Bacterial Bioluminescence: Equilibrium Association Measurements, Quantum Yields, Reaction Kinetics, and Overall Reaction Scheme†

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ABSTRACT: The characteristics of the bioluminescence reactions with bacterial luciferase from two different cell types, *Photobacterium fischeri* and *Beneckea harveyi,* are reported. The reduced flavine mononucleotide ($FMNH_2$)-luciferase association constant, directly measured by equilibrium dialysis and gel filtration is the same for both luciferases, $3 \times 10^4\ M^{-1}$ at room temperature, and is significantly different from the kinetic reciprocal Michaelis-Menten constant. The luciferase bioluminescence quantum yield for the highest activity preparations is the same as for the aldehyde. Rapid stopped-flow observations show that the oxidation of $FMNH_2$ in the presence of sufficient luciferase to outcompete autoxidation, is bimodal. A long-lived intermediate, formed before reaction with aldehyde, has an activation energy for decay of 35 kcal mol^{-1}, much greater than for the light reaction, 14 kcal mol^{-1}. The ratio of bioluminescence quantum yields with respect to aldehyde and $FMNH_2$ is independent of temperature, however, and also of aldehyde chain length longer than octanal, pH (6.5-8), and type of luciferase and its specific activity. Even when the aldehyde concentration limits the rate of the light reaction, the quantum yield of the long-lived intermediate is unchanged, and together these data mean that, under the optimal conditions chosen for quantum yield measurements, no dark side reactions effectively compete with the main reaction leading to light emission. A series of reactions involving one-electron steps and the sequential oxidation of two $FMNH_2$ molecules is postulated for the formation of the long-lived intermediate.

The in vitro reaction of bacterial bioluminescence is a complex sequence of events involving three substrates, $FMNH_2$,[1] a long chain aliphatic aldehyde (RCHO), and molecular oxygen (Cormier and Strehler, 1953; Strehler and Cormier, 1953, 1954; Strehler et al., 1954; McElroy and Green, 1955). The products of the reaction are FMN, formed quantitatively (Cormier and Totter, 1957; Lee and Seliger, 1965), H_2O_2 (Lee, 1972), and the carboxylic acid of the same carbon chain length as the aldehyde (Shimomura et al., 1972; Dunn et al., 1973; McCapra and Hysert, 1973; Vigny and Michelson, 1974). The emitted light has a spectral emission maximum (λ_B) near to 490 nm, the value depending slightly on the type of bacterium from which the luciferase was purified (Seliger and McElroy, 1965; Seliger and Morton, 1968; Hastings et al., 1973a; Cline and Hastings, 1974; Lee et al., 1974). It has been postulated that the emitting species is the flavine cation, $FMNH^+$, formed in an excited state by some exergonic step in the reaction (Eley et al., 1970; Lee and Murphy, 1973a).

The aim of this work is to find a series of reactions which will reasonably describe the bioluminescence process. The first step in the sequence is the interaction of $FMNH_2$ with native luciferase. This is a weak complex and reacts with oxygen in several steps to form an intermediate which looks like an oxidized flavoprotein (Lee and Murphy, 1973b; Murphy et al., 1974a), and then this reacts with RCHO to yield the luminescence (Hastings and Gibson, 1963).

The stoichiometry of the reaction has been derived from measurement of direct utilization of the substrates and yield of one of the products, based on the quantum yield of bioluminescence of each reactant and product (Lee, 1972):

$$2FMNH_2 + 2O_2 + RCHO \longrightarrow 2FMN + H_2O_2 + [H_2O] + RCOOH$$

The square brackets are used since H_2O has not yet been identified as a reaction product. This stoichiometry, however, is at variance with that derived from a formal analysis of the steady-state kinetics (Edsall and Wyman, 1958). The initial light intensity is first order in the initial concentration of $FMNH_2$ and a simple kinetic model would suggest the involvement of one $FMNH_2$ (Meighen and Hastings, 1971; Watanabe and Nakamura, 1972), not two.

Clearly any proposed reaction scheme must encompass this utilization of two $FMNH_2$'s but leading to a first-order steady-state kinetics. In this paper we show that the two-$FMNH_2$ utilization is not due to multiple pathways, since it is unchanged by variations in external parameters such as temperature, pH, substrate concentration, etc. A simple Michaelis-Menten model does not describe the transient kinetics of the oxidation of $FMNH_2$ on the luciferase. We propose a series of one-electron oxidation steps involving both flavine and a sulfhydryl group on the luciferase, leading finally to the long-lived flavoprotein intermediate of composition HOOSE-FMN. Some of the results presented here have been described in preliminary communications elsewhere (Lee and Murphy, 1973a,c; Murphy et al., 1974b).

Experimental Section

Chemicals. FMN was obtained from Fluka, A. G., Buchs, Switzerland, and was their "purum" grade, nominally 86% FMN. It was further purified by chromatogra-

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[1] Abbreviations used are: FMN, flavine mononucleotide; RCHO, long-chain aliphatic aldehyde; DEAE, diethylaminoethyl-cellulose; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; PF, *Photobacterium fischeri;* BH, *Beneckea harveyi.*

phy on DEAE-cellulose (Whatman 32) (Massey and Swoboda, 1963); four minor bands separate on the column from the bulk of the material (FMN). On purification the Q_B ($FMNH_2$)[2] increased about 10%, consistent with the stated nominal purity. We assume these preparations to have at least 95% purity. Concentrations of FMN were calculated from OD(445) (ϵ 12500 M^{-1} cm^{-1}, Whitby, 1953). We have found Fluka to be the best commercial source of FMN. It was noted by Lee (1972) for instance, that for FMN from Sigma Chemical Co. (St. Louis, Mo.), $Q_B(FMNH_2)$ increases 1.4 times on purification. This material gives seven or more bands on a DEAE column. The $FMNH_2$ was prepared by photoreduction of FMN in phosphate (0.05 M) buffer pH 7, containing EDTA (0.02 M) (Radda and Calvin, 1964). The aldehydes except tridecanal (Fluka) were from Chemical Sample Corporation (Columbus, Ohio) and were purified by vacuum distillation immediately before use. All other chemicals were the best available commercial grades. All buffers and solutions were made up in glass-distilled water.

Luciferase Preparation. The luciferase used in these experiments was from two types of bacteria. The first was *Photobacterium fischeri,* abbreviated PF, the cell culture originally being obtained from M. J. Cormier, who had derived it from type 7744 of the American Type Culture Collection. The other was "MAV" from J. W. Hastings, which has recently been assigned to *Beneckea harveyi* by Reichelt and Baumann (1973) and so henceforth will be referred to by the abbreviation BH. The method for purification of the luciferase from these bacteria does not vary significantly from those reported by others (Hastings et al., 1965; Kuwabara et al., 1965; Gunsalus-Miguel et al., 1972). For PF the method used by Lee (1972) was extended by adding a DEAE-Sephadex A50 chromatography step, equilibrating the column with phosphate buffer (0.25 M, pH 7) and loading with the luciferase eluate in 0.25 M phosphate from the first DEAE step, then eluting the A50 with a linear phosphate gradient, 0.35–0.5 M. A significant increase in specific activity could often be obtained at this point by concentrating the solution to about 5 mg/ml, adding ammonium sulfate until just cloudy and let stand overnight. An inactive precipitate results. For BH the ammonium sulfate precipitate of crude luciferase was thoroughly dialyzed, loaded to DEAE 32, washed with 0.05 M then 0.15 M phosphate buffer, and then eluted with 0.25 M. This eluate was loaded onto DEAE-Sephadex A50, washed (0.25 M), and eluted with 0.35 M phosphate (Gunsalus-Miguel et al., 1972).

