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Chemistry

Simple synthesis of a 4a-hydroperoxy adduct of a 1,5-dihydroflavine: Preliminary studies of a model for bacterial luciferase

(4a-hydroperoxyflavine/chemiluminescence/bioluminescence)

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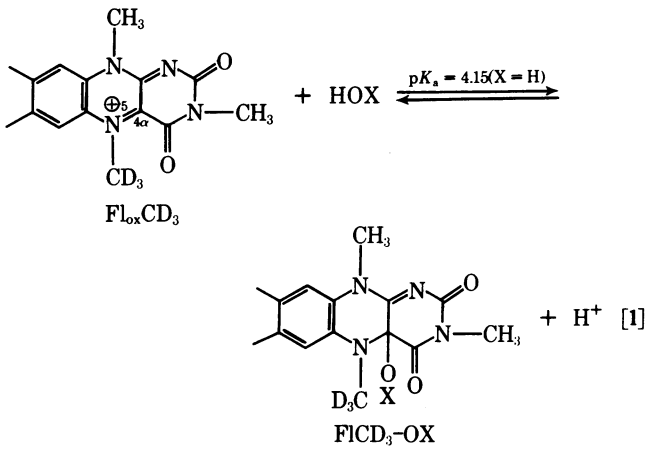
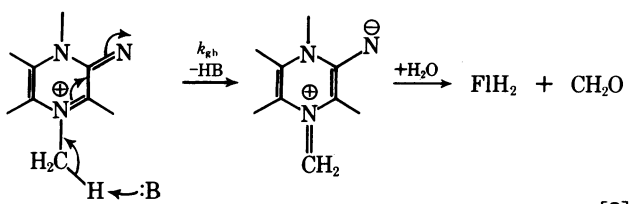
ABSTRACT **The solution chemistry of N(5)-alkyl flavinium cations and radical species formed by their $1e^-$ reduction are discussed. Previously unknown, the 4a-flavine hydroperoxides are established to be formed on reaction of N(5)-alkyl flavinium cations with H_2O_2 or on reaction of N(5)-alkyl-1,5-dihydroflavines with $^3O_2$. The stability of the 4a-flavine hydroperoxide species is exemplified in the isolation and characterization of 4a-hydroperoxy-N(5)-ethyl-3-methyllumiflavine. 4a-Flavine hydroperoxide compounds are shown to be stronger oxidants than H_2O_2, and to undergo a chemiluminescent reaction in the presence of an aldehyde. Preliminary observations on the chemiluminescent reaction of 4a-flavine hydroperoxides + RCHO are provided, and these are compared to those in the literature dealing with the bioluminescence of bacterial luciferase in the presence of $^3O_2$ and RCHO.**

Peroxy adducts of oxidized flavine have been proposed (1) as intermediates in the $^3O_2$ oxidation of 1,5-dihydroflavines (2), the mechanism of external flavomonooxygenases (3, 4), and the mechanism of bacterial luciferase (5). Proposals of mechanisms (1, 2, 3, 5) have dealt most often with the 4a-hydroperoxy flavine. Studies directed toward the chemistry of peroxy flavine compounds have not been forthcoming for the simple reason that none were known. We report herein the simple synthesis of 4a-hydroperoxy adducts of 5-ethyl-3-methyllumiflavine (FlC_2H_5-OOH) and analogs (FlR-OOH) and report evidence for their involvement as intermediates in the $^3O_2$ oxidation of N(5)-alkyl-1,5-dihydrolumiflavine (6). Also of considerable interest is our finding of light emission on reaction of FlR-OOH compounds with aldehydes (models for bacterial luciferase).

