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Bacterial Bioluminescence. Quantum Yields and Stoichiometry of the Reactants Reduced Flavin Mononucleotide, Dodecanal, and Oxygen, and of a Product Hydrogen Peroxide†

John Lee

ABSTRACT: Conditions are described for obtaining the maximum bioluminescence quantum yields for each component of the *in vitro* bacterial bioluminescence reaction. With optimum concentration of *Photobacterium fischeri* luciferase the quantum yields are: for reduced flavin mononucleotide 0.048, dodecanal 0.1, oxygen 0.048, and for an identified product hydrogen peroxide 0.12. Under optimum conditions the reaction of reduced flavin and luciferase is probably the sole chemical pathway and its overall stoichiometry can be inferred from the quantum yield ratios as: $2FMNH_2 + 2O_2 + RCHO \rightarrow 2FMN + H_2O + H_2O_2 +$ product. The quantum yield ratio 2:1 of dodecanal to reduced flavin mononucleotide is strong evidence for aldehyde utilization in the reaction. Present evidence is unable to distinguish whether this overall reaction represents a single pathway or is the sum of two independent enzyme reactions which proceed with equal velocities. The maximum flavin quantum yield is independent of oxygen concentration and of the purity of the luciferase preparation used (60% to crystalline). Both the flavin and dodecanal maximum quantum yields are reduced at very high luciferase concentration and both are unaffected by the presence of catalase which excludes free peroxide as a reaction intermediate.

A chemiluminescence reaction may be represented by a molecule A reacting with B to give the excited state of the product, C*:

$$\begin{array}{l} A + B \longrightarrow C^* \longrightarrow C + h\nu \\ \downarrow \\ D \end{array} \qquad (1)$$

Competing with this light path may be a dark reaction yielding a product D. The observed chemiluminescence quantum yield with respect to A, $Q_c(A)$[1] will depend on the number of A molecules which disappear by the light over the dark path.

If the concentration of B is sufficiently high the dark reaction rate will be negligible in comparison to the light reaction and $Q_c(A)$ will approach a maximum value. Under this condition it can be predicted for reaction 1 that $Q_c(A) = Q_c(B) = Q_c(C)$.

As a corollary the relative maximum Q_c of each component in a reaction will be a measure of the stoichiometry of that component. This stoichiometry is unity for each component in reaction 1 but if instead two molecules of A were consumed for each C produced then $Q_c(A) = 0.5Q_c(B)$.

Chemiluminescence and bioluminescence reactions usually take place under vigorous oxidizing conditions and the presence of dark reactions cannot be neglected. In a study of the luminol chemiluminescence, reaction conditions were found to fall into two groups according to the magnitude of Q_c (luminol) (Lee and Seliger, 1972). Aqueous hydrogen peroxide oxidation (pH > 11) or oxidation under basic aprotic conditions led to maximum values of Q_c, around 0.0125. Under these conditions various factors such as temperature, solvent polarity, or reactant concentration were without effect on Q_c, from which it was inferred that there were no competing dark chemical reactions. These factors strongly influence Q_c under other oxidizing conditions such as ferricyanide, where Q_c was much lower and competing chemical reactions were suggested. The Methylene Blue photosensitized chemi-

† From the Department of Biochemistry, University of Georgia, Athens, Georgia 30601. *Received January 18, 1972.*

[1] The quantum yield of chemiluminescence or bioluminescence with respect to a reaction component is the total number of photons emitted divided by the total change as a result of the reaction in the number of molecules of that component.

luminescence oxidation of luminol was analyzed in some detail in terms of branched chemical pathways (Matheson and Lee, 1970).

The *in vitro* reaction of bacterial bioluminescence involves $FMNH_2$,[2] oxygen, bacterial luciferase, and a long-chain aliphatic aldehyde such as dodecanal (Strehler, 1953; Cormier and Strehler, 1953; Strehler and Cormier, 1954; Strehler *et al.*, 1954). Since the discovery of the aldehyde requirement by Cormier and Strehler (1953) the question of whether it is consumed in the reaction or has a catalytic function has been argued back and forth (for recent reviews, see Cormier and Totter, 1968; Hastings, 1968; Airth *et al.*, 1970). Certainly products of aldehyde reaction at the concentration levels likely to be found would be very difficult to isolate and characterize. In addition the whole kinetic picture has been confused by the dominance of side chemical pathways.

In this work the component concentration conditions for producing maximum bioluminescence quantum yield Q_B are first determined. Under these conditions the Q_B's are found to be in the ratio of 1:1:2:2 for $FMNH_2$, O_2, dodecanal, and hydrogen peroxide, which is identified as a product. Since the Q_B (dodecanal) is comparatively high (0.1) it seems reasonably likely that all the components react on luciferase under these concentration conditions and the stoichiometry of this *overall* reaction can be written as

$$2FMNH_2 + 2O_2 + RCHO \xrightarrow{E} 2FMN + H_2O_2 + [H_2O] + [\text{product}]$$

The square brackets represent an unidentified component. This overall reaction may result from the sum of two separate reactions on the luciferase which occur with equal rates. The first produces light and the second hydrogen peroxide by direct oxygen oxidation of bound reduced flavin. An alternative view is that there is only one reaction occurring and that the light emission requires two reduced flavins and yields hydrogen peroxide. Peroxide is also a product in the suggested mechanism of the chemiluminescence of certain aldehydes (Rapaport *et al.*, 1972).

The overall negative free energy of this reaction assuming that the "product" is RCOOH is more than sufficient to satisfy the energetic requirement for photon production. The definite stoichiometry of RCHO:$FMNH_2$ makes fairly certain that RCHO is consumed in the reaction. The consumption of only 2 moles of oxygen and the production of 1 mole of peroxide eliminates the decarboxylation mechanism which has been proposed for the excitation mechanism in the bioluminescence of higher organisms (for recent reviews, see McCapra, 1970; White *et al.*, 1971).

Experimental Section

Chemicals. Flavin mononucleotide was a gift from the Sigma Chemical Co. and was purified by DEAE-cellulose column chromatography according to the method of Massey and Swoboda (1963). Methanol, cyclohexane, and dimethyl sulfoxide were spectroscopic grade solvents dried over Zeolite (Norton Co., Akron, Ohio). Luminol (3-aminophthalhydrazide, Eastman Chemicals, Rochester, N.Y.) was recrystallized until white from acetic acid. All other chemicals used were the best grades available commercially.

Absorption Measurements. All spectra were measured on a Cary 14 spectrophotometer.

Light Measurements and Calibration. For all determinations except $Q_B(O_2)$ and $Q_B(H_2O_2)$ reactions were carried out in a cylindrical scintillation vial at room temperature (23°) and a photomultiplier viewed the reaction through the bottom of the vial. The arrangement was similar to that used for the luminol Q_c determination (Lee and Seliger, 1965). For the measurements of $Q_B(O_2)$ and $Q_B(H_2O_2)$ the same photomultiplier viewed the reaction (20°) through the side of the chamber (Figure 1) in an otherwise unchanged geometrical arrangement.

