[H_2O]$ so that at constant pH and buffer concentration and under the experimental condition where $[Fl_{ox}]_0 = 5 \times 10^{-5}$ M $\ll$ [CH] $\cong 3 \times 10^{-3}$ M, the value of the zero-order rate constant ($k_{obsd}$ of eq 5) is simply provided by eq 6.

$$k_{obsd} = \frac{\Delta A}{\epsilon_{Fl_{ox}} \times \text{time}} \tag{5}$$

$$k_{obsd} = (k_{gb}[B] + k_{HO}[HO^-])[CH] \tag{6}$$

Plots of $k_{obsd}$ vs. buffer concentration (Figure 4) exhibit an intercept at zero buffer establishing that at pH values below 7.5 the kinetic term $k_{HO}$-$[HO^-]$ is insignificant.

$$k_{obsd} = k_{gb}[B][CH] \tag{7}$$

To establish that the basic species of the buffer is the catalytic entity, eq 7 may be written as eq 8 which then provides eq 9

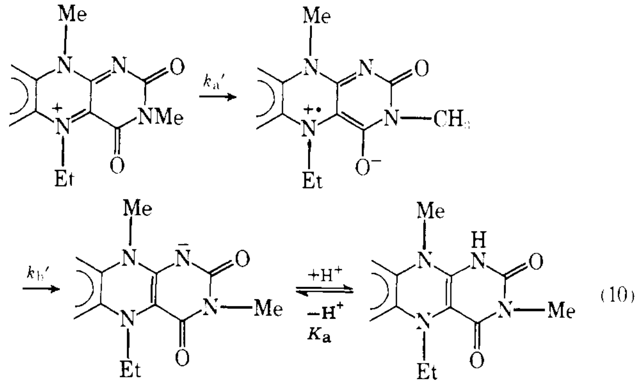
$$k_{obsd} = k_{gb}\frac{K_a}{K_a + a_H}[B_T][CH] \tag{8}$$

$$\frac{1}{k'_{obsd}} = \frac{1}{k_{gb}} + \frac{a_H}{k_{gb}K_a} \tag{9}$$

$$k'_{obsd} = k_{obsd}/[CH][B_T]$$

where $K_a$ is the acid dissociation constant for the buffer and $[B_T]$ represents total buffer concentration. From the slope and intercept of a plot of $1/k'_{obsd}$ vs. $a_H$ for the reaction of DCI with furoin in phosphate buffer (seven pH values), the p$K_a$ of the phosphate buffer is calculated to be 6.6 (tritrimetric = 6.6). Employing furoin and furoin-$\alpha$-$d$ a value of the kinetic deuterium isotope effect ($k^H/k^D$) of 3.5 was observed which is in accord with furoin ionization being rate determining. The results of these studies of the oxidation of furoin and phenacyl alcohol by 7- and 8-$CNFl_{ox}$ and DCI are provided in Table I.

*The reaction of $Fl_{ox}{}^+Et$ ($7 \times 10^{-5}$ M) with furoin ($0.78$-$3.13 \times 10^{-3}$ M) and phenacyl alcohol ($3.13$-$15.6 \times 10^{-3}$ M)* was investigated between pH 6.4 and 8.0 ($H_2O$, 0.02-0.2 M phosphate buffer, 30 °C, $\mu = 1$ with KCl). These reactions were followed spectrophotometrically and were found to be characterized by the appearance of FlEt· (585 nm) in high yield followed by its reduction to 1,5-FlEtH (eq 10).



(10)

In Figure 6 there is plotted $A_{585}$ vs. time for the reaction of furoin ($1.56 \times 10^{-3}$ M) with $Fl_{ox}{}^+Et$ at pH 6.85. Examination of Figure 6 reveals that the appearance of FlEt· ($k_a' = \Delta A_{585}/\epsilon_{FlEt\cdot}$, $\epsilon_{585} = 4000\ cm^{-1}\ M^{-1}$) is zero order in $[Fl_{ox}{}^+Et]$ and the disappearance of FlEt· ($k_b'$) is zero order in the concentration of this radical species. Similar plots were obtained with furoin at other pH values and for the reaction of phenacyl alcohol with $Fl_{ox}{}^+Et$ at pH 6.45 and pH 7.4. The apparent zero-order rate constants ($k_a'$ and $k_b'$) were found to be identical under strictly anaerobic conditions (as may be seen from Figure 6). Insufficient degassing of the reaction mixture was found not to influence the values of $k_a'$ but decreased the values of $k_b'$. Plots of $k_a'$ vs. phosphate buffer concentration

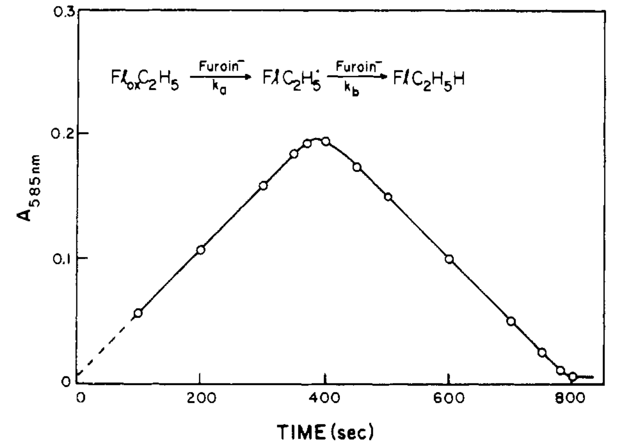


FIGURE 6: Plot of absorbance (585 nm) of FlEt· vs. time for the reaction of furoin ($1.56 \times 10^{-3}$ M) with $Fl_{ox}^+Et$ ($5.0 \times 10^{-5}$ M) in phosphate buffer (0.18 M, pH 6.85, $\mu = 1$ with KCl) at 30 °C. One might note that this marks a very unusual experimental finding of a consecutive A → B → C zero-order process.