By SDS gel electrophoresis (Brewer and Ashworth, 1969) the luciferase was judged to be about 80% pure at this point, since several minor bands could be detected. For higher purities the luciferase was passed down Sephadex G-150 and/or crystallized which resulted in about 10% increase in specific activity. The overall purification on the basis of protein is 40 and on OD(280) about 200, in agreement with that reported by Gunsalus-Miguel et al. (1972). For calculating enzyme concentrations we have assumed a mol wt 80,000 (Hastings et al., 1969) and that a solution of unit OD(280) contains 1 mg of luciferase/ml. Biuret determinations of protein are higher than this (1.1–1.2 mg ml^{-1} OD^{-1}) so allowing for impurities, this approximation is sufficient.

Light Reactions and Measurement. The light reactions were carried out as described before (Lee, 1972) with absolute light calibration made by using the luminol chemiluminescence reaction (Lee et al., 1966). The specific activity, defined as the initial light intensity maximum (I_0) per mg of luciferase, was determined for PF as before (Lee, 1972) using dodecanal. It was usually around 6×10^{13} $h\nu$ sec^{-1} mg^{-1} for most preparations but small amounts of much higher activities (~20) were obtained by the ammonium sulfate procedure. For BH luciferase, specific activity was measured with decanal at not too high a concentration as to show RCHO inhibition (Hastings et al., 1969). Optimum specific activity was obtained with 10 μl of a 0.1% saturated solution of decanal in methanol, added to the reaction mixture. The specific activity of apparently homogeneous preparations of BH luciferase was found to be more variable than PF. An activity around 3×10^{13} $h\nu$ sec^{-1} mg^{-1} was routine, but preparations around 2×10^{13} were used in some experiments where the activity was not critical. The highest activity which we could make reproducibly was 5.5×10^{13}. A large variation is also to be noted in the BH luciferase preparations used in J. W. Hastings' laboratory (Mitchell and Hastings, 1969; Meighen and Hastings, 1971; Gunsalus-Miguel et al., 1972; Hastings et al., 1973b).

Instrumentation. Stopped-flow kinetics were measured with a Durrum instrument (Palo Alto, Calif.) interfaced to a Nova computer (DeSa, 1972). Absorption spectra were taken with a Cary 14 spectrophotometer.

Results

Association of Luciferase and $FMNH_2$. The binding of $FMNH_2$ by luciferase was measured either by equilibrium dialysis or by filtration of luciferase through Sephadex G-25 equilibrated with $FMNH_2$, a method introduced by Hummel and Dreyer (1962). Equilibrium dialysis was performed by placing a dialysis sack containing luciferase (3 ml, 4 mg/ml) into a solution of FMN (25 μM, 15 ml) in Tris buffer (0.1 M, pH 7) containing EDTA (0.02 M) and dithiothreitol (0.005 M) in a glass vessel which could be attached to a vacuum line via a stopcock. The solution was degassed by applying a vacuum (10^{-4} Torr) and stirring vigorously for several hours, then the FMN photoreduced and the system closed to the vacuum and to air. During the 24–30-hr dialysis period the $FMNH_2$ was maintained in the reduced state by ambient light, as judged by the absence of yellow color. The temperature was maintained at 17°. In an alternative technique the solution was first partially degassed by bubbling N_2, then a slight excess of solid sodium dithionite added to reduce the FMN; the system was then evacuated as before and closed to air. The value obtained for the equilibrium constant was the same by both methods. No photolytic degradation of the FMN occurred during the experiment since if an aliquot was reduced to $FMNH_2$, the $Q_B(FMNH_2)$ was unchanged.

Equilibrium was attained in 17–20 hr. The partitioning of $FMNH_2$ between the enzyme and surroundings was measured by opening the system to air, thereby allowing all the $FMNH_2$ to oxidize, and removing the dialysis bag from the surrounding solution. The concentration of FMN was measured 0.5 hr later to ensure complete dissipation of any long-lived intermediates formed on oxidation of $FMNH_2$ in the presence of luciferase (Lee and Murphy, 1973b). Vol-

[2] The quantum yield of bioluminescence, $Q_B(A)$, with respect to a reactant or product A, is the number of photons emitted by the reaction per molecule of A disappearing as a reactant or appearing as a product. The dimensions of Q_B are photons per molecule or einsteins per mole.

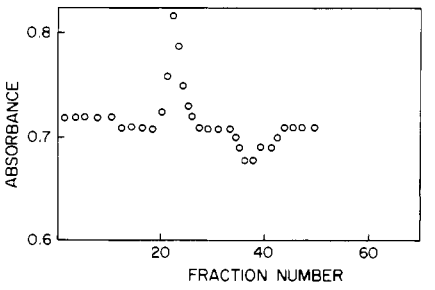

FIGURE 1: Association of $FMNH_2$ and BH luciferase demonstrated by gel filtration. The points are the absorbance (445 nm) in each fraction collected from the G-25 Sephadex column. The first tube is the fraction collected as the luciferase solution was loaded. The peak in absorbance is from the $FMNH_2$ as it coelutes through association with the luciferase. The $FMNH_2$ is measured by letting it oxidize to FMN and then measuring the absorbance at 445 nm.

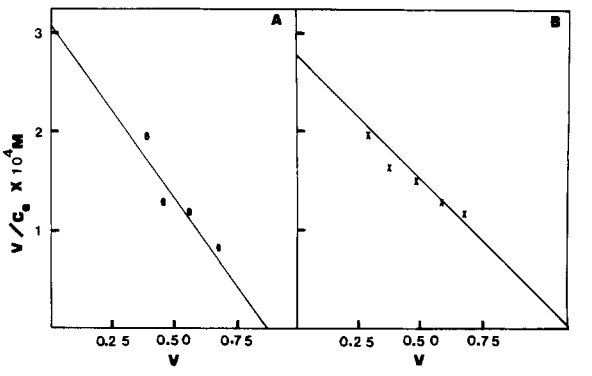

FIGURE 2: Scatchard plot of the gel filtration data. The percent $FMNH_2$ enzyme-bound (ν) is divided by the equilibrium concentration of $FMNH_2$ (C_e) and plotted vs. ν. (A) PF luciferase; (B) BH luciferase. The lines drawn are least-squares fitted to the data. The intercept of the line on the x axis at a value of nearly one indicates a one-to-one stoichiometry for the equilibrium. The intercept on the y axis divided by the x intercept yields the binding constant.

ume changes, $FMNH_2$ adsorbed to the dialysis sack, Donnan membrane correction terms, and light scattering by luciferase in the absorbance determination of FMN, have been taken into account in the data. The initial specific activities were PF 7.5×10^{13} and BH 2.2×10^{13} $h\nu$ sec^{-1} mg^{-1} and decreased 20–30% in the course of the experiment.

