The Kinetics and Mechanisms of 1,5-Dihydroflavin Reduction of Carbonyl Compounds and Flavin Oxidation of Alcohols. 3. Oxidation of Benzoin by Flavin and Reduction of Benzil by 1,5-Dihydroflavin

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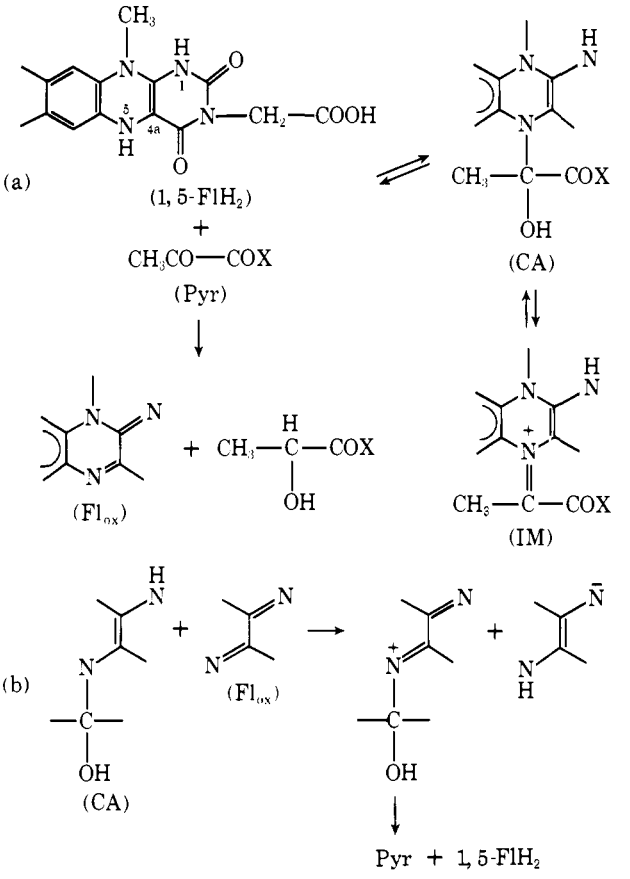
Abstract: The oxidation of benzoin by lumiflavin-3-acetic acid (Fl_{ox}) to provide benzil and 1,5-dihydrolumiflavin-3-acetic acid (FlH_2) is a readily reversible reaction. It has been established that the mechanism involves general base ionization of benzoin carbon acid (α-ketol) to yield endiolate anion, followed by partitioning of the endiolate anion back to benzoin through general acid proton donation and to benzil by reaction with Fl_{ox}. The reaction of endiolate anion with Fl_{ox} is not subject to acid or base catalysis. Evidence that ionization of benzoin precedes its oxidation by Fl_{ox} stems from the observation that the rate attributed to the latter process possesses a constant equal to that for racemization of (+)-benzoin and O_2 oxidation of benzoin and that this rate constant is characterized by a primary deuterium kinetic isotope effect ($k^{benzoin}/k^{\alpha\text{-}^2H\text{-}benzoin}$) of 7.24 ± 1.5. Reduction of benzil to benzoin by FlH_2 is pH and buffer insensitive below the pK_a of FlH_2. These results are consistent with either general acid catalyzed attack of benzoin carbanion at the 4a-position of Fl_{ox}, followed by a specific base catalyzed collapse of adduct to diketone and dihydroflavin (Scheme III), or to the uncatalyzed reaction of carbanion (endiolate anion) with flavin to provide a semidione–flavin radical pair which then goes on to diketone and dihydroflavin in a non-acid–base catalyzed reaction (Scheme V). These mechanisms are discussed in terms of the kinetics of reaction of other carbanion species with flavin.

Introduction

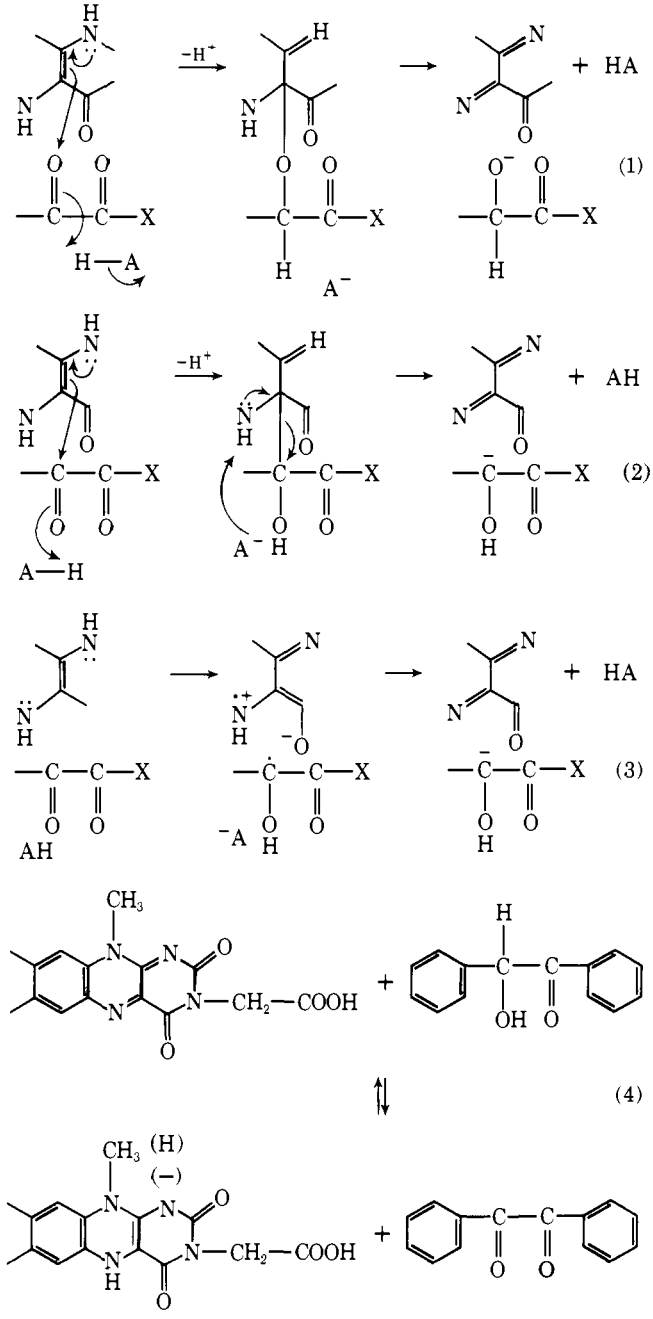
In the preceding paper[1] we describe the kinetics for the reduction of ethyl pyruvate, pyruvamide, and pyruvic acid by 1,5-dihydroflavin (1,5-FlH_2 and 1,5-FlH^-). The salient conclusions which were reached were that the formation of α-keto addition products at the N(5) position [carbinolamine (CA) and imine (IM)] arise from equilibrium reactions which are not productive to α-keto group reduction and that reduction occurred (Scheme I) via a general acid catalyzed reaction of 1,5-FlH_2 and pyruvate (Pyr). The requirement of the mechanism for product formation to be in competition with a slowly reversible equilibrium (a of Scheme I) plus feedback of starting reactants via a redox reaction between a product of reduction and a product of the nonproductive equilibria (b of Scheme I) provide rather unique requirements for the time dependence of Fl_{ox} appearance. The trapping of CA and analog fitting of the time dependence for Fl_{ox} appearance, to within that of the precision of the spectrophotometer employed, provide the evidence for the invoking of the reactions of Scheme I. Our primary interest in this and the preceding study resides in the mechanism whereby 1,5-FlH_2 + -CO-CO- → Fl_{ox} + -CH(OH)-CO-. Three mechanisms, not involving CA and IM as intermediates along the reaction path, have been considered. These involve enamine attack upon oxygen (Brown and Hamilton;[2] eq 1) and enamine attack upon carbon and a radical mechanism (Bruice,[3] eq 2 and 3, respectively). In the preceding study (see also ref 3 and 4) we point out that the $\Delta G°$ of formation of the radical intermediates of eq 3 do not exceed the $\lambda G^{\ddagger}_{exptl}$ for the reaction. The present report deals with an investigation of the reaction of eq 4 which has been studied in both the forward and retrograde direction.