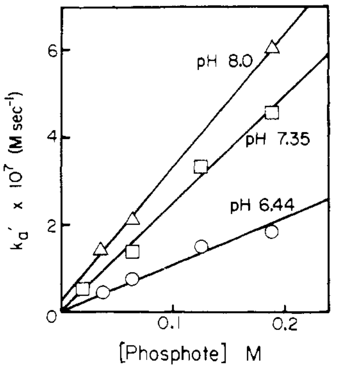
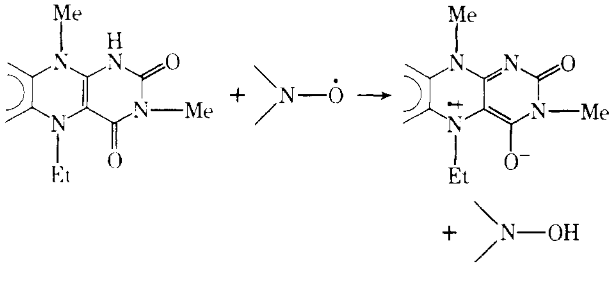


FIGURE 7: Plots of ($k_a'$) vs. [phosphate] at various pH values for the reaction of furoin ($3.13 \times 10^{-3}$ M) with $Fl_{ox}^+Et$ ($5.50 \times 10^{-5}$ M).

at various pH values are linear (Figure 7) and exhibit a small intercept at only the highest pH value (pH 8). This may be attributed to hydroxide ion catalysis of the ionization of the carbon acid. Plots of $k_a'$ vs. carbon acid concentrations are also linear (Figure 8). These findings suggest (as in the reactions with 7- and 8-$CNFl_{ox}$ and DCI) rate-determining carbanion (enediolate) formation by general base catalysis. Equation 9 can be rewritten as

$$\frac{1}{k_a} = \frac{1}{k_{gb}} + \frac{a_H}{k_{gb}K_a} \qquad (11)$$

where $k_a = k_a'/([CH][B_T])$. A plot of $1/k_a$ vs. $a_H$ (at eight pH values) provides a straight line with a positive intercept. From the slope and intercept, the $pK_a$ of the phosphate buffer and $k_{gb}$ are calculated to be 6.58 and $6.67 \times 10^{-4}$ M$^{-1}$ s$^{-1}$, respectively. The rate constants $k_a = k_b$ are summarized in Table II and the various mechanisms which are compatible with these findings are considered in the Discussion section. At completion of the reaction sequence of eq 10, the addition of nitroxide (2,2,6,6-tetramethylpiperidinol-1-oxy) regenerates FlEt· to a concentration equal to that of the $Fl_{ox}^+Et$ initially employed (see Chan & Bruice, 1977).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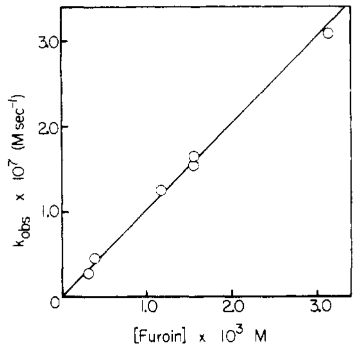
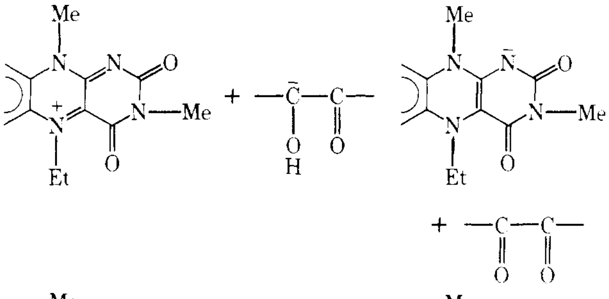


FIGURE 8: Plot of $k_{obsd}$ vs. [furoin] for the reaction of $Fl_{ox}^+Et$ ($3.50 \times 10^{-5}$ M) with furoin in phosphate buffer (0.18 M, pH 7.0, $\mu$ = with KCl) at 30 °C.

TABLE II: Values of $k_a$ (30 °C, $H_2O$, $\mu = 1$) for the Reaction of $Fl_{ox}^+Et$ (~$7 \times 10^{-5}$ M) with Furoin ($7.8 \times 10^{-4}$ to $3.13 \times 10^{-3}$ M) and Phenacyl Alcohol ($3.13 \times 10^{-3}$ to $1.56 \times 10^{-2}$ M).

| | $k_a$ (M$^{-1}$ s$^{-1}$) | |
|---|---|---|
| pH | furoin, $\times 10^4$ | phenacyl alcohol, $\times 10^5$ |
| 5.63 | 1.17 | |
| 5.99 | 1.65 | |
| 6.45 | 3.46 | 4.45 |
| 6.85 | 4.25 | |
| 7.00 | 5.37 | |
| 7.40 | 8.05 | 9.80 |
| 8.00 | 10.50 | |