The determination of the equilibrium constants from the gel filtration of the protein was carried out in the following manner: a G-75 Sephadex column (1 × 47 cm) was equilibrated with $FMNH_2$ (15–80 μM, depending on the experiment) produced by photoreduction in nitrogen deaerated buffer containing sodium phosphate (0.05 M, pH 7), dithiothreitol (10^{-4} M), and EDTA (5×10^{-3} M). The column was equilibrated with $FMNH_2$ by flowing down 250 ml of the solution before anaerobically loading the luciferase, which was in a solution containing the same concentration of $FMNH_2$. The $FMNH_2$ was maintained in the reduced state at all times by keeping the column under illumination and it could be observed that the eluent did not develop the characteristic FMN yellow color until it passed through the transfer tube and into the fraction collector. These experiments were done at room temperature (23°). The absence of photolytic degradation of FMN was again established by the quantum yield method as before.

The results of a typical experiment are shown in Figure 1. The peak is a result of $FMNH_2$ being dragged along in the column front by its association with luciferase. The peak and trough areas are about the same, which indicates that the equilibrium had time to establish itself during the course of the experiment. The specific activities of the luciferases were the same as for the equilibrium dialysis measurements but no loss occurred in the gel filtration experiments.

The equilibrium constants determined for the two luciferases by the two methods are listed in Table I. There is no significant difference between the equilibrium constants determined by the two methods, but the gel filtration data are more precise. In the equilibrium dialysis a control experiment with bovine serum albumin (5 mg/ml) replacing luciferase in the dialysis bag showed no binding ($K < 10^3$ M^{-1}). Catalase (0.1 mg/ml) added to remove traces of H_2O_2 formed by reoxidation of $FMNH_2$ by oxygen, or dodecanal (10 μl of methanol saturated as in the specific activity assay), in the PF luciferase experiment, did not affect the final result. With FMN no association with BH luciferase was detectable ($K < 10^3$ M^{-1}), in contrast to our earlier

Table I: Equilibrium Association Constants.

$$E + FMNH_2 \rightleftarrows E\text{–}FMNH_2$$

Luciferase (E)	Equilibrium Dialysis[a] (M^{-1})	Gel Filtration[b] (M^{-1})
PF	$1.6 \pm 1 \times 10^4$	$3.4 \pm 0.3 \times 10^4$
BH	$2 \pm 1 \times 10^4$	$2.6 \pm 0.4 \times 10^4$

[a] 17°. [b] 23°; results from the Scatchard plots (Figure 2) assuming exactly one-to-one association. Solution conditions are described in the text.

observations (Lee and Murphy, 1973c) and recent reports of Baldwin (1974) and Waters et al. (1974).

The Scatchard plots in Figure 2 are made from the gel filtration data and give an intercept on the abscissa of nearly unity, indicating a one-to-one association between luciferase and $FMNH_2$. There is no significant difference between the association constants of the two types of luciferase in spite of the threefold difference in specific activity and differences in other kinetic properties. However, it is remarkable that the equilibrium constant, $K^{-1} \sim 30$ μM, greatly exceeds K_m (<1 μM, Meighen and Hastings, 1971; Watanabe and Nakamura, 1972).

Insufficient amounts of the very high activity luciferases were available to make a Scatchard plot for comparison, but an equilibrium determination assuming a one-to-one stoichiometry gave about the same value for the equilibrium constant.

Initial Rate of $FMNH_2$ Oxidation. In order to directly test the assumption that under a sufficient luciferase concentration to obtain maximum $Q_B(FMNH_2)$, all $FMNH_2$ is oxidized by an enzyme mediated process, rapid stopped-flow observations of the oxidation of $FMNH_2$ were carried out. $FMNH_2$ in phosphate buffer (0.05 M, 0.02 M EDTA, pH 7) was loaded into one syringe of the stopped-flow device and the other syringe was filled with air-saturated buffer with or without luciferase. The reactions were carried out at 5° and the final concentrations are given in the legend of Figure 3.

The slower rate ($k = 0.78$ sec^{-1}) in the main part of Figure 3 is for OD (445 nm) appearance in the presence of BH luciferase. The change accounts for only 50% of the expected OD change based on the amount of $FMNH_2$ added.

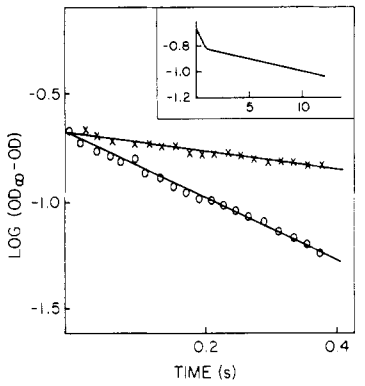

FIGURE 3: Oxidation of $FMNH_2$ (14 μM) by molecular oxygen (190 μM) in the presence (x) or absence (O) of BH luciferase (58 μM). The insert is the luciferase reaction on a longer time scale. The appearance of FMN is measured by OD (445 nm); all reactions are in phosphate buffer, pH 7, 5°.

Present evidence suggests that at this time (~1 sec) one $FMNH_2$ has been completely oxidized to free FMN, since the mixture has a fluorescence corresponding to free FMN, but of only one-half the intensity expected from the total $FMNH_2$ added (Lee and Murphy, 1973b). A similar picture is found for PF luciferase but with somewhat faster rates (results not shown). A scanning stopped-flow study of the reaction will be reported elsewhere.

After 1 sec and utilization of the first $FMNH_2$, the reaction slows down and a slower first-order rate (k = 0.02 sec^{-1}) takes over. The insert in Figure 3 is a longer time scale and the biphasic nature of the reaction is clearly evident.

The faster rate, the circles in Figure 3 (k = 2.6 sec^{-1}), is the autoxidation of $FMNH_2$ (no luciferase). In the presence of luciferase the slower rate seen for the initial change is first order and shows no contribution from the faster autoxidation rate. In the experiments reported by Yoshida et al. (1973,1974) a biphasic rate of oxidation was also seen, but insufficient luciferase concentration was used to ensure utilization of all the $FMNH_2$ through the enzyme path, and their initial rate is, therefore, dominated by autoxidation. The same can be said of the experiments of Gibson et al. (1966) since the luciferase used there was of low purity.

Quantum Yields of $FMNH_2$ and RCHO. The $Q_B(FMNH_2)$ was determined as before (Lee, 1972) by rapidly injecting $FMNH_2$ (0.1 ml) into buffer solution (1 ml) containing aldehyde and luciferase (>40 μM). The added $FMNH_2$ was varied in amount from 1 to 16 nmol with no significant change in the final result. Table II presents these results averaged for 3–5 determinations each. The amount of aldehyde used depended on the type of luciferase. For PF we added 10 μl of an aldehyde saturated methanol solution for undecanal or the longer chain lengths, and 10 μl of a 10% saturated solution for shorter chain lengths. For BH luciferase the aldehyde concentration range for which I_0 is maximum was first determined. For octanal through decanal it is 5–60 μM (final concentration), undecanal 5–120 μM, dodecanal and tridecanal 25–120 μM, and tetradecanal 40–100 μM. The concentration was maintained in excess of $FMNH_2$ but in the optimum range for the $Q_B(FMNH_2)$ determination. It was found for decanal that $Q_B(FMNH_2)$ was maximized in the same range as I_0. We shall show later that if aldehyde is added to the BH luciferase reaction subsequent to the addition of $FMNH_2$, the inhibition disappears.

