

BACTERIAL BIOLUMINESCENCE AND ITS APPLICATION
TO ANALYTICAL PROCEDURES

John Lee, Charles L. Murphy, George J. Faini
and Terry L. Baucom

Department of Biochemistry, University of Georgia
Athens, Georgia 30602, U. S. A.

INTRODUCTION

Bioluminescence techniques using scintillation counters for detection are finding increasing applications in biological trace analysis. One of the most sensitive chemical assay procedures known is the determination of adenosine triphosphate (ATP) by measuring the light emitted when it is added to purified extracts of the firefly (1-5). With suitable precautions less than 10^{-15} moles of ATP can be measured (2).

Assay procedures based on the bioluminescence system from certain marine bacteria have also been developed (5-9). Changes in the bioluminescence from whole cells have been used to monitor the effects of ionizing radiation (10,11), air pollutants (12) and anaesthetics (13), not to mention the classical work on the effect of temperature, pH, pressure and narcotics on whole cells (14).

The bacterial bioluminescence reaction is quite different from that of the firefly. It involves a reaction between reduced flavin mononucleotide (FMNH₂), a long-chain aliphatic aldehyde such as dodecanal (RCHO), oxygen and the enzyme (E) bacterial luciferase. By measuring the quantum yields of each component, that is the number of photons emitted per molecule involved, it has been established that the complete reaction is (15,16):



The FMNH₂'s add with O₂ in two sequential steps to make some type of oxidized flavoprotein, which subsequently reacts with the aldehyde to give luminescence (17). The light emission appears to come from a protonated flavin molecule (FMNH⁺), since the spectral emission distribution precisely corresponds to the fluorescence from this species (18,19). This implies that the FMN is bound to a site on the luciferase having a high acidity, a finding of some consequence for the theory of enzyme catalysis.

With partially purified luciferases procedures have been devised for the rapid and accurate assay of FMN, flavin adenine dinucleotide (FAD) and nicotine adenine dinucleotide (NAD) (6,8,9). The sensitivity is not as good as for ATP in the firefly assay, being only about 10⁻¹⁴ moles for NADH (8). Still, the method is remarkably sensitive and specific, and the results of some of our recent work on the mechanism show that it possesses the capacity of being improved.

In this paper we show how the choice of type of luciferase and degree of purification can be quite important in developing assay procedures. Using a highly purified, high specific activity luciferase, together with another protein fraction, which is not yet characterized but is separated in the purification of luciferase, we demonstrate that an improved sensitivity to NADH, below 10⁻¹⁵ moles, may be achieved.

EXPERIMENTAL METHODS

Types of Bacteria. All the bioluminescent bacteria are of marine origin and may be found either free-living or symbiotic with certain fish. The type we have used for many of our studies is *Photobacterium fischeri* (PF) which was obtained from M. J. Cormier (University of Georgia) in 1965, which was derived from type number 7744 of the American Type Culture Collection (ATCC), Washington, D.C. The type *Achromobacter fischeri* (AF) we obtained from F. H. Johnson (Princeton University) and the type 7744 was originally derived from this. The type MAV was obtained from J. W. Hastings (Harvard University) but he is unsure of its origin. The type A13 is a symbiotic bacteria from the "silver macrourid" fish, isolated by J. Paxton (Australian

Museum) and J. Fitzgerald (Monash University).

Preparation of Luciferase. Bacterial strains were maintained on solid agar medium (18). For enzyme extraction the cells were inoculated into liquid medium (250 cc), grown for 24 hours and used as an inoculum for 400 liters (Fermacell, New Brunswick Scientific Co., New Brunswick, N.J.). After 24 hours growth the cells were harvested on a Sharples refrigerated centrifuge. The cells were then disrupted in a continuous-flow French press, the debris removed by centrifugation and the proteins salted out with ammonium sulfate (80% saturation). The luciferase was purified by column chromatography on Sephadex G-75, DEAE-cellulose (DEAE-32) and DEAE-Sephadex (A-50). The detailed procedures are described elsewhere (19). The luciferase gave a single band on disc electrophoresis, two subunit bands on SDS gels and very close to one equilibrium binding stoichiometry with pure FMNH₂ (20).

