Proc. Nat. Acad. Sci. USA
Vol. 70, No. 12, Part I, pp. 3468–3472, December 1973

Spectral Properties of an Oxygenated Luciferase–Flavin Intermediate Isolated by Low-Temperature Chromatography

(bioluminescence/enzyme intermediates/oxygen)

J. W. HASTINGS*, CLAUDE BALNY, CHRISTIAN LE PEUCH, AND PIERRE DOUZOU

Ecole des Hautes Etudes, Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie, Paris 5 France

Communicated by I. C. Gunsalus, July 27, 1973

ABSTRACT **Bacterial luciferase catalyzes the oxidation of reduced flavin mononucleotide by molecular oxygen; long-chain aldehyde is required for light emission. At 20° the bioluminescence has a lifetime of tens of seconds, while excess reduced flavin is removed by way of nonenzymatic autoxidation in less than a second. This observation indicates the existence of a long-lived enzyme intermediate, which has been postulated to be a peroxide of the enzyme-bound reduced flavin. This intermediate was isolated and studied at low temperature (−20°), where it has a lifetime measured in days. It has an absorption with a single band peaking at 372 nm, and fluorescence emission centered at about 485 nm, which might be expected for the postulated flavin peroxide. Upon conversion to product, flavin mononucleotide-like absorption and fluorescence appear, supporting the postulate that flavin turns over in the reaction. Upon injection into buffer at 20° with added aldehyde, bioluminescence occurs. Based on a stoichiometry of one flavin per luciferase molecule, the specific activity of the intermediate is equal to that of pure luciferase.**

The oxidation of reduced FMN by molecular oxygen, catalyzed by bacterial luciferase, involves an enzymatic intermediate whose lifetime at 20° is measured in tens of seconds (1). This intermediate (*II*) reacts further, with concomitant bioluminescence, in the presence of a long-chain aldehyde (Fig. 1); in its absence, further reaction also occurs, yielding, it is postulated, oxidized FMN and H_2O_2, with little or no light emission.

The fact that intermediate II has reacted with oxygen was deduced from the observation that bioluminescence will occur subsequent to the removal of free molecular oxygen. However, in spite of its relatively long lifetime, intermediate II has not been well characterized. Spectral studies are made difficult by the fact that a major fraction of the reduced flavin substrate is oxidized nonenzymatically (Fig. 1) during the first second, thus remaining in the mixture as a background absorption and fluorescence. Even at high enzyme concentrations, for example, where according to calculation greater than 90% of the $FMNH_2$ should have been bound, only about 25% was found to be tied up in the complex (2). It was, nevertheless, concluded (2) that in the intermediate II that is formed, the flavin is *not* in the fully oxidized state, since concomitant with its emission of light, an increase in both absorption (450 nm) and fluorescence emission (530 nm) occurred.

The most important requirement for a detailed study of the enzyme intermediate is that it be separated from the free flavin. In recent years, techniques for studying enzymes and their reactions at low temperatures have been developed in our laboratory (3). Since the lifetime of intermediate II at low temperatures (−20° to −50°) is measured in hours or days (4), separation by column chromatography at −20° was attempted and achieved. Intermediate II was found to have an absorption maximum at 370 nm and a fluorescence emission peaking at 485 nm, consistent with the hypothesis that it involves a flavin-peroxide (5).

MATERIALS AND METHODS

Bacterial luciferase, isolated from *Achromobacter fischeri*, strain MAV (6), was purified (7) and stored at −20° before use. Since it was prepared by a short procedure, there remained some impurities, especially some absorbance in the visible range. These were not large enough, however, to interfere with the observations. Its specific activity with $FMNH_2$, determined at the time these experiments were done, was 4.6×10^{13} quanta sec^{-1} mg^{-1}, with dodecanal at 22°. Electrophoresis on acrylamide gels indicated a purity of about 80%. Bioluminescence was measured with a photometer (8) calibrated with the standard of Hastings and Weber (9).

As already described for other enzymes (3), a medium appropriate for studies at low temperatures must be an innocuous organic solvent mixed with an aqueous system to depress the freezing point, and adjusted to suitable ionic environment and proton activity. For luciferase, a suitable medium in which the enzyme is active and not denatured at low temperatures is 50% ethylene glycol–10 mM phosphate buffer (pH 7) (10). Under these conditions the actual "proton activity" (pH*) of the medium is 7.6 at +20° and 7.8 at −20° (11) in the range where the quantum yield of the bioluminescence reaction is relatively independent of pH (12).

