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# Kinetics of Light Emission and Oxygen Consumption by Bioluminescent Bacteria

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Oxygen plays a key role in bacterial bioluminescence. The simultaneous and continuous kinetics of oxygen consumption and light emission during a complete exhaustion of the exogenous oxygen present in a closed system has been investigated. The kinetics are performed with *Vibrio fischeri*, *V. harveyi*, and *Photobacterium phosphoreum* incubated on respiratory substrates chosen for their different reducing power. The general patterns of the luminescence time courses are different among species but not among substrates. During steady-state conditions, substrates, which are less reduced than glycerol, have, paradoxally, a better luminescence efficiency. Oxygen consumption by luciferase has been evaluated to be ≈17% of the total respiration. Luciferase is a regulatory enzyme presenting a positive cooperative effect with oxygen and its affinity for this final electron acceptor is about 4–5 times higher than the one of cytochrome oxidase. The apparent Michaelis constant for luciferase has been evaluated to be in the range of 20 to 65 nM $O_2$. When $O_2$ concentrations are as low as 10 nM, luminescence can still be detected; this means that above this concentration, strict anaerobiosis does not exist. By *n*-butyl malonate titration, it was clearly shown that electrons enter the luciferase pathway only when the cytochrome pathway is saturated. It is suggested that, in bioluminescent bacteria, luciferase acts as a free-energy dissipating valve when anabolic processes (biomass production) are impaired.

**KEY WORDS:** *Vibrio fischeri*; *Vibrio harveyi*; *Photobacterium phosphoreum*; oxygen; bioluminescence; kinetics; luciferase; cytochrome oxydase; biomass; anaerobiosis.

## INTRODUCTION

It is generally accepted that in all bioluminescent systems molecular oxygen ($O_2$) is directly or indirectly involved. In bacterial bioluminescence, $O_2$ oxidizes both $FMNH_2$ and a long-chain aliphatic aldehyde and the energy liberated by this process is not used for $H^+$ gradient building, osmotic work, nor ATP synthesis, but directly dissipated in the form of light. An overview of the major biochemical reactions involved in this phenomenon is shown on Fig. 1. The free energy produced during these specific oxidations has been evaluated to be 115 kcal/mol. This vast amount of energy is largely sufficient to populate the singlet excited state of the FMN 4a-hydroxide emitter (68 kcal/mol) and, finally, to generate a 490-nm photon (59 kcal/mol) (Merenyi *et al.*, 1992; Tu and Mager, 1995).

At the cellular level, there exists several similarities between the respiratory chain of bioluminescent bacteria and the mitochondrial one. For example, bioluminescent bacteria are "oxidase$^+$" (able to oxidize trimethyl-*p*-phenylenediamine), which means that oxygen reduction is catalyzed by a periplasmic cytochrome *c* oxidase (Pelmont, 1993). They also possess the equivalent of mitochondrial complexes I and II, called, respectively, NDH-1 and NDH-2 (Yagi, 1993). In addition, it is likely that a $Na^+$-dependent NADH:quinone oxidoreductase coexists with the NDH-1 system (Wada *et al.*, 1992). For about two decades, bacterial luminescence was viewed as an alternate electron transport pathway in which luciferase acts as a terminal oxidase (Hastings, 1983).