Chemicals. FMN (87%) was obtained from Fluka, A. G., Buchs, Switzerland and was further purified on a DEAE column (21). Four minor bands separated from the bulk of the material (FMN) and two had slight activities in the light reaction at about the level expected for riboflavin and FAD. The FMNH₂ was prepared by photoreduction of FMN in the presence of ethylenediamine tetra-acetic acid (EDTA, 20 mM, pH 7) taking care to exclude oxygen. The aldehydes were from Chemsamp Co., Columbus, Ohio and were repurified by vacuum distillation. All other chemicals were the best available commercial grades and were used without further purification.

Light Reactions and Measurement. The bioluminescence was initiated by rapid addition of FMNH₂ to a cuvette containing luciferase and aldehyde in aerated buffer (1 cc). The light was detected by a photomultiplier in an optical setup the same as or similar to that previously described (15,22). For intensity measurements the photomultiplier output signal was amplified by a picoammeter (Keithley 414 A) which drove a strip chart recorder (Esterline Angus Speedservo) and total light was measured by passing the output of the photomultiplier to an operational integrating amplifier circuit. Both systems were calibrated for absolute light intensity (photons sec⁻¹ μA⁻¹ or photons volt⁻¹)

by use of the luminol chemiluminescence reaction as a light standard (22,23).

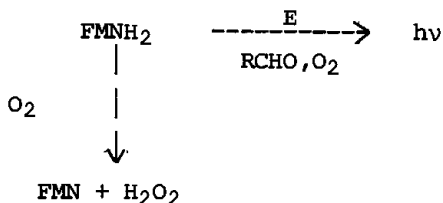
A typical chart recording of a light reaction is shown in Figure 1. This was obtained with the luciferase type A13. The insert shows the light intensity (I) decays approximately logarithmically with time only over the first decade.

For the NADH stimulated reaction, NADH was added to the cuvette containing luciferase, aldehyde, NADH dehydrogenase and a yellow protein fraction to be described later. A light flash was obtained which was similar to Fig. 1 but on about a ten times longer time scale.

Emission spectra were measured on an absolutely calibrated fluorescence monochromator and the details of the measurements will be described elsewhere (24).

RESULTS

Quantum Yields. The oxidation of FMNH₂ by molecular oxygen is both fast and complex (25). When the FMNH₂ is added to the luciferase (E) to initiate the light (hv) reaction, there will be competition for it between O₂ and E:



The dashed lines are used to indicate that this is composed of a series of chemical steps.

At a sufficiently high concentration of E, the light path can be made to outcompete the autooxidation route and the luminescence utilization of FMNH₂ will then be optimal. It has been shown that this simple scheme represents reality since as the concentration of E is increased, the quantum yield of bioluminescence with respect to FMNH₂, $Q_B(\text{FMNH}_2)$, increases to a maximum saturating value (15,

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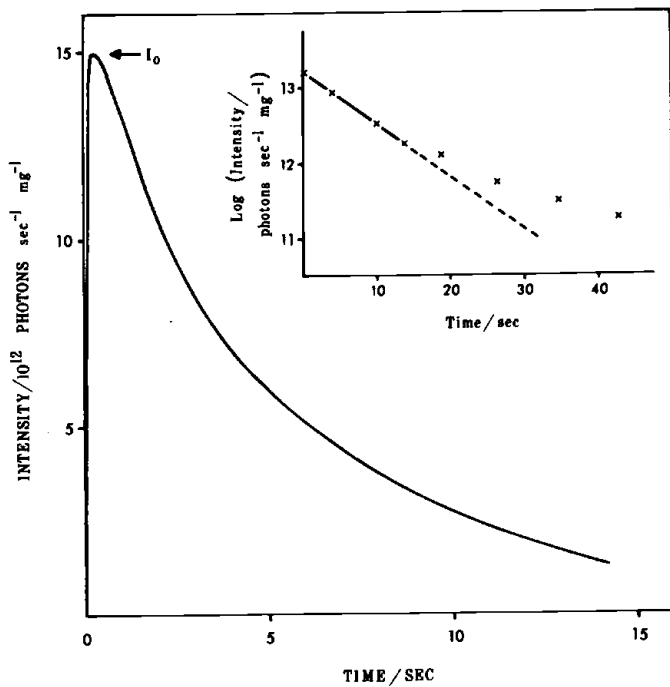