*The reversibility of the oxidation of furoin by flavin and $N^5$-blocked flavin* was assessed by examining the reactions of furil (~$3 \times 10^{-4}$ M) with 3-methyl-1,5-dihydrolumiflavin ($2 \times 10^{-5}$ M) and $N^5$-ethyl-3-methyl-1,5-dihydrolumiflavin ($6 \times 10^{-5}$ M) at pH 7.4 (0.1 M Mops buffer, $H_2O$, $\mu = 1$ at 30 °C). The nonblocked 1,5-dihydroflavin was oxidized by furil in the stopped-flow time range, whereas FlEtH remained unchanged after 24 h. These results establish that the reaction of eq 13 possesses a much greater negative $\Delta G°$ than the reaction of eq 14.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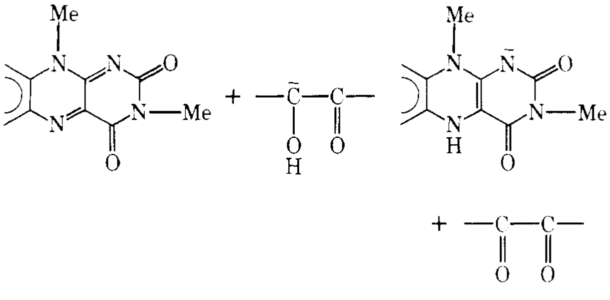
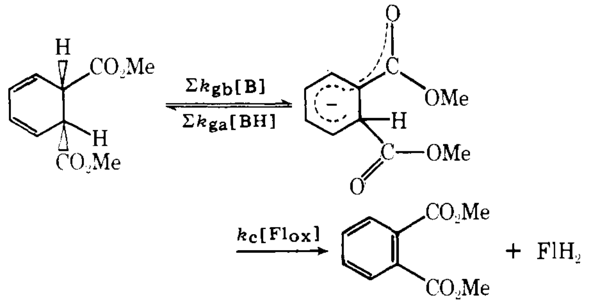

(14)

## Discussion

Model studies of carbon acid oxidations carried out in this laboratory have established that flavin reduction by ionizable carbon acids involves the carbanion species as reductant (eq 15, Main et al., 1972; eq 16, Shinkai et al., 1974; Bruice & Taulane, 1976; this study; eq 17, Yokoe & Bruice, 1975; eq 18, Chan & Bruice (unpublished); eq 19, Novak & Bruice, 1978).



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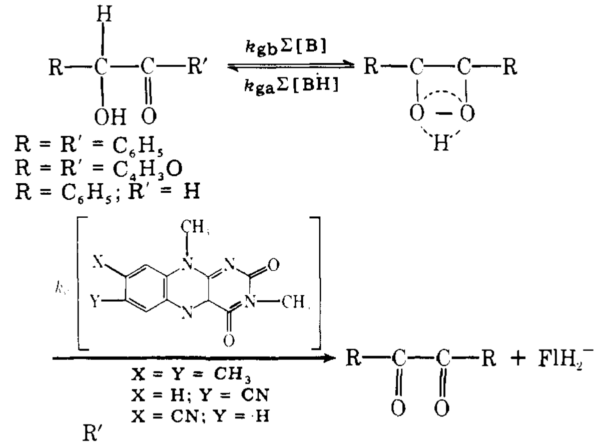

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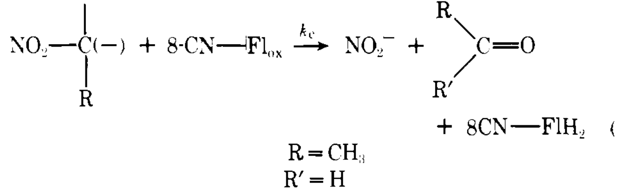

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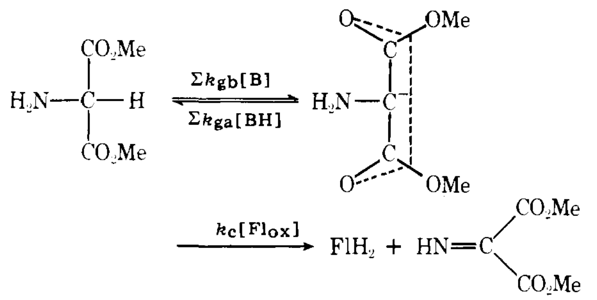

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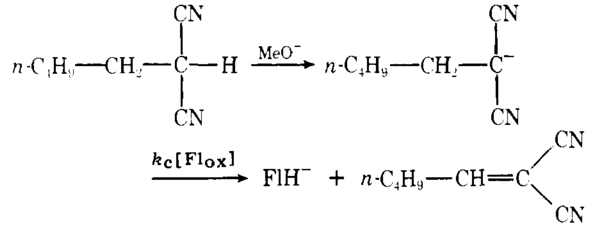
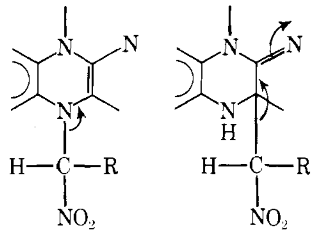


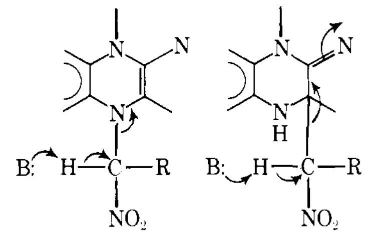
(19)

Evidence for carbanion species being the immediate reductant of the flavin ($Fl_{ox}$) includes the zero-order dependence of the disappearance of $Fl_{ox}$ upon its concentration when 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 for reactions of eq 15, 16, and 18). In the instance of nitroalkanes, the low $pK_a$ of the carbon acids has allowed separate investigation of carbanion and carbon acid. This establishment of carbanion oxidations by $Fl_{ox}$ in model systems is in accord with the most reasonable interpretation of results from flavoenzyme studies (see introductory section). The problem remaining is the determination of whether the carbanion undergoes oxidation via 2e⁻ transfer by formation of a covalent adduct (4a, 5, or other) or whether the oxidation is radical in nature.