Scheme I



Experimental Section

Materials. Benzil (Eastman Reagent Grade) twice recrystallized from 95% ethanol, pale-yellow needles, mp 95–95.5 °C (lit.[5] 95.0–96.0 °C). **Benzoin** (Aldrich Reagent Grade) twice recrystallized from 95% ethanol very pale-yellow needles, mp 133–134 °C (lit.[6] 133 °C). **Deuteriobenzoin** ($C_6H_5CD(OH)COC_6H_5$) was prepared by the reduction of purified benzil with the binary system of magnesium–magnesium iodide, followed by hydrolysis of the reaction mixture with 1 N D_2SO_4 (Merck MD-39 lot D-455) in D_2O (Thompson Packard 99.8%), as given by Hammond and Wu.[7] After two sublimations at 110 °C and 1 mm Hg, pale-yellow powder was obtained, mp 133–134 °C, 65% C-D by NMR in CD_3CN. **(+)-Benzoin** was obtained by resolution of purified (±)-benzoin after Kenyon and Patel[8] via fractional crystallization of the (+)-quinidine (Norse Labs) salt. The (+)-benzoin so obtained melted at 131–134 °C, 94% resolved by optical rotation ($[\alpha]_{589}$ = 110.37 in acetone, [lit.[8] $[\alpha]_{589}$ = 118.4]). **Furoin** (Eastman) was purified by the method of Huntress[6] to buff-colored crystals, mp 137–138 °C (lit.[6] 138–139 °C). Furil (Aldrich)



was purified by the method of Huntress to yellow-orange crystals, mp 162.5–163.5 °C (lit.[6] 163 to 165 °C). **Lumiflavin** was synthesized as described by Hemmerich,[9] [mp 324–325 °C (lit.[9] 325 °C)] and converted to **lumiflavin-3-acetic acid** as described by Hemmerich.[10] The latter was extensively purified by chromatography on silica to one spot on TLC (silica gel, Eastman) with 12:1 (v/v) $CHCl_3/CH_3OH$ (λ_{max} in H_2O 367 nm, 443 nm), mp 316 to 319 °C with decomposition (lit.[10] ca. 300 °C). This material was recrystallized from absolute ethanol–diethyl ether in the dark at −20 °C to provide (3 weeks) dark-orange micro needles, properties unchanged. All melting points are uncorrected.

Kinetic Measurements. Buffers were made up with double glass distilled water employing reagent grade acids and salts. All kinetic studies were carried out at 30 ± 0.1 °C in a mixed solvent of 30% CH_3CN–H_2O (v/v) at μ = 0.7 with KCl. pH measurements were carried out at 30 ± 0.1 °C employing a Radiometer 26 pH meter and Radiometer Type GK 2302 combined calomel-glass electrode. The pK_a values of all buffer acids were determined in the solvent of this study by half-neutralization.

Racemization of Benzoin. The loss of optical activity of (+)-benzoin was followed at 365 nm with a Perkin-Elmer Model 141 recording polarimeter employing a 10-cm thermostated cell. The racemization reactions were slow enough to allow preparation of reaction solutions [3 ml of a stock benzoin solution (1.29×10^{-2} M in CH_3CN) added to 7 ml of appropriate buffer], and thermostating for 15 min within the cell prior to recording of the change of rotation with time. The final concentration of benzoin (3.9×10^{-3} M) was that employed in the kinetic studies of the reaction of benzoin with oxidized flavin.

Reduction of lumiflavin-3-acetic acid by benzoin was followed under anaerobic conditions in Thunberg cuvettes at 443 nm in a Cary 118C spectrophotometer. For a typical run 1 ml of benzoin stock solution (2.0×10^{-2} M in CH_3CN) was mixed with 1 ml of appropriate buffer in the top reservoir of the cuvette and 0.5 ml of flavin stock solution (4×10^{-4} M in CH_3CN) and 2.5 ml of buffer placed in the bottom of the cuvette. The solutions were then deoxygenated with a stream of argon scrubbed of traces of O_2 by means of a vanadous ion trap. After closing and equilibrating the cuvette at 30 °C the reaction was initiated by mixing its contents. Mixing time was on the order of 10 s. For a typical reaction, the concentration of benzoin was 4×10^{-3} M, flavin 4×10^{-5} M, and the buffer concentration between 0.5 to 0.05 M final.

Oxidation of 1,5-dihydrolumiflavin-3-acetic acid by benzil was followed by means of a Durrum stopped-flow spectrophotometer at 443 nm. Anaerobic conditions were maintained by enclosing the entire spectrophotometer in a glovebox under a N_2 atmosphere. Typically, 1 ml of flavin stock solution (4×10^{-4} M in CH_3CN) was mixed with 4 ml of appropriate buffer (0.25 to 0.025 M final concentration containing 10- to 50-fold excess EDTA over flavin). The solution was deoxygenated with O_2 free argon and the flavin photoreduced in one of the storage syringes of the stopped-flow spectrophotometer. Stock benzil solution (2 ml) (1×10^{-2} M in CH_3CN) was added to 3 ml of 1 M aqueous KCl solution and the total deoxygenated with O_2 free argon prior to loading of the second storage syringe. On mixing in the stopped-flow spectrophotometer, the initial concentration of dihydroflavin was ~3.8×10^{-5} M and that of benzil was 2×10^{-3} M (μ = 0.7). Since the reaction was found to be multiphasic (first order followed by complex processes), the reaction was also studied in Thunberg cuvettes under argon employing the same solutions. The initial first-order reaction could be obtained under stopped-flow observations while the last ½ or ¼ of the initial reaction plus following spectral changes were obtainable from the experiments using Thunberg cuvettes.

Oxidation of Benzoin by O_2. The concentration of O_2 in solution was found not to be critical since the reaction is zero order in this component. The oxidation of benzoin to benzil was monitored by following the increase OD at 373 nm (shoulder of the benzil spectra). The buffer/acetonitrile solutions were oxygenated for 30 min and brought to 30 °C, and then typically 100 μl of a stock solution of benzoin (8.27×10^{-2} M in CH_3CN) was added to 2.9 ml of aqueous buffer/acetonitrile [30% CH_3CN (v/v)] and change in absorbance recorded.

Results

The reduction of lumiflavin-3-acetic acid (Fl_{ox}) by benzoin is characterized by the bleaching of the Fl_{ox} absorbance (443 nm) accompanying its complete conversion to the corresponding 1,5-dihydroflavin anion (1,5-FlH^-). At completion of reaction the admittance of O_2 immediately restores the characteristic Fl_{ox} spectra. The kinetics of the reaction were investigated in the alkaline pH range with [benzoin] exceeding [Fl_{ox}] by 100-fold. These conditions are, in the case of bimolecular reactions, referred to as pseudo-first-order:

$$-\frac{d[Fl_{ox}]}{dt} = k_r[\text{benzoin}][Fl_{ox}]$$

$$k_{obsd} = k_r[\text{benzoin}]$$

$$-\frac{d[Fl_{ox}]}{dt} = k_{obsd}[Fl_{ox}] \qquad (5)$$

In Figure 1 there is plotted ΔA_{443} vs. time for the reaction at several pH values. Examination of Figure 1 reveals that the rate of disappearance of Fl_{ox} may be independent, at least to 80% completion of reaction, upon the % Fl_{ox} remaining. The reaction is therefore initially zero order in [Fl_{ox}], and for this reason eq 5 cannot apply to the reduction of Fl_{ox} by benzoin, and the reaction does not occur by direct reaction of benzoin