Figure 1. Time dependence of light intensity from reaction of FMNH₂, RCHO and O₂ with luciferase of the type A-13 (23°C, pH 7). I₀ is the initial light intensity.

16).

Figure 2 shows how $Q_B(\text{FMNH}_2)$ increases with increasing concentrations of luciferase of the type A13 and AF. The maximum Q_B 's are compared in Table I, with those previously reported for luciferases of the type PF and MAV and are all seen to be quite different (15,16).

Other properties of the bacterial *in vivo* and *in vitro* light reactions are also compared in Table I. Although A13 has an *in vivo* spectral emission maximum considerably different from the others, the *in vitro* spectral maxima all cluster around the same value. This has been also noted for a large number of other bioluminescent bacteria by Seliger and Morton (26). These emission spectra will be presented in detail elsewhere (24).

The flash height I_0 observed under the FMNH_2 assay conditions is a measure of the specific activity of the luciferase. The specific activity of AF luciferase is thirty times that of MAV, yet the Q_B 's differ only by a factor of two. Thus Q_B is a minor factor in determining the flash height, and the nature and velocity of the rate-determining step are more important and differ between the different types.

TABLE I. Characteristics of Bioluminescence from the Different Bacteria.

Bacteria Type	Light per cell (<i>in vivo</i>) hv sec^{-1}	Spectral Emission Maximum (cm^{-1})		I_0 10^{12} hv $\text{sec}^{-1} \text{mg}^{-1}$	Q_B (FMNH_2)
		<i>In vivo</i>	<i>In vitro</i>		
PF	60	20400	20400	90	0.05
MAV	50	20400	20200	5	0.027
AF	300	20300	20000	140	0.057
A13	2000	21000	20000	15	0.021

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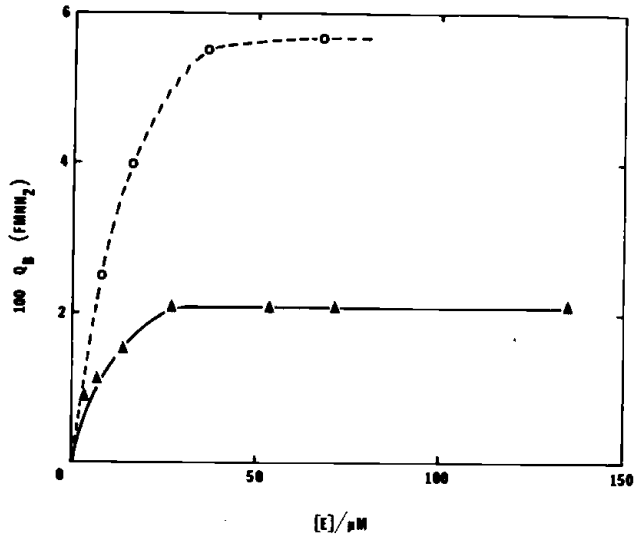


Figure 2. Change of quantum yield with respect to $FMNH_2$, $Q_B(FMNH_2)$ with luciferase (E) concentration for type A-13 (▲) and AF(o).

If the luciferase reactions differ in the nature of the rate determining step, the stationary state concentrations of certain intermediates in the chemical pathway will not be the same. Thus the light reactions may differ in susceptibility to the influence of external agents, such as temperature, pH or concentrations of radical scavengers.