Table II: The Ratio of $Q_B(RCHO)/Q_B(FMNH_2)$ under Different Experimental Conditions.

Aldehyde	$Q_B(FMNH_2)$	$Q_B(RCHO)$	$Q_B(RCHO)/Q_B(FMNH_2)$
PF at 10°			
Octanal	0.047	0.110	2.11
Nonanal	0.053	0.115	1.98
Decanal	0.052	0.100	1.79
Undecanal	0.039	0.088	2.26
Dodecanal	0.038	0.082	2.16
Tridecanal	0.035	0.080	2.29
Tetradecanal	0.034	0.077	2.15
$Q_B(RCHO)/Q_B(FMNH_2)$ = 2.11 ± 0.17			
BH at 10°			
Octanal	0.026	0.059	2.27
Nonanal	0.029	0.055	1.89
Decanal	0.021	0.046	2.19
Undecanal	0.028	0.053	1.89
Dodecanal	0.029	0.053	1.83
Tridecanal	0.033	0.054	1.64
Tetradecanal	0.031	0.057	1.84
$Q_B(RCHO)/Q_B(FMNH_2)$ = 1.94 ± 0.17			
PF with Dodecanal			
Temp (°C)	$Q_B(FMNH_2)$	$Q_B(RCHO)$	$Q_B(RCHO)/Q_B(FMNH_2)$
2 ± 1	0.034	0.067	1.98
10 ± 1	0.038	0.082	2.16
25 ± 1	0.047	0.100	2.13
$Q_B(RCHO)/Q_B(FMNH_2)$ = 2.09			
PF at 10° with Dodecanal			
pH	$Q_B(FMNH_2)$	$Q_B(RCHO)$	$Q_B(RCHO)/Q_B(FMNH_2)$
6.5	0.035	0.079	2.25
7.0	0.038	0.082	2.16
7.5	0.038	0.078	2.08
$Q_B(RCHO)/Q_B(FMNH_2)$ = 2.16			

The $Q_B(RCHO)$ was determined exactly as described before (Lee, 1972) using a limiting quantity of RCHO (2–5 nmol) and repeated addition of $FMNH_2$ (20 nmol, 0.2 ml) into the solution of RCHO and luciferase (PF 100 μM, specific activity 4 × 10^{13} $h\nu$ sec^{-1} mg^{-1}; BH 20–50 μM, specific activity 2 × 10^{13} $h\nu$ sec^{-1} mg^{-1}, phosphate 0.05 M). Results were corrected for aldehyde blank (a few percent of the total light obtained with RCHO could be elicited in its absence, presumably arising from contaminating traces of RCHO), and self-absorption (Lee, 1972). There was no effect on $Q_B(RCHO)$ by change in the BH luciferase concentration in the range 20–50 μM. A decrease in $Q_B(RCHO)$ at higher PF luciferase reported by Lee (1972) was not confirmed; this quenching was probably due to the presence of ammonium sulfate which we find to quench the reaction, and more care was taken here to thoroughly dialyze the luciferase preparations.

The quantum yields are all listed in Table II. For BH luciferase the absolute values are only about half that for PF and are constant with temperature, 0–25°, pH 6.5–7.5 (not shown) and aldehyde carbon chain length except decanal. In contrast, for PF luciferase the absolute quantum yields are significantly affected by temperature change and aldehyde carbon chain length, but in all cases the ratio $Q_B(RCHO)/Q_B(FMNH_2)$ remains constant at 2.0.

Reaction of Hexanal. The integral ratio of 2.0 for $Q_B(RCHO)/Q_B(FMNH_2)$ is not maintained when heptanal or shorter carbon chain length aldehydes are used (Lee and Murphy, 1973a); the I_0's also drop abruptly (Hastings et al., 1963, 1969). For PF luciferase, Q_B(hexanal) is only 0.003 (10°) instead of about 0.12 which would be expected by extrapolation of the data in Table II. Catalase has no effect on the result.

The question arises whether hexanal is still consumed by the reaction but with a lower efficiency for light production, or whether hexanal is simply a poor substrate. Since catalase does not enhance Q_B(hexanal) then the aldehyde is evidently not being depleted by reaction with H_2O_2 in free solution.

We find that hexanal does not react in the process, since the yield of H_2O_2, measured by a comparison of oxygen consumption in the presence and absence of catalase as described before (Lee, 1972), is the same with or without hexanal. To 2 ml of PF luciferase (9 mg/ml, specific activity 6 $\times 10^{13}$ $h\nu$ sec^{-1} mg^{-1}) in buffer (0.05 *M* Tris, pH 7) at 20° and excess RCHO (100 nmol) was added 6.0 nmol of $FMNH_2$ (0.1 ml). The yield of H_2O_2 was 2.4 ± 0.4 times greater for hexanal as when dodecanal was used, per mole of $FMNH_2$ added. For decanal the enzyme reaction is

$$RCHO + 2FMNH_2 + 2O_2 \longrightarrow RCOOH + H_2O_2 + 2FMN + H_2O$$

so that for hexanal we have no reaction

$$RCHO + FMNH_2 + O_2 \longrightarrow RCHO + H_2O_2 + FMN$$

and not

$$RCHO + FMNH_2 + O_2 \longrightarrow RCOOH + H_2O + FMN$$

This is the reason for the apparently low quantum yield of the hexanal. It is possible that the small amount of hexanal that is consumed in the light path does so with the same Q_B as the higher aldehydes.

Quantum Yield of $FMNH_2$ on Preincubation with Luciferase. Since the association of $FMNH_2$ and luciferase is so weak, the question arises whether the complex lies on the reaction pathway or not. The rate of association is difficult to measure directly, but at least we are able to conclude from the following experiments, that the equilibrium is established rapidly in comparison to the rate of autoxidation, and therefore more rapidly by far than the overall light reaction.

The $Q_B(FMNH_2)$ vs. E concentration was measured both by the method where $FMNH_2$ is added to the luciferase–RCHO mixture, and where luciferase and $FMNH_2$ are first preincubated, and the RCHO added secondarily. For the preincubation reaction a nitrogen deaerated solution (0.05 *M* phosphate–0.02 *M* EDTA, pH 7) of luciferase and FMN was drawn into a gas-tight syringe (Hamilton, 1 ml) photoreduced, and added to a solution (1 ml) of decanal of the same concentration as used for the $Q_B(FMNH_2)$ determination by the regular method.