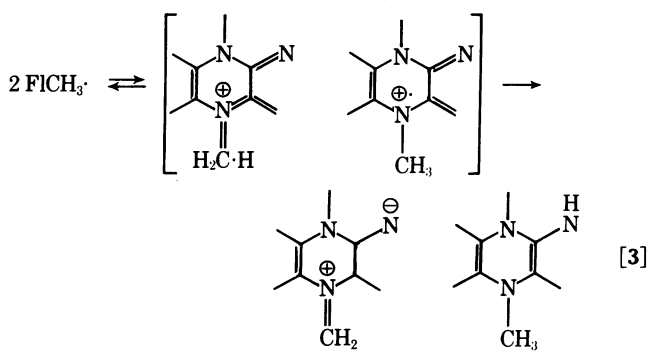
It has been appreciated for some time that N(5)-alkyl flavinium cations exist in solution in equilibrium with their *pseudo*-base forms (7). For N(5)-CD_3 lumiflavine ($Fl^{\oplus}_{ox}CD_3$), the equilibrium of Eq. 1 (X = H) has been established (8). It occurred to us that FlR-OOH could be prepared simply by the addition of H_2O_2 to an $Fl^{\oplus}_{ox}R$ species (Eq. 1, X = OH). Since FlR-OOH would be in equilibrium with $Fl^{\oplus}_{ox}R$, a knowledge of the chemistry of the latter was desired. In brief, our findings (8) on this aspect of the problem follow. Under anaerobic conditions, $Fl^{\oplus}_{ox}CH_3$ (below pH 8.0) undergoes a general base catalyzed (Bronsted β constant = 0.6) conversion to a 1:2 mixture of 3-methyllumiflavine (Fl_{ox}) and N(3,5)-dimethylmonohydrolumiflavine ($FlCH_3\cdot$) (Eq. 2). The deuterium isotope effect for k_{gb} (i.e., $k_{Fl^{\oplus}_{ox}CH_3}/k_{Fl^{\oplus}_{ox}CD_3}$) is 10.5 when B = H_2O and 14.0 when B = CH_3COO^-. The general base catalyzed solvolysis of $Fl^{\oplus}_{ox}C_2H_5$ occurs at a rate about 50-fold slower than does $Fl^{\oplus}_{ox}CH_3$. Under anaerobic conditions the radical species, $FlCH_3\cdot$, disproportionates *via* a mechanism which involves the formation of a dimeric complex followed by $1e^- + H^+$ or H· transfer. That H^+ or H· moiety is transferred within the dimeric complex from one $FlCH_3\cdot$ to the other (Eq. 3) was established through the observation that the rate of disproportionation is not sensitive to change of $[HO^-]$ (i.e., pH 2–8) nor the concentration of buffer bases, and that the C-H(D) kinetic isotope effect ($k_{FlCH_3\cdot}/k_{FlCD_3\cdot}$) = 35 at pH 7.0.





[2]

$$FlH_2 + 2\,Fl_{ox}CH_3^{\oplus} \longrightarrow Fl_{ox} + 2\,FlCH_3\cdot$$



[3]

Abbreviations: Fl_{ox}, 3-methyllumiflavine; FlH_2, 3-methyl-1,5-dihydrolumiflavine; $Fl^{\oplus}_{ox}R$, N(5)-alkyl substituted 3-methyllumiflavine (R = CH_3, CD_3, C_2H_5); FlHR, N(5)-alkyl substituted 3-methyl-1,5-dihydroflavine; FlR·, N(5)-alkyl substituted 3-methylmonohydrolumiflavine; FlR-OH and FlR-OOH, N(5)-alkyl substituted 4a-hydroxy and 4a-hydroperoxy-3-methyllumiflavine, respectively.

MATERIALS AND METHODS

Synthesis of 4a-Hydroperoxy-5-ethyl-3-methyllumiflavine (FlC_2H_5-OOH). Fifty milligrams of 5-ethyl-3-methyllumiflavine perchlorate ($[Fl^{\oplus}{}_{ox}C_2H_5]ClO_4^-$) dissolved in about 0.5 ml of acetonitrile (purified by distilling over P_2O_5 three times) was added to 2 ml of a 4.0 M H_2O_2 solution of 0.25 M phosphate buffer (pH = 6.1). Light green-yellow crystals of the flavine hydroperoxide immediately form. The crystals were washed with 5 ml of 4.0 M aqueous H_2O_2 solution (no buffer, pH adjusted to neutrality with KOH) and then with a little methanol, and dried overnight at about 25° at 0.2 torr (26 pascals). Yield was >80%. Elemental analysis: Found: C, 58.17; H, 6.38. Calculated: C, 57.82; H, 6.07. The crystals melt at 121–122° with decomposition and are stable for several weeks in a sealed vial away from light. $FlCH_3$-OOH and $FlCD_3OOH$ were synthesized in a similar fashion.