Radical scavengers such as butylated hydroxy-toluene (BHT; 2,6-di(*t*-butyl) *p*-cresol) and sodium formate are seen in Figure 3 to provide strong quenching of the light reaction with A13 luciferase. The quenching constant K is obtained from the relation $I_0/I_0^C = 1 + KC$. Such a strong sensitivity to these quenchers is not seen with the other luciferases.

In culture the light emission reaches a maximum at the end of the logarithmic growth phase (27). At this point the light emission per cell also reaches a maximum and these are tabulated as photons sec^{-1} cell $^{-1}$ in Table I. Although the cells are approximately all the same size the remarkable fact is that one cell of A13 produces 2000 hv sec^{-1} whereas its nearest competitor is 300. Yet the I_0 's are in the reverse order which raises the question of whether the substrates might not be different between the *in vivo* and *in vitro* situations. We shall present some preliminary data in the following section to show that this may be so.

Spectral Properties of Luciferases. Previous workers studying the purification of bacterial luciferases have established that the enzyme contains no bound metals or other co-factors (28-30). Nevertheless the absorption spectra of luciferase reveals the presence of minor amounts of pigments and some of these have been separated and partially characterized (31,32). The absorption spectrum of purified PF luciferase (before the final column step) is shown in Figure 4. Basically the same features are shown here as reported by others (28-30) except that here the spectrum is run against a high concentration of bovine serum albumin (BSA) in the reference cuvette to compensate for the very high degree of light scattering exhibited by luciferase. This technique provides an absorption spectrum much less distorted by scattering than reported by others.

In Figure 4 the absorption of the oxidized and

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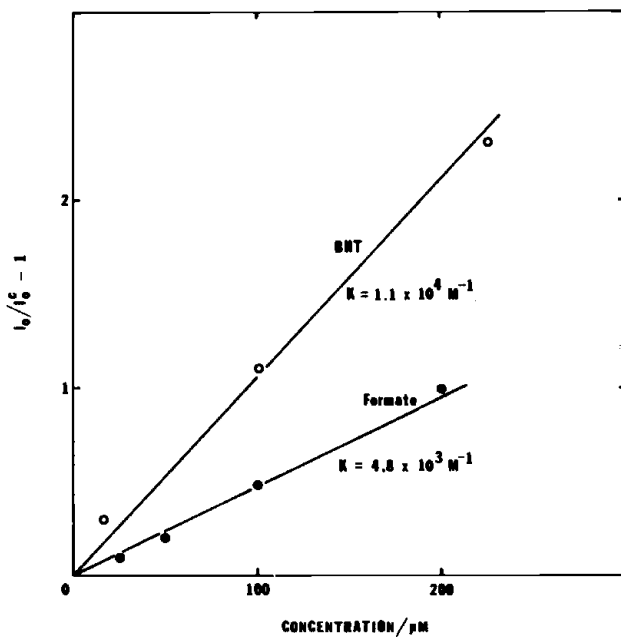


Figure 3. Quenching of initial light intensity I_0 with A-13 luciferase by radical scavengers. I_0^C is the I_0 at scavenger concentration C.