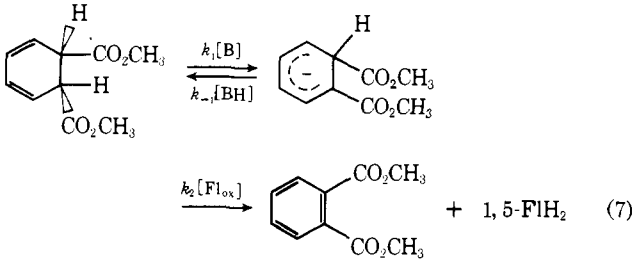
Table I. Constants Determined from Analog Computer Fit of Eq 8b to ΔA_{443} on Reaction of Benzoin [$\sim 4 \times 10^{-3}$ M] with Lumiflavin-3-acetic Acid [4×10^{-5} M]

pH	$[HCO_3^- + CO_3^{2-}]$ M	$k_1' \times 10^{-3}$ min^{-1}	$\alpha \times 10^{-6}$
11.25	0.5	8.52	6.25
	0.5	8.59	5.25
	0.375	6.65	4.20
	0.375	6.72	4.30
	0.25	5.86	4.35
	0.25	5.83	4.05
	0.125	4.58	3.55
	0.125	3.94	3.85
	0.07	4.49	3.9
	0.07	4.85	3.9
	0.052	4.03	4.15
	0.052	3.85	4.2
	0.050	2.95	3.50
	0.050	3.01	3.05
	0.035	3.10	3.95
	0.035	3.10	3.95
	0.0175	3.54	3.90
	0.0175	3.34	4.0
10.50	0.50	4.05	23.2
	0.50	4.28	17.1
	0.375	4.14	16.2
	0.375	3.90	15.1
	0.25	2.63	5.9
	0.25	2.59	5.4
	0.125	2.0	0.63
	0.05	1.46	0.61
10.30	0.070	1.23	4.8
	0.052	1.00	4.55
	0.052	1.00	4.55
	0.035	0.758	4.0
	0.035	0.837	4.55
	0.0175	0.544	4.1
	0.0175	0.488	4.25
10.2	0.250	1.88	13.0
	0.250	1.97	13.4
	0.125	1.49	11.2
	0.125	1.41	10.4
	0.050	0.716	4.13
	0.050	0.725	4.85

with Fl_{ox}. From the initial slopes of plots of A_{443} vs. time there can be approximated first-order constants from the expression of

$$k = \frac{\Delta A_{443} \Delta \text{time}^{-1} [\text{benzoin}]^{-1}}{\epsilon_{443}^{Fl_{ox}}} \qquad (6)$$

Values of k, so determined, were found to exhibit a positive dependence on increase in pH and total carbonate-bicarbonate buffer concentration $[B_T]$ at constant pH. These results are reminiscent of the flavin-mediated dehydrogenation of dimethyl *trans*-1,2-dihydrophthalate as studied by Main, Kasperek, and Bruice (eq 7).[11] The mechanism of eq 7 involves



(7)

general base catalyzed formation of carbanion which is then rapidly oxidized by Fl_{ox}. As in the present case of benzoin oxidation, the reaction was found to be initially zero order in $[Fl_{ox}]$—k_1 rate determining—changing to first order in $[Fl_{ox}]$ as the concentration of this species is depleted:

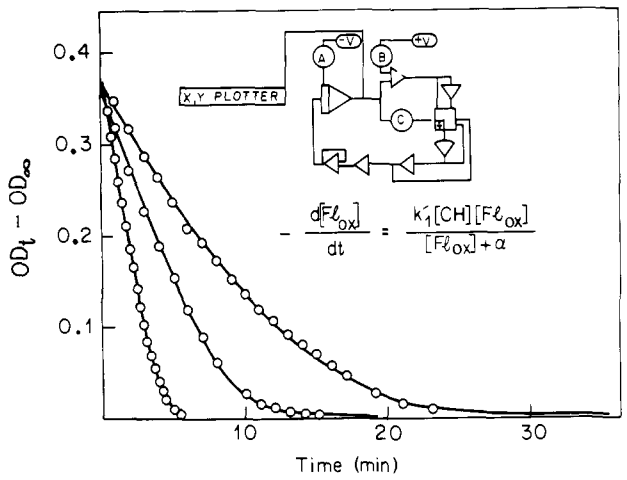


Figure 1. Decrease in absorbance of lumiflavin-3-acetic acid on reaction with benzoin at pH values 11.25, 10.5, and 10.2 (fastest to slowest rates). $[Fl_{ox}] = 4 \times 10^{-5}$ M, [benzoin] $= 4 \times 10^{-3}$ M carbonate buffer at 0.25 M. The points are experimental and the lines computer generated via the indicated program (± V = 10 volt, A = $[Fl_{ox}]_{t=0}$, B ≡ α (see text), C ≡ k_1' [benzoin]) to the rate equation which is included.

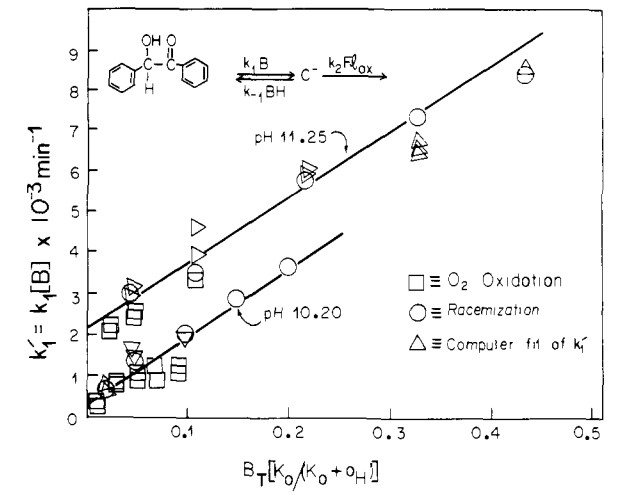


Figure 2. Plot of the apparent first-order rate constant (k_1') for the oxidation of benzoin vs. the concentration of buffer base (i.e., $[CO_3^{2-}]$).

$$CH \underset{k_{-1}[BH]}{\overset{k_1[B]}{\rightleftharpoons}} C^- \xrightarrow{k_2[Fl_{ox}]} C_{ox} + FlH^- \qquad (8a)$$

$$v = \frac{k_1 k_2 [CH][B][Fl_{ox}]}{k_{-1}[BH] + k_2[Fl_{ox}]} = \frac{k_1'[CH][Fl_{ox}]}{\alpha + [Fl_{ox}]} \qquad (8b)$$

$$v_{initial} = k_1[CH][B] = k_1'[CH] \qquad (8c)$$

$$v_{final} = \frac{K_1 k_2 K_{a_{BH}} [CH][HO^-][Fl_{ox}]}{K_w} \qquad (8d)$$

The ability to determine the rate constant for carbon acid ionization (k_1) accurately from initial rates is dependent upon $k_{-1}[BH]/k_2$, i.e., α but is assuredly obtained by analog computer fitting of the differential expressions of eq 8a to the ΔA_{443} vs. time course of the reaction. For this purpose the program included as an insert to Figure 1 has been employed. The points of Figure 1 are experimental and the lines computed for the best values of k_1' and α of eq 8b (Table I). Plots of the values of k_1' determined at pH 10.20 and 11.25 vs. the concentration of buffer base (i.e., $[CO_3^{2-}]$) are shown in Figure 2. The slopes

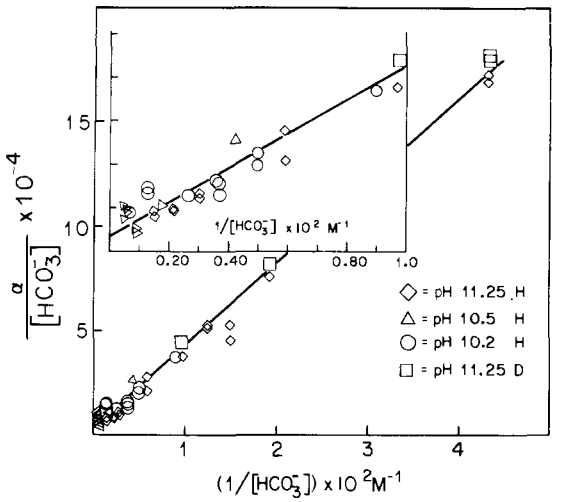


Figure 3. Plot of the apparent partition coefficient (α) for conversion of endiolate anion to benzoin and benzil divided by buffer acid concentration vs. the reciprocal of buffer acid concentration. The inset is an expansion of the plot for the experimental points between $1/[HCO_3^-] = 0$ and 1×10^2 M^{-1}. The slopes of the lines for the inset is identical with that for the major plot.