The absolute light calibration of each arrangement was determined by using the luminol chemiluminescence reactions in aqueous solution and in dimethyl sulfoxide as secondary light standards (Lee *et al.*, 1966). The calibrations were corrected for differences in overlap between the phototube spectral sensitivity and the emission from the luminol and bioluminescence reactions, and for differences in solvent refractive index (Lee and Seliger, 1965).

At high light levels nonlinear phototube effects were avoided by optical attenuation using a color filter (Corning C.S. 3-70). The transmission of the filter for the overlap of the bacterial bioluminescence and the phototube response was determined using a low level emission from an *in vivo* culture of *Photobacterium fischeri* bacteria. The solution was used in a 1:5 dilution (3% NaCl) and the light signal with and without the filter in position was used to determine the filter attenuation. The *in vivo* emission spectrum is known to be the same as *in vitro* (Seliger and Morton, 1968).

The absolute accuracy of the Q_B reported here is ±20%. This includes the uncertainties in the Q_c(luminol), the calibration procedure, precision of the measurements, and purity of the reaction components. Recent comparisons with the absolute fluorescence yield of quinine (Lee and Seliger, 1972) and with the absolute light intensity from the O–NO gas phase reaction (Fontijn and Lee, 1972) show the luminol reaction to be a reliable secondary standard.

Preparation and Purification of Luciferase. Cells from a strain of *P. fischeri* originally derived from ATCC 7744 were grown in 400-l. batches in the basal medium developed by Farghaly (1950) supplemented with bactopeptone (Difco, 10 g/l.) at pH 7.4, 25°. Cells were harvested at the peak of light emission (100–200 $h\nu$ sec^{-1} $cell^{-1}$) and density (2–4) × 10^9 cells ml^{-1}. Purification of luciferase was by procedures modified from those described previously (Kuwabara *et al.*, 1965; Hastings *et al.*, 1965). The cells were lyzed, and the protein was fractionally precipitated with ammonium sulfate and chromatographed on Sephadex G-75 and then passed once or twice down DEAE-cellulose using a shallow gradient of NaCl in Tris (0.01 M) buffer for elution. This procedure is similar to a recently published three-step technique which was stated to produce luciferase in a state of 95% purity (Gunsalus *et al.*, 1972). Crystalline luciferase was also prepared by dialysis against ammonium sulfate.

The absolute specific activities of these luciferase preparations were in the range (4–6) × 10^{13} $h\nu$ mg^{-1} sec^{-1} for the less pure to the crystalline preparations and correspond to the activity measured previously for crystalline luciferase (Eley *et al.*, 1970). The level of impurities was estimated by acrylamide gel electrophoresis using 50 μg on the gel. From the intensity of several minor bands in the less pure luciferase preparations the major component was estimated to be 60–

[2] Abbreviations used are: FMN, riboflavin 5′-monophosphate; $FMNH_2$, fully reduced FMN; RCHO, long-chain aliphatic aldehyde; $Q_B(A)$, bioluminescence quantum yield with respect to a reactant A, $Q_B'(D)$ with respect to a product D.

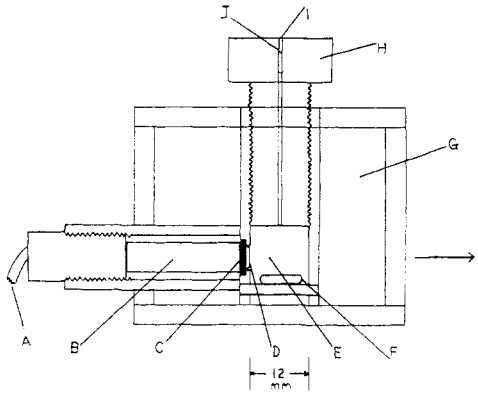


FIGURE 1: Oxygen quantum yield Lucite reaction vessel. Volume of chamber E is 1.5 ml. Light is emitted and viewed by the photomultiplier in the direction indicated by the arrow: A, electrode leads to potentiometer; B, barrel of oxygen electrode; C, "O" ring seal; D, membrane; E, chamber; F, magnetic stir bar; G, constant temperature water; H, reaction volume control plug; I, 18-gauge capillary; J, liquid level.

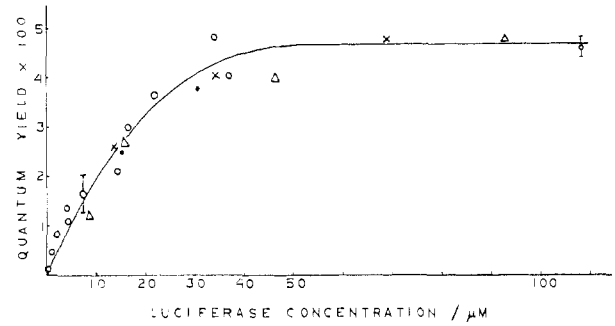


FIGURE 2: Bioluminescence quantum yield of $FMNH_2$ dependence on the concentration and purity of the bacterial luciferase in air-saturated solution at pH 7.3, 23°. Estimated purities of the luciferase preparations are x, 95% (crystalline); ○, 90%; ●, 70%; △, 60%. Reaction mixture: 1 ml of luciferase, 10 μl of dodecanal in methanol, 0.1 ml of $FMNH_2$ of known concentration in the range 40–160 μM.

70% of the total. The preparations of different apparent purities were used for quantum yield measurements.

The characteristics of this crystalline luciferase correspond to those reported for *P. fischeri* ATCC 7746 by Hastings *et al.* (1969) except for the absolute specific activity.

Light Reactions. An aliquot of luciferase in Tris or phosphate buffer (pH 7) of known concentration from OD (280) (1 OD $cm^{-1} \sim 1$ mg/ml) was added to 2 ml of buffer in the vial. If the final luciferase concentration was below 0.1 mg/ml, bovine serum albumin (1 mg/ml) was also added (Hastings *et al.*, 1966). A 10-μl volume of air-saturated methanol solution saturated with dodecanal was then added (Eley, 1968). This quantity of dodecanal is about 400 nmoles, sufficient to saturate the requirement for maximum light intensity. Methanol did not interfere with the reaction (<100 μl), and the same luciferase activity was obtained as by using a sonicated aqueous suspension of aldehyde (Hastings *et al.*, 1965).