Determination of the Equivalent Weight of FlC_2H_5-OOH. A weighed amount of FlC_2H_5-OOH (typically 0.3–0.6 mg) was dissolved in 10 ml of 1 M HCl and the concentration of $Fl^{\oplus}{}_{ox}C_2H_5$ produced determined from the measured absorbance at 546 nm ($\epsilon_{546} = 7.95 \pm 0.05 \times 10^3\ M^{-1}\ cm^{-1}$). Assuming quantitative conversion of FlC_2H_5-OOH to $Fl^{\oplus}{}_{ox}C_2H_5$ (Eq. 1, X = OH), a molecular weight of 333 ± 5 was calculated for the hydroperoxy flavine (actual molecular weight = 332.36).

Peroxidation Equivalent of FlC_2H_5-OOH was determined by a colorimetric triiodide method (9). A weighed amount of FlC_2H_5-OOH was dissolved in 0.12 M methanolic iodide solution (N_2 atmosphere, $[FlC_2H_5\text{-OOH}] \simeq 5 \times 10^{-5}$ M) and the absorbance at 349 nm measured. After correcting for the flavine absorbance ($\epsilon_{349} = 6800\ M^{-1}\ cm^{-1}$), the yield of I_3^- ($\epsilon_{349} = 2.29 \pm 0.03 \times 10^4\ M^{-1}\ cm^{-1}$) was determined to be 86 ± 3% of the flavine hydroperoxide consumed. In a control experiment, the 4a-hydroxy flavine was found to give no I_3^-.

Chemiluminescence intensity was measured with a Nuclear Chicago liquid scintillation counter with the coincidence circuit inactivated. Fluorescence and chemiluminescence emission spectra were measured with a Perkin-Elmer MPF-3 spectrophotofluorometer.

RESULTS

The 4a-OOH group of FlR-OOH (R = CH_3, CD_3, C_2H_5) was found to be labile in protic solvents, being replaced by -OH and -OCH_3 in water and methanol, respectively. That H_2O_2 was released into the solution was confirmed by the fact that the addition of I^-, after the end of the exchange reaction, resulted in the appearance of I_3^- at a rate equal to that obtained with authentic H_2O_2 (see below). In methanol, in contrast to water, the replacement reaction was sufficiently slow ($k_{obs} = 5.1 \times 10^{-4}\ sec^{-1}$ with FlC_2H_5-OOH, 27°) to allow an accurate spectrum of the flavine hydroperoxide to be recorded (Fig. 1). In the conversion of FlC_2H_5-OOH to FlC_2H_5-OCH_3, the λ_{max} of the former (370 nm) gives way to that of the latter (356 nm) with an isosbestic point at 349 nm. In absolute dioxane, FlR-OOH was found to be very stable (lifetime measured in days).

In methanol and dioxane, the reaction of 3,5-dimethyl-1,5-dihydrolumiflavine ($FlHCH_3$) with oxygen provides the corresponding 4a-hydroperoxy flavine (Eq. 4), as shown by the fact that the spectral properties of the product are identical with those of the flavine hydroperoxide synthesized by reacting H_2O_2 with $Fl^{\oplus}{}_{ox}CH_3$ (see *Materials and Methods*). When $FlHCH_3$ was dissolved in methanol saturated with oxygen ($[O_2]$ about 10^{-3} M, $[FlHCH_3] = 2 \times 10^{-5}$ M) immediate formation of the flavine hydroperoxide could be shown to occur (spectrophotometrically) to 80% (I^- oxidation) based on $[FlHCH_3]$ employed. Small amounts (about 15%) of the flavine radical, $FlCH_3$·, were also formed by this procedure. FlR-OOH was found to be a much better oxidizing agent than H_2O_2 toward iodide. In methanol, a pseudo first-order rate constant of $1.5 \times 10^{-3}\ sec^{-1}$ was obtained for the reaction of H_2O_2 (about 5×10^{-5} M) with I^- (0.12 M). In contrast, the FlC_2H_5-OOH (5×10^{-5} M) oxidation of I^- (0.12 M) was over during the time of mixing (15 sec).