Table II. Constants Determined from Analog Computer Fit of Eq 8b to ΔA_{443} vs. Time on Reaction of α-Deuteriobenzoin (65% D) [$\sim 4 \times 10^{-3}$ M] with Lumiflavin-3-acetic Acid [4×10^{-5} M]

pH	$[HCO_3^-] + [CO_3^{2-}]$ M	$k_1'^D \times 10^{-3}$ min^{-1}	$\alpha^D \times 10^{-6}$
11.25	0.50	4.25	13.2
	0.50	3.71	12.8
	0.07	1.71	4.55
	0.07	1.76	4.55
	0.07	1.76	4.55
	0.052	1.50	4.20
	0.035	1.21	4.25
	0.0175	1.35	4.15
	0.0175	1.35	4.20
10.30	0.070	0.41	4.25
	0.070	0.39	3.75
	0.052	0.36	5.30
	0.052	0.36	5.30
	0.035	0.16	4.05
	0.035	0.16	4.05
	0.0175	0.154	3.75
	0.0175	0.173	4.50

of the plots are seen to be parallel and provide the second-order rate constant for CO_3^{2-} catalyzed ionization of the carbon acid substrate. From the pH dependent intercepts there can be calculated the second-order rate constant for HO^- mediated ionization of benzoin. These results dictate the expression of eq 9.

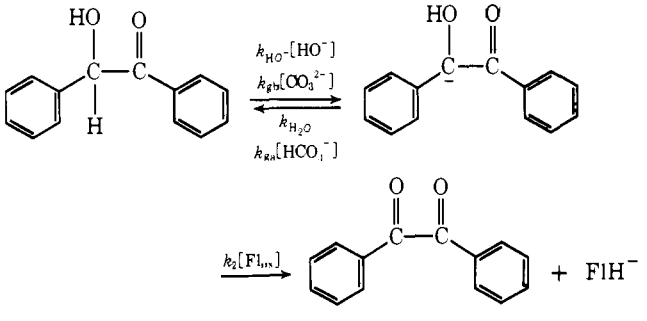
$$k_1[B] = k_{HO^-}[HO^-] + k_{gb}[CO_3^{2-}] \quad (9)$$

In Figure 3 there are plotted the various values of $\alpha/[HCO_3^-]$ vs. $1/[HCO_3^-]$. Inspection of Figure 3 reveals that intercept and slope are pH independent which requires that H_2O solvent as well as HCO_3^- act as general acids:

$$\frac{\alpha}{[HCO_3^-]} = \frac{k_{H_2O}}{[HCO_3^-]k_2} + \frac{k_{ga}}{k_2} \quad (10)$$

From the plot of Figure 3, k_{H_2O}/k_2 is obtained as the slope and k_{ga}/k_2 as intercept. These experiments establish the kinetics of Scheme II to pertain to the base-catalyzed oxidation of

Scheme II



$k_{HO^-} = 1.1 \pm 0.2$ M^{-1} min^{-1}
$k_{gb} = 1.6 \times 10^{-2}$ M^{-1} min^{-1}
$k_{H_2O}/k_2 = 4 \times 10^{-6}$ M
$k_{ga}/k_2 = 3.57 \times 10^{-5}$
$k_{ga}/k_{H_2O} = 8.93$ M^{-1}

benzoin by flavin in $HCO_3^- - CO_3^{2-}$ buffered solutions.

Replacement of α-protio- by α-deuteriobenzoin (65% deuterium label) results in a decided dimunition of the values of k_1' for reduction of Fl_{ox} (Table II). Inclusion of the values of α determined with deuterated substrate (α^D of Table II) in the plot of $\alpha/[HCO_3^-]$ v. $1/[HCO_3^-]$ (Figure 3) reveals that there is no primary deuterium isotope effect upon α. This observation is in accord with the kinetic path of Scheme II.

Base-catalyzed racemization of (+)-benzoin was studied (aerobically) in the same solvent, with the same carbonate buffers and in the same pH range employed in the kinetic investigation of the reduction of Fl_{ox} by benzoin. The racemization was found to follow first-order kinetics to 4 to 6 $t_{1/2}$. The determined rate constants are included in Figure 2 of k_1' vs. $[CO_2^{2-}]$ for reduction of Fl_{ox} by benzoin under anaerobic conditions. The values of k_1' determined by racemization are seen to fit the plot as well as those values determined from the reduction of Fl_{ox} by benzoin.

Oxidation of benzoin with $^3O_2$ was followed spectrophotometrically by observation of the time dependence for benzil formation at 375 nm. The reaction was studied over the same pH range, with the same buffers, and in the same solvent as employed for oxidation of benzoin by Fl_{ox}. Solutions were saturated with O_2 prior to initiation of the reaction. Previous investigations[12] have shown that the O_2 oxidation of benzoin is zero order in O_2 and first order in benzoin. The experimentally determined first-order rate constants are included in the plot of k_1' vs. $[CO_3^{2-}]$ of Figure 2 and are seen to fit the plots as well as the k_1' constants for oxidation of benzoin by Fl_{ox}. Both O_2 and Fl_{ox} oxidation of benzoin must, therefore, involve oxidation of benzoin carbanion (endiolate anion).

Reduction of Benzil by 1,5-Dihydrolumiflavin-3-acetic Acid. Kinetic investigations were carried out under the condition of $[benzil]$ in 50-fold excess over $[FlH_2 + FlH^-] = 4 \times 10^{-5}$ M. The reactions were found to be multiphasic. On mixing solutions of reactants, a first-order increase in A_{443} in the stopped-flow time range was followed by a slow increase in absorbance which continued for 20 to 30 min. In experiments carried out below pH 10 there were noted no further changes in absorbance. Above pH 10 a third phase of reaction becomes evident. At pH values between 10 and 11.2, there occurs, at about 100 min after mixing, a sudden decrease in A_{443} which continues until (300 to 400 min) all Fl_{ox} formed in phase I and II had been consumed. Admittance of O_2 at completion of phase III brings about the very rapid appearance of Fl_{ox} to 95+% based on the initial concentration of FlH^- employed. The onset of phase III is independent of the age of the deoxygenated solutions of dihydroflavin and benzil at their time of mixing. The reactions occurring in phase III would appear,

from the sudden onset at ca. 100 min and the lack of a simple kinetic order (a plot of A_{443} vs. time is sigmoid), to be due to a free radical chain reaction. In any event, phase III has nothing to do with the reduction of benzil by 1,5-dihydroflavin and need not receive further concern. This statement is supported by the observation that when dihydroflavin is replaced by oxidized flavin, all other conditions remaining invariant, there occurs at ca. 100 min the onset in disappearance of Fl_{ox} which continues to completion. Admittance of O_2 restores $[Fl_{ox}]$ to ~100% of that initially present.

Phase I and II of the time course for appearance of $Fl_{ox}(A_{443})$ resembles those previously observed in the reaction of dihydroflavins with CH_2O,[13] $CH_3COCO_2C_2H_5$,[1,13] $CH_3COCONH_2$,[1] and CH_3COCO_2H.[1] In the reduction of CH_2O and CH_3COCOX compounds by dihydroflavins, an initial first-order appearance of Fl_{ox} is followed by a slower process in which the rate of appearance of Fl_{ox} has a degree of independence upon the % completion of reaction. From pH 2.15 to 10 the % Fl_{ox} formed during the course of phase I and II was ~100%. In analogy with previous studies,[1] phase I may be taken to be direct reduction of carbonyl compound by dihydroflavin (reaction a of Scheme I) while phase II is best ascribed to a comproportionation reaction of oxidized flavin and carbinolamine (reaction b of Scheme I). In Table III there is presented the first-order rate constants for phase I divided by the concentration of benzil employed. These second-order rate constants show little dependence upon pH (~2 to 7.6) nor buffer type and concentration. In the calculation of the rate constants of Table III, A_∞ values were chosen to provide good first-order kinetics to at least 2 to 4 $t_{1/2}$.