The reaction was initiated by rapid injection of 0.5 ml of $FMNH_2$. The $FMNH_2$ was conveniently produced by photoreduction of an FMN solution in the presence of EDTA (5 mM). The FMN solution for assay usually was of unit OD (445 nm) which had a nominal concentration of 8×10^{-5} M (ε 12,500, Koziol, 1971; Mayhew, 1971). Photoreduction was achieved by illuminating a syringe containing the solution with a 40-W tungsten light source. If the FMN solution had been previously deoxygenated by bubbling about 5 min with N_2, about 15 sec was usually sufficient for completely decolorizing the FMN and full reduction. This was verified by the fact that the solution would titrate mole for mole with oxygen (see Results). Use of a higher EDTA concentration (20 mM) or longer irradiation times did not change either the assay or the quantum yield results. If the solution warmed during irradiation it was brought to room temperature before addition. If the FMN was reduced by H_2/Pt no different results were obtained. However the photoreduction technique had the advantage of allowing more accurate and convenient solution volume measurements.

Measurement of Oxygen Concentration. A reaction chamber (Figure 1) was built to hold a Clark oxygen probe (Yellow Springs Instruments, Yellow Springs, Ohio, Model 4004). The solution to be reacted (1.5 ml) was placed in the cell, and the plug was screwed down until the liquid filled the central capillary. The cell was maintained at 20 ± 0.2°. A gas-tight syringe (Hamilton Co., Whittier, Calif.; 1000 μl), containing $FMNH_2$ both in the barrel and the needle, was placed with its needle down the central capillary, and the reaction was initiated. The light output was monitored by a phototube on the side opposite the electrode and the oxygen concentration changes by the electrode output operating into a simple potentiometric circuit.

Results

Quantum Yield of $FMNH_2$. The first step in the reaction leading to bacterial bioluminescence is believed to be binding of $FMNH_2$ to luciferase (E) (Hastings and Gibson, 1963; Gibson *et al.*, 1966; Meighen and Hastings, 1971). For the purpose of discussion a simplified competitive situation can be imagined with binding occurring at a rate k_1 and homogeneous oxidation with molecular oxygen which does not lead to light emission at a rate k_4.

$$FMNH_2 + E \xrightarrow{k_1} [E\text{-}FMNH_2] \quad (2)$$

$$FMNH_2 + O_2 \xrightarrow{k_4} FMN + H_2O_2 \quad (3)$$

At a sufficiently high value of luciferase concentration [E], $k_1[E] \gg k_4[O_2]$ and $Q_B(FMNH_2)$ would be expected to approach a maximum value. This is shown in Figure 2. Each point is the average of three to five determinations using an $FMNH_2$ concentration for initiation of about 5 μM in the reaction mixture. The reactions were carried out with luciferase preparations of different purities as determined by disc gel electrophoresis and the [E] determined from the purity using a molecular weight of 80,000 (Hastings *et al.*, 1969). The maximum $Q_B(FMNH_2)$ is seen to be uninfluenced by the extent of impurities present in the luciferase preparations which demonstrates the unimportance of other enzymatic $FMNH_2$ reactions in these measurements (Gibson *et al.*, 1966). At [E] = 10 μM, where the Q_B should be most sensitive to these changes, the effect of higher [EDTA] or longer photoreduction times or dithionite reduction was tested and found to give the same value for $Q_B(FMNH_2)$. Also at this concentration the [$FMNH_2$] was varied over a small range 2–10 μM. At the higher end the $Q_B(FMNH_2)$ would decrease slightly readily explained by a lack of turnover of the luciferase (Hastings *et al.*, 1965). At the lower end a drop also

occurred presumably due to the difficulty of maintaining the $FMNH_2$ quantitatively in the reduced state due to traces of oxygen. Optimum levels of [$FMNH_2$] were therefore around 5 μM in the reaction mixture. The maximum $Q_B(FMNH_2)$ ([E] > 50 μM) was independent of [$FMNH_2$] over the range 4–16 μM. The amount of dodecanal added was determined to be saturating at all [E] used.

The reactions were carried out at 23° by rapid injection of the $FMNH_2$ (0.1 μl). The light intensity typically rose to a maximum in about 1 sec and then decayed over a period of about 30 sec. The decay was not strictly exponential in contrast to the reports of other workers (Hastings and Gibson, 1963; Hastings, 1966, 1968; Hastings *et al.*, 1965, 1966). The $Q_B(FMNH_2)$ was then calculated by dividing the total integrated light yield by the total $FMNH_2$ added.

If the FMN from the Sigma Chemical Co. was used without purification a maximum $Q_B(FMNH_2)$ of only 0.030 was obtained. On purification this increased by 1.4 times. As a result of his study of the binding of FMN to flavodoxin, Mayhew (1971) suggested that the DEAE-cellulose purified FMN is only about 85% pure. In calculating Q_B therefore the purity has been assumed to be 90% with an uncertainty of ±10% and the ϵ (445 nm), 12500 M^{-1} cm^{-1}.

The maximum $Q_B(FMNH_2)$ was found to be independent of [O_2] 10–280 μM. Figure 3 shows however that in the lower [O_2] range, 10–150 μM, there was an optimum value for [E]. At higher concentrations Q_B decreases. This optimum [E] is approximately twice the [$FMNH_2$]. Different [$FMNH_2$] were not examined and this "stoichiometry" may be purely fortuitous. However an indication of a luciferase stoichiometry also arises from the Q_B(RCHO) data (Figure 6). In calculating [E] for Figure 3 and subsequent figures an average luciferase purity of 75 ± 15% was assumed.

The crosses in Figure 3 are the results from reactions carried out in the presence of catalase. The $Q_B(FMNH_2)$ is not significantly lowered showing that free hydrogen peroxide generated from oxidation of $FMNH_2$ is not an intermediate in the bioluminescence pathway.

The final maximum value for $Q_B(FMNH_2)$ is estimated from the data of Figures 2 and 3 as 0.048 ± 0.005. The error represents the overall precision of measurement, *i.e.*, the repeatability and uncertainty in concentration.

Quantum Yield of Dodecanal. The purity of the dodecanal was judged to be at least 90% on the basis of refractive index, n_D^{20} 1.4350 (Handbook of Chemistry and Physics, 1969; 1.435) and extinction coefficient ϵ in cyclohexane 16 M^{-1} cm^{-1} at 280 nm (Perkampus *et al.*, 1966; ϵ 16). Likely impurities are aldehydes of other chain lengths, dodecanoic acid, and peroxides. By gas chromatography there are only small amounts of the other aldehydes in these samples. The level of acid is negligible since it does not contribute to ϵ (280 nm). Dodecanoyl peroxide is insoluble in methanol but its ϵ (cyclohexane) is 16 ± 2 (Perkampus *et al.*, 1966, ϵ 16). The presence of traces of water in methanol makes ϵ in this solvent less precise, presumably due to aldehyde hydration. From these criteria it was decided that the purity of the dodecanal was satisfactory for the present measurements, the uncertainty being ±10%.