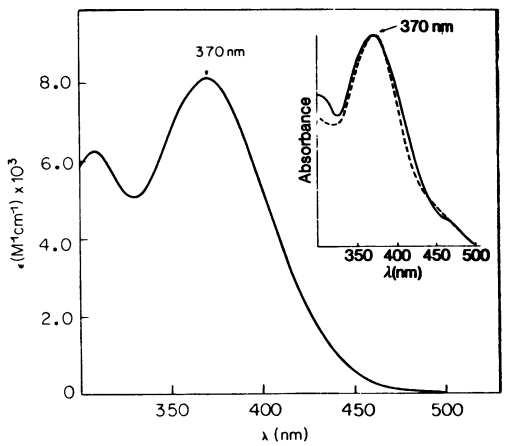
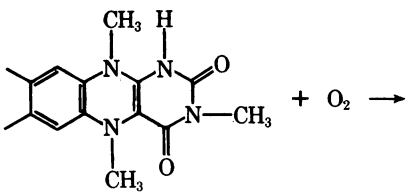
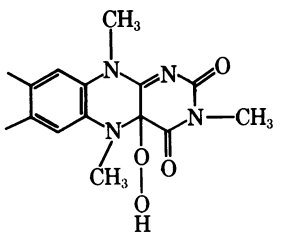


FIG. 1. Spectrum of FlC_2H_5-OOH in MeOH. Inset: comparison of the spectrum of $FlCH_3$-OOH (— —, in dioxane) with that of the bacterial luciferase bound oxygenated flavine (Enz-FMN-OOH) intermediate (- - -, ref. 5). The two curves were normalized at the absorption peak at 370 nm. The shoulder at about 460 nm in the $FlCH_3$-OOH spectrum is due to about 5% Fl_{ox} impurity. It is likely that Enz-FMN-OOH is contaminated with a few percent of FMN.



($FlHCH_3$)



($FlCH_3$-OOH) [4]

The addition of an aldehyde to a solution of FlR-OOH was found to induce chemiluminescence *which was visible to the dark adapted eye.* Light emission was not dependent upon the mode of formation of the flavine hydroperoxide, being observed not only on reaction of presynthesized FlR-OOH with aldehyde but on reaction of aldehyde with FlHR dissolved in oxygenated solvent or on addition of aldehyde to a solution of $Fl^{\oplus}{}_{ox}R$ in H_2O_2 solution. In addition, light emission was obtained with a number of aldehydes (e.g., formaldehyde, acetaldehyde, chloral, hexanal, heptanal, decanal, benzaldehyde, and salicaldehyde). The time course for light emission in the reaction of formaldehyde (0.21 M) with FlC_2H_5-OOH (1.9×10^{-4} M) in dioxane (containing

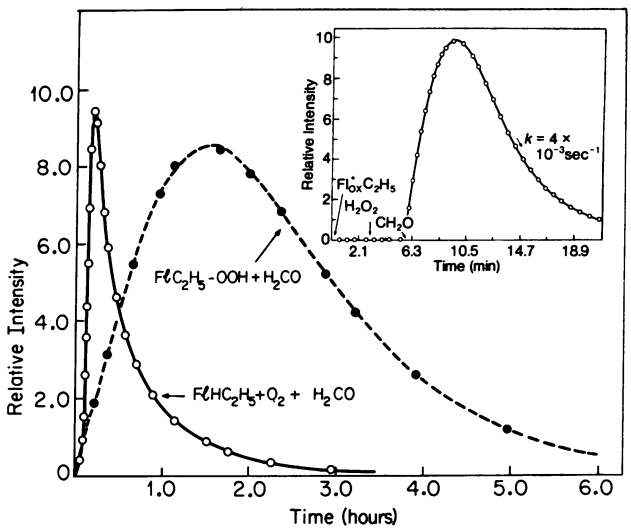


FIG. 2. Time course of light emission observed (23°) from the reactions "FlC_2H_5-OOH (1.9×10^{-4} M) + H_2CO (0.21 M)" and "$FlHC_2H_5$ (1.9×10^{-4} M) + O_2 + H_2CO (0.21 M)". In both experiments, a weighed amount of flavine was added to a 6.1 ml solution of H_2CO in dioxane (which had been saturated with O_2 in the latter case). Inset: chemiluminescence observed (23°) upon step-wise addition of H_2O_2 (0.32 M) and CH_2O (1.3 M) to a 9×10^{-5} M $Fl^{\oplus}_{ox}C_2H_5$ solution in acetate buffer (pH = 4.5, 0.5 M).