FMN was obtained from Sigma and was used without further purification. It was reduced catalytically by bubbling hydrogen in the presence of platinized asbestos, in either phosphate buffer or in the ethylene glycol–phosphate buffer. Aldehyde (dodecanal) was obtained from Aldrich and dissolved in ethanol to provide a stock solution (0.1% v/v, about 5 mM).

Chromatography with Sephadex LH20 was done at −20° in a specially designed column (2.5 × 15 cm) and cold chamber.

Abbreviations: FMN and $FMNH_2$, flavin mononucleotide and its reduced form.

* Permanent address: The Biological Laboratories, Harvard University, Cambridge, Mass. 02138.

Temperature is kept constant at any desired value between −25° and +4°, and homogeneous along the full length of the column, by a thermal regulating system. The samples were applied and then eluted with the same ethylene glycol buffer. 2 hr were required for elution, with a positive pressure of nitrogen of 0.7 bar to maintain the flow of the solvent, which is highly viscous at the low temperature, and a peristaltic pump to regulate the flow rate at about 0.5 ml min^{-1}. The fractions were collected and maintained at −20° (or lower) during all subsequent manipulations and transfer to absorption (Cary 15) and fluoresence (Aminco-Bowman) instruments, both modified as described (13) to permit measurements of samples at temperatures as low as −65°.

RESULTS

The separation of intermediate II from free flavin is shown in Fig. 2. The intermediate was produced by initiating the reaction at +4°, and then trapped by lowering the temperature to −20°. Luciferase (0.8 ml, 10 mg ml^{-1}) without added aldehyde was mixed with 0.2 ml of 2 mM catalytically reduced $FMNH_2$ at +4°, both in the 50% ethylene glycol–phosphate buffer. After 10 sec, the reaction mixture was cooled to −20° by transferring it to another tube containing 1 ml of the same buffer at −35°, thereby stopping the reaction and trapping a substantial percentage of the luciferase in the intermediate II stage (4, 10). This mixture was promptly applied to the column and eluted with the same buffer at −20°. The isolation and characterization has been repeated many times, with results similar to those described below.

The free oxidized flavin (450 nm) is well separated from the protein (280 nm). The latter presumably includes both intermediate II (370 nm) and any unreacted or contaminant protein; the oxidized flavin presumably includes both that which reacted by way of the nonenzymic pathway and any which had already been formed as product by way of enzymic pathways before the time the reaction was stopped.

Aliquots of each tube were assayed for bioluminescence capacity by injecting 0.1 ml into 2 ml of phosphate buffer at 22° (pH 7) with 50 μM dodecanal. The bioluminescence activity coincides with the protein peak and corresponds kinetically (Fig. 3) to intermediate II, with a decay that is accurately exponential and independent of enzyme concentration (1, 6).

The absorption spectrum of the peak tube (No. 11) kept at −24° is shown in Fig. 4. With the exception of the absorption contributed by any protein contaminant or unreacted luciferase, this spectrum should represent the absorption of intermediate II itself. According to the postulate of Fig. 1, if the intermediate is then simply allowed to warm to +20°, the reaction goes to completion by way of the nonaldehyde path-

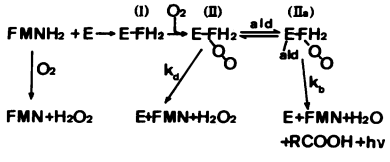

FIG. 1. Hypothetical scheme depicting the pathways and intermediates in the luciferase-catalyzed oxidation of $FMNH_2$ by molecular oxygen. Intermediates II and IIa are in reversible equilibrium; the apparent first-order rate constants for the decay of II (k_d) and IIa (k_b) are similar but not identical, and may differ considerably depending on many factors and conditions. *E*, enzyme.