The light reactions were carried out under limiting concentrations of dodecanal and excess of all other components. The most reproducible technique for adding the dodecanal was to take a 100-μl volume of pure dodecanal, which from density corresponds to 454 μmoles, and dilute it to 5 ml in cyclohexane. A fifty times dilution of this into methanol would then be made, and a 25-μl volume added to the reaction.

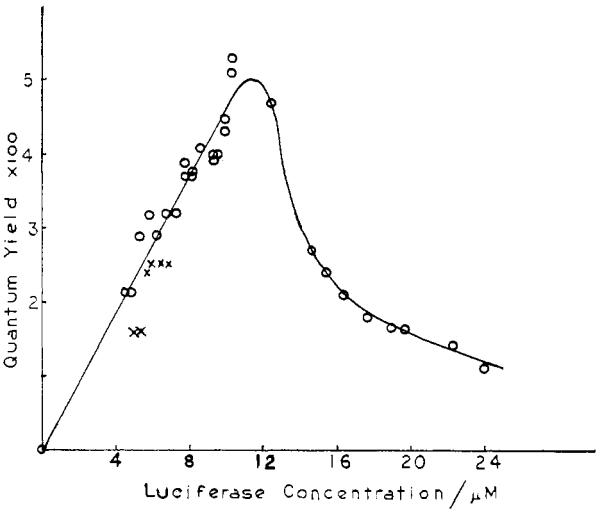


FIGURE 3: Bioluminescence quantum yield of $FMNH_2$ dependence on the concentration of bacterial luciferase in a solution at low oxygen concentration (<150 μM) (pH 7.3, 20°). The crosses indicate reaction in presence of catalase (1 mg/ml). Reaction mixture: 1 ml of luciferase, 10 μl dodecanal in methanol, 0.1 ml of $FMNH_2$ of known concentration in the range 40–160 μM.

Methanol was used here since cyclohexane inhibits the reaction. Both solvents were flushed with N_2 prior to use to remove oxygen which could deplete the aldehyde by autooxidation. Separate experiments showed that hydrate formation as judged by loss of OD (280) in methanol did not affect the Q_B(RCHO) or the activity of the dodecanal in the luciferase assay procedure. For this reason a direct estimate of [RCHO] in methanol cannot be made by OD (280) since the formation of hydrate causes the available aldehyde to be underestimated. Although the light yield from the methanol solution was unchanged at least overnight (Table I), fresh dilutions from pure dodecanal were made before each determination. Using different volumes for dilution and addition to the light reaction gave consistent results.

The light reactions were initiated by rapid addition of 0.5 ml of $FMNH_2$ (30 nmoles) into a 1.5-ml volume of luciferase in air-saturated buffer at room temperature. Although the amount of aldehyde (second column, Table I) is limiting in the reaction, it was not all reacted on the first addition of $FMNH_2$ under any of the conditions examined. Addition of another 0.5 ml of $FMNH_2$ produced more light and this could be repeated until, presumably, the limiting component, dodecanal, was completely exhausted. Figure 4 shows the results of a typical series of additions when the total light obtained on each addition was plotted against the accumulated [FMN]. For all reactions studied the individual light yields fall logarithmically with increasing [FMN] after the first one or two additions. The first addition where the [FMN] is calculated on the basis of the total added in that first reaction was usually a maximum, indicated by the broken line, and contained 30–50% of the total light yield. At the highest [E] used about 80% of the total light could be obtained on the first addition. Presumably all the aldehyde could be completely reacted in this way by going to sufficiently high [E].

All light yields have been corrected for self-absorption of the bacterial emission by overlap with the long-wavelength tail of the FMN absorption. This approximate correction, f_a, applies to the light that escapes the reaction cell and varies with [FMN] by the relation given by Parker (1968), f_a =

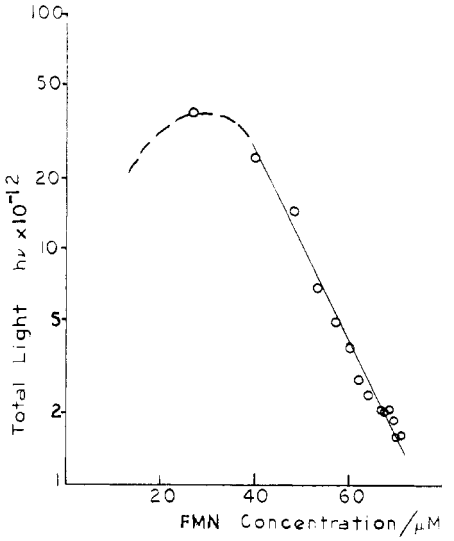


FIGURE 4: Total light obtained on each addition of $FMNH_2$ (30 nmoles) to the luciferase reaction mixture under limiting amounts of dodecanal (10 nmoles). The initial reaction (1.5 ml) increases in FMN concentration on each addition of $FMNH_2$.

$(2.303\overline{OD}x)/(1 - \text{antilog}(-\overline{OD}x))$, where $\overline{OD}$ (cm^{-1}) is the optical density of the solution averaged with the bioluminescence spectral overlap and x is the depth of the bioluminescence solution viewed by the detector. The range of f in these experiments was 1.03–1.47. In addition, trapped light will be absorbed and reemitted as FMN fluorescence (Fontijn and Lee, 1972), and the data have also been adjusted for this small enhancement.

Table I lists the values of Q_B(RCHO) under optimum and other conditions. The Q_B is calculated by summing the light q_i obtained on each addition of $FMNH_2$ and then adding the remaining area under the line shown, for instance, in Figure 4 by extrapolating i to infinity. In a typical experiment 15 additions were made

$$Q_B = \sum_{i=1}^{15} q_i + \int_{i=16}^{\infty} q_i di - EQ_E$$

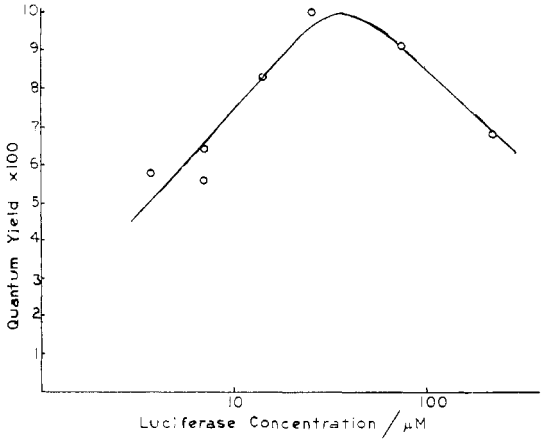


FIGURE 5: Bioluminescence quantum yield of dodecanal dependence on the concentration of luciferase in the initial reaction volume (2 ml, pH 7.3, 23°). Initial amount of dodecanal was 9 nmoles. Luciferase blank light yield has been subtracted.