In Figure 4 the results for the preincubation technique (filled points) are compared with the regular method (crosses) for BH luciferase. A lower E concentration is required for half-maximum $Q_B(FMNH_2)$ in the former case. This is a simple result of the one-half initial oxygen concentration for the preincubated reaction (Lee, 1972) which lessens the competition by O_2 over E for $FMNH_2$. After this has been allowed for, it can be concluded that there is no significant difference between the two sets of results. Therefore the equilibrium must be rapidly established in comparison to the rate of autoxidation. Similar results are obtained for PF luciferase (results not shown).

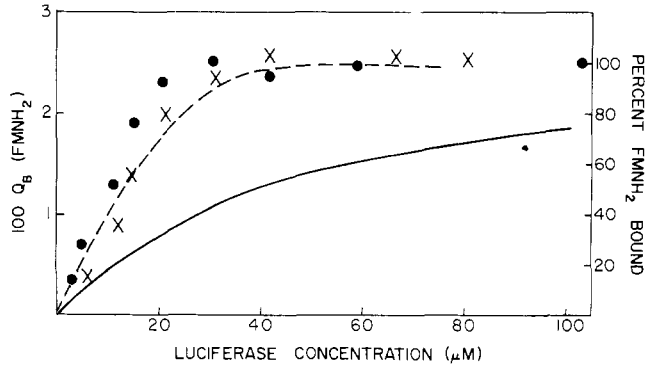

FIGURE 4: Quantum yield of $FMNH_2$ dependence on the concentration of luciferase (BH) with the preincubation method (●) or the regular method (X) of reaction (see text). The solid line is the calculated percentage of the total $FMNH_2$ (3.5 μ*M*) bound to luciferase. All reactions are at room temperature, pH 7.

Table III: Quantum Yield of BH Luciferase.[a]

Temp (°C)	$I_0(10^{13} h\nu\ sec^{-1}\ mg^{-1})$[b]	$k\ (sec^{-1})$[c]	$100Q_B(E)$
37	3.3	0.53	1.1
23	2.4	0.39	1.1
10	0.53	0.092	1.0
5	0.33	0.05	1.2

[a] Phosphate buffer 0.05 *M*, pH 7.0, 10^{-4} *M* dithiothreitol, 1 mg/ml of bovine serum albumin, decanal 20 μ*M*. [b] Specific activity = I_0. [c] Observed first-order decay of light emission.

The full line on Figure 4 is the percent bound $FMNH_2$ calculated from the equilibrium constant. It is notable that the $Q_B(FMNH_2)$ reaches its constant value at a point where only half the $FMNH_2$ is initially bound. A model based on the premise that full utilization of $FMNH_2$ to give light requires one $FMNH_2$ to be initially oxidized on the luciferase with the other in free solution, which will be elaborated more fully later, predicts the relationship shown by the dashed curve.

Quantum Yield of Luciferase. The $Q_B(E)$ was measured by the total light emitted in the normal assay for specific activity divided by the moles of luciferase, which is of course now the reactant present in limiting concentration. Variations in the assay procedure, such as the preincubation of luciferase with dithionite reduced $FMNH_2$, introduced by Meighen and Hastings (1971) and Meighen and MacKenzie (1973), did not give any different results. The $Q_B(E)$ for BH luciferase of this specific activity at different temperatures are listed in Table III. It is seen that I_0 and k, the apparent first-order rate of decay of light intensity, change in a compensatory way such that the total light is unaffected by change in temperature. The same behavior is found for PF luciferase (results not shown).

The pH dependence of $Q_B(E)$, I_0, and k exhibits some interesting features (Figure 5). For BH luciferase $Q_B(E)$ is constant from pH 6.1 to 7.9, I_0 and k ($t_{1/2}$ is plotted in the figure) changing in this region again in a compensatory way to maintain a constant total light. The fall of $Q_B(E)$ on the basic side is not due to denaturation since the luciferase has a half-life of 5 min at pH 9, and the determination is made within several seconds of preparing the luciferase in the solution of required pH. The decrease evidently corresponds

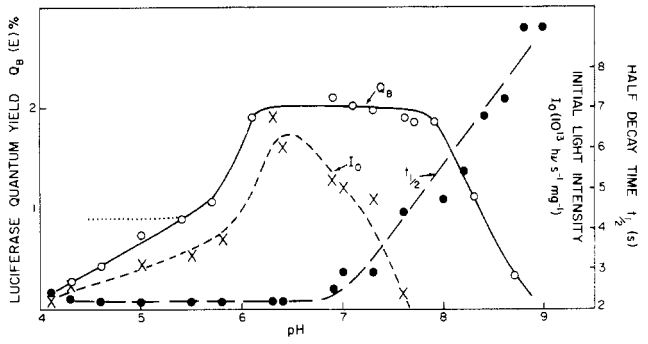

FIGURE 5: Dependence of BH luciferase quantum yield, Q_B(E), initial light intensity I_0 per mg of luciferase, and half-time of the light decay $t_{1/2}$, on pH. Specific activity of luciferase (pH 7) is 5.0×10^{13} $h\nu$ sec^{-1} mg^{-1} and the measurements were made by the routine assay procedure in buffer (23°): pH 4–5.8 (0.1 *M* phthalate), 5.8–7.5 (0.1 *M* phosphate), 7.5–9.0 (0.1 *M* Tris).

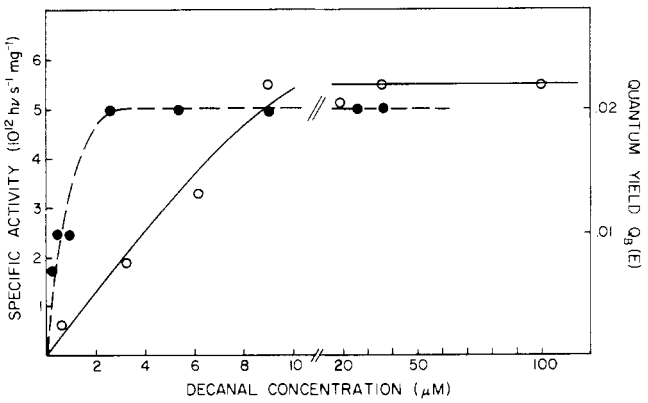

FIGURE 6: Quantum yield (●) and initial reaction rate, measured by specific activity (○), dependence on decanal concentration. The BH luciferase, specific activity at 23° 4×10^{13} $h\nu$ sec^{-1} mg^{-1}, was used at a final concentration 0.3 μ*M*. All reactions were done at 5° in phosphate buffer (0.05 *M*, pH 7) solution containing bovine serum albumin (0.5 mg/ml) and $FMNH_2$ (24 μ*M*).

to a genuine acid–base equilibrium, and either cysteine (p*K* = 8.3), as suggested by Nicoli et al. (1974), or flavine semiquinone (p*K* = 8.4) would be likely candidates.

On the acid side there is a sharp decrease in both Q_B(E) and I_0 below pH 6.0 which is, of course, the same as the p*K* of the imidazole side group of histidine. No change in k ($t_{1/2}$) occurs here so that it would seem that this ionization is not allowing a different *chemical* pathway but may be causing a change in the *efficiency* of light emission. All parameters appear to remain constant with further increasing acidity, at least as far as can be measured, since denaturation is quite rapid at pH 4 ($t_{1/2} \sim 30$ sec). The dotted line is the Q_B(E) corrected for extent of denaturation during the time of the measurement, with the assumption that the bound intermediates denature at the same rate as native luciferase.