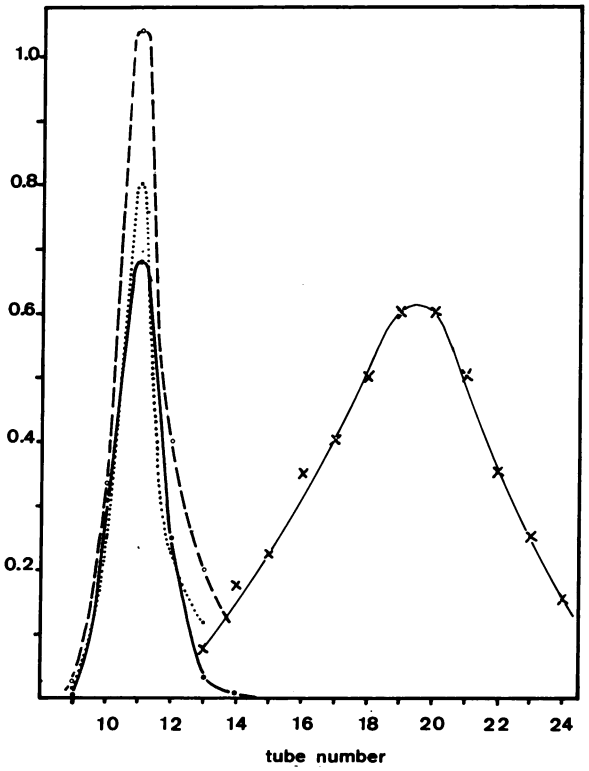

FIG. 2. Chromatography at −20° of luciferase-flavin intermediate on Sephadex LH20. *Ordinate:* absorbance at 280 (- - -), 370 (· · ·), or 450 (×——×; FMN) nm; bioluminescence (·——·) (multiply by 5 × 10^{12} to obtain initial intensity in quanta sec^{-1} for a 0.1-ml sample). *Abscissa:* tube number, 1.9 ml per tube.

way, yielding free oxidized FMN, H_2O_2, and luciferase. The spectrum of the same sample after it was warmed to +20° (Fig. 4, also determined at −24°) indicates the production of oxidized FMN, consistent with the postulated scheme.

When the absorption attributable to the luciferase is subtracted from these two spectra, one obtains the absorption attributable to the flavin moiety in intermediate II (372-nm peak) and, after warming, to the oxidized flavin itself (Fig. 5). The spectrum of this flavin is actually more nearly like that of authentic FMN (14) than was the starting material, which was not a fresh bottle and contained impurities, presumably degradation products. Since such impurities presumably do not react with luciferase (15), the isolated product is purified.

Based on its absorption at 450 nm (Fig. 5) and assuming a millimolar absorption coefficient of 12.2 (14), the flavin in the tube (No. 11; Fig. 2) was 7.5 μM. Similar calculations for luciferase, assuming an absorption coefficient (0.1%, 1-cm path) of 0.94 (16) and a molecular weight of 79,000, gives 12 μM luciferase. The excess of luciferase over flavin (1.6 to 1) is reasonable, since unreacted luciferase, or non-luciferase protein impurities in the preparation were not separated from intermediate II in these experiments. Such a separation will be required to determine directly the luciferase–flavin stoichiometry in intermediate II.

The bioluminescence of intermediate II was also slightly better than the starting material, based on a stoichiometry of one flavin per luciferase. The activity of tube no. 11 was 3.4 × 10^{13} quanta sec^{-1} ml^{-1}; based on its FMN concentration (7.5 μM), this corresponds to an activity of 5.7 × 10^{13} quanta