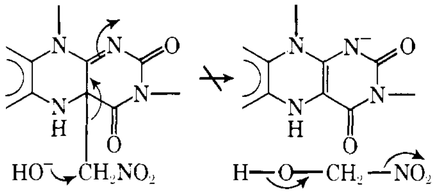
Insofar as substrates for $Fl_{ox}$ are concerned, nitromethane anion may be recognized as being very special. Formation of dihydroflavin by transfer of the nitromethane anion's electron pair to the flavin moiety could not occur regardless of the intervention of a radical mechanism or the formation of $N^5$- or 4a-nitroalkane flavin adducts as intermediates. Thus, $N^5$ or 4a adduct could only provide by an elimination reaction an impossibly unstable nitroalkane carbonium ion (VIII) or by base catalysis a nitrocarbene (IX). This is, of course, not true of the oxidation of the carbanions of $\alpha$-$NH_2$- and $\alpha$-HO-carboxylic acids, succinic acid, etc. (see structures I, II, III, IV of introductory section) wherein transfer of the electron pair from carbanion to $Fl_{ox}$ may be coupled with the loss of a proton. About the only means that can be envisioned for the oxidation of a nitroalkane anion by $Fl_{ox}$ would be: (i) through a mechanism involving radical intermediates; (ii) by a $S_N2$ displacement upon a covalent adduct with hydroxide ion (eq 20);



a VIII b



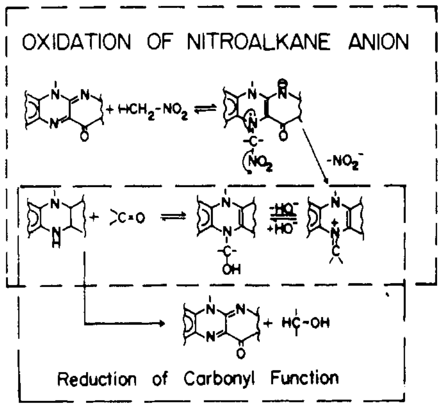
a IX b



(20)

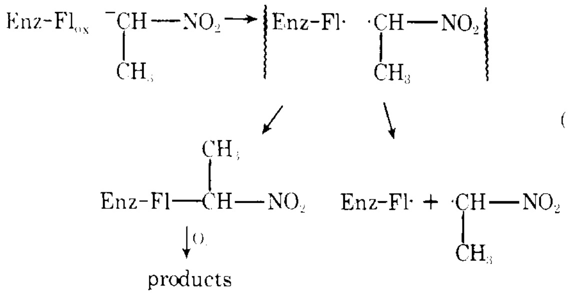
and (iii) through the formation of a $N^5$ adduct followed by elimination of $NO_2^-$ to yield an imine which could then be hydrolyzed through its carbinolamine to yield $FlH_2$ and $R_2C{=}O$ (Scheme I). The results of the present study establish that the 4a adduct of nitromethane anion and $Fl_{ox}^+Et$ is stable to base. This, of course, removes the mechanism (ii) of eq 20 from contention. The finding that the 4a-nitroalkane adduct spontaneously dissociates in acidic media to regenerate nitroalkane and $Fl_{ox}^+Et$ and that it does not yield $FlEt^-$ in base establishes with definity that the 4a-nitroalkane adduct of flavin does not reside along a reaction path leading to reduced flavin and oxidized nitroalkane. Mechanism iii involves the

SCHEME I



reaction sequence suggested by Porter et al. (1973) for oxidation of nitroalkane anions by D-amino acid oxidase (Scheme I). Included in Scheme I are the competing reactions which are observed in the rapid reaction of 1,5-dihydroflavin with carbonyl compounds (Williams & Bruice, 1976). Inspection of Scheme I reveals that dihydroflavin on reaction with carbonyl compound partitions to $N^5$-carbinolamine plus imine and to oxidized flavin and alcohol. The formation of the $N^5$-covalent adducts (carbinolamine + imine) is a reversible but dead-end equilibrium which does not provide oxidized flavin product. Now, it is into this normally nonproductive equilibrium that the $N^5$-nitroalkane adduct feeds on its conversion into imine by loss of $NO_2^-$ (Scheme I). Therefore, though the nitroalkane anion is a substrate for oxidation by certain flavoenzymes and by electron deficient isoalloxazines (Yokoe & Bruice, 1975), its mechanism of oxidation through $N^5$ addition to $Fl_{ox}$ has little more bearing upon the mechanism or reductions of $Fl_{ox}$ by the carbanions of $\alpha$-amino acids and $\alpha$-hydroxy acids than does the reduction of $Fl_{ox}$ by hydrogen over palladium.

The formation of the $N^5$-nitroalkane flavin adduct may be free radical in nature. Porter & Bright (1977) report that nitroethane reacts with glucose oxidase to provide an enzyme-substrate species which partitions 65% to a $N^5$ adduct and 35% to enzyme bound flavin radical. The process could involve the sequence of eq 21.


$$\begin{tikzcd}[column sep=small] \text{Enz-Fl}_{ox}\ {}^{-}\underset{\text{CH}_3}{\text{CH}}\text{—NO}_2 \arrow[r] & \left|\text{Enz-Fl}\cdot\ \ \cdot\underset{\text{CH}_3}{\text{CH}}\text{—NO}_2\right| \arrow[dl] \arrow[d] \\ \text{Enz-Fl—}\overset{\text{CH}_3}{\text{CH}}\text{—NO}_2 \arrow[d, "\text{O}_2"] & \text{Enz-Fl}\cdot + \cdot\underset{\text{CH}_3}{\text{CH}}\text{—NO}_2 \\ \text{products} & \end{tikzcd} \tag{21}$$

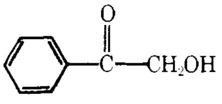
In this study we have shown that nitromethane anion reacts with $Fl_{ox}{}^+Et$ to provide a 4a adduct plus about 5% of $FlEt\cdot$. Previous studies (Russell & Danen, 1966; Kornblum et al., 1966) established that the substitution of chloride ion from *p*-nitrobenzyl chloride by nitroalkane anion involves a radical chain process and the product was predominantly that of "C"