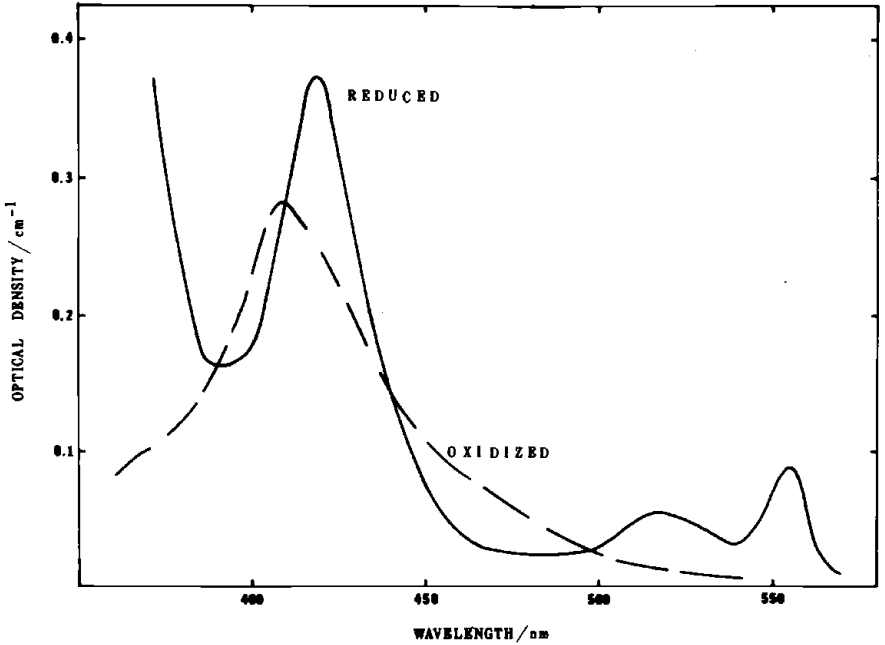


Figure 4. Absorption spectrum of purified bacterial luciferase (10 mg/ml). The reference cuvette contains BSA (10 mg/ml) to remove the distortion due to scattering.

dithionite reduced luciferase preparation are observed to be different. First there is a loss of absorbance at 450 nm. There is also a weak fluorescence in the region of 520 nm (450 nm excitation) for the oxidized but not reduced material so that we can attribute this contribution to a flavoprotein, present at the level of about one mole percent of the luciferase. This impurity is not detectable in preparations of the MAV luciferase which would place it at a level less than 0.1 mole percent.

A second feature is the appearance of a narrow peak around 419 nm and minor bumps at 518 and 555 nm in the dithionite reduced spectrum. These are characteristic of cytochrome and indeed a cytochrome band does follow the luciferase down the G75 column and no doubt contaminates it a little. The absorption spectrum of this dithionite reduced cytochrome (Fig. 5) is characteristic of cytochrome c_1 , but estimates of its molecular weight place it at 30,000-50,000, rather different from values of 300,000 measured for other c_1 (33).

Comparison of Light Reactions. It is generally believed that FMNH₂ and aldehyde act as substrates *in vivo* as they have been shown to *in vitro*, although their presence in these bacteria has not been firmly established. Indeed the presence of free FMN/FMNH₂ in the cytoplasm would not be expected in principle, although, as we have shown, quantities of flavins bound as flavoproteins are certainly there.

An NADH dehydrogenase linked to FMN can be extracted and purified (34) and contaminates the luciferase preparations. Thus the FMNH₂ may be generated in the cell by



This reaction may also be used for the *in vitro* assay, i.e. the addition of NADH and FMN to luciferase and RCHO, generates a persistent low level luminescence. Under the normally used reaction conditions the $Q_B(\text{FMNH}_2)$ in this reaction is less than 10^{-4} , since most of the FMNH₂ is oxidized by O₂ before it reacts with the luciferase.