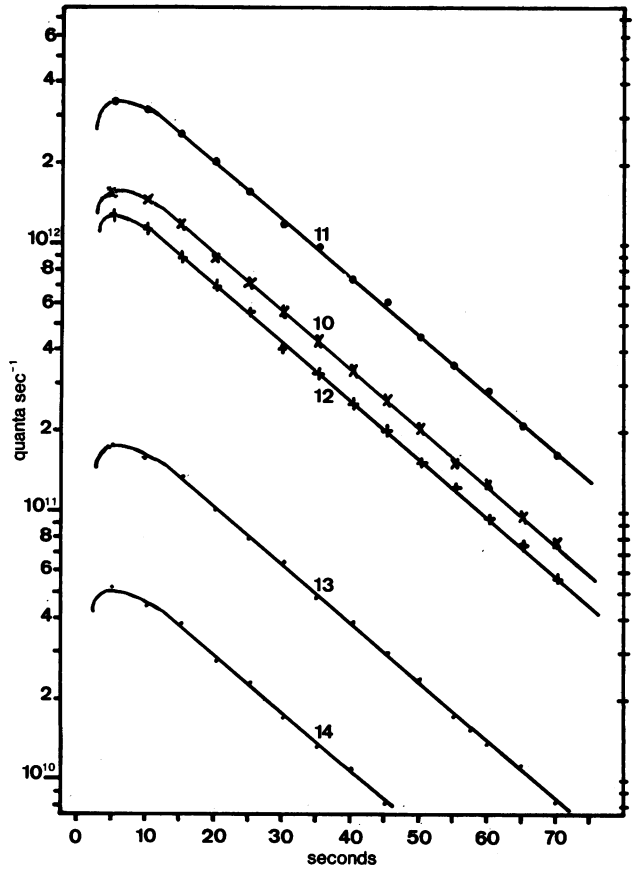

FIG. 3. Time course of the bioluminescence of individual tubes from the column (Fig. 2), assayed by injecting the sample into buffer with aldehyde at 22°. The apparent first-order rate constant for the decay is 0.05 sec^{-1}.

sec^{-1} mg^{-1} of luciferase, compared to an activity of 4.5×10^{13} quanta sec^{-1} mg^{-1} for the starting material. Here again one is evidently dealing with a "purified" material, since the calculation is based on only those molecules to which flavin was bound. When the activity of the starting material is adjusted for purity, the 20% higher value gives 5.5×10^{13} quanta sec^{-1} mg^{-1}, very close to the measured activity of intermediate II. In a second experiment, the agreement was good but not as close: intermediate II had an activity of 7.8×10^{13} quanta sec^{-1} mg^{-1} of luciferase.

If, instead, the activity of intermediate II is calculated on the basis of two flavins per luciferase, which was indicated in the experiments of Lee (17), its specific activity would be 11.4×10^{13} for the first experiment and 15.6×10^{13} quanta sec^{-1} mg^{-1} for the second experiment. A stoichiometry of one flavin per luciferase, as previously reported in other experiments (18, 19), is, therefore, in better agreement with the present work.

The fluorescence of tube no. 11 was measured at −24° before and after warming. Before warming there was a fluorescence emission peaking at about 485 nm using excitation at 370 nm. If we assume an absorption coefficient for the flavin intermediate to be equal to that of flavin itself, the quantum yield of this fluorescence (of intermediate II) was estimated to be at least 35% of that of FMN, or about 0.1. After warming, fluorescence typical of FMN was observed, both in its excitation and emission spectra, and with regard to its fluorescence quantum yield.

The combination of luciferase with reduced flavin was postulated to form a complex, intermediate I, which has only a transient existence in the presence of oxygen (1). However, McCapra and Hysert have recently suggested (20) that our intermediates I and II are the same, and should both be identified with the luciferase-bound reduced flavin. Therefore, mixtures of reduced flavin and luciferase were prepared, excluding oxygen, and examined spectrally at +24° and −24°, to see if the species with absorption at 370 nm, identified above with intermediate II, might in any way be attributable to the luciferase-reduced flavin complex, intermediate I. No indication of this was found. 2 ml of a solution containing luciferase (10 μM) and FMN (20 μM) with mM EDTA and Dow Corning Antifoam was degassed in an anaerobic cuvette (1 cm × 1 cm) by bubbling with prepurified nitrogen for 40 min, and then subjected to a brief (30 sec) irradiation from a 100 W xenon lamp to bring about the photoreduction of FMN. After absorption measurements at +24°, the sample was cooled within 3 min to −24° and similar measurements were recorded. The above procedure was repeated with a lower FMN concentration (4 μM), and also by reducing catalytically with hydrogen and platinized asbestos instead of photochemically. In no case was there any indication of a new absorption band in the 300- to 400-nm range.

Attempts were also made to determine if there are any changes in the absorption when intermediate II is converted to intermediate IIa. At −24° aldehyde was added to a sample from tube no. 11 (Fig. 2). After 30 min a slight change had