TABLE I: Bioluminescence Quantum Yields of Dodecanal. Concentrations of Luciferase [E] is the Value in the Initial Reaction Volume of 1.5 ml (23°).

[E] (μM)	RCHO (nmoles)	Q_B(RCHO)	Condition
1.7	3.8	0.103	Maximum conditions, average = 0.10
8.4	7.0	0.108	
8.4	6.6	0.102	
8.4	6.6	0.103	
8.4	6.6	0.092	
26	6.6	0.102	
26	6.6	0.092	
8.4	18.2	0.090	
1.7	18.2	0.050	
1.7	9.1	0.050	
1.7	9.5	0.049	Methanol–RCHO solution stirred over glass beads 16 hr
23	9.5	0.096	
23	9.5	0.088	Catalase (1 mg/ml)
20	4	0.099	Dithioerythritol (3×10^{-4} M)
0.1	50	0.004	
0.1	50	0.001	KI (10^{-4} M)
0.1	50	0.001	BHT (10^{-4} M)

where E is the amount of luciferase (mg) present and Q_E the aldehyde blank, that is the total light obtained without the addition of dodecanal. Typically $Q_E = 8 \times 10^{12}\ h\nu\ mg^{-1}$ and is only 1–2% of Q_B in most experiments. The extrapolated area given by the integral usually amounts to about 5% of Q_B.

Figure 5 shows that the Q_B(RCHO) depends on the [E] in a manner like $Q_B(FMNH_2)$ (Figure 3). Comparison of some results in Table I with Figure 5 shows that the maximum Q_B(RCHO) is usually achieved when [E]/[RCHO] is at least unity. Table I lists the maximum values of Q_B(RCHO) and compares these with results obtained under other conditions. A high concentration of catalase produced a lowering but it is not significant. This demonstrates that free hydrogen peroxide derived from aldehyde cannot be intermediate in the light reaction.

No loss of dodecanal occurs on standing the methanol solution overnight or by adsorption on the glass surfaces as demonstrated by the addition of the glass beads.

It would be expected that hydrogen peroxide generated by the homogeneous oxidation of the excess $FMNH_2$ would remove dodecanal in a nonlight reaction competing with the enzyme path. This would explain the drop-off in Q_B(RCHO) at low [E] (Figure 5) but is in disagreement with the assumptions of McElroy and Green (1953) and Cormier and Totter (1957) who suggested that aldehyde is utilized *only* by a luminescent pathway.

Table II shows the difference in dodecanal utilization in the presence and absence of luciferase. Since dodecanal is limiting it can easily be demonstrated that at any given concentration of luciferase and FMN, the initial flash height and, more precisely, the q_i is proportional to the amount of dodecanal present. The sum $q_6 + q_7 + q_8$ is presented in each column as a

TABLE II: Differences in Dodecanal Utilization.

Number of Additions of 30 nmoles of $FMNH_2$ i	Light Yield ($q_i/10^{12}$ $h\nu$)[a]		
	A	B	C(FMN)
1	52.7	0	0
2	49.4	0	0
3	38.5	0	0
4	33.6	0	0
5	25.8	0	0
Total	200.0		
6	19.5	23.6	28.2
7	16.9	17.7	23.5
8	13.5	17.6	22.0
Total	49.9	59.9	73.7

[a] Utilization of aldehyde in : (A) complete reaction with dodecanal (11 nmoles), luciferase (20 nmoles); (B) dodecanal (11 nmoles), luciferase not added until after the $i = 5$ addition of $FMNH_2$; (C) dodecanal (11 nmoles), luciferase (20 nmoles), FMN added for $i = 1$–5. Reaction concentrations are initial values in a 2.5-ml initial volume (pH 7.3, 23°). Luciferase in the absence of aldehyde produces a blank light yield at a level of 1–2% and this has been subtracted from each measurement.

measure of the amount of aldehyde left after each reaction, $i = 1$–5. In the complete system A, 200×10^{12} $h\nu$ are emitted in the first five additions. In B the luciferase was not added until after q_5 and it is seen that the aldehyde ($q_6 + q_7 + q_8$) is lower than in C where the same concentrations of FMN not $FMNH_2$ had been used for the first five additions. Therefore, the $FMNH_2$ does react with aldehyde by a homogeneous path at a rate which must be less than half the enzymatic rate at this luciferase concentration. For optimum Q_B(RCHO) the luciferase must be increased five to ten times, and under these conditions the homogeneous reaction can be neglected. Under optimal conditions it is proposed that aldehyde is utilized entirely by the light reaction, but under other conditions a dark utilization path may compete. From the data in Table II, the amount of dodecanal utilized by the emission of 200×10^{12} $h\nu$ is [(73.7 − 49.9)/73.7]11 = 3.55 nmoles, and $Q_B \cdot$(RCHO) is therefore 0.085, a value quite consistent with the total light results in Table I and Figure 5.

The homogeneous oxidation path is by a free-radical mechanism, and it was expected therefore that the addition of radical traps KI or BHT would inhibit this process and favor the light path. Table I shows that this did not happen. The $Q_B \cdot$(RCHO) was decreased, which might be taken to mean that free radicals are also involved in the light reaction. In the luminol chemiluminescence these quenchers in ten times higher concentration are without effect on the Q_c(luminol) (Lee and Seliger, 1972). This shows that there are no luminol free radicals involved in the chemiluminescence although the rate of this reaction is decreased by the inhibitors. The sulfhydryl stabilizer dithioerythritol, which should be also a potent free radical inhibitor by virtue of its thiol groups, is without influence on the maximum Q_B(RCHO). This argues against free-radical involvement in this bioluminescence reaction. These questions are under current study in this laboratory.

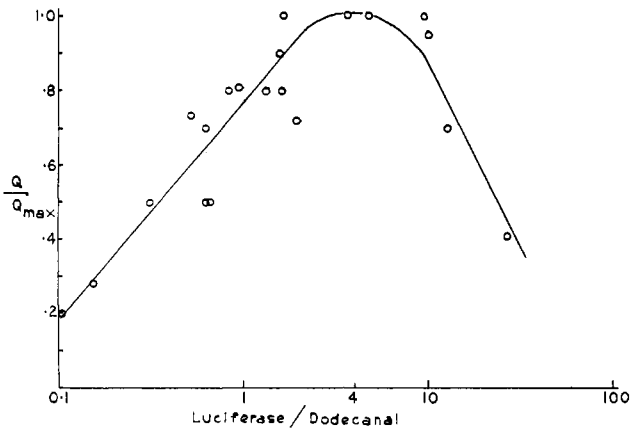


FIGURE 6: Dependence of the ratio of the bioluminescence quantum yield of dodecanal to the maximum observed value (Figure 5) on the ratio of the amount of luciferase to dodecanal in the reaction mixture.