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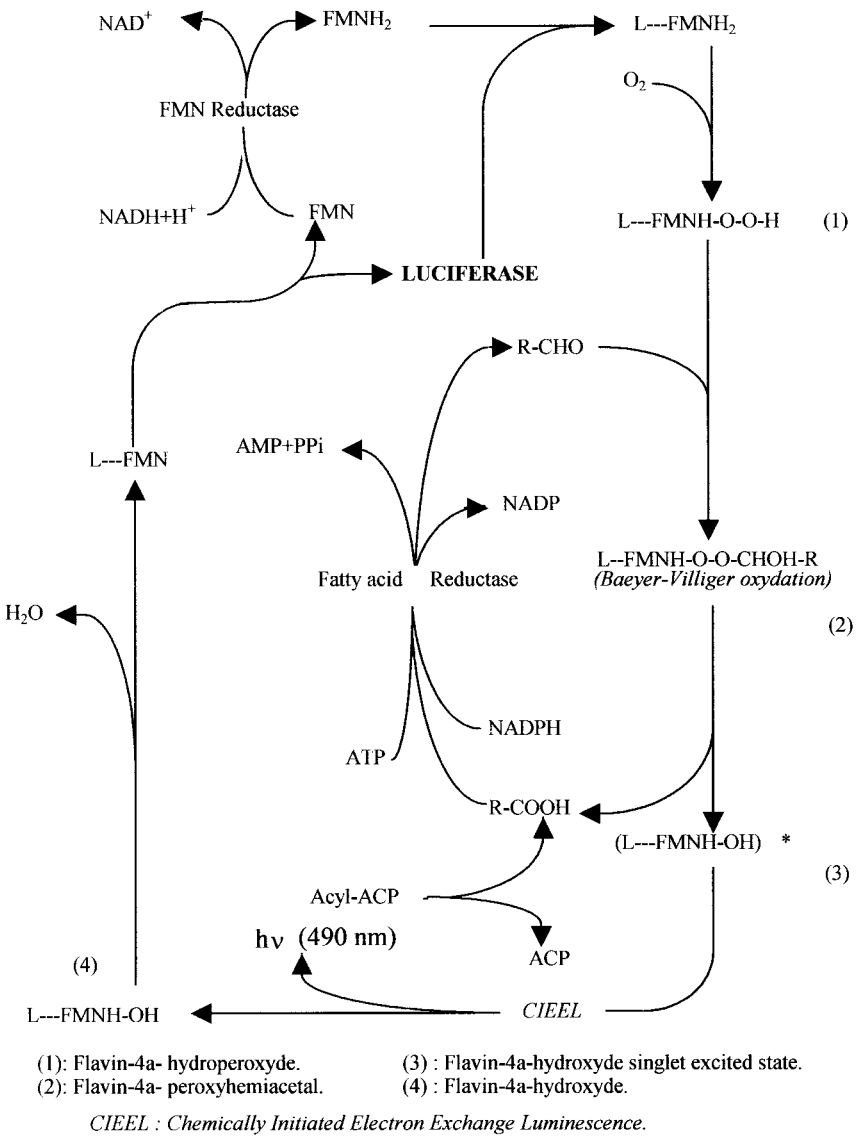


**Fig. 1.** General overview of the biochemical pathway of the light-emitting system of bioluminescent bacteria. (ACP, acyl carrier protein). Three enzymatic complexes are involved in this system: FMN reductase, luciferase, and fatty acid reductase.

Indeed, luciferase can be considered an "alternative oxidase," but it does not oxidize ubiquinol (ubiquinol-oxygen oxydoreductase), as it is in the case of plant, fungi (Day *et al.*, 1995), and protozoa mitochondria (Jarmuszkiewicz *et al.*, 1998).

The possible function of bacterial luciferase can be understood by considering the primary habitat of bioluminescent bacteria. *Vibrio fischeri* and *Photobacterium phosphoreum* are mostly symbiotic microorganisms and live at a very high density in specific organs of marine bioluminescent fishes (Haygood, 1993) and squid (Ruby and McFall-Ngai, 1992). In these particular conditions, luciferase is synthetized in large amounts according to a quorum-sensing regulatory system called autoinduction (Greenberg, 1997) and oxygen is always kept at low levels, chronically imbalancing the cellular redox potential. This situation generates an excess of reducing equivalents in the cell that slows down intermediary metabolism. It has been suggested that the luciferase pathway could scavenge the excess reducing equivalents, helping the reoxidation of reduced coenzymes (Makemson and Hastings, 1986).

The possible connections that exist between the respiratory chain and the luciferase pathway have been investigated by several authors. Some key aspects, such as, the accurate determination of the affinities of cytochrome *c* oxidase and luciferase for oxygen and the contribution of the luciferase pathway to the global $O_2$ consumption remain controversial. Moreover, the influence of the reduction state of the respiratory substrate on the luminous efficiency and the factors regulating the partitioning of the electrons between both pathways have, thus far, not been studied.

In the present work, we simultaneously analyze the kinetics of light production and $O_2$ consumption by three bioluminescent bacteria (*Vibrio harveyi*, *V. fischeri*, and *Photobacterium phosphoreum*) incubated on various respiratory substrates with different reducing power (glycerol > lactate > pyruvate > malate). This provides us with valuable informations concerning the efficiency of the luciferase pathway in steady-state conditions and its contribution to global $O_2$ consumption. The regulation of the electronic flux distribution between both pathways (partitioning) is achieved employing a novel experimental methodology using *n*-butyl malonate titration (Jarmuszkiewicz *et al.*, 1998) and precise determination of the affinity of bacterial cytochrome *c* oxidase for $O_2$ by measurement of its partial pressure at half-maximal respiration (Gnaiger *et al.*, 1995).

The evaluation of the affinity of luciferase for $O_2$ is performed by a new HPLC technology allowing essays of dissolved $O_2$ down to the nanomolar level (Stubauer *et al.*, 1997).

## EXPERIMENTAL PROCEDURES

### Bacterial Cultures

*Vibrio harveyi* (Johnson & Shunk, 1936) strain LMG 4044, *V. fischeri* (Beijerinck, 1889) strain LMG 4414, and *Photobacterium phosphoreum* (Cohn, 1878) strain LMG 4231 are supplied by the Laboratorium voor Mikrobiologie Culture Collection, University of Ghent, Belgium.