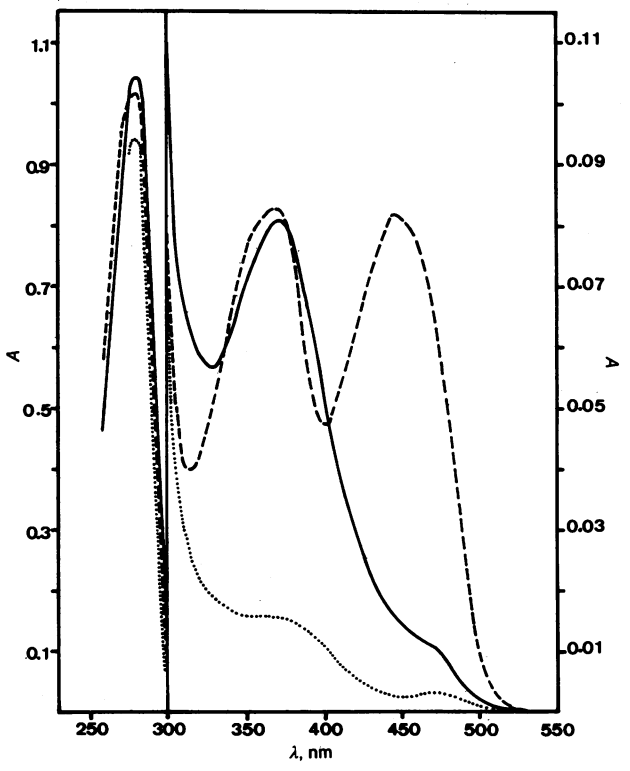

FIG. 4. Absorption spectra of tube no. 11 (Fig. 2) determined at −24°, both before it was warmed (*solid line*) and after it was warmed (*dashed line*); about 5 min elapsed between measurements. The absorption of the same amount of luciferase, without additions, at −24°, is also shown (*dotted line*).

occurred, appearing however to be due simply to the production of some oxidized flavin; intermediate IIa appears to react to form product at lower temperatures than does intermediate II (4). The sample was then warmed up relatively rapidly, but in steps, over a period of 15 min. Spectra at several intermediate temperatures (−5°, +8°, and +15°) showed increasing amounts of oxidized flavin. The final spectrum was identical to the one recorded in the absence of aldehyde (Fig. 4). The yield and character of the flavin is thus apparently the same in the two pathways, assuming that most of the intermediate actually reacted by way of the intermediate IIa pathway. This result also gives no indication of a spectral difference between intermediates II and IIa. But, of course, the actual proportion of the intermediate in the IIa form at any given time was not known.

DISCUSSION

The isolation and characterization, by low-temperature technology, of the long-postulated intermediate II provides support for the proposed reaction scheme and spectral evidence concerning its chemical nature, which is hypothesized to be an oxygenated intermediate in the enzymatic oxidation of reduced flavin. Some years ago, Massey *et al.* (21) suggested that oxidation of reduced flavin might proceed by way of addition to either its 4a or 1a position. Based on this and other considerations, we postulated (5) a luciferase reaction mechanism starting with oxygen addition to the 4a position to give the peroxide, identified with intermediate II of Fig. 1. Evidence concerning the expected spectral properties of such an intermediate (22–24) is compatible with the spectra that we have reported here. For example, Jefcoate *et al.* (23) have observed that the spectrum of reduced 1,3,7,8-tetramethylalloxazine is similar to that of reduced FMN; when substituted in position 4a a new band with a peak of 360 nm occurs. This observation thus supports (but certainly does not prove) the postulate that our intermediate II with a band at 372 nm is oxygenated in position 4a. It will also be of value to know the fluorescence properties of these model flavin compounds, compared to intermediate II.

Spector and Massey (25) recently reported spectral evidence for an oxygenated intermediate in the reoxidation of the flavin in the reduced enzyme–substrate complex of *p*-hydroxybenzoate hydroxylase. From absorption measurements made during the very early stages in reoxidation (15 msec after mixing), they found a species with an absorption band peaking at about 410 nm, which they believe is the oxygenated intermediate. Since the spectrum is not in agreement with that expected for the 4a position, they suggested that this intermediate could be the oxygen adduct at position 1a. Irrespective of the specific position where oxygen may add, both in this and in the luciferase, if oxygenated species are indeed the intermediate in these two cases, it is quite interesting that the two systems apparently add oxygen in different positions.