Table III. Second-Order Rate Constants (k_{obsd}/[benzil]) for Appearance of Lumiflavin-3-acetic Acid on Reaction of Benzil (2×10^{-3} M) with 1,5-Dihydrolumiflavin-3-acetic Acid [Solvent CH_3CN-H_2O, 30% (v/v), 30 °C, μ = 0.7 with KCl]

Buffer	B_T, M	pH	Benzil, $M \times 10^{-3}$	k_{obsd}/[benzil], $M^{-1}\,s^{-1}$
H_3O^+		2.15	1.96	11.2
		2.47	2.026	17.5
		3.57	2.026	10.9
Formate	0.25	4.15	2.026	14.46
	0.25	4.12	2.026	9.47
	0.175	4.12	2.026	8.68
	0.125	4.13	2.026	9.67
	0.075	4.10	2.026	7.45
	0.025	4.07	2.026	8.29
Acetate	0.250	5.32	2.026	10.80
	0.250	5.23	2.00	9.35
	0.175	5.35	2.026	9.77
	0.125	5.36	2.026	15.25
	0.07	5.35	2.026	9.42
	0.025	5.37	2.026	11.5
Bes[a]	0.25	6.85	2.026	11.89
Tes[b]	0.10	7.65	2.06	12.13

[a] *N,N*-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid. [b] *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

Discussion

In aqueous solution and in the presence of $HCO_3^- - CO_3^{2-}$ buffer, the oxidation of benzoin by lumiflavin-3-acetic acid (Fl_{ox}) is described quantitatively by Scheme II. In the sequence of Scheme II general base catalyzed ionization of benzoin is rate limiting at high $[Fl_{ox}]$ and reaction of benzoin carbanion (endiolate anion) with Fl_{ox} rate limiting at low $[Fl_{ox}]$. The rate constants for benzoin ionization, determined by the base-catalyzed oxidation of benzoin by O_2 and flavin as well as base catalyzed racemization of (+)-benzoin concur. In addition, the oxidation of α-deuteriobenzoin by oxidized flavin is associated with a kinetic isotope effect of the magnitude that might be expected for rate-determining carbon-acid ionization (eq 8c). Employing the eight comparable constants for oxidation of benzoin and α-deuteriobenzoin (65% D) of Tables I and II a value of $k^H/k^{100\%\,D} = 7.2 \pm 1.5$ ($k^H/k^{65\%\,D} = 3.0 \pm 0.5$) is obtained. The requirement that ionization of the α-CH moiety of benzoin be rate limiting at high $[Fl_{ox}]$ is not met by the mechanism of Brown and Hamilton (eq 1).

The rate of reduction of benzil by 1,5-dihydrolumiflavin-3-acetic acid (FlH_2) is not affected by pH nor the concentration of buffer acids and bases below the pK_a of FlH_2 (Table III). Carbinolamines have been shown to be produced in nonproductive equilibria in the reduction of formaldehyde,[13] pyruvamide, ethyl pyruvate, pyruvic acid, and pyruvate by FlH_2 (Scheme I).[1] In the present instance carbinolamine and imine could not be trapped by $NaCNBH_3$. This may be due to steric hindrance to approach of cyanoborohydride or to a sterically unfavorable conversion of carbinolamine to imine. Both possibilities are eminently reasonable. Attention is now drawn to the means by which the carbanion (endiolate) derived from benzoin undergoes a $2e^-$ transfer to flavin to yield benzil and FlH_2. This may occur via $1e^-$ transfer reactions or through the formation of a covalent intermediate of benzoin ion and Fl_{ox}, followed by a base-catalyzed elimination reaction.

The reaction sequence of eq 11 and 12 is kinetically competent if it is assumed that for eq 11 product formation from intermediate (k_B) is rate limiting ($\Sigma k_{-a}B > k_B[HO^-]$), and for eq 12 intermediate formation (k_b) is rate limiting. In eq 11 and 12, $\Sigma k_{gb}[B] = k_{CO_3}[CO_3^{2-}] + k_{HO}[HO^-]$, $\Sigma k_{ga} = k_{HCO_3}[HCO_3^-] + k_{H_2O}[H_2O]$, $\Sigma k_a[Fl_{ox}][BH] = [Fl_{ox}](k[HCO_3^-] + k[H_2O])$ etc. Assumption of steady state in $[C^-]$, [int], and [int'] provides eq 13 for the sequence of eq 11 and eq 14 for the sequence of eq 12:

$$\text{benzoin} \underset{\Sigma k_{ga}[BH]}{\overset{\Sigma k_{gb}[B]}{\rightleftharpoons}} C^- \underset{\Sigma k_{-a}[B]}{\overset{\Sigma k_a[Fl_{ox}][BH]}{\rightleftharpoons}} \text{int} \underset{k_{H_2O}}{\overset{k_B[HO^-]}{\rightleftharpoons}} \text{benzil} + FlH_2 \quad (11)$$

$$\text{benzoin} \underset{\Sigma k_{ga}[BH]}{\overset{\Sigma k_{gb}[B]}{\rightleftharpoons}} C^- \underset{k_{-b}}{\overset{k_b[Fl_{ox}]}{\rightleftharpoons}} \text{int}' \underset{k_{-c}}{\overset{k_c}{\rightleftharpoons}} \text{benzil} + FlH_2 \quad (12)$$

$$-\frac{d[Fl_{ox}]}{dt} = \frac{\Sigma k_{gb}[B] \cdot \Sigma k_a \cdot k_B[\text{benzoin}][Fl_{ox}][HO^-]}{\Sigma k_{ga}\Sigma k_{-a}[B] + \Sigma k_a \cdot k_B[HO^-][Fl_{ox}]} \quad (13a)$$

$$= \frac{\Sigma k_{gb}[B][\text{benzoin}][Fl_{ox}]}{\frac{\Sigma k_{ga}\Sigma k_{-a}K_{BH}[BH]}{k_B\Sigma k_a K_w} + Fl_{ox}} = \frac{\Sigma k_{gb}[B][\text{benzoin}][Fl_{ox}]}{\alpha + Fl_{ox}} \quad (13b)$$

$$-\frac{d[Fl_{ox}]}{dt} = \frac{\Sigma k_{gb}[B] \cdot k_b[\text{benzoin}][Fl_{ox}]}{\Sigma k_{ga}[BH] + k_b[Fl_{ox}]} \quad (14a)$$

$$= \frac{\Sigma k_{gb}[B][\text{benzoin}][Fl_{ox}]}{\Sigma \frac{k_{ga}}{k_b}[BH] + [Fl_{ox}]} = \frac{\Sigma k_{gb}[B][\text{benzoin}][Fl_{ox}]}{\alpha + Fl_{ox}} \quad (14b)$$

When the term of the denominator containing $[Fl_{ox}]$ predominates, eq 13 and 14 reduce to:

$$-\frac{d[Fl_{ox}]}{dt} = \Sigma k_{gb}[B][\text{benzoin}]$$

$$= (k_{CO_3^{2-}}[CO_3^{2-}] + k_{HO^-}[HO^-])[\text{benzoin}] \quad (15)$$

while at lower concentrations of Fl_{ox} eq 13 and 14 provides:

$$-\frac{d[Fl_{ox}]}{dt} = k_B\left(\frac{k_{gb}}{k_{ga}}\right)\left(\frac{k_a}{k_{-a}}\right)[\text{benzoin}][HO^-][Fl_{ox}] \quad (16)$$