The cell could overcome the auto-oxidation problem in

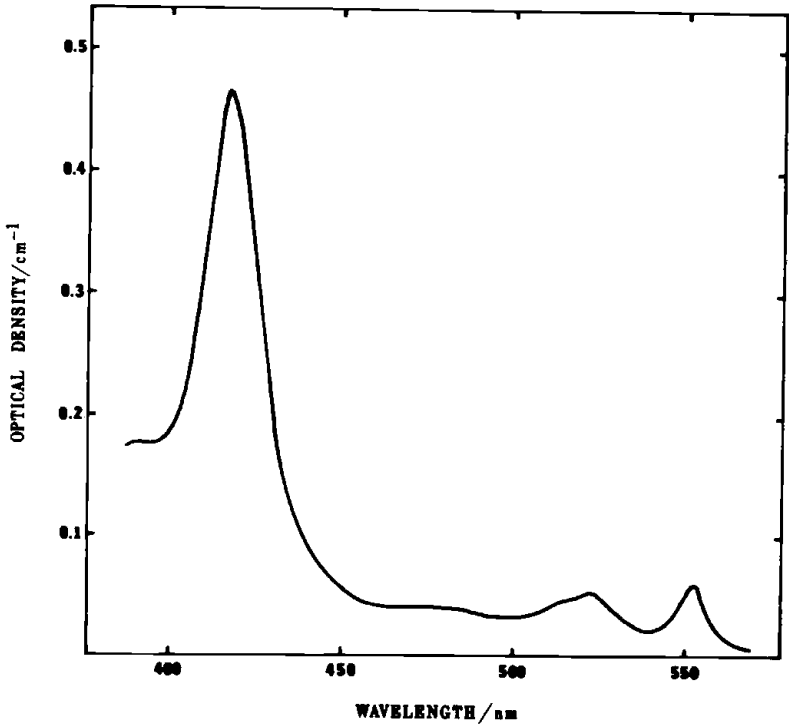


Figure 5. Absorption spectrum of a soluble cytochrome isolated from *Photobacterium fischeri* reduced with dithionite.

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two ways. The luciferase comprises more than 1% of the total soluble protein in the cell. This places its concentration in the cytoplasm at values adequate to outcompete the O_2 for $FMNH_2$ and so achieve the maximum $Q_B(FMNH_2)$ as shown in Fig. 2 (15). Alternatively, perhaps the cell does not use FMN at all and this possibility prompted us to look for a flavoprotein in the extracts which might show light activity with luciferase.

A yellow band elutes from DEAE at a 0.15 M salt concentration in the purification scheme of PF luciferase. It has not been further purified but the absorption and fluorescence of this fraction show the presence of flavoproteins.

This fraction substitutes for FMN in the light reaction with NADH, dehydrogenase and luciferase. It is not reduced under the conditions of EDTA/light showing that its activity is not due to release of free FMN.

In Table II the activities of luciferases are compared. The advantage of using a fresh preparation either partially or fully purified is readily seen to give rise to many times greater activity in both $FMNH_2$ and NADH assays.

TABLE II. Comparison of Luciferase Activities.

Luciferase	Specific Activity/ 10^{12} hv sec ⁻¹ OD(280) ⁻¹		
	FMNH ₂ reaction	NADH reaction with FMN yellow fraction ^b	
PF	90	0.3	1.2
Sigma ^a	0.6	.005	
Worthington ^a	3	.014	

^aCommercial preparations.

^bIsolated during luciferase purification.

The yellow fraction greatly stimulates the NADH activity and part of this comes from an increased level of dehydrogenase in this fraction. The details of this reaction are still being investigated.

A number of other FMN flavoproteins (flavodoxin, dihydro-orotic dehydrogenase and glycollate oxidase) were substituted for FMN in the NADH assay and were found not to show activity except for what was attributable to free FMN from denatured material.

Bioluminescence Assay for FMN and NADH. Having established conditions for optimizing Q_B 's, we thought these techniques could be adapted to improve the bioluminescence assay for FMN and NADH. In Figure 6 is shown the I_0 obtained as a function of added FMNH₂ or NADH under the following conditions. The FMN is determined in a 1 cc mixture of FMN, luciferase (PF, 0.5 mg/ml), dodecanal (10 λ , methanol saturated) by the addition of dithionite or NaBH₄ in optimal amounts (9). The sensitivity is limited by the presence of an enzyme blank even in the purest preparations, equivalent to about 10⁻¹² moles of FMN.

The NADH is added to 1 cc of luciferase (PF, 0.5 mg/ml), dodecanal (10 λ , methanol saturated), yellow fraction (0.5 cc), FMN (5 μ M). There is no enzyme blank and the method appears only limited at this point by photomultiplier noise, which is equivalent to 10⁻¹⁶ moles NADH. This can probably be reduced another two orders of magnitude.