With regard to the other postulates concerning the reaction, as set forth in Fig. 1, the suggestion that the corresponding acid is produced in the luminescent pathway has received experimental support from several laboratories (20, 26, 27). The results described here indicate that the product of the reaction is unmodified FMN. This hypothesis is in agreement with earlier studies showing a yield of more than one photon per FMN in a system with turnover (28). A more definitive proof will require that all, or the majority, of the intermediate

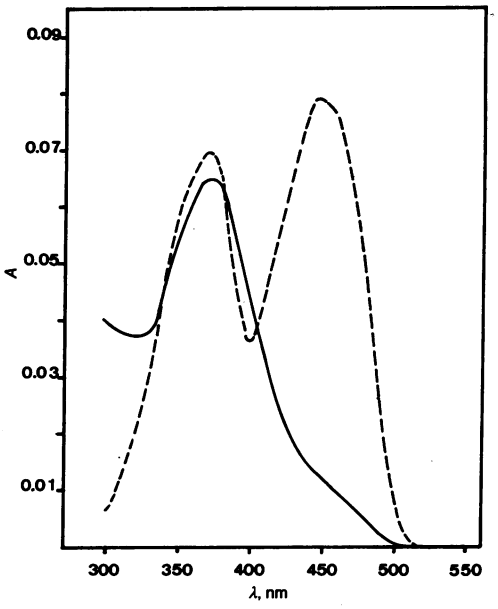

FIG. 5. Absorption attributable to the flavin moiety of intermediate II (*solid line*), peak at 372 nm, and the oxidized flavin (*dashed line*) released after the breakdown of intermediate II.

be shown to react by way of the IIa pathway, and that the product be identified unambiguously as FMN.

The results do show unambiguously that intermediate II does not require aldehyde for its formation, and that it can be distinguished from intermediate I. To accommodate the heresy of McCapra and Hysert (20) one might suggest that in intermediate II, $FMNH_2$ and oxygen are bound independently and noncovalently. Although attempts to demonstrate an oxygen-binding site on luciferase have not met with success, this cannot be cited as definitive proof, since $FMNH_2$ binding might "induce" oxygen binding. Nevertheless, the spectral band at 370 nm, the fact that intermediate IIa is capable of emission in the absence of free molecular oxygen, and that its combination with aldehyde is reversible, all indicate that oxygen is present in intermediate II in covalent combination, as originally postulated (1).

We thank Dr. A. M. Michelson, S. Ghisla, J.-M. Lhoste, and P. Hemmerich for helpful discussions and Ann Gunsalus-Miguel for the luciferase. J. W. H. was the recipient of an NIH Special Research Fellowship (1 FO3 GM53274-01). The research received support from NSF (GB31977X), the CNRS (equipe associee 262), the INSERM, and the DGRST (71-7-3091).

1. Hastings, J. W. & Gibson, Q. H. (1963) "Intermediates in the bioluminescent oxidation of reduced flavin mononucleotide," *J. Biol. Chem.* **238**, 2537–2554.
2. Gibson, Q. H., Hastings, J. W., Weber, G., Duane, W. & Massa, J. (1966) in *Flavins and Flavoproteins*, ed. Slater, E. C. (Elsevier Publishing Co., Amsterdam), pp. 341–359.
3. Douzou, P. (1973) "Enzymology at sub-zero temperatures," *Mol. Cell. Biochem.* **1**, 15–27.
4. Hastings, J. W., Gibson, Q. H. & Greenwood, C. (1964) "On the molecular mechanism of bioluminescence. I. The role of long-chain aldehyde," *Proc. Nat. Acad. Sci. USA* **52**, 1529–1535.
5. Eberhard, A. & Hastings, J. W. (1972) "A postulated mechanism for the bioluminescent oxidation of reduced flavin mononucleotide," *Biochem. Biophys. Res. Commun.* **47**, 348–353.
6. Hastings, J. W., Weber, K., Friedland, J., Eberhard, A.,