The bacteria are grown in batch culture at 22–23°C on a complete broth (CB) containing distilled water with the addition of: NaCl 385 mM, KCl 10 mM, $MgSO_4 \cdot 7\ H_2O$ 50 mM, $CaCl_2 \cdot 2\ H_2O$ 10 mM, $NH_4Cl$ 19 mM, $KH_2PO_4$ 0.7 mM, arginine–HCl 1 mM, Bacto-Casitone 10 g/l, "LabLemco" powder (meat extract) 10 g/l, Tris–HCl 1 M, pH 7.5, 50 ml/l.

After 18 h of growth, the cell density is spectrophotometrically checked, considering that an $OD_{660\,nm}$ of 1 corresponds to $\approx 1.5 \times 10^9$ cells; at that time, the culture has reached the stationary phase (Bourgois *et al.*, 1994). A 20-ml sample is then centrifuged ($1800 \times g$, 15 min) and the resulting bacterial pellet resuspended in 1 ml of the original supernatant to avoid any regrowth; such a concentrated cell suspension has proved to be stable at room temperature for several hours.

### Assay Procedures

#### *Simultaneous Monitoring of Oxygen Concentration and Luminescence*

Experiments were performed using a slightly modified apparatus described earlier (Mallefet and Baguet, 1989). Briefly, it consists of an oxygen respirometer fitted with a Clark-type electrode (Eschweiler, Kiel, Germany) and a light guide. This guide leads the light to a 1P 21 photomultiplier tube (EMI, Ruislip, England) calibrated with a tritium-irradiated phosphor emitting at 470 nm (Saunders-Roe Betalight, Hayes, England). In our experimental conditions, a photomultiplier output of 1 V corresponds to a flux of $570 \times 10^6$ quanta/sec at the tip of the optical fibers. In this kind of experimental setup, unavoidable oxygen back-diffusion (Seppi *et al.*, 1997) occurs at low dissolved oxygen levels; this side-effect has been evaluated to be ($134 \pm 20$) nM/min.

A 9.5-$\mu$1 aliquot of the bacterial suspension is injected into the 0.95-ml measuring chamber of the apparatus filled with an air-saturated mineral medium (CB devoid of Bacto-Casitone and "LabLemco") supplemented or not with the following substrates: glycerol, lactate, pyruvate or malate (final concentration: 25 mM). Due to their high salinity, the initial oxygen concentration in the incubation media is estimated at 220 $\mu$M at 25°C (Gilbert *et al.*, 1967). Zero calibration of the Clark electrode is achieved by reducing all dissolved $O_2$ with solid sodium dithionite.

For each bacterial species and each substrate, the time course of the oxygen concentration $[O_2]$ in the medium and the light output are monitored on a two-channel stripchart recorder. The experiment is stopped when the electrode signal becomes undetectable.

#### *Oxygen Consumption by Luciferase*

Bacterial cells were incubated in a mineral medium supplemented with 25 mM glycerol, citrate, and malate. The $O_2$ consumption (in steady-state conditions), due to luciferase, consists in the slope of the time course of

$O_2$ concentration in a medium containing 1 mM KCN. Decanol (0.1 mM), an inhibitor of the fatty acid reductase complex and, by the same method of the luminescent system, is added at the end of the essay in order to check the existence of any residual cyanide-insensitive respiration. For the three investigated bacteria, the result appeared to be negative. The final result is expressed in percentage of the total consumption (essayed in a medium without inhibitors).

### *Metabolic Regulation of Bacterial Bioluminescence (Electron Partitioning)*

The methodology of this experiment is based on the fact that *n*-butyl malonate acts as a nonpenetrating inhibitor of particular respiratory substrates (e.g., succinate and other multifunctional carboxylic acids) uptake in mitochondria (Jarmuszkiewicz *et al.*, 1998; Genchi *et al.*, 1999). Here, the experiment is performed with *Photobacterium phosphoreum* suspended in the mineral medium supplemented with the $\alpha$-hydroxy acid lactate (25 mM) as the oxidizable substrate. We assumed that *n*-butyl malonate controls the electronic flux into the respiratory chain in oxidase$^{plus}$ bacteria by blocking substrate uptake (like it does in mitochondria). We observed that *n*-butyl malonate at a concentration of 12 mM inhibits both $O_2$ uptake and luminescence. Steady-state total $O_2$ uptake rate and luminescence intensity are then plotted against decreasing concentrations of *n*-butyl malonate (12–0 mM). This procedure allows one determine which pathway is chosen preferentially by the electrons at low, medium, and maximal input (partitioning).