about 0.5 M H_2O) is shown in Fig. 2. Inspection of Fig. 2 reveals that on initiation of reaction, the light intensity reaches a maximum in 88 min, and then decreases in intensity. The decrease in light emission follows the first-order rate law ($k_{obs} = 2.12 \times 10^{-4}$ sec^{-1} at 23°). These results dictate that FlC_2H_5-OOH and CH_2O react to provide a species whose further reaction is accompanied by light emission. Since addition of the singlet oxygen quencher (10) 1,3-diphenylisobenzofuran (final concentration = 5×10^{-5} M) had no effect on the light intensity, the involvement of $^1O_2$ was ruled out. Chemiluminescence was found to occur equally well under *anaerobic* or aerobic conditions. The major, if not sole, flavine product of the reaction of formaldehyde with FlC_2H_5-OOH appears to be the 4a-hydroxy adduct of $Fl^{\oplus}_{ox}C_2H_5$ as identified by its spectrum (λ_{max}'s at 345, 305, and 282 nm in dioxane).

The time course of the chemiluminescence observed upon $^3O_2$ addition to a dioxane solution of $FlHC_2H_5$ (1.9×10^{-4} M) and H_2CO (0.21 M) is shown in Fig. 2, which also contains, for comparison, the time course of chemiluminescence upon reacting FlC_2H_5-OOH (1.9×10^{-4} M) with CH_2O (0.21 M). As can be seen in Fig. 2, both the increase and the decrease in light intensity occur severalfold faster in the $^3O_2$ reaction. The faster rate of increase in light intensity is likely to be due to the trapping of FlC_2H_5-$O_2^{\ominus}$ by the aldehyde, prior to its protonation. Any of the three species, $FlHC_2H_5$, FlC_2H_5·, and O_2^{-}·, which are known (6) to be present in a reaction mixture containing $FlHC_2H_5 + O_2 + H_2CO$ (but not in the reaction of FlC_2H_5-OOH with H_2CO) may be considered the cause of the increased rate of decay of chemiluminescence. When the reaction of FlC_2H_5-OOH (1.8×10^{-4} M) with H_2CO (0.24 M) was studied under anaerobic conditions in the presence of $FlHC_2H_5$ (10^{-4} M), the time course of the chemiluminescence was found to be almost identical to that obtained in the absence of $FlHC_2H_5$. Since FlC_2H_5· is produced in the reaction of $FlHC_2H_5$ with FlC_2H_5-OOH (see below), both $FlHC_2H_5$ and FlC_2H_5· can be ruled out as the species most responsible for the increased rate of decay. In separate experiments (in MeOH, anaerobic conditions, 30°) the reaction of FlC_2H_5-OOH (1.8×10^{-4} M) with $FlHC_2H_5$ (1.3×10^{-4} M) was found to be over in about 3 min, whereas, the reaction of the pseudo-base, FlC_2H_5-OH (1.5×10^{-4} M), with $FlHC_2H_5$ (1×10^{-4} M) took about 2 hr to go to completion. The stoichiometry of the former reaction was consistent with a Haber–Weiss (11) mechanism (Eq. 5). In agreement with the mechanism of Eq. 5, one flavine radical (FlC_2H_5·) is formed per one reduced flavine ($FlHC_2H_5$) when [FlC_2H_5-OOH] > [$FlHC_2H_5$]. This is in contrast to the reaction of FlC_2H_5-OH with $FlHC_2H_5$ which produces two FlC_2H_5· per limiting flavine reactant (Eq. 6). Consideration of Eqs. 5 and 6 reveals that the reaction of FlC_2H_5-OOH with $FlHC_2H_5$ should be biphasic when $FlHC_2H_5$ is in excess and that the final yield of FlC_2H_5· should be three times the initial concentration of the flavine hydroperoxide. This was experimentally verified. The reaction depicted in Eq. 5 was determined to be about 25-fold slower in dioxane than in methanol. As with $FlHC_2H_5$, FlC_2H_5· was found to react with the flavine hydroperoxide. It will be of interest to ascertain the influence of O_2^{-}· on the light emission (i.e., reaction with FlR-OOH).