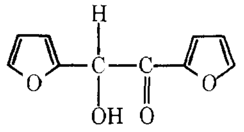
$$\begin{tikzcd}[column sep=small] \text{Fl}_{ox}{}^{+}\text{Et} + {}^{-}\text{CH}_2\text{NO}_2 \arrow[r] & \left|\text{FlEt}\cdot\ \ \cdot\text{CH}_2\text{NO}_2\right| \arrow[dl] \arrow[dr] & \\ \text{FlEt}\cdot + \text{other products} & & \text{4a adduct} \end{tikzcd} \tag{22}$$

$$\begin{gathered} \text{Fl}_{ox}{}^{+}\text{Et} + {}^{-}\text{CH}_2\text{NO}_2 \longrightarrow \text{FlEt}\cdot + \cdot\text{CH}_2\text{NO}_2 \\ \text{FlEt}\cdot + {}^{-}\text{CH}_2\text{NO}_2 \longrightarrow [\text{FlEt-CH}_2\text{NO}_2]^{-}\cdot \\ [\text{FlEt-CH}_2\text{NO}_2]^{-}\cdot + \text{Fl}_{ox}{}^{+}\text{Et} \longrightarrow \text{4a adduct} + \text{FlEt}\cdot \end{gathered} \tag{23}$$

alkylation. The present experimental results find explanation in either the collapse of a radical pair (eq 22) or a radical chain reaction (eq 23).

In this study we find that the $N^5$-blocked flavin, 5-ethyl-3-methyllumiflavin ($Fl_{ox}{}^+Et$), is reduced by the resonantly stabilized carbanions (enediolates) of phenacyl alcohol and furoin.


phenacyl alcohol


furoin

In the carbanion reduction of $Fl_{ox}$ one observes the disappearance of $Fl_{ox}$ to be accompanied by the appearance of 1,5-dihydroflavin ($FlH_2$), while the carbanion reduction of $Fl_{ox}{}^+Et$ is accompanied by the formation of radical ($FlEt\cdot$) which is in turn reduced to FlEtH. These differing observations do not indicate anything about the means by which the nonbonding electron pair of the carbanion are transferred to $Fl_{ox}$ and $Fl_{ox}{}^+Et$ nor do they suggest that the mechanism of carbanion reduction of $Fl_{ox}$ need differ from that for $Fl_{ox}{}^+Et$ (see below).

SCHEME II[a]

$$\text{CH} \underset{k_{-1}'}{\overset{k_1'}{\rightleftharpoons}} \text{C}^-$$

$$\text{C}^- + \text{Fl}_{ox} \xrightarrow{k_2} \text{C}_{ox} + \text{FlH}_2$$

[a] Where $k_1' = k_1[\text{B}]$ and $k_{-1}' = k_{-1}[\text{BH}]$.

SCHEME III

$$\text{CH} \underset{k_{-1}'}{\overset{k_1'}{\rightleftharpoons}} \text{C}^-$$

$$\text{C}^- + \text{Fl}_{ox} \xrightarrow{k_2} \text{C}\cdot + \text{Fl}\cdot$$

$$\text{C}^- + \text{Fl}\cdot \xrightarrow{k_3} \text{C}\cdot + \text{FlH}_2$$

$$\text{C}\cdot + \text{Fl}\cdot \xrightarrow{k_4} \text{C}_{ox} + \text{FlH}_2$$

$$\text{C}\cdot + \text{Fl}_{ox} \xrightarrow{k_5} \text{C}_{ox} + \text{Fl}\cdot$$

$$2\text{Fl}\cdot \xrightarrow{k_6} \text{Fl}_{ox} + \text{FlH}_2$$

The kinetics for the reduction of $Fl_{ox}$ or DCI by carbanions are in accord with either a 2e⁻ transfer from the carbanion to $Fl_{ox}$ or DCI (Scheme II) or a 1e⁻ transfer from the carbanion to $Fl_{ox}$ (or DCI) to provide flavin (or DCI) radical which then disproportionates to give the reduced isoalloxazine (Scheme III). (What follows applies to DCI as well as to $Fl_{ox}$.)

The rate equation for Scheme II assuming steady state in $[C^-]$ is provided by eq 24

$$\frac{-d[\text{Fl}_{ox}]}{dt} = \frac{k_1'k_2[\text{CH}][\text{Fl}_{ox}]}{k_{-1} + k_2[\text{Fl}_{ox}]} \tag{24}$$

Zero-order disappearance of $Fl_{ox}$ (or DCI) requires that $k_2[\text{Fl}_{ox}] \gg k_{-1}$ and eq 24 is reduced to eq 25

$$-\frac{d[\text{Fl}_{ox}]}{dt} = k_1'[\text{CH}] = k_1[\text{B}][\text{CH}] \tag{25}$$

so that the rate of disappearance of flavin is equal to that for the general base (B) catalyzed ionization of CH to yield carbanion. Assumption of a steady state in $[C^-]$, $[C\cdot]$, and $[Fl\cdot]$ for Scheme III leads to the rate eq 26.

$$-\frac{\mathrm{d}[\mathrm{Fl_{ox}}]}{\mathrm{d}t}=\frac{k_1'k_2[\mathrm{Fl_{ox}}]+k_3[\mathrm{Fl\cdot}])[\mathrm{CH}]}{k_{-1}+k_2[\mathrm{Fl_{ox}}]+k_3[\mathrm{Fl\cdot}]} \tag{26}$$

Zero order dependence of the disappearance of $\mathrm{Fl_{ox}}$ on $[\mathrm{Fl_{ox}}]$ would result if $k_2[\mathrm{Fl_{ox}}] + k_3[\mathrm{Fl\cdot}] \gg k_{-1}$ and the rate of disappearance of $\mathrm{Fl_{ox}}$ which is given by eq 27

$$-\frac{\mathrm{d}[\mathrm{Fl_{ox}}]}{\mathrm{d}t}=k_1'[\mathrm{CH}]=k_1[\mathrm{B}][\mathrm{CH}] \tag{27}$$

is equal to the rate of general base catalyzed ionization of CH to yield carbanion.