### *Affinities of Bacterial Cytochrome* c *Oxidase and Luciferase for Oxygen*

*Cytochrome* c *oxidase.* In many cell types, respiratory control by oxygen can be described by a hyperbolic relation in the low-oxygen range. Therefore, oxygen affinity ("apparent" Michaelis constant for oxygen) is conveniently expressed as the oxygen partial pressure ($p_{O_2}$) at half-maximal steady state respiration ($p_{50}$) (Gnaiger *et al.*, 1995). The accurate determination of this parameter was performed using high-resolution respirometry. The methodology of this technique is based on the application of a computerized two-channel titration-injection respirometer with back-diffusion control (Oroboros Oxygraph, Paar, Graz, Austria). In the present experimental setup, the bacterial cells ($\approx 400 \times 10^6$ cells/ml) are incubated in a medium containing 0.1 mM *n*-decanol. Acquired data are processed and analyzed by the accompanying DatLab software (Oroboros, Innsbruck, Austria) (Steinleichner-Maran *et al.*, 1996). The final result, representing the plot of the volume-specific oxygen flux ($J_{O_2}$) *versus* $p_{O_2}$ (expressed in kPa), is displayed and a hyperbolic fitting of this plot gives, after appropriate corrections (atmospheric pressure, solubility factor of $O_2$ in the medium, etc.…), the exact values of $p_{50}$ (converted in $\mu$M) and $J_{O_{2\,max}}$ (Fig. 2).

*Luciferase.* Previous work, based on indirect evaluations, has shown that $O_2$ concentrations needed to produce half-maximal light emission range between 0.015 and 0.700 $\mu$M (Lloyd *et al.*, 1985).

For direct and accurate essays at such low levels, a conventional Clark electrode is inappropriate and a novel method based on HPLC has been applied. Details concerning this new technology are accurately explained in Seppi *et al.* (1997) and Stubauer *et al.* (1997). The apparatus called "Oxytron" (IT-V, Innsbruck, Austria) enables detection down to $[O_2] \cong 10$ nM, but it does not allow continuous monitoring. Needless to say that, at such very sensitive levels of detection, extreme care is taken to avoid $O_2$ back-diffusion (gas-tight Hamilton syringes for sampling, quartz capillaries, etc.…). The analog output of the photomultiplier tube is digitalized through a Velleman acquisition card and displayed in real time on a computer screen and saved for further analysis.

Bacteria are injected in a medium containing 1 mM KCN to block complex IV. Optimal measurements are performed when the cell density is $\cong 250 \times 10^6$ cells/ml. The affinity (apparent $K_m$ of luciferase for oxygen $[O_2]$, producing half-maximal light intensity) is evaluated graphically by extrapolation. An example of such evaluation is shown in Fig. 3.

## Chemicals

Mineral salts, glycerol, arginine, and KCN are from Merck. Bacto-Casitone is from Difco. LabLemco powder is from Oxoid. Lactate, pyruvate, citrate, and malate (as sodium salts) are from Sigma. *n*-Decanol is from Fluka and *n*-butyl malonate is from Aldrich.

## Statistics

Unless otherwise stated, results are expressed following sextuplicate experiments; error bars represent confidence limits of the mean ($p < 0.05$).

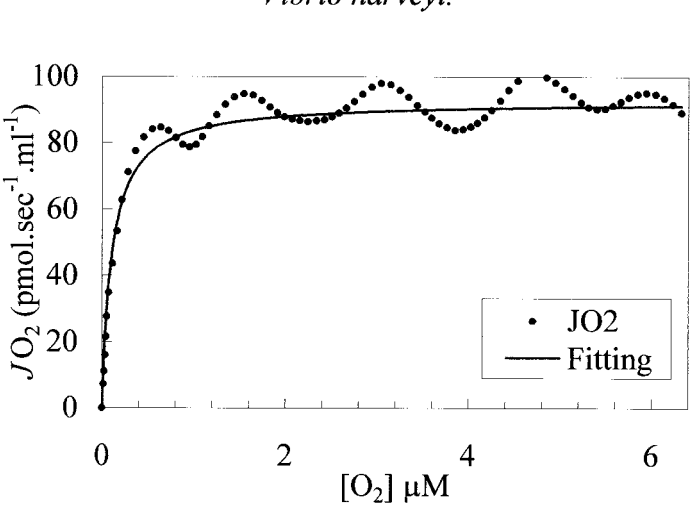


**Fig. 2.** High resolution respirometry performed on *Vibrio harveyi* growing on 25 mM glycerol, citrate, and malate. Hyperbolic fitting of the volume specific oxygen flux ($J_{O_2}$) plotted *vs.* [$O_2$] when luminescence is inhibited by 0.1 mM decanol. Oscillations are due to slight unstabilities of the polarographic sensor current at low oxygen levels. The $p_{50}$ values (apparent Michaelis constant) of cytochrome oxidase of the three bacterial species are listed in Table II.