$$FlHC_2H_5 + FlC_2H_5\text{–}OOH \longrightarrow FlC_2H_5\cdot + FlC_2H_5\text{–}OH + \cdot OH \quad [5]$$

$$FlHC_2H_5 + FlC_2H_5\text{–}OH \longrightarrow 2\,FlC_2H_5\cdot + H_2O \quad [6]$$

We also investigated chemiluminescence in water using $Fl^{\oplus}_{ox}R$, H_2O_2, and an aldehyde. Very weak chemiluminescence is obtained on aerobic solvolysis of 5-alkylflavinium ions. The intensity is drastically increased on the addition of hydrogen peroxide and formaldehyde. For example, addition of H_2O_2 (final concentration = 0.32 M) to a 9×10^{-5} M solution of $Fl^{\oplus}_{ox}C_2H_5$ in 0.5 M acetate buffer (pH = 4.5) increases the light intensity about 10-fold. When formaldehyde is also added (final concentration = 1.3 M), the light intensity goes up another 250-fold in about 4 min, and then decays with a first-order rate constant of 4×10^{-3} sec^{-1} (23°, Fig. 2, inset). The decay of light intensity was found to parallel the disappearance of $Fl^{\oplus}_{ox}R$. In these experiments, [$(Fl^{\oplus}_{ox}R)ClO_4^{-}$] = 1.5×10^{-4} M was added to a premixed solution of H_2CO (1.16 M) and H_2O_2 (0.036 M) in 0.23 M formate buffer (pH = 4.04). With $Fl^{\oplus}_{ox}CH_3$, both chemiluminescence and flavine absorbance changes yielded almost the same first-order rate constants (3.3×10^{-3} sec^{-1} at 23.3° and 4.3×10^{-3} sec^{-1} at 27°, respectively), the slight difference between the two constants being due to the 4° (approximate) difference in temperature of the counter and spectrophotometer. The length of time over which light emission occurs parallels the hydrolytic (Eq. 2) stability of the $Fl^{\oplus}_{ox}R$ species employed to generate FlR-OOH ($Fl^{\oplus}_{ox}C_2H_5$ > $Fl^{\oplus}_{ox}CD_3$ > $Fl^{\oplus}_{ox}CH_3$). However, although the chemiluminescent lifetime observed with $Fl^{\oplus}_{ox}C_2H_5$ in the presence of H_2O_2 and CH_2O is longer, the initial intensity of light observed is only about half of that obtained when $Fl^{\oplus}_{ox}CH_3$ and $Fl^{\oplus}_{ox}CD_3$ are employed. This result finds explanation in the observation that the rate of exchange of the peroxy moiety of FlR-OOH with OCH_3^{-} (in methanol) is about two times faster with FlC_2H_5-OOH than with $FlCH_3$-OOH (5.1×10^{-4} sec^{-1} and 2.2×10^{-4} sec^{-1}, respectively, 27°). Undoubtedly, the equilibrium concentrations of individual flavine hydroperoxides and the resistance of the $Fl^{\oplus}_{ox}R$ species to general base catalyzed solvolysis (Eq. 2), etc. are important in the experiment in H_2O. As in the reaction of FlC_2H_5-OOH + CH_2O, the addition of the singlet oxygen

quencher 1,3-diphenylisobenzofuran to a reaction mixture of $Fl^{\oplus}_{ox}C_2H_5 + H_2O_2 + CH_2O$ was found to have no effect on the light intensity. Employing $[Fl^{\oplus}_{ox}CH_3] = 1.9 \times 10^{-4}$ M and $[H_2O_2] = 0.04$ M in water (pH 4.0, 0.24 M formate), it was found that substitution of D_2CO for H_2CO (0.45 M) did not result in a kinetic isotope effect in the first-order rate constants for decrease in light emission [$(k^H = 3.3 \times 10^{-3}$ $sec^{-1})/(k^D = 3.2 \times 10^{-3}$ $sec^{-1}) \simeq 1$]. For the same experiment, the total integrated light intensity provided the deuterium isotope effect (k^H/k^D) of 2.8 ± 0.2. A similar result was obtained with presynthesized FlC_2H_5-OOH in dioxane. With both H_2CO and D_2CO the rate of decrease in light intensity was about the same; however, about three times as much light was obtained with the former. These results suggest that the oxidation of formaldehyde to formic acid occurs concomitant to FlR-OOH reduction and light emission, but that this reaction comprises a minor pathway for the disappearance of FlR-OOH (see *Discussion*). After 5 days of reaction time, only a few percent of benzoic acid was isolatable from a reaction of $FlCH_3$-OOH (6×10^{-3} M) and benzaldehyde (50-fold excess) in dioxane. However, since a control run without $FlCH_3$-OOH gave comparable results, the yield of the acid was low (<2%) under the conditions employed.