On the basic side I_0 falls to half-maximum at quite a significantly lower pH than Q_B(E). The ionization affecting the rate-limiting step is evidently not the same as that which controls the overall efficiency of light production.

The Q_B(E) for PF luciferase is constant over the range pH 6.7–8.0 (Table II) and the other parameters have a behavior similar to that for BH luciferase in Figure 5 (results not shown).

At room temperature, Q_B(E) at pH 7 is proportional to luciferase specific activity but independent of its concentration, 2.5×10^{-4} to 2.5 mg/ml, and aldehyde concentration and carbon chain length, octanal through tetradecanal. For BH luciferase the aldehyde concentration for the normal assay was kept in the range 8–40 μ*M* because of the aldehyde inhibition. Based on the stoichiometry of the reaction and the single turnover condition, Q_B(E) is predicted to be the same as Q_B(RCHO). In fact, a measurement of Q_B(E) should determine the amount of active enzyme in a given protein preparation as suggested by Ward and Seliger (1974), who propose the term "specific light yield" as a more realistic measure of luciferase "purity" than specific activity or protein homogeneity.

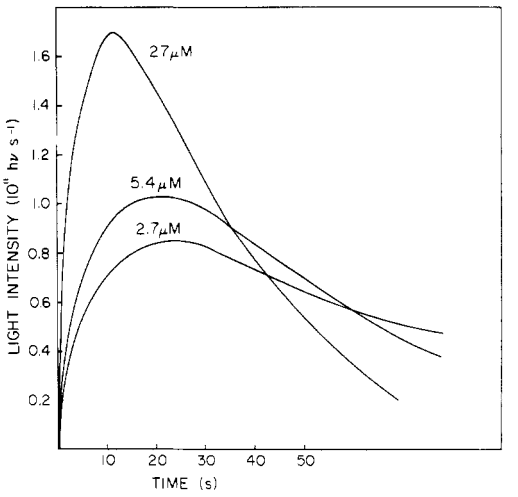

FIGURE 7: The light intensity vs. time for various decanal concentrations, when BH luciferase (0.3 μ*M*) is reacted with $FMNH_2$ (24 μ*M*) at 5° in buffer as in Figure 6.

The maximum observed specific activity of PF luciferase is 25×10^{13} $h\nu$ sec^{-1} mg^{-1} using dodecanal, and Q_B(E) is equal to 0.09, in good agreement with Q_B for dodecanal (Table II). High activity luciferase is unstable in solution and the activity decreases two to three times to a fairly constant level in a few days.

Quantum Yield of the Long-Lived Intermediate. The bioluminescence may also be initiated by adding aldehyde subsequent to the addition of $FMNH_2$ to E and O_2, and this fact has been interpreted as evidence of a long-lived intermediate, formed by the combination of oxygen, $FMNH_2$, and luciferase (Hastings and Gibson, 1963). In the absence of RCHO this intermediate decays in such a way that it is inactive for the light reaction. If the amount of decay in the period of delay before RCHO addition is corrected for, the quantum yield of the long-lived intermediate is the same as Q_B(E).

When RCHO is added last to the BH luciferase reaction it no longer shows the effect of inhibiting the reaction at higher concentration. Figure 6 shows the dependence of Q_B(E) and initial light intensity (specific activity) on RCHO concentration when the RCHO is added about 2 sec after the $FMNH_2$. The Q_B(E) reaches half-maximum at about 1 μ*M* RCHO, whether the RCHO is added subsequently or not. In the language of Michaelis–Menten, the RCHO K_m ($\sim$1 μ*M*) is the same for both reaction conditions. In contrast, the K_m for the reaction when RCHO is added last is about five times that for when it is there initially.

Figure 7 shows the time dependence of the light intensity for the reaction of the long-lived intermediate with RCHO. When RCHO is present in amounts above about the 27 μ*M*

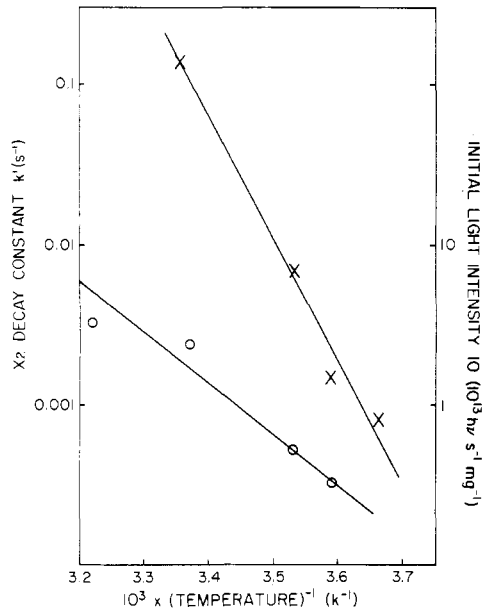

FIGURE 8: Arrhenius plot of first-order decay constant (×) of the BH luciferase intermediate X_2, and for the initial light intensity (○) from Table III.

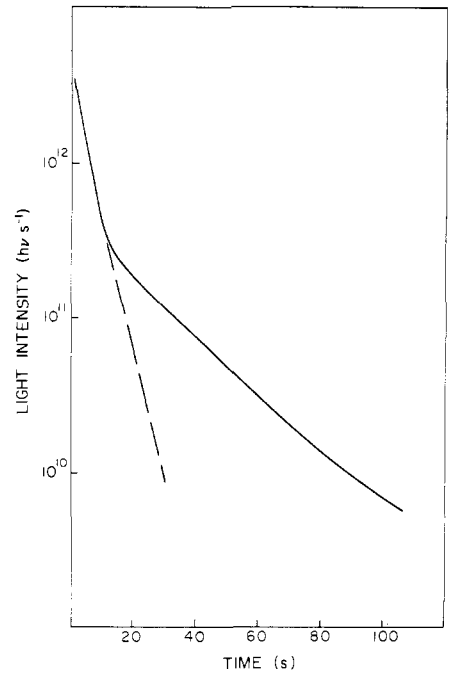

FIGURE 9: Decay of light intensity when BH luciferase (0.65 μM) is reacted at 23° with $FMNH_2$ (30 μM) in buffer (0.05 M phosphate, pH 7) containing bovine serum albumin (0.5 mg/ml) and a mixture of two aldehydes, decanal (25 μM) and dodecanal (25 μM). The rapid equilibrium kinetic model predicts the dashed line (see text).

shown in Figure 7 up to a saturation concentration, it is the same as when RCHO is present initially but within the optimum concentration range (4–40 μM). As RCHO concentration is decreased both I_0 and k decrease in a compensatory way to leave the quantum yield of the long-lived intermediate unchanged. That is, the areas under the curves of Figure 7 are all the same. Even though the reaction rate is clearly limiting in RCHO concentration, there can be no *effective* competition from the decay of an intermediate, even at room temperature or higher, otherwise a quantum yield decrease would be observed. Only when RCHO concentration <2 μM at room temperature or above is a quantum yield decrease to be observed, and so it is only below this concentration that a dark pathway becomes competitive.