## RESULTS

### Luminescence during Oxygen Depletion

Typical kinetics of $O_2$ concentration and luminescence during a total exhaustion of the dissolved $O_2$ in the respirometer chamber are shown on Fig. 4. Glycerol, as oxidable substrate, is given as an example for each species, since the same pattern is observed with other substrates. Immediately upon injection in the air-saturated medium, the three species of bacteria emit light and consume $O_2$ in a specific manner. *Vibrio harveyi* is typically a tonic bioluminescent bacteria, i.e., it keeps the intense light produced initially at the same level as long as $O_2$ is present in the medium. *Photobacterium phosphoreum* is a phasic species: a light flash lasting ± 10 min is first emitted, which is followed by a steady glow persisting until the exhaustion of $O_2$ in the medium. *Vibrio fischeri* exhibits an intermediate pattern.

For all tested substrates, the decreasing order of maximal luminescence is: *P. phosphoreum* > *V. harveyi* > *V. fischeri*; the reducing power of the substrate does not induce significant variations of this parameter.

The relationship existing between the intensity of light emission and $O_2$ uptake rate is best observed when the smoothed derivatives of the oxygen concentration time courses are superimposed on the luminescence curves (Fig. 5). Three phases could be observed: (I) a initial high $O_2$ uptake rate occurring at the burst of luminescence, followed by steady-state conditions for both luminescence and $O_2$ consumption (II) , which duration is species- and substrate-dependent (Table I). Finally, there is a sharp decrease in both parameters (III).

When the Clark electrode does no longer detects $O_2$ ([$O_2$] < 0.5 $\mu$M) (Lloyd *et al.*, 1985), a rapid luminescence decrease occurs, followed by stabilization at a level representing oxygen back-diffusion.

### Efficiency of the Luciferase Pathway

The efficiency of this alternative pathway can be expressed by considering either the steady-state conditions (Fig. 6A) or a whole time course (Fig. 6B). Among the

**Table I.** Duration of Steady-State Conditions Corresponding to Phase II, Fig. 5

| Substrate (25 mM) | Time(min). *Vibrio harveyi* | *Vibrio fischeri* | *Photobactereum phosphoreum* |
|---|---|---|---|
| Control | 7 ± 1 | 25 ± 4 | 45 ± 7 |
| Glycerol | 11 ± 2 | 7 ± 2 | 16 ± 4 |
| Lactate | 10 ± 2 | 17 ± 3 | 9 ± 2 |
| Pyruvate | 5 ± 1 | 11 ± 2 | 11 ± 3 |
| Malate | 11 ± 3 | 10 ± 2 | 25 ± 5 |

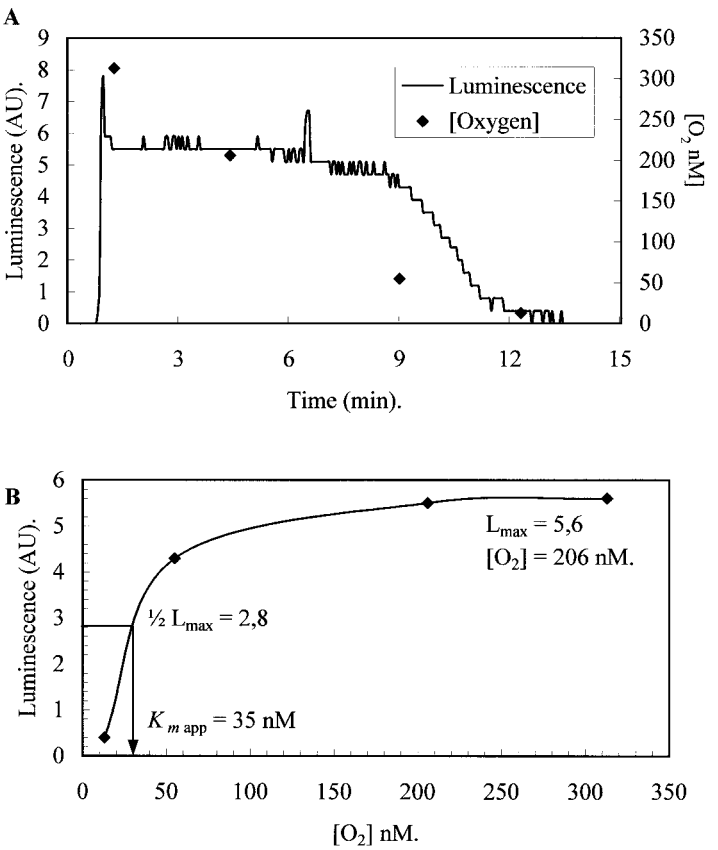


**Fig. 3.** (A) Representation of the time course of luminescence (expressed here in arbitrary units) and $[O_2]$ in the "Oxytron" measuring chamber containing *Vibrio fischeri* cells, incubated with 1 mM cyanide to block cytochrome oxidase. Although oxygen could not be monitored continuously, it was possible to plot luminescence vs. $[O_2]$ (Fig.3 B) and to determine the apparent $K_m$ of luciferase by a direct graphical method.

three bacteria investigated, the quantity of photons produced for the same initial amount of oxygen consumed can be ranked in the following order: *P. phosphoreum* >>> *V. harveyi* > *V. fischeri*. The effect of the reduction state of the substrate on the luminous efficiency is only marked when the steady-state efficiency is considered and the substrates, which, are less reduced than glycerol, perform better.