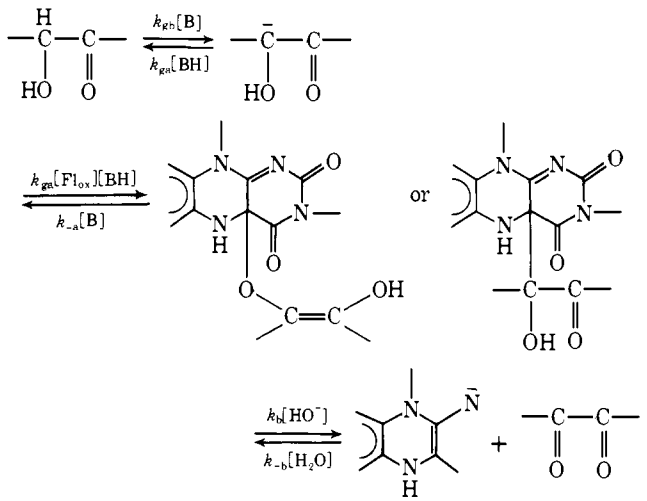
$$-\frac{d[Fl_{ox}]}{dt} = k_b\left(\frac{k_{gb}}{k_{ga}}\right)\left(\frac{[B]}{[BH]}\right)[\text{benzoin}][Fl_{ox}]$$

$$= k_b\left(\frac{k_{gb}}{k_{ga}}\right)\left(\frac{K_{BH}}{K_w}\right)[\text{benzoin}][Fl_{ox}][HO^-] \quad (17)$$

(where K_{BH} is the acid dissociation constant of BH), respectively. Comparison of the experimentally established rate equations to those derived from the reaction sequence of eq 11 and 12 (i.e., 8b vs. 13a and 14a; 8c vs. 15; and 8d vs. 16 and 17) reveals their identity. The sequences of eq 11 ($k_B[HO^-]$ rds) and eq 12 ($k_b[Fl_{ox}]$ rds) are also in accord with the lack of pH and buffer dependence of the rate of reduction of benzilby 1,5-dihydroflavin.

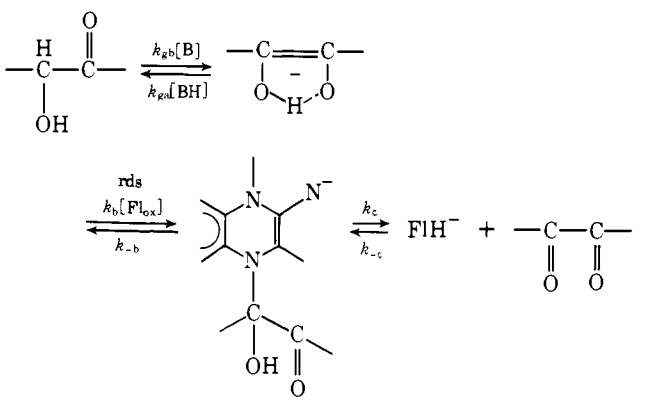
The mechanism of Scheme III is in agreement with the al-

Scheme III



lowed kinetic path of eq 11 and Schemes IV and V are in ac-

Scheme IV

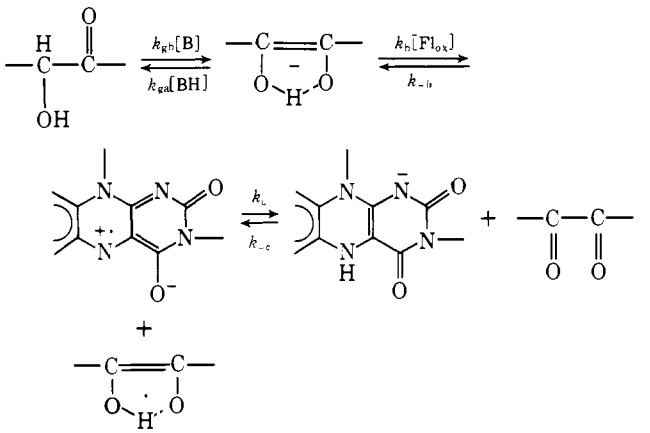


cord with the allowed kinetic path of eq 12. Scheme III involves addition of carbanion (endiolate) to the 4a-position of Fl_{ox} (eq 2) followed by hydroxide ion catalyzed elimination of ketone from 4a-adduct. Substitution of $k_B[HO^-]$ by $k_B[B]$ in the terminal step of eq 11, all other assumptions remaining invariant, provides the rate law of the following equation

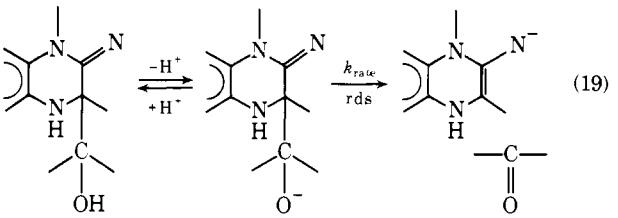
$$-\frac{d[Fl_{ox}]}{dt} = \frac{k_{gb}k_ak_B[B][CH][Fl_{ox}]}{k_{ga}(k_B + k_{-a}) + k_Bk_a[Fl_{ox}]} \quad (18)$$

which is not kinetically equivalent to the experimental rate law. Therefore, for the mechanism of Scheme III to be kinetically competent the formation of 4a-adduct must be general acid catalyzed and its decomposition to products specific base

Scheme V



catalyzed. It has been established that 4a-addition of a base species to oxidized flavin involves general acid protonation of the N(5) position.[14] General acid catalysis of 4a-nucleophilic addition is anticipated from a consideration of the change in pK_a of the N(5) nitrogen of the flavin moiety on going from oxidized flavin to adduct ($\Delta pK_a > 14$)[14]—the libido rule of Jencks.[15] The oxidation of thiols by Fl_{ox} serves as a useful example of flavin oxidations which involve general acid catalysis of the formation of an intermediate 4a-adduct.[16] For the termination step, the requirement for specific base catalysis of elimination ($k_B[HO^-]$, Scheme III) to yield ketone and dihydroflavin is at least reasonable. A specific base catalysis of this process dictates the mechanism of eq 19. This is ac-



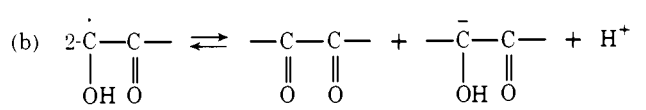
(19)

ceptable on the basis that proton removal from an oxygen acid is involved, the pK_a of the oxy acid exceeds the pH and also neither base nor acid catalysis is observed in the direction of reduction of benzil by 1,5-dihydroflavin. From these considerations the mechanism of Scheme III must be deemed as most reasonable.

In Scheme IV carbanion and Fl_{ox} provide the $N(5)$-carbinolamine adduct. It could be shown, in the reduction of CH_2O and CHCOCO-X (X = -OH, $-O^-$, $-OC_2H_5$, $-NH_2$) by 1,5-dihydroflavin, that $N(5)$-carbinolamine species are formed. However, it was also established that these carbinolamines are not intermediates leading to the production of Fl_{ox} and alcohol.[1,13] In these examples, the free-energy barrier to $N(5)$-carbinolamine yielding oxidized flavin and alcohol must reside in the k_{-b} step of Scheme IV. The reaction of CH_2O and pyruvates with FlH_2 and FlH^- could be studied only at pH values below neutrality because the direct reduction of carbonyl compounds by FlH^- and FlH_2 as well as carbinolamine formation were found to be acid catalyzed. At these pH values the free energies of formation of carbanions ($\Delta G°$) exceed the free-energy content of the transition state ($\Delta G^‡$) for an observed alternate path for the oxidation-reduction reaction. This path was proposed to involve radical intermediates. In the present investigation, carbanion is an intermediate in the oxidation of benzoin to benzil. The free-energy content of benzoin carbanion (endiolate) does not exceed $\Delta G^‡$ for reaction with Fl_{ox} and, therefore, the $N(5)$-carbinolamine may not be excluded from being along the reaction path. For carbinolamine to be a competent intermediate it must go on to benzil and dihydroflavin at a rate exceeding that for benzoin ionization

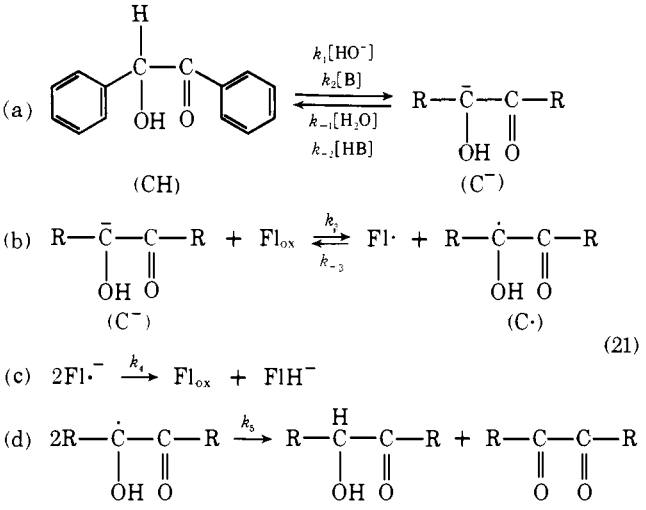
(Scheme II). At present there is no means of ascertaining if this requirement is met.