The kinetics for the reduction of $\mathrm{Fl_{ox}^+Et}$ by carbanion ($\mathrm{C^-}$) allow either an overall $2\mathrm{e^-}$ transfer reaction yielding FlEtH which then provides FlEt· on disproportionation with remaining $\mathrm{Fl_{ox}^+Et}$ (Scheme IV) or may involve $1\mathrm{e^-}$ transfer to yield FlEt· directly (Scheme V). Experimentally the reduction of $\mathrm{Fl_{ox}^+Et}$ is characterized by a zero-order formation of FlEt· (phase 1) followed by a zero-order disappearance of FlEt· (phase 2) as shown in Figure 6 for the substrate furoin. Assumption of a steady state in [FlEtH], [$\mathrm{C^-}$], and [C·] for phase 1 of Scheme IV leads to kinetic eq 28. The

SCHEME IV[a]

$$\mathrm{CH} \underset{k_{-1}'}{\overset{k_1'}{\rightleftharpoons}} \mathrm{C^-} \tag{a}$$

$$\mathrm{C^-} + \mathrm{Fl_{ox}^+Et} \xrightarrow{k_2} \mathrm{FlEtH} + \mathrm{C_{ox}} \tag{b}$$

$$\mathrm{FlEtH} + \mathrm{Fl_{ox}^+Et} \xrightarrow{k_3} 2\mathrm{FlEt\cdot} \tag{c}$$

$$\mathrm{FlEt\cdot} + \mathrm{C^-} \xrightarrow{k_4} \mathrm{FlEtH} + \mathrm{C\cdot} \tag{d}$$

$$\mathrm{Fl_{ox}^+Et} + \mathrm{C\cdot} \xrightarrow{k_5} \mathrm{FlEt\cdot} + \mathrm{C_{ox}} \tag{e}$$

$$\mathrm{FlEt\cdot} + \mathrm{C\cdot} \xrightarrow{k_6} \mathrm{FlEtH} + \mathrm{C_{ox}} \tag{f}$$

[a] $k_1' = k_1[\mathrm{B}]$ and $k_{-1}' = k_{-1}[\mathrm{BH}]$.

SCHEME V[a]

$$\mathrm{CH} \underset{k_{-1}'}{\overset{k_1'}{\rightleftharpoons}} \mathrm{C^-} \tag{a}$$

$$\mathrm{C^-} + \mathrm{Fl_{ox}^+Et} \xrightarrow{k_2} \mathrm{FlEt\cdot} + \mathrm{C^-} \tag{b}$$

$$\mathrm{FlEt\cdot} + \mathrm{C} \xrightarrow{k_3} \mathrm{Fl_{ox}EtH} + \mathrm{C_{ox}} \tag{c}$$

$$\mathrm{FlEt\cdot} + \mathrm{C^-} \xrightarrow{k_4} \mathrm{FlEtH} + \mathrm{C\cdot} \tag{d}$$

$$\mathrm{C\cdot} + \mathrm{Fl_{ox}^+Et} \xrightarrow{k_5} \mathrm{FlEt\cdot} + \mathrm{C_{ox}} \tag{e}$$

$$\mathrm{Fl_{ox}^+Et} + \mathrm{FlEtH} \xrightarrow{k_6} 2\mathrm{FlEt\cdot} \tag{f}$$

[a] Where $k_1' = k_1[\mathrm{B}]$ and $k_{-1}' = k_{-1}[\mathrm{BH}]$.

$$-\frac{\mathrm{d}[\mathrm{FlEt\cdot}]}{\mathrm{d}t}=\frac{2k_1'(k_2[\mathrm{Fl_{ox}^+Et}]+k_4[\mathrm{FlEt\cdot}])[\mathrm{CH}]}{k_{-1}'+k_2[\mathrm{Fl_{ox}^+Et}]+k_4[\mathrm{FlEt\cdot}]} \tag{28}$$

zero-order dependence of the appearance of FlEt· upon $[\mathrm{Fl_{ox}^+Et}]$ would result if $k_2[\mathrm{Fl_{ox}^+Et}] + k_4[\mathrm{FlEt\cdot}] \gg k_{-1}'$ so that the appearance of [FlEt·] is provided by eq 29

$$-\frac{\mathrm{d}[\mathrm{FlEt\cdot}]}{\mathrm{d}t}=2k_1'[\mathrm{CH}]=2k_1[\mathrm{B}][\mathrm{CH}] \tag{29}$$

and the rate of appearance of flavin radical is twice that for the general base (B) catalyzed ionization of CH to yield carbanion. The disappearance of flavin radical (phase 2) is initiated when $[\mathrm{Fl_{ox}^+Et}] = 0$ so that the reactions b and c of Scheme I are unimportant and, as before, $k_4[\mathrm{FlEt\cdot}] \gg k_{-1}'$ so that (eq 30)

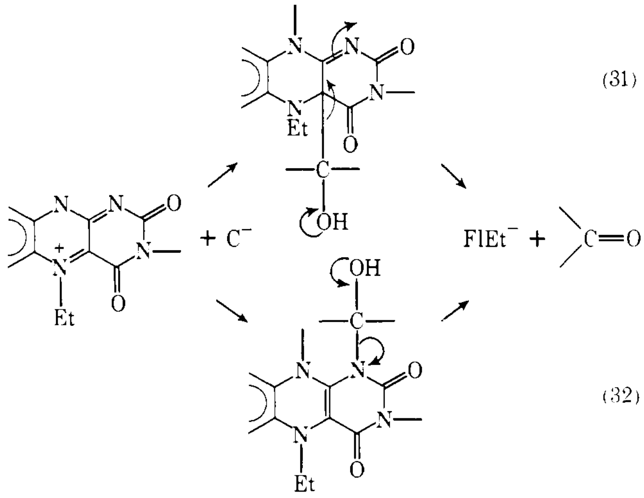
$$\frac{-\mathrm{d}[\mathrm{FlEt\cdot}]}{\mathrm{d}t}=\frac{2k_4k_1'[\mathrm{FlEt\cdot}][\mathrm{CH}]}{k_{-1}'+k_4[\mathrm{FlEt\cdot}]} \tag{30}$$