## Contribution of the Luciferase Pathway to the Total $O_2$ Consumption

The measurement of the cyanide-insensitive, decanol-sensitive respiration gives a good estimation of the $O_2$ consumption by luciferase. The proportion of the luciferase pathway to the total oxygen consumption has been evaluated to be $11.7 \pm 2.1\%$. No significant difference could be detected between the three bacteria (ANOVA, $p < 0.05$).

## $P_{50}$ Determination of Bacterial Luciferase and Cytochrome Oxidase

Half-maximal oxygen consumption by luciferase (cyanide-insensitive $O_2$ consumption) and the respiratory chain (decanol insensitive respiration) is summarized on Table II. It clearly appears that the affinity of luciferase for oxygen is about 4–5 times higher than cytochrome oxidase. The affinity of cytochrome oxidase for oxygen in *P. phosphoreum* is significantly lower than the one of the *Vibrios* (Student's *t*-test, $p < 0.05$); this observation seems to be also applicable for luciferase, but the lack of sufficient replicates does not allow for this conclusion.

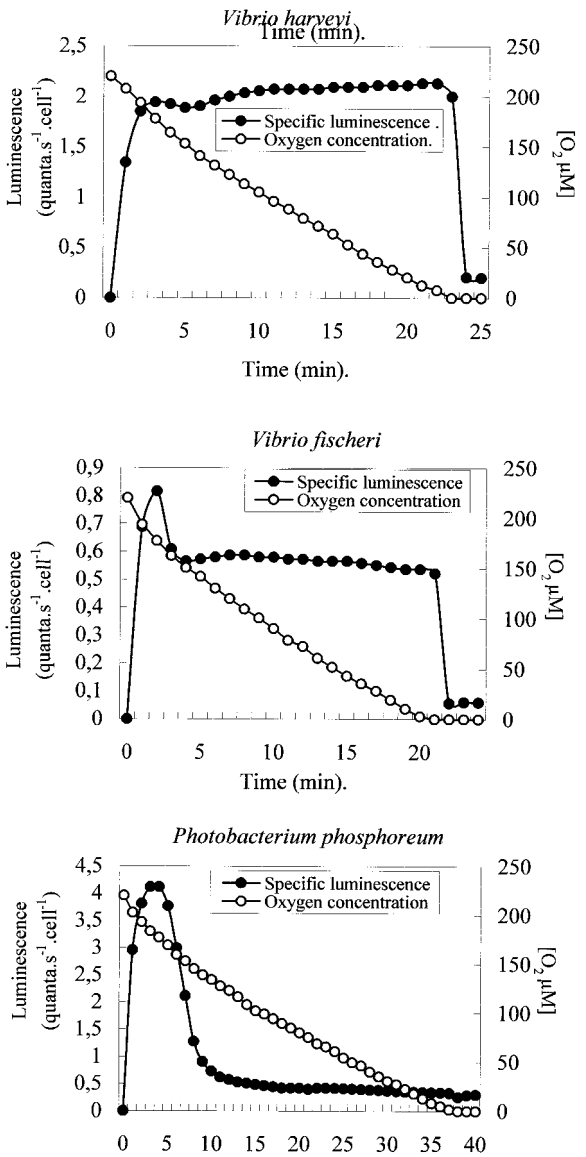


**Fig. 4.** Time course of the specific luminescence and oxygen concentration in the 0.95 ml respirometer chamber. The shapes of the luminescence curves are clearly different for the three species, whatever the oxidizable substrate used for respiration (here glycerol). A sharp dimming always occurs when luminescence becomes $[O_2]$ dependent. This happens at $[O_2] < 0.5\,\mu$M; such low $O_2$ levels are undetectable with Clark electrodes. The residual luminescence corresponds to oxygen back-diffusion in the system.