In Scheme V, carbanion (endiolate anion) is proposed to transfer $1e^-$ to flavin in a non-acid-base catalyzed reaction to provide a semidione-flavin radical pair which undergoes an intracomplex $1e^- + H^+$ transfer from semidione to the flavin radical anion to yield α-diketone and dihydroflavin. In the preceding manuscript[1] we established that the standard free energies of formation of radical intermediates on $1e^-$ transfer from 1,5-dihydroflavin to pyruvic acid, pyruvate, or pyruvamide are not large. Though $E^{\circ\prime}$ values for benzoin-benzil semidione are not known, there can be little doubt that they are more positive than the $E^{\circ\prime}$ values for formation of pyruvate radical from pyruvate. These considerations make the radical mechanism of Scheme V very attractive. Assuming the correctness of Scheme V, the semidione-flavin radical pair, once formed, must either partition to reactants and products or if significant leakage from the radical pair occurs both flavin radical and semidione radical must comproportionate as in eq 20. (This is to say that two semidione radicals must yield one



(20)

enediolate anion and one ketone.) If this were not the case then the experimentally determined rate constants for benzoin ionization would not equal the rate constants for benzoin oxidation by flavin at high flavin concentration. This can be shown by consideration of the sequence of eq 21 in which compro-



(21)

portionation of semidione yields ketone and carbon acid rather than ketone and carbanion as in eq 20b. The assumption of steady state in carbanion ($[C^-]$) and flavin radical ($[Fl\cdot^-]$) provides eq 22 and 23, respectively.

$$[C^-] = \frac{(k_1[HO^-] + k_2[B])[CH] + k_{-3}[Fl\cdot^-][C\cdot]}{(k_{-1}[H_2O] + k_{-2}[HB]) + k_3[Fl_{ox}]} \quad (22)$$

$$k_3[Fl_{ox}][C^-] = k_{-3}[Fl\cdot^-][C\cdot] + k_4[Fl\cdot^-]^2 \quad (23)$$

Substitution of eq 22 into eq 23 provides eq 24.

$$k_3[Fl_{ox}]\frac{(k_1[HO^-] + k_2[B])[CH] + k_{-3}[Fl\cdot^-][C\cdot]}{k_{-1}[H_2O] + k_{-2}[HB] + k_3[Fl_{ox}]} = k_{-3}[Fl\cdot^-][C\cdot] + k_4[Fl\cdot^-]^2 \quad (24)$$

If the rate-determining step of the sequence of Scheme III is ionization of benzoin then $k_3[Fl_{ox}] \gg (k_{-1} + k_{-2}[HB])$ so that eq 24 reduces to:

$$(k_1[HO^-] + k_2[B])[CH] = k_4[Fl\cdot^-]^2 \quad (25)$$

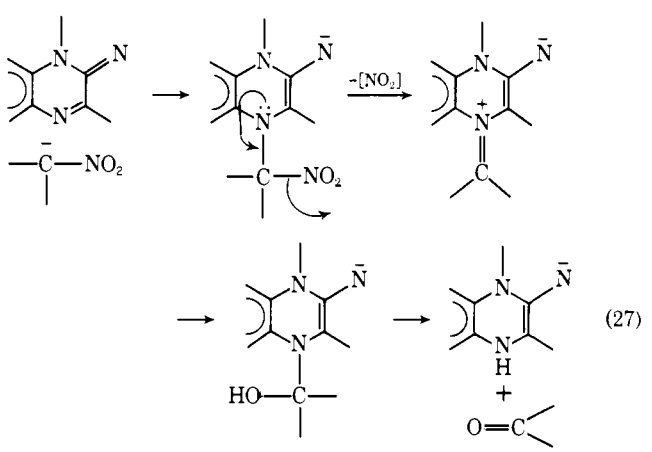
From eq 25 it then follows that the rate of reduction of Fl_{ox} (i.e., formation of reduced flavin) is one-half that for ionization of benzoin:

$$+\frac{d[FlH^-]}{dt} = \frac{k_4[Fl\cdot^-]}{2} = \frac{(k_1[HO^-] + k_2[B])[CH]}{2} \quad (26)$$

If semidione radical comproportionated to benzoin carbanion and to benzil (eq 20 instead of eq 21d), eq 26 will not hold and, in this instance, conversion of the radical pair to products and the separate comproportionation of the radical species are not distinguishable.

Three types of flavin mediated carbon acid oxidation reactions have now been investigated in some detail: α-ketol oxidation to α-diketone, nitroalkane oxidation to nitrite and carbonyl compound,[16a] and the dehydrogenation of dimethyl *trans*-dihydrophthalate to yield dimethylphthalate (eq 7).[11] All proceed through the carbanion species and most importantly each carbanion has been established to react with oxidized flavin *in a process which is pH and buffer base and acid independent.* Also, each type reaction has an enzymatic counterpart. The D-amino acid oxidase catalyzed oxidation of nitroalkane anions to nitrite and carbonyl compound has been suggested by Porter, Voet, and Bright[17] to involve the formation of N(5) adducts, though with glucose oxidase[17c] flavin radical appears as an intermediate. Lactic acid oxidase and amino acid oxidase have been established to involve ionization of carbon acid (α-CH) substrate prior to its reaction with enzyme bound oxidized flavin.[18]

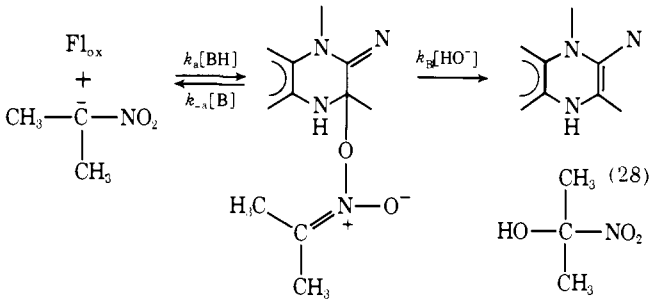
It is possible, in both the model and enzyme reactions that the mechanisms of Scheme III, IV, and V are all involved in carbon acid oxidation (dependent upon carbon acid, enzyme etc.). Porter, Voet, and Bright[17] have proposed the sequence of eq 27 as being kinetically competent for the oxidation of



(27)

nitroalkane ions by D-amino acid oxidase. The C-alkylation of nitroalkane anions has been known since the early studies of Kornblum[19] to be of a free radical nature. Formation of the N(5) adduct may, therefore, occur either by a radical or nucleophilic mechanism. Arguments have been presented[3] that support the mechanism of eq 28 as the only reasonable prospect for oxidation of nitroalkane anion if said oxidation proceeds through a 4a-adduct. In eq 28, HO^- performs the role of nucleophile rather than as a specific base catalyst as in Scheme III. However, the mechanism of eq 28 suffers in that various oxygen and nitrogen bases did not act as nucleophilic catalysts in the Fl_{ox} oxidation of nitroalane ions.[16a]

In accord with eq 11, dehydrogenation of dimethyl *trans*-dihydrophthalate anion, if it should occur through a 4a-adduct, requires that ionization of the intermediate 4a-carbon acid is specific base catalyzed (eq 29). This requirement would appear unreasonable for the ionization of a carbon acid[20] and for E2 or E1cB elimination reactions.[21] There is no chemistry of