Finally Figure 6 presents the Q_B(RCHO) from a number of measurements at different concentrations and enzyme:aldehyde ratios. It shows again that the condition of maximum Q_B is at a luciferase concentration suggestive of a stoichiometric ratio of two to four over dodecanal. When the luciferase is in excess of four times the aldehyde, the Q_B decreases. A similar behavior has been already noted for $Q_B(FMNH_2)$ and these results are probably connected.

The maximum value of Q_B(RCHO) is 0.10 ± 0.012 (Table I). The error represents the overall precision from the reproducibility of the measurements and the uncertainty in the purity of the dodecanal.

Previous estimates of Q_B(RCHO) are 0.03 (Cormier and Totter, 1957), 0.3 for both Q_B(RCHO) and $Q_B(FMNH_2)$ (Hastings *et al.*, 1965; Hastings, 1966), and 0.01–0.02 for $Q_B(FMNH_2)$ (Eley *et al.*, 1970). The requirement for optimum luciferase:aldehyde ratio was not recognized by these previous workers, and it is now apparent that the measurements of Hastings are subject to systematic error (Hastings and Reynolds, 1966; Lee and Seliger, 1972; Fontijn and Lee, 1972).

Quantum Yield of Oxygen. The oxygen utilization in the bioluminescence reaction was found by simultaneously measuring the light emission and the change in oxygen concentration, $[O_2]$, on a single addition of 0.10 ml of $FMNH_2$ standard solution (5–10 nmoles) to 1.5 ml of a solution of luciferase and dodecanal in Tris buffer (pH 7.3, 20°). The reactions were carried out in a constant volume (Figure 1), and oxygen from the air was found not to diffuse down the capillary at a significant rate.

The volume of added solution was a significant part of the total volume, and the $[O_2]$ was therefore found to change both by dilution and reaction. An absolute $[O_2]$ calibration technique was devised to include this dilution effect. This was done by observing the electrode potential change on addition of 0.10 ml of Tris buffer thoroughly equilibrated with air at 20°. The electrode calibration was carried out either in buffer or *in situ* in the reaction mixture itself at the end of an experiment. Results were consistent and corresponded to an approximate calibration calculated from the reading on air-saturated buffer and a 1/15 dilution factor. The calibration technique used takes both the dilution effect and any nonlinearity of the oxygen electrode into account. Calibrations were made before and at the end of each experiment.

The zero level of the oxygen electrode was determined by

TABLE III: Oxygen Utilization (Ratio to Added $FMNH_2$) in Control Solutions 1.5 ml of buffer (Tris, pH 7.3, 20°).

Solution		
Bovine Serum Albumin (mg/ml)	Catalase (mg/ml)	$O_2/FMNH_2$
0	0	1.0
2	0	0.99
5	0	0.94
2	0	0.98[a]
0	1	0.51
2	1	0.56
5	1	0.45
2	1	0.61[a]
5	1	0.51[a]

[a] Dodecanal (10 μl) was added.

TABLE IV: Oxygen Stoichiometry to $FMNH_2$ in the Presence of Luciferase and the Light Reaction (Dodecanal, 10 μl).

$[O_2]$ (μM)	Luciferase [E] (μM)	$FMNH_2$ Added (nmoles)	$O_2/FMNH_2$
100	1.3	4.0	1.02
100	12	4.0	0.93
100	12[a]	4.6	1.0
90	12	7.9	0.93
20	12	8.4	0.95
150	12	8.4	1.06
125	14	9.6	0.95
105	14	9.6	1.01
100	30	6.6	1.0
90	30	7.4	1.03
		Average =	1.0

[a] No dodecanal.

passing N_2 over the stirred solution in the cell for about 30 min. Nitrogen bubbling could not be used with protein solutions but even without this almost complete deoxygenation could be achieved in this time. The last 1–2% O_2 were removed by addition of 0.1–0.2 ml of $FMNH_2$. No further change in the electrode output could be achieved by further addition of $FMNH_2$. The zero level was found to hold constant and was the same for protein or buffer solution.

Table III shows a series of control experiments on oxygen utilization in the presence and absence of protein, dodecanal, and catalase. The results are condensed for presentation into stoichiometric ratios of O_2 to $FMNH_2$ and each is the result of 5–10 separate determinations except for the plain buffer case which was done many more times. The precision of the results was better than 10%, which limits the otherwise good accuracy (±3%) achievable by this internal standardization technique.

In calculating the stoichiometric ratio the $FMNH_2$ was considered 100% pure (ϵ 12500 M^{-1} cm^{-1} at 445 nm) in terms of reducing ability. Separate experiments were done to show that the results did not depend on $[FMNH_2]$ or on the volume of added solution, provided of course the calibration was made in the same way to eliminate the dilution effect.

When $FMNH_2$ is oxidized by oxygen homogeneously, according to reaction 3 the stoichiometry should be 1.0 O_2 to $FMNH_2$. In the presence of catalase the stoichiometry reduces

$$H_2O_2 \longrightarrow H_2O + 0.5O_2$$

to 0.5. These predictions are verified in Table III for all cases. In the presence of catalase there may be some catalase-induced peroxidation of the aldehyde leading to a ratio a little in excess of that predicted. The fourth result shows that when a low amount of $FMNH_2$ is added in a single addition there is no significant oxygen utilization *via* a homogeneous reaction with aldehyde since the ratio is not increased above unity.

Table IV shows that in the overall luciferase reaction the $O_2/FMNH_2$ remains unity. Reactions were carried out both under luciferase concentrations for optimum Q_B(RCHO) and otherwise (Figure 3). Each result is the average of 5–10 determinations. When the reaction is carried out in the absence of dodecanal or when the oxygen concentrations are varied, the $O_2/FMNH_2$ ratio remains the same. Oxygen concentrations higher than 150 μM were not used since the error in the dilution factor is too large for reliable estimate of the oxygen disappearing by reaction.

Under the optimal light yield conditions $FMNH_2$ is limiting and presumably all reacts through the luciferase path, reaction 2. Therefore all the O_2 is reacting by this path too and the maximum $Q_B(O_2)$ must be the same as for $Q_B(FMNH_2)$, $Q_B(O_2) = 0.048 \pm 0.007$, where the error includes that contributed by $Q_B(FMNH_2)$ plus a 10% uncertainty in estimating the oxygen consumed.

Quantum Yield for Hydrogen Peroxide Production. Hydrogen peroxide was detected as a product of the luciferase reaction by two assay procedures. The first made use of induction of luminol chemiluminescence which is specific for the presence of hydrogen peroxide. To the completed reaction mixture (1.5 ml) reacted under optimum $Q_B(FMNH_2)$ conditions was added NaOH (1 ml, 0.1 N) to bring the final pH to 11.6. To this was immediately added a 1-ml volume of luminol solution (10^{-5} M in 0.1 M K_2CO_3) containing a trace of hemoglobin. A chemiluminescence light flash was observed. The intensity was about the same if the same volume of $FMNH_2$ was added to buffer alone and treated in the same way. This flash was about ten times higher than the blank level without any added $FMNH_2$.