Arrhenius plots for the rate of decay of the intermediate, k' (see scheme below) and for the rate represented by I_0, are shown in Figure 8. The activation energy for k' is 35 kcal mol^{-1} and for I_0, 14 kcal mol^{-1}. Therefore, branching at the point of the long-lived intermediate cannot be equally effective in reducing quantum yields at all temperatures over the range studied (Tables II and III).

Hastings et al. (1966) claimed that the addition of RCHO to a long-lived intermediate (II) required a model in which RCHO and II are in equilibrium with IIA:

$$\begin{array}{l} \text{II} + \text{RCHO} \rightleftharpoons \text{IIA} \dashrightarrow \text{light} \\ \quad\downarrow \\ \text{dark decay} \end{array}$$

This was based on an observation that when the long-lived intermediate from PF luciferase was reacted with a mixture of decanal and dodecanal, the rate of decay of light intensity was closely first-order with a value which was the mean of those obtained with decanal or dodecanal alone.

We are unable to reproduce this effect with PF luciferase. With BH luciferase, the difference in k's for decanal and dodecanal is much greater (Hastings et al., 1969), and Figure 9 shows that the addition of a mixture of the two in equal amounts yields a double exponential decay, one rate corresponding to the decanal k and the other to the dodecanal. The irreversible addition of RCHO is evidently the correct model and the results of the last two sections shows that competition from the rate of decay (k') of the intermediate (X) can therefore be neglected for practically all reaction conditions studied.

$$\begin{array}{l} \text{X} + \text{RCHO} \xrightarrow{k''} \text{X}' \dashrightarrow \text{light} \\ \quad\downarrow k' \\ \text{decay} \end{array} \qquad k' < 10^{-6}k''$$

Discussion

The quantum yield approach to stoichiometry (Lee, 1972) suffers from the ambiguity that the deduction of the stoichiometry is valid only if there is a single reaction pathway. Reaction branching would lead to utilization of one component through a nonlight pathway and consequently lower its quantum yield. Different reactions routes would be expected to change in a different way with alteration of external conditions and lead to variation in quantum yield ratios. Two main branching possibilities have been pointed out by Meighen and MacKenzie (1973) and by Hastings et al. (1973a). The first step, the binding of $FMNH_2$ to luciferase (E), has to compete with the rapid autoxidation of $FMNH_2$ (Gibson and Hastings, 1962; Hastings and Gibson, 1963; Gibson et al., 1966):

$$\begin{array}{l} \text{E} + \text{FMNH}_2 \rightleftharpoons \text{E–FMNH}_2 \xrightarrow[\text{RCHO}]{\text{O}_2} \text{light} \\ \quad\downarrow \text{O}_2 \\ \text{FMN} + \text{H}_2\text{O}_2 \end{array}$$

The broken arrow is used to signify that there are a number of further steps before light is produced. The stopped-flow results of Figure 3 show without doubt that when the luciferase concentration is sufficiently in excess to produce a

maximum $Q_B(FMNH_2)$, the production of FMN by autoxidation is no longer seen. The oxidation of $FMNH_2$ to FMN by O_2 on the luciferase is bimodal and each phase occurs to an equal extent.

The second possibility is the decay of a long-lived intermediate (X) formed by the reaction of O_2 with the E-$FMNH_2$ complex (Hastings and Gibson, 1963). This decay has been suggested to compete with the RCHO reaction (Hastings et al., 1973a) but both the temperature and the RCHO concentration independence of $Q_B(E)$ invalidate this possibility.

Lee (1972) suggested that loss of one luciferase bound $FMNH_2$ or intermediate in any of the steps of the reaction sequence at a rate equal to its utilization by the light reaction, would be an unlikely coincidence, and therefore proposed the two flavine overall reaction for light emission. The present results strengthen this proposal. It is not feasible that separate utilization of $FMNH_2$ would have the same rate dependence on external parameters such as temperature, type of luciferase, its specific activity, aldehyde, etc., as the rate of the light reaction. These factors change the overall bioluminescence rate by a factor of ten or more (McElroy and Green, 1955; Hastings et al., 1963; Hastings et al., 1969). Clearly, however, branching may be introduced under some conditions, such as by using a very low aldehyde concentration, or with shorter chain length aldehydes (hexanal), or with insufficient luciferase concentration, or at high pH.

The pH dependence of $Q_B(E)$, I_0, and k is very instructive. The sharp change at pH 6 of $Q_B(E)$ and I_0, but not k, suggests that it is not the chemical mechanism that is being affected at this point but the efficiency of light production in the last step of the reaction sequence, either through an effect on the efficiency of the production of the excited state or through some change in the fluorescence quantum yield of the emitter. The spectral emission has not been studied in detail in this region, but preliminary studies show no major shift in emission maximum. On the other hand, there is a change in the chemical rate in the basic direction since both I_0 and k decrease. This is not such as to introduce branching, however, until above pH 8, since $Q_B(E)$ and $Q_B(RCHO)/Q_B(FMNH_2)$ remain constant. Above pH 8 a new chemical reaction probably takes over. If an intermediate in the reaction is a luciferase-bound flavine semiquinone (E-$FMNH\cdot$), the situation is simply described by a dissociation:

$$\begin{array}{ccc} \text{E–FMNH}\cdot & \rightleftharpoons & \text{E–FMNH}\cdot^- \\ \downarrow & & \downarrow \\ \text{light} & & \text{dark} \end{array}$$

We have previously shown (Lee and Murphy, 1973b) that when the $FMNH_2$ is oxidized on the luciferase, free FMN is released early in the time sequence. In the presence of excess reducing agent this free FMN could be rapidly rereduced and be able to recycle back through the reaction. This was the reaction condition used by Meighen and Hastings (1971) and it is feasible that the rereduction process would lead to their observation of a first-order dependence of I_0, reflecting the steady-state rate of the light emission on the initial $FMNH_2$ concentration. It should also be noted that the inhibition effect of excess aldehyde is no longer seen in the reaction under reducing conditions (dithionite; Meighen and MacKenzie, 1973), the same behavior as the subsequent aldehyde addition demonstrated here. The lifetimes of certain quenchable species in the reaction sequence must be different for these two cases than in the normal reaction with aldehyde there from the start. We shall return to this point later.

Excess reducing agent was not present in the experiments of Watanabe and Nakamura (1972) who obtained the $FMNH_2$ by photoreduction. The ubiquity of traces of oxygen does not allow the extension of this technique below about 10^{-7} M ($FMNH_2$) with any reliability. This would probably not be low enough to render the reaction rate limiting in $FMNH_2$ concentration and thus show up a second-order dependence.

The utilization of oxygen in the light reaction is also biphasic (Lee and Murphy, 1973b). The slower rate is first order, but the faster one is too rapid for precise measurement with the oxygen electrode. The second mole of oxygen is taken up after the 2 mol of $FMNH_2$ have been oxidized to FMN and 1 mol of H_2O_2 formed. The slower oxygen rate is at a $t_{1/2}$ of 1.5 min (5°) and corresponds to the rate of formation of the long-lived intermediate (X_2). There must be an intermediate(s) before X_2, corresponding to the fully oxidized FMN state, and we shall refer to this (these) as X_1.