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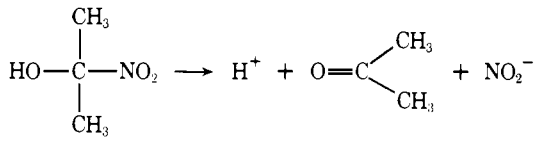
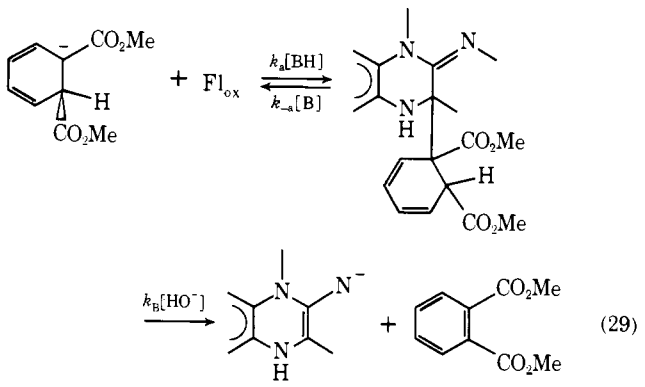
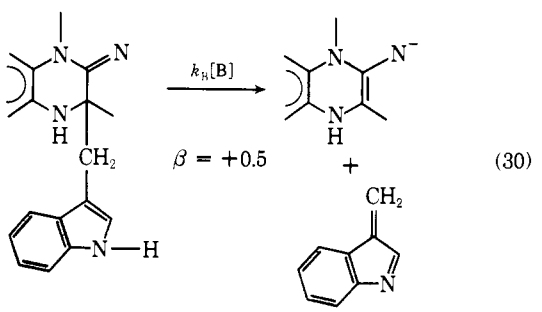




(29)

4a-adducts that would tend to dictate a specificity for HO^- in the formation of FlH_2 by a base-catalyzed elimination. The general base catalyzed reaction of eq 30 has been established

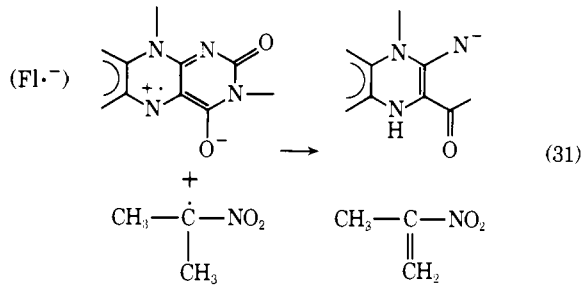


(30)

by Clerin and Bruice.[22] Thus, Scheme III is most reasonable for benzoin oxidation but not for dihydrophthalate dehydrogenation.

If a general mechanism for the oxidation of resonantly stabilized carbanions is sought then this mechanism would appear to be a radical mechanism as employed (in Scheme V) for benzoin oxidation and for the reduction of CH_2O and pyruvate derivatives. The radical pairs of eq 31 and 32 may arise in the flavin oxidation of the carbanions of nitroalkanes and dimethyl *trans*-dihydrophthalate, respectively, and proceed to products via H· transfer to the flavin radical. The pK_a of formation of $Fl\cdot^-$ from FlH· is 8.4 so that both flavin radical species may be present in the alkaline pH range.

Results published in preliminary form by Shinkai, Kunitake, and Bruice[12] established that the first-order rate constants



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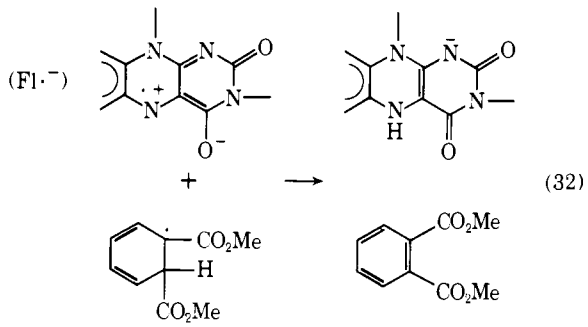

(32)

calculated from initial rates for oxidation of furoin and benzoin by $^3O_2$, 2,6-dichlorophenolindophenol and lumiflavin were comparable at pH 10.33 in carbonate buffer. This paper provides the required extension of the previous study.

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References and Notes

(1) R. Williams and T. C. Bruice, preceding paper in this issue.
(2) L. E. Brown and G. A. Hamilton, *J. Am. Chem. Soc.*, **92**, 7225 (1970).
(3) T. C. Bruice in "Progress in Bioorganic Mechanisms", E. T. Kaiser and F. J. Kezdy, Ed., Wiley-Interscience, New York, N.Y., 1976, p 1.
(4) R. F. Williams, S. Shinkai, and T. C. Bruice, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 1763 (1975).
(5) "Handbook of Chemistry and Physics", 53d ed, Chemical Rubber Publishing Co., Cleveland, Ohio.
(6) E. H. Huntress, "Identification of Organic Compounds", Wiley, New York, N.Y., 1941.
(7) G. S. Hammond and C.-H. S. Wu, *J. Am. Chem. Soc.*, **95**, 8215 (1973).
(8) J. Kenyon and R. I. Patel, *J. Chem. Soc.*, 435 (1965).
(9) P. Hemmerich, *Helv. Chim. Acta*, **39**, 1243 (1956).
(10) P. Hemmerich, *Helv. Chim. Acta*, **47**, 464 (1964).
(11) L. Main, G. J. Kasperek, and T. C. Bruice, *Biochemistry*, 11, 3991 (1972).
(12) S. Shinkai, T. Kunitake, and T. C. Bruice, *J. Am. Chem. Soc.*, **96**, 7140 (1974).
(13) S. Shinkai and T. C. Bruice, *J. Am. Chem. Soc.*, **95**, 7526 (1973).
(14) T. C. Bruice, L. Hevesi, and S. Shinkai, *Biochemistry*, **12**, 2083 (1973).
(15) W. P. Jencks, *Chem. Rev.*, **72**, 705 (1972).
(16) (a) I. Yokoe and T. C. Bruice, *J. Am. Chem. Soc.*, **97**, 450 (1975); (b) E. L. Loechler and T. C. Hollocher, *ibid.*, **97**, 3255 (1975).
(17) (a) D. J. T. Porter, J. G. Voet, and H. J. Bright, *J. Biol. Chem.*, **247**, 1951 (1972); (b) *ibid.*, **248**, 4400 (1973); (c) D. V. T. Porter and H. Bright in "Flavines and Flavoenzymes", T. P. Singer, Ed., Elsevier, Amsterdam, 1976, p 235.
(18) (a) C. T. Walsh, A. Shonbrunn, and R. H. Abeles, *J. Biol. Chem.*, **246**, 6855 (1971); (b) C. T. Walsh, E. Krodel, V. Massey, and R. H. Abeles, *J. Biol. Chem.*, **248**, 1946 (1973); (c) C. T. Walsh, O. Lockridge, J. Massey, and R. H. Abeles, *J. Biol. Chem.*, **248**, 7049 (1973).
(19) (a) R. C. Kerber, G. W. Urry, and N. Kornblum, *J. Am. Chem. Soc.*, **87**, 4520 (1965); (b) N. Kornblum, R. C. Kerber, and G. W. Urry, *ibid.*, **86**, 3904 (1964); (c) G. A. Russell and W. C. Dannen, *ibid.*, **88**, 5663 (1966); (d) N. Kornblum, R. E. Michel, and R. C. Kerber, *ibid.*, **88**, 5660, 5662 (1966).
(20) R. F. Pratt and T. C. Bruice, *J. Org. Chem.*, **37**, 3563 (1972).
(21) (a) B. Holmquist and T. C. Bruice, *J. Am. Chem. Soc.*, **91**, 3003, 3009 (1969); (b) R. F. Pratt and T. C. Bruice, *ibid.*, **92**, 5956 (1970).
(22) D. Clerin and T. C. Bruice, *J. Am. Chem. Soc.*, **96**, 5571 (1974).