An emission spectrum consisting of a broad band extending from about 480 to above 600 nm, with a λ_{max} at about 570 nm (uncorrected for phototube sensitivity; emission slit = 40 nm) was obtained from the "$Fl^{\oplus}_{ox}CD_3 + H_2O_2 + CH_2O$" reaction in 50% aqueous dioxane (vol/vol). Employing presynthesized $FlCH_3$-OOH and H_2CO in dioxane, the broad emission possessed $\lambda_{max} = 530$ nm. The fluorescence spectra of the 4a-hydroperoxy and 4a-hydroxy flavines were obtained in glassy frozen solutions at 77 K (no fluorescence could be detected in solution). FlC_2H_5-OH was found to have an emission peak at 460 nm (uncorrected) in ethylene glycol-H_2O (1:1), whereas FlC_2H_5-OOH had an emission peak at 475 nm (uncorrected) in ethanol. These fluorescence spectra are very similar to that of 4a-ethoxy-5-ethyl-3-methyllumiflavine for which a λ_{max} of 486 nm was reported (12).

DISCUSSION

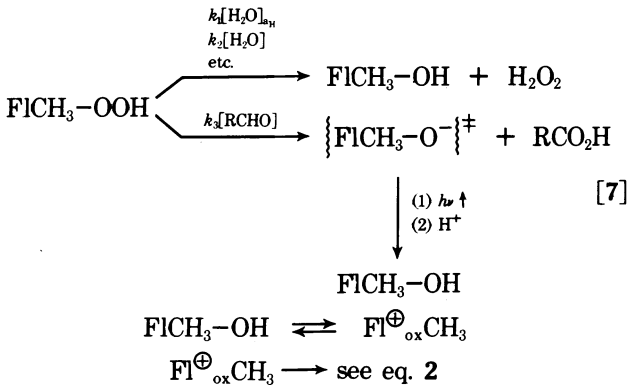
The following similarities are found to exist between synthetic FlR-OOH and the bacterial luciferase bound oxygenated FMN intermediate (Enz-FMN-OOH) described by Hastings and coworkers (5, 13, 14): (*a*) both react with aldehydes to produce light; and (*b*) aldehyde is converted to the corresponding acid in the bacterial luciferase catalyzed reaction (15–17). Evidence [e.g., C-H(D) isotope effect on the light yield, see below] supports a like oxidative mechanism for the model; (*c*) as is the case with the enzymic reaction (5), chemiluminescence occurs equally well under anaerobic and aerobic conditions; (*d*) in the reaction of FlR-OOH with aldehydes, the time course of light emission is characterized by an initial increase in light intensity followed by an exponential decay. The same behavior, on a much faster time scale, is observed in the reaction of Enz-FMN-OOH with aldehydes (13); (*e*) in the absence of aldehyde, both Enz-FMN-OOH (13) and FlR-OOH yield H_2O_2; (*f*) the absorption spectra of FlR-OOH and Enz-FMN-OOH are remarkably similar (Fig. 1, inset); (*g*) the fluorescence emission spectrum of Enz-FMN-OOH (14) resembles that of FlR-OOH. In addition to the above, the recent finding by Hastings and coworkers* that bioluminescence also occurs on combination of the reactants FMN, H_2O_2, and aldehyde with bacterial luciferase finds direct analogy in our finding of chemiluminescence in the reaction of $Fl^{\oplus}_{ox}R$ with H_2O_2 and aldehyde.