This luminol test gave a qualitative indication of the presence of hydrogen peroxide and could no doubt be made more quantitative by adopting the procedures of Armstrong and Humphreys (1965). However as Table III shows measurements of the oxygen consumption in the presence of catalase could also provide this quantitative information.

Figure 7 shows the oxygen consumption as a ratio to $FMNH_2$ added (9.6 nmoles nominal) in buffer as a control (open circles) and the consumption under maximum Q_B ($FMNH_2$) conditions in the presence of luciferase and dodecanal (crosses), and luciferase and dodecanal plus catalase (filled circles). The presence of catalase is seen to reduce the stoichiometry in the luciferase reaction to an average of 0.81 ± 0.08. Production of 1 mole of hydrogen peroxide per mole of $FMNH_2$ should yield 0.5 in the presence of catalase (Table III). Therefore it can be concluded that 1 mole of hydrogen peroxide must be produced per 2 moles of $FMNH_2$ reacting with luciferase.

TABLE V: Summary of Quantum Yields.

Component	Maximum Q_B	Stoichiometry in the Luciferase Reaction
Dodecanal	0.10 ± 0.012	0.94 ± 0.14
$FMNH_2$	0.047 ± 0.005	2.0
O_2	0.047 ± 0.007	2.0 ± 0.4
H_2O_2	0.12 ± 0.03	1.1 ± 0.3

Using the figure 0.81 then the maximum $Q_B(H_2O_2)$ can be calculated with reference to the $Q_B(FMNH_2)$: $Q_B(H_2O_2)$ = 0.12 ± 0.03. This is obtained by comparing the luciferase–catalase $O_2/FMNH_2$ with the average of the controls and multiplying by $Q_B(FMNH_2)$. The large error mainly arises from the uncertainty in estimating the small differences in $[O_2]$ shown in Figure 6. The precision is sufficient to suggest an equality between the maximum values of $Q_B(H_2O_2)$ and Q_B(RCHO).

Discussion

The maximum bioluminescence quantum yields for each of the components of the reaction studied here are summarized in Table V. On the basis of the inverse relation referred to in the beginning of the paper the stoichiometry of each component is calculated from Q_B in the table using for reference $FMNH_2$ as 2.0 moles.

The simplest overall reaction which fits these stoichiometries is eq 4. Since 1 mole of oxygen is used by 1 mole of $FMNH_2$ to

$$2FMNH_2 + 2O_2 + RCHO \longrightarrow 2FMN + H_2O_2 + [H_2O] + [RCHO + O] \quad (4)$$

make H_2O_2 it would appear required that the other $FMNH_2$ should produce H_2O. By the fact that 0.5 mole of oxygen is now left over to be accounted for, and in view of the stoichiometric ratio 2:1 $FMNH_2$:RCHO it would appear reasonable that aldehyde reacts in the light path with this 0.5 mole of oxygen to produce dodecanoic acid. This suggestion has been in the literature for 17 years (McElroy and Green, 1955). It can only finally be established by the difficult experiment of isolating the product and showing it to have the same Q_B as aldehyde.

Recent studies of the kinetics of the light reaction by Meighen and Hastings (1971) have shown that the rate of the light reaction is first order in $FMNH_2$. This can be reconciled with the above stoichiometry by suggesting that (4) is the sum of two independent pathways of flavin utilization, a light reaction (5) and a dark reaction (6). Both would need to proceed with equal velocities under the maximum Q_B conditions. It should then be possible to independently change these velocities by

$$FMNH_2 + O_2 + RCHO \longrightarrow FMN + [H_2O] + [RCHO + O] \quad (5)$$

$$FMNH_2 + O_2 \longrightarrow FMN + H_2O_2 \quad (6)$$

alterations in temperature, pH, aldehyde chain length, type of luciferase, etc.

A point of branching can be considered in terms of a reaction scheme based on that proposed by Hastings and Gibson (1963) and Meighen and Hastings (1971).

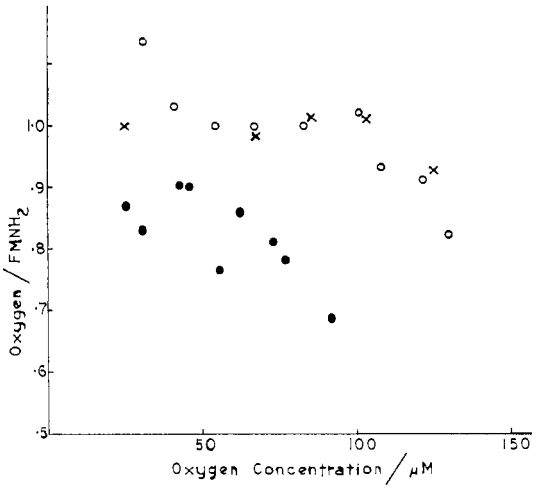
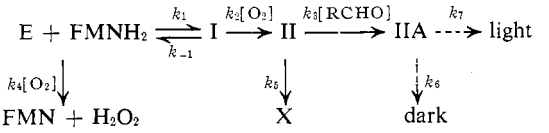


FIGURE 7: Ratio of oxygen reacted to $FMNH_2$ added at various oxygen concentrations in buffer alone (○), in the presence of luciferase (12 μM) and aldehyde (×), and with luciferase, aldehyde, and catalase (1 mg/ml) (●).



Luciferase reacts with $FMNH_2$ reversibly to form an intermediate I which reacts irreversibly with oxygen to produce a long-lived intermediate II, which is further stabilized at high enzyme concentration. This can decay to dark products X or react with aldehyde to produce IIA and eventually light.

This rate k_3 is probably slow and rate determining in the light reaction. It is for this reason that the aldehyde quantum yield experiments are carried out in a manner different from the $FMNH_2$. For the latter excess enzyme and aldehyde are used to ensure the production of a stoichiometric amount of IIA from the limiting $FMNH_2$. On the other hand, when aldehyde is limiting the slow rate of reaction of aldehyde with II means that all the aldehyde may not have time to react with it before it disappears *via* the k_5 pathway. If $FMNH_2$ is repeatedly added to the luciferase, II is regenerated and in this way all the aldehyde can be exhausted, *i.e.*, there is not further light emission on addition of $FMNH_2$.

At high enzyme concentration II is known to be stabilized and about 80% of the aldehyde (9 nmoles) can be made to react on the first addition of $FMNH_2$. For a study of the variation of Q_B(RCHO) on [RCHO] and [E] the repeated addition technique was adopted since it was found to be more precise. The estimate of Q_B(RCHO) is the same by either technique taking into account the amount of aldehyde remaining after reaction if only one addition is used.