We can combine the present results of this paper with the oxygen utilization data and recent spectral studies and quantum yields of the reaction mixture (Murphy et al., 1974), to formulate the following reaction sequence (excess E):

$$\begin{gathered} \text{E} + \text{FMNH}_2 \rightleftharpoons \text{E–FMNH}_2 \\ \text{E–FMNH}_2 + \text{FMNH}_2 + \text{O}_2 \longrightarrow \text{X}_1 + \text{FMN} + \text{H}_2\text{O}_2 \\ \text{X}_1 + \text{O}_2 \longrightarrow \text{X}_2 \\ \text{X}_2 \rightleftharpoons \text{apoX}_2 + \text{FMN} \\ \text{X}_2 + \text{RCHO} \longrightarrow \text{light} \end{gathered}$$

The formation of X_1 is accompanied by the release of free FMN and H_2O_2. If the amount of X_2 is calculated as one-half the added $FMNH_2$, we find that $Q_B(X_2)$ is equal to $Q_B(RCHO)$ which is approximately equal to $Q_B(E)$ the uncertainty arising from the difficulty of making 100% active luciferase.

Before we can formulate a reaction sequence we need to know the nature of the intermediate X_2. This has been investigated by Murphy et al. (1974) who quantitatively converted BH luciferase to X_2 at 0°, where it has a half-life of about 15 min, then subjected the mixture to rapid chromatography on Sephadex G-25 to remove free FMN. Surprisingly it was found that practically all the FMN was removed by this procedure. By warming the eluted protein to destroy intermediates, the content of FMN was found to be less than 10 mol % of the original X_2. This preparation was called apo-X_2 since the holo-X_2, an FMN flavoprotein apparently, could be regenerated spectrally by adding back FMN. The equilibrium dissociation constant was 1.4 μM which is much closer to the observed K_m than the dissociation constant of $FMNH_2$ from native luciferase. Even on the assumption that only active luciferase binds $FMNH_2$ and the results of Table I are corrected for this, the dissociation constant of $FMNH_2$ from PF luciferase is about 10 μM, still ten times too large to correspond to K_m. The reports of binding of FMN to native luciferase could well arise from traces of the intermediate present in preparations, particularly of the BH type.

It was also found that $Q_B(\text{apo-}X_2) = Q_B(X_2)$, and that the specific activities (I_0 on adding aldehyde) of both were

the same as with the original luciferase. With the apo-X_2 the light reaction still requires at least a trace of FMN, since Q_B(apo-X_2) is significantly reduced once the concentration of FMN falls below a few tenths of a mole percent of apo-X_2. It is not known whether RCHO reacts first with X_2 or apo-X_2, but FMN evidently plays some role in the light emission process, as evidenced by the property of apoflavodoxin in quenching the reaction. It was suggested that FMN sensitizes the light emission.

The long-lived intermediate then is a mixture of apo-X_2 and X_2, and since the X_2 is a flavoprotein which breaks down to free FMN and H_2O_2, we suggest it has a composition FMN-E-OOH. The oxidizing power is in some hydroperoxide group on the luciferase. Hastings et al. (1973) claim from spectral evidence that the hydroperoxide is on the flavine, E-FHOOH, but this would clearly not allow the FMN to be removed while still preserving activity. In their study a time interval of only 10 sec at 5° was allowed before stopping the reaction by rapidly lowering the temperature to −20°, an insufficient time as we have shown, for complete oxygen uptake, and so their spectra probably correspond to an intermediate(s) somewhere in the region of X_1, or even to a mixture of FMN and $FMNH_2$.

While the details of the reactions remain to be investigated, the data available at the present time allow a reasonable conjecture to be made, at least for the reaction in the absence of RCHO up to the formation of X_2. We will write HSE for native luciferase to propose a role for a sulfhydryl group which Nicoli et al. (1974) have found to be essential for the reaction

$$\text{HSE–FMNH}_2 + \text{O}_2 \longrightarrow \text{HSE–FMNH}\cdot + \text{O}_2\cdot^- + \text{H}^+$$

$$\text{HSE–FMNH}\cdot + \text{FMNH}_2 + \text{O}_2 \longrightarrow X_1 + \text{FMN} + \text{O}_2\cdot^- + \text{H}^+$$

$$X_1 + \text{O}_2 \longrightarrow \text{HOOSE–FMN} + \text{O}_2\cdot^- + \text{H}^+$$

Preliminary evidence shows that E-FMNH· is formed in the first step of reaction (G. J. Faini, R. J. DeSa, and J. Lee, unpublished results) and this then reacts with unbound $FMNH_2$ or FMNH·, to make X_1. H_2O_2 is formed by the dismutation of $O_2\cdot^-$:

$$2\text{O}_2\cdot^- + 2\text{H}^+ \longrightarrow \text{H}_2\text{O}_2 + \text{O}_2$$

but neither H_2O_2 (Lee, 1972) nor $O_2\cdot^-$ are involved in the reaction pathway. Superoxide dismutase fails to quench the rate of the light reaction even at pH 9 where the $O_2\cdot^-$ is much more stable (J. Lee, unpublished observation). With crude luciferase no H_2O_2 is found (J. Lee, unpublished observation). A *c*-type cytochrome has been isolated from these bacterial extracts (Lee et al., 1974) and possibly some contamination remains in preparations to reduce the H_2O_2 yield by intercepting $O_2\cdot^-$. Superoxide dismutase has been isolated from luminous bacteria (Puget and Michelson, 1974) and the system has been found by Cormier and Totter (1954) to reduce menadione, a good $O_2\cdot^-$ acceptor.

Aldehyde appears to be able to act as a specific radical quencher if present early in the reaction sequence. The initial oxidation on PF luciferase is much faster than BH, and the aldehyde quenching is consequently much less noticeable with PF. If RCHO is added last, the process has probably gone through to X_2, which is no longer so readily quenchable. Quenching ability depends very much on the lifetime of intermediates, which is clearly a function of the type of luciferase. For instance nonspecific radical quenchers, such as hydroxylated cresols, show a marked effect with one type of luciferase "A13", but not with BH or PF (Lee et al., 1974).

Aldehyde must play a second role early in the reaction sequence besides this quenching. The steady state of light emission (Figure 7) is achieved at a time shorter than required for oxygen utilization or complete $FMNH_2$ oxidation in the absence of RCHO. Since the formation of X_2 must be irreversible, we suggest that RCHO acts in the same way as found for the second substrate in other hydroxylases (White-Stevens et al., 1972). The presence of the second substrate in salicylate hydroxylase accelerates the reduction of the flavoprotein by NADH. Since we find that $Q_B(E) = Q_B(X_2)$ we conclude that the presence of RCHO cannot change the overall result up to X_2, but what the real function of the RCHO is here and what the real nature of these postulated intermediates is in reaction sequence, is the subject of ongoing investigation.

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