Some differences between the model and enzymatic systems have also been noted. For example, the emission spectra obtained from the reactions "$Fl^{\oplus}_{ox}CD_3 + H_2O_2 + H_2CO$ in 50% aqueous dioxane (vol/vol)" and "$FlCH_3$-OOH + H_2CO in dioxane," differ from that of the bacterial luciferase system. The enzymatic reaction emits in the blue-green (λ_{max} about 490 nm) (18), whereas the models emit at longer wavelengths (λ_{max} about 570 nm and 530 nm, respectively). This difference is not too surprising because it has been found that the color of luminescence with bacterial luciferase depends on the flavine analogue employed (19) and in the model reaction on the solvent. Unlike Enz-FMN-OOH, FlR-OOH is found to be less discriminating toward the aldehyde which is required for light emission. Thus, whereas bacterial luciferase functions only with long chain aldehydes (18), the model system also works with short chain and aromatic aldehydes. The specificity of the enzyme is, of course, ascribable to specific binding requirements. Although the reaction between Enz-FMN-OOH and RCHO is slow as enzyme catalyzed reactions go, it is much faster than the reaction of FlR-OOH with aldehydes. This also is not surprising because the enzyme is undoubtedly designed to catalyze the light emitting reaction. With the model system, the light intensity reaches a maximum usually in 1–2 hr (14 hr with chloral hydrate!), whereas with the enzyme the maximum is reached in less than 1 sec (25°) (18). The decay of the light intensity has a half-life of 5–10 sec (25°) with bacterial luciferase (18), whereas with the model system it is usually in hours.

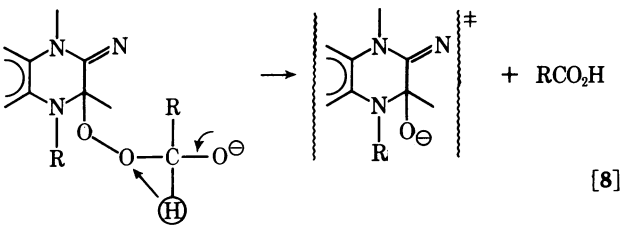
Despite many years of research, the mechanism of light emission by bacterial luciferase is not completely understood. Thus, the emitting species has yet to be identified. Our preliminary results establish, as suggested by Hastings for bacterial luciferase (5), that the reaction of 4a-hydroperoxyflavine with aldehyde leads to a chemically excited state. However, the details of the mechanism for the model reaction are yet to be elucidated. The finding that, in dioxane containing 0.5 to 1.0 M H_2O or in H_2O at pH 4.0, replacement of H_2CO by D_2CO results in no deuterium isotope effect on the first-order rate of diminution of light emission but results in an isotope effect of about 3 (i.e., k^H/k^D) on the integrated light emission finds explanation in the scheme of Eq. 7 (written for $FlCH_3$-OOH but also pertaining to FlC_2H_5-OOH). If the major path for destruction of $FlCH_3$-OOH is not that associated with k_3, no kinetic isotope effect should be observed. However, if the light emitting reaction (k_3) is associated with a C-H(D) isotope effect, then the integrated light emission should exhibit an isotope effect. It follows that the first-order rate constants obtained for the decay of the light intensity (see *Results*) pertain to the steps describing the major reaction (e.g., $k_1 + k_2$ + solvolysis rate + etc.) and not to k_3 (which constitutes a minor path for the consumption of $FlCH_3$-OOH). The present results are not in discord with a Baeyer–Villiger oxidation as previously suggested by Eberhard and Hastings (20) with the reservation that for the *N*(5)-alkyl flavine peroxides of this study the emitting species would be required to be the pseudo-base

* Becvar, J. E., Tu, S.-C. & Hastings, J. W. (1976) *Fed. Proc.*, in press.

(Eq. 8). However, FlR-OH does not provide a fluorescence emission spectrum unless in frozen solution. Further investigation into the chemistry of FlROOH compounds may be expected to provide a better understanding of this important class of enzymes.



[7]



[8]

As with bacterial luciferase, 4a-hydroperoxy-FMN is thought to be an intermediate in the reactions catalyzed by external flavomonooxygenases (4, 21, 22). And for these enzymes, the absorption spectra of the $^3O_2$ dihydroflavine enzyme are similar to the model FlR-OOH compounds of this study.

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