the rate of radical disappearance is also equal to two times the rate of carbanion formation. A mathematically identical rate expression is derivable from Scheme V. Experimentally, the rates of radical appearance and disappearance are found to be equal in the absence of $\mathrm{O_2}$ (Figure 6). That disappearance of FlEt· ($k_\mathrm{b}'$) becomes slower than FlEt· appearance ($k_\mathrm{a}'$, eq 10) in the presence of minute traces of $\mathrm{O_2}$ may be attributed to the trapping of C· by $\mathrm{O_2}$ and reoxidation of FlEtH. During the formation of FlEt· (phase 1) any free semidione radical (C·) that is present is rapidly trapped by the highly reactive $\mathrm{Fl_{ox}^+Et}$. Thus, the rate of FlEt· appearance is not affected by the presence of traces of oxygen. During phase 2, $\mathrm{Fl_{ox}^+Et}$ is no longer present and FlEt· is a relatively stable species; thus, oxygen is able to compete with FlEt· for any free semidione molecules. By considering only Schemes II–V, it is apparent that if the ionization of the carbon acid is the rate-limiting step, the rate constants determined from FlEt· appearance should be twice that obtained from $\mathrm{Fl_{ox}}$ or DCI reduction. This is found to be the case (Table III).

TABLE III: Relative Rate of Reduction of $\mathrm{Fl_{ox}^+Et}$, DCI, 7-$\mathrm{CNFl_{ox}}$, and 8-$\mathrm{CNFl_{ox}}$ by Furoin and Phenacyl Alcohol at pH 6.4 and 7.4.

| | pH | $k_{\mathrm{Fl_{ox}^+C_2H_5}}$ | $k_{\mathrm{DCI}}$ | $k_{\mathrm{7CNFl_{ox}}}$ | $k_{\mathrm{8CNFl_{ox}}}$ |
|---|---|---|---|---|---|
| furoin | 6.45 | 2 | 0.81 | 0.81 | 0.83 |
| | 7.40 | 2 | 0.99 | 0.83 | 0.86 |
| phenacyl alcohol | 6.45 | 2 | 0.97 | | |
| | 7.40 | 2 | 0.95 | 0.82 | 0.88 |

If $2\mathrm{e^-}$ reduction of $\mathrm{Fl_{ox}^+Et}$ by carbanion (reaction b of Scheme IV) were in effect, it is probably safe to assume that $\mathrm{N^5}$ addition to this $\mathrm{N^5}$-blocked flavin is not involved. This [with our previous finding that the $\mathrm{N^5}$ adduct is off the reaction path in the reduction of carbonyl compounds to carbon acids (Scheme I) and the implication of microscopic reversibility] seems to allow the statement that these carbon acids (and presumably related structures as $\alpha$-$\mathrm{NH_2}$- and $\alpha$-HO-carboxylic acids, esters, etc.) are not oxidized by flavins through $\mathrm{N^5}$ adducts. One might then consider the covalent addition mechanisms of eq 31 and 32. The $\mathrm{N^1}$ addition of the carbanions of furoin and phenacyl alcohol would be accompanied by for-

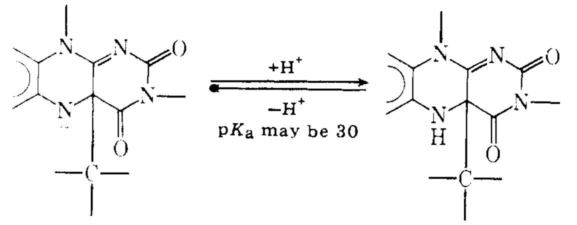


(31)

(32)

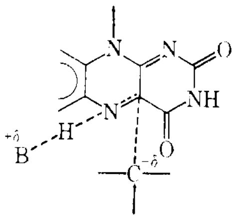
bidding steric hindrance. An $N^1$ adduct (carbinolamine) apparently does arise in the course of reduction of $CH_2O$ by $FlH_2$ (Williams et al., 1977). However, this carbinolamine species does not reside along the reaction path to methanol. Left in contention as mechanisms for normal flavins (unblocked) are the 4a addition and radical pathways.

Concerning the general competence of the 4a pathway one may recall past results (Bruice & Taulane, 1976). It has been established, for the few reactions whose kinetics have been investigated under conditions wherein carbanion formation is not rate limiting (eq 14 and 15), that the oxidation of carbanions by $Fl_{ox}$ is an apparently uncatalyzed process (eq 33).

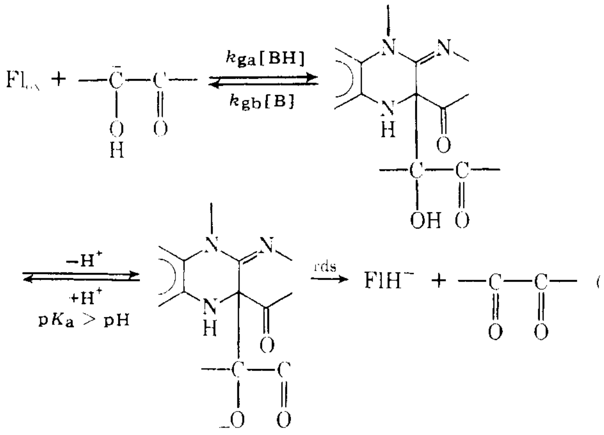


(33)