Mitchell, G. W. & Gunsalus, A. (1969) "Structurally-distinct bacterial luciferases," *Biochemistry* **8**, 4681–4689.
7. Gunsalus-Miguel, A., Meighen, E. A., Ziegler-Nicoli, M., Nealson, K. H. & Hastings, J. W. (1972) "Purification and properties of bacterial luciferases," *J. Biol. Chem.* **247**, 398–404.
8. Mitchell, G. W. & Hastings, J. W. (1971) "A stable inexpensive solid state photomultiplier photometer," *Anal. Biochem.* **39**, 243–250.
9. Hastings, J. W. & Weber, G. (1963) "Total quantum flux of isotropic sources," *J. Opt. Soc. Amer.* **53**, 1410–1415.
10. Lavelle, F., Henry, J.-P. & Michelson, A. M. (1970) "Etudes de bioluminescence. Etude en milieu fluide et à basse température de la réaction d'emission de bioluminescence de *Photobacterium leiognathi*," *C. R. Acad. Sci.* **270**, 2126–2129.
11. Hui Bon Hoa, G. & Douzou, P. (1973) "Ionic strength and proton activity of supercooled solutions used to study enzyme systems," *J. Biol. Chem.* **248**, 4649–4654.
12. Nicoli, M. (1972) *Active center studies on bacterial luciferase.* Ph.D. Thesis. Department of Biological Chemistry, Harvard University.
13. Balny, C., Hui Bon Hoa, G. & Travers, F. (1972) "Dispositif de regulation de temperature (de − 150° à + 100°C) adaptable à différents spectrophotomètres," *J. Chim. Phys.* **2**, 366–368.
14. Whitby, L. G. (1953) "A new method for preparing flavin adenine dinucleotide," *Biochem. J.* **54**, 437–442.
15. Mitchell, G. W. & Hastings, J. W. (1969) "The effect of flavin isomers and analogs upon the color of bacterial bioluminescence," *J. Biol. Chem.* **244**, 2572–2576.
16. Meighen, E. A., Ziegler-Nicoli, M. & Hastings, J. W. (1971) "Hybridization of bacterial luciferase with a variant produced by chemical modification," *Biochemistry* **10**, 4062–4068.
17. Lee, John (1972) "Bacterial bioluminescence. Quantum yields and stoichiometry of the reactants reduced flavin mononucleotide, dodecanal, and oxygen, and of a product hydrogen peroxide," *Biochemistry* **11**, 3350–3359.
18. Meighen, E. A. & Hastings, J. W. (1971) "Binding site determination from kinetic data: reduced flavin mononucleotide binding to bacterial luciferase," *J. Biol. Chem.* **246**, 7666–7674.
19. Watanabe, T. & Nakamura, T. (1972) "Studies on luciferase from *Photobacterium phosphoreum*. II. Substrate specificity and stoichiometry of the reaction *in vitro*," *J. Biochem.* **72**, 647–653.
20. McCapra, F. & Hysert, D. W. (1973) "Bacterial bioluminescence—Identification of fatty acid as product, its quantum yield and suggested mechanism," *Biochem. Biophys. Res. Commun.* **52**, 298–304.
21. Massey, V., Müller, F., Feldberg, R., Schuman, M., Sullivan, P. A., Howell, L. G., Mayhew, S. G., Mathews, R. G. & Foust, G. P. (1969) "The reactivity of flavoproteins with sulfite. Possible relevance to the problem of oxygen reactivity," *J. Biol. Chem.* **244**, 3999–4006.
22. Walker, W. H., Hemmerich, P. & Massey, V. (1967) "Reductive photoalkylierung des flavinkerns flavinkatalysierte photodecarboxylierung von phenylacetat," *Helv. Chim. Acta* **50**, 2269–2279.
23. Jefcoate, C. R., Ghisla, S. & Hemmerich, P. (1971) "Studies in the flavins series, Part XVI Alkylation and rearrangement reactions of dihydroalloxayines," *J. Chem. Soc. C* 1689–1694.
24. Hemmerich, P., Ghisela, S., Hartman, U. & Müller, F. (1971) in *Flavins and Flavoproteins, Third International Symposium*, ed. Kamin, H. (University Park Press, Baltimore, Med.), pp. 83–105.
25. Spector, T. & Massey, V. (1972) "*p*-hydroxybenzoate hydroxylase from *Pseudomonas* fluorescence. Evidence for an oxygenated flavin intermediate," *J. Biol. Chem.* **247**, 5632–5636.
26. Shimomura, O., Johnson, F. H. & Kohama, Y. (1972) "Reactions involved in bioluminescence systems of limpet (*Latia neritoides*) and luminous bacteria," *Proc. Nat. Acad. Sci. USA* **69**, 2086–2089.
27. Michaliszyn, G. A., Dunn, D. K. & Meighen, E. A. (1973) "Conversion of aldehyde to acid by bacterial luciferase," *Fed. Proc.* **32**, 627abstr.
28. Cormier, M. J. & Totter, J. (1957) "Quantum efficiency determinations on components of the bacterial bioluminescence system," *Biochim. Biophys. Acta* **25**, 229–237.