The sensitivity for NADH using this assay is about 100 times better than reported previously (5,7,8) and is even better than for ATP using the firefly assay (2).

DISCUSSION

In our studies of the mechanism of bacterial bioluminescence we have developed techniques of optimizing the light output. First of all the luciferase must be sufficiently purified and present in sufficient concentration to outcompete the very rapid auto-oxidation of FMNH₂. Second, luciferases from different sources differ greatly in initial light output, so that if the light flash (I_0) method is to be used for assay it is very important to choose an enzyme that has a maximum activity. Total light however

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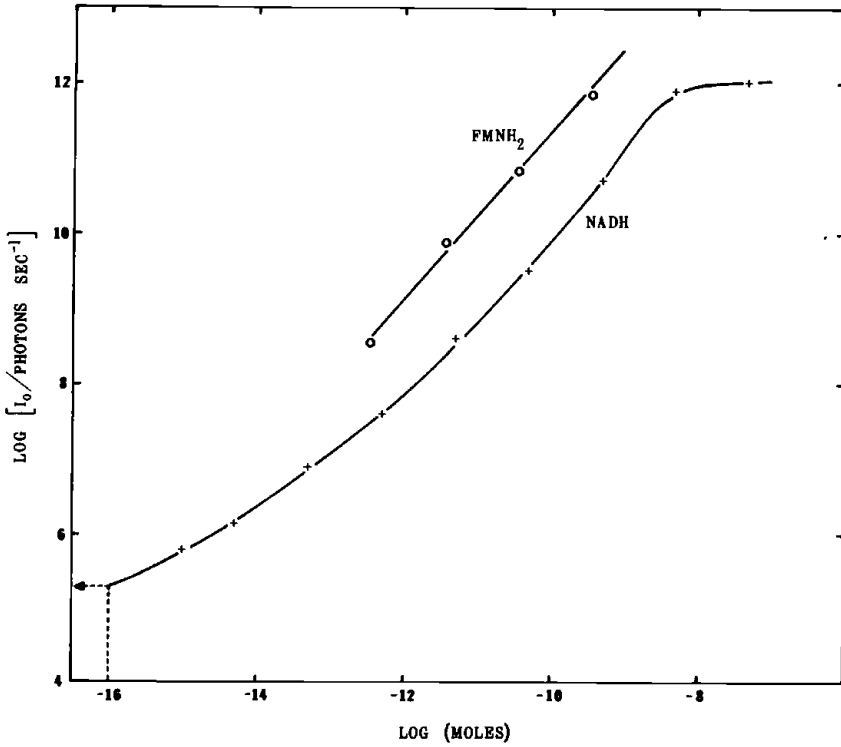


Figure 6. Initial light intensity I_0 on addition of FMNH_2 (o) or $\text{NADH}(+)$ to PF luciferase under the appropriate assay conditions. The arrow indicates the photomultiplier noise level and corresponds to 10^{-16} moles NADH .

does not vary as much from one type to another.

Practical considerations also would make the susceptibility to external quenchers a less desirable feature. The types MAV and A13 are strongly inhibited by excess aldehyde for instance and this may be the same radical scavenging effect as is definitely observed with A13.

However the application of luminous bacteria to the detection of air pollutants (12) and other substances which have a strong propensity towards radical type reactions, would make A13 the organism of choice. *In vitro* at least, its light reaction is much more susceptible than the others tested to the presence of one-electron acceptors.

In the use of coupling techniques, which may generate FMNH₂ directly but more usually via NADH and NADH-dehydrogenase, past workers have not been sufficiently aware of the competition for the FMNH₂ between O₂ and luciferase. Again this competition may be overcome by high concentrations of luciferase but we have proposed here that there may be a substrate that the bacteria itself uses, possibly a flavoprotein which is less readily reoxidized from the reduced state by oxygen, and consequently provides a more efficient coupling between NADH dehydrogenase and luciferase.

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