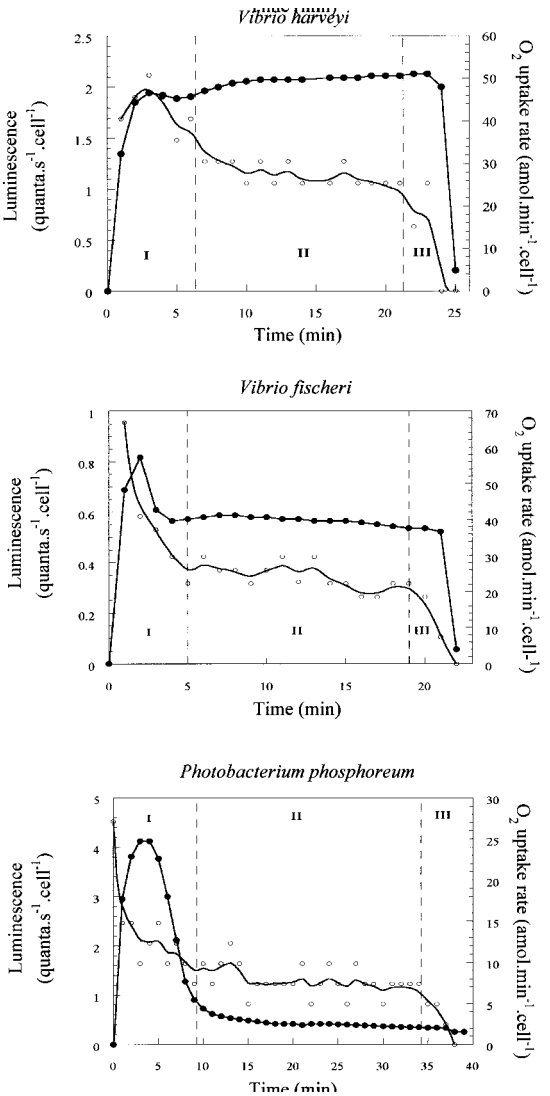


**Fig. 5.** This figure is a modified version of Fig. 4. where the derivative of the $[O_2]$ time course is overlayed on the luminescence curve. This is done to accurately determine the time lapse of steady-state conditions. These correspond to phase II on the graphs. See Table I for values.

Moreover, the graphs (performed with the three bacteria), like the ones displayed on Fig. 3B, are clearly pseudohyperbolic and the $R_s$ value ($[O_2]$ at 90% of $I_{max}/[O_2]$ at 10% of $I_{max}$) of luciferase can be evaluated to 6.6. This indicates that luciferase is a regulatory enzyme presenting a positive cooperative effect with oxygen.

## Metabolic Regulation of Bacterial Bioluminescence

*n*-Butyl malonate (NBM) titration, a methodology used to study the partitioning of the electron flux between the respiratory chain and alternative pathways in mitochondria (Jarmuszkiewicz *et al.*, 1998), allowed us to investigate the effect of the intensity of the electron flux on luminescence and oxygen consumption. We first observed

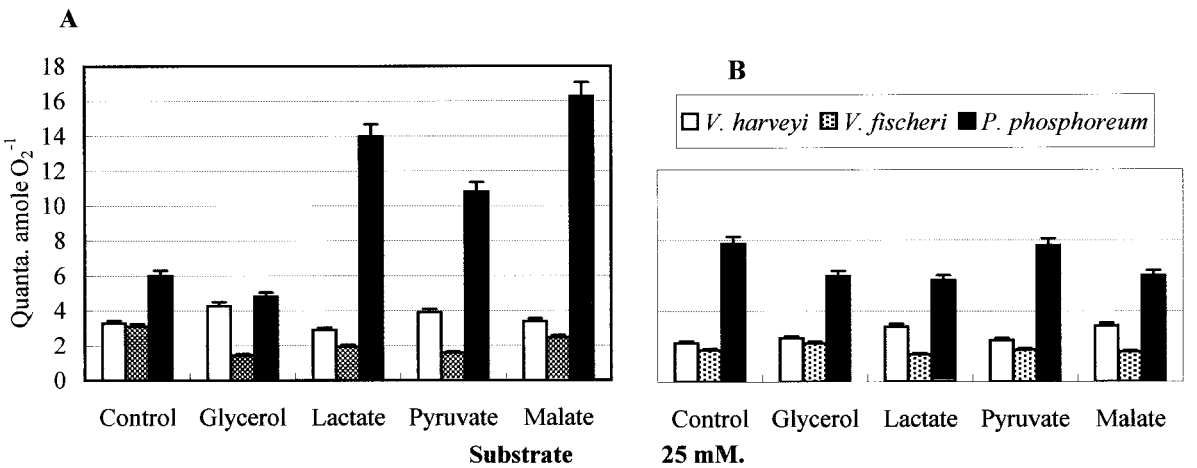


**Fig. 6.** "Luminous efficiency" in steady-state conditions (Fig. 6A) and during an entire time course (Fig. 6B). Results are expressed in quanta of light produced per attomole of *total* oxygen consumed and are computed following processed data obtained, like on Fig. 4. In all cases, *P. phosphoreum* performs better. The respiratory substrate has no effect on the average luminous efficiency, but does have an effect on the steady-state luminous efficiency. Oxidized substrates like lactate, pyruvate and, especially, malate are the most efficient.

that (as it does in mitochondria), NBM acts as a inhibitor of electron input into the respiratory chain. In *P. phosphoreum*, incubated on 25 mM lactate, a concentration of 12 mM NBM inhibits both respiration and luminescence. When the NBM concentration decreases, the availability of reducing equivalents increases and our results (Fig. 7) show that, when [NBM] <8 mM, the respiratory chain nears saturation and a sharp increase in luminescence is observed, indicating that excess electrons begin to be delivered to the luciferase pathway (overflow).