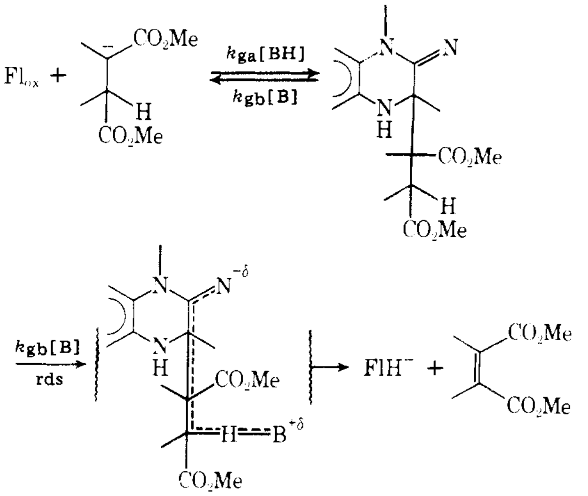
The addition of carbanion at the 4a position of $Fl_{ox}$ would create a base whose conjugate acid would possess a very high $pK_a$ (eq 34). The aniline type anion of eq 34 would be too unstable at neutrality to exist in aqueous solution. Additions at the 4a position should, therefore, require general-acid catalysis through the transition state of X. (These same considerations would apply to addition at the $N^1$ position of $Fl_{ox}$ but not to the $N^5$ position.) These considerations are embodied in the libido rule (Jencks, 1976) and may be appreciated from MAR diagrams (More O'Ferral, 1970; Jencks, 1972; Bruice, 1976). Further, it has been established that $N^5$ addition of $SO_3{}^{2-}$ to $Fl_{ox}$ is a noncatalyzed reaction, whereas 4a addition of $SO_3{}^{2-}$ to $Fl_{ox}$ is general-acid catalyzed (Bruice et al., 1973). As previously stated, carbanion oxidation by $Fl_{ox}$ requires no observable catalysis. This might be taken to mean that the oxidation does not involve a 4a-covalent intermediate. However, there is a mechanistic offering that would allow a 4a adduct of enediolate carbanion and $Fl_{ox}$ to exist along the reaction path. A general-acid catalysis of 4a-adduct formation could be made experimentally invisible if the 4a-covalent intermediate provided products upon rate-determining specific base catalyzed breakdown (eq 34; (BH/B) × $(K_w/a_H) = K_w/K_a$) (Bruice & Taulane, 1976).The mechanism of eq 34 suffices as a kinetically competent mechanism for the oxidation of $-C^-(OH)—CO-$ carbanions. It is, however, not suitable for the formation of carbon-carbon double bonds through carbanion oxidation by flavins. For succinic acid oxidase and the model reaction of eq 14, the specific base (ionization step) of eq 34 would have to be replaced by general base proton abstraction in the rate determining step (eq 35).



X



(34)



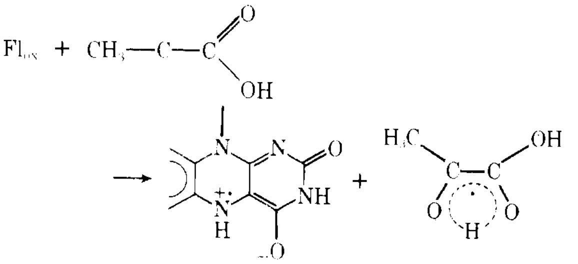
(35)

This is so because the ionization of carbon acids is a general-base catalyzed process. The sequence of eq 35 is not kinetically competent (Main et al., 1972) since it would require the experimental observation of an apparent general acid catalysis [i.e., $(k_{ga}[BH]/k_{gb}[B])k'_{gb}[B] = (k_{ga}k_{gb}'/k_{gb})[BH])$]. Additional model reactions involving $Fl_{ox}$ oxidative carbon-carbon double bond formation must be investigated. However, on the basis of findings with the reaction of eq 14 it would appear that a "4a-adduct mechanism" will not serve as a *general* mechanism for carbanion oxidation by flavins.

One electron transfer from carbanion to $Fl_{ox}{}^+Et$ is favorable. Trialkyl aminium cation radicals possess much greater stability than do dialkyl aminium cation radicals (Taft, 1975) and for this reason $N^5$-ethyl substitution provides a great stabilizing influence to the flavin radical (Bruice & Yano, 1975; Kemal & Bruice, 1976). This is easily appreciated when one considers that the reaction of eq 36 proceeds only to the right so that the FlEt· radical

$$\text{FlEt}^- + \text{Fl}_{ox}{}^+\text{Et} \rightleftharpoons 2\text{FlEt}\cdot \qquad (36)$$

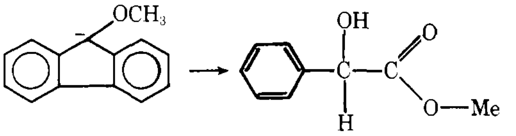
is thermodynamically more stable than is the reduced or oxidized $N^5$-alkyl flavin. It has been pointed out, in the case of unblocked flavins (Williams & Bruice, 1976; Williams et al., 1977), that the standard free energies of formation of radicals (eq 37)



(37)

in the flavin oxidation of lactic acid and its acyl derivatives (or reduction of formaldehyde) does not exceed the experimental free energy of activation for the oxidation of these substrates to pyruvic acid and its derivatives. Thus, the radical pairs must be considered as energetically allowed intermediates.

The decision as to whether carbanions from 4a-covalent intermediates on oxidation by flavin and flavoenzyme or whether a radical mechanism is involved is likely best made on the basis of intermediate trapping experiments. Actually, the oxidation of the carbanion of 9-methoxyfluorene by an isoalloxazine has been shown (Novak & Bruice, 1977) to be radical in nature (eq 38)



(38)

by identification of radical coupling products and the trapping out of the radical species by $O_2$ and nitroxide. A very similar investigation establishing the formation of radical intermediates in the flavin oxidation of methyl mandelate will be published shortly (Novak & Bruice, 1978).

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