The measurements of $Q_B(FMNH_2)$ use an aldehyde concentration about fifty times greater than in the aldehyde-limiting experiment. All the $FMNH_2$ can be assumed to react to IIA since there is no effect on $Q_B(FMNH_2)$ of changing the [RCHO], showing that the aldehyde is really saturating.

The curve of Figure 2 obviously is the result of the competition for reduced flavin in the first step. Using the rate values of Gibson and Hastings (1963) the autooxidation rate $[k_4(O_2)]$ can be estimated to be about 10 sec^{-1} under air-saturated con-

ditions. This must be equal to the luciferase reaction at the point of half-maximum quantum yield, [E] = 12 μM. From ferricyanide competition experiments, Gibson *et al.* (1966) have obtained estimates of k_1 (10^7 M^{-1} sec^{-1}) and k_{-1} (0.4 sec^{-1}) for *P. fischeri* type luciferase. The value of k_2 is not known but a stationary-state analysis for equal velocities at [E] = 12 μM, air saturated (Figure 2), and [E] = 5 μM, [O_2] = 150 μM (Figure 3), yields estimates of k_2 in the range 100–400 M^{-1} sec^{-1}. At a sufficiently high [E] all the $FMNH_2$ should react on the luciferase and a negligible portion by autooxidation.

The final branching point appears to be at intermediate IIA, after all the substrates have reacted with the luciferase. There is at present insufficient evidence to determine if there are two processes here or not. As mentioned previously it should be possible to alter the rate k_6 independently of k_7 by alteration in reaction conditions.

The emitting chromophore in bacterial bioluminescence has been proposed to be the flavin cation $FMNH^+$. This suggestion is based on the similarity of the fluorescence emission spectra of $FMNH^+$ (Muller, 1971) to the bioluminescence spectra and on the appearance of a new chromophore formed to a small extent when FMN is mixed with luciferase in high concentration. This new chromophore has fluorescence, excitation, and absorption characteristics similar to those of $FMNH^+$ (Eley *et al.*, 1970; Eley, 1970). It is proposed that this arises from the interaction

$$E + FMN + H^+ \rightleftharpoons E\text{-}FMNH^+ \quad (7)$$

which is a weak association in the ground state at pH 7. If the right-hand side is formed as the excited state product of the reaction, its radiative rate must be faster than dissociation.

The minimum free energy required for a chemical reaction to populate $FMNH^+$ in its first excited electronic singlet state is that corresponding to the O–O′ transition of $FMNH^+$, 445 nm or 64 kcal $mole^{-1}$. A bond energy calculation for reaction 8 predicts an overall enthalpy change more negative than −100 kcal $mole^{-1}$ (Cox and Pilcher, 1970).

$$FMNH_2 + RCHO + O_2 \longrightarrow FMNH^+ + RCOO^- + H_2O \quad (8)$$

This should be sufficient to satisfy the above energetic requirement provided the entropy contributions are not too large. Enzymic binding differences between reactants and products may also modify this estimate slightly.

The fluorescence quantum yield Q_F of $FMNH^+$ in a rigid boric acid glass at room temperature is around 0.2 (Eley *et al.*, 1970). It may be somewhat less on the luciferase. In reaction 8 an excitation efficiency (Q_E) of at least 0.5 is calculated therefore from the relation $Q_B = Q_E Q_F$ (Lee and Seliger, 1972) if Q_F is assumed to be 0.2 and Q_B to be 0.1, since the $FMNH_2$ in the light reaction 5 must have the same Q_B as aldehyde.

If an accurate measurement of Q_F shows it to be less than 0.1 then the light reaction must involve the two-flavin process (4). The first-order dependence of the rate of this light reaction would then need to be explained away by a modification of the simple reaction scheme above. The drop in both Q_B·(RCHO) and $Q_B(FMNH_2)$ at high [E] and the apparent stoichiometric relation of the luciferase already cannot be explained by this simple scheme.

On the basis that luciferase catalyzes the oxygen oxidation of a substrate and produces a mole of water, it would appear that it functions as a hydroxylase.

It is noted also that in the bacterial cell itself, since the luciferase can be extracted in yields greater than 1% of the total soluble protein (Hastings *et al.*, 1965; Kuwabara *et al.*, 1965; Nakamura and Matsuda, 1971) the *in vivo* luciferase concentrations must be quite high. This can be calculated to be of the order of magnitude found here for the optimum quantum yields. The high concentrations of luciferase used here thus can be taken to represent the true condition within the cell.

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Added in Proof

The aldehyde to $FMNH_2$ optimum quantum yield ratio remains the same for changes in pH, temperature, type of luciferase, and aldehyde chain lengths longer than octanal, suggesting that $FMNH_2$ is utilized by only one pathway and that the light reaction is represented by reaction 4 (J. Lee and C. L. Murphy, unpublished data).

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Mutationally Altered Bacterial Luciferase. Implications for Subunit Functions†

Thomas W. Cline‡ and J. W. Hastings*

ABSTRACT: In order to determine the functional relationship between the two nonidentical subunits of bacterial luciferase, we have isolated two classes of mutationally altered enzymes and determined the subunit location of the lesions. One class, the temperature-sensitive luciferases, includes those enzymes with lesions primarily altering thermal stability. Various mutants differ considerably with respect to this parameter. Often they also have a decreased ability to recover after treatment with 4 M guanidine hydrochloride. Some of the mutant enzymes are fully active at the low temperature. The second class of mutants, the altered-kinetics luciferases, possess lesions altering a wide assortment of catalytic parameters, usually with no concomitant effect upon thermal stability. Lesions which alter catalytic properties occur exclusively in the α subunit, whereas lesions decreasing thermal stability occur in either of the subunits. We conclude that only the α subunit contributes residues to the active site, but that the β subunit is required to maintain the active conformation of the catalytic subunit, α.

One way to evaluate the contribution of individual subunits, and indeed of individual amino acid residues, to specific functional aspects of proteins is to map the location of lesions responsible for specific types of enzyme defects. Toward this end, we have isolated mutationally altered luciferases by two techniques. One allows us to identify luciferases with lesions primarily affecting the thermal stability of the enzyme. With the other we can isolate luciferases with lesions primarily affecting the enzyme's catalytic parameters. Due to several unique features of the bacterial bioluminescence system, the procedures for mutant selection are simple and provide for the rapid isolation of a large number and variety of altered luciferases.

Among those mutants whose *in vivo* bioluminescence is

† From the Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138. *Received March 27, 1972.* This research was supported in part by National Science Foundation Research Grants GB 16512 and GB 31977X.
‡ U. S. Public Health Trainee, GM 00138-13.
* To whom to address correspondence at The Biological Laboratories, Harvard University, Cambridge, Mass. 02138.