## DISCUSSION

### Luminescence during Oxygen Depletion

The nature of the carbon source present in the incubation medium has an impact on the light intensity and on the $O_2$ flux only under steady-state conditions; this effect is species dependent. However, it cannot be directly related to the reduction state of the respiratory substrate. Three major reasons could explain these observations: (1) many reagents penetrate the outer membrane of Gram-negative bacteria at different rates, (2) cells are frequently difficult to starve of endogenous substrates (Nicholls and Ferguson, 1992), and (3) the connections existing between the respiratory chain and the luciferase pathway could be more complex, as was usually thought. Indeed, $FMNH_2$, which provides reducing equivalents, is bound to the cell membrane system as a part of the NDH-1 complex in the respiratory chain, but has appeared to be mostly free when it is involved in the luminescent system (Inouye, 1994; Wada *et al.*, 1994). These last hypothesis is supported by the fact that luciferase has proved to have a strictly cytoplasmic localization (Colepicolo *et al.*, 1989) in *V. harveyi*.

**Table II.** $p_{50}$ Values of Luciferase and Cytochrome Oxidase of the Three Bacteria Obtained Respectively, by HPLC-Based Oxygen Essays and High-Resolution Respirometry[a]

| | $p_{50}$ (nM) Luciferase | $p_{50}$ (nM) Cytochrome oxidase |
|---|---|---|
| *V. harveyi* | $21.6 \pm 0.4$ ($n = 3$) | $138 \pm 25$ ($n = 6$) |
| *V. fischeri* | 34.25 ($n = 2$)<br>35.0 | $127 \pm 23$ ($n = 6$) |
| *P. phosphoreum* | 43.0 ($n = 2$)<br>65.2 | $202 \pm 42$ ($n = 6$) |

[a] Despite the lack of sufficient replicates for luciferase's $p_{50}$, it is possible to assess that the affinity of luciferase for oxygen is about four-fold higher than the one of cytochrome oxidase.

### Efficiency of the Luciferase Pathway

The accurate determination of steady-state conditions for both luminescence and oxygen consumption (Table I) was necessary to characterize important aspects of the luciferase pathway, like its efficiency, its contribution to the total oxygen consumption, and its $P_{50}$.

The luminescent system of *P. phosphoreum* is obviously more efficient (i.e., the specific luminescence is stronger for the same concentration of dissolved $O_2$) than the one of the two other bacteria. This explains earlier similar observations on the "bioluminescent potential" of bioluminescent bacteria (O'Kane *et al.*, 1985); thus,

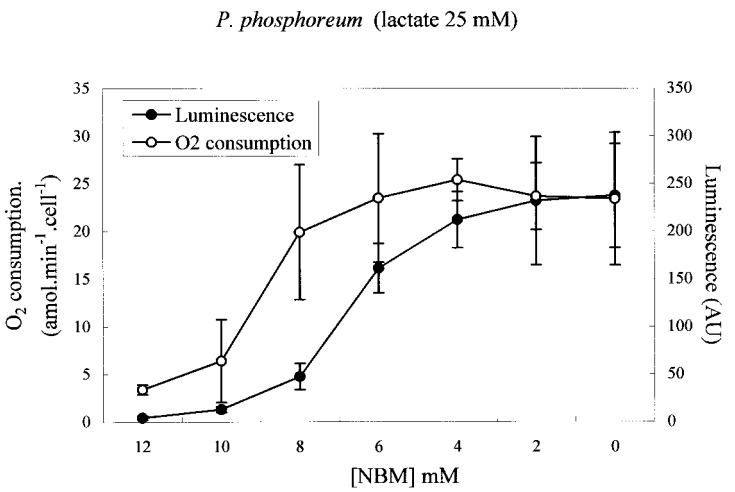


**Fig. 7.** *n*-Butyl malonate (NBM) titration of $O_2$ consumption and luminescence. NBM (12 mM) is sufficient to block both respiration and luminescence. Decreasing [NBM] in the incubating medium first restores respiration. When [NBM] = 8 mM, respiration is already optimal, while luminescence remains inhibited; at a concentration of 4 mM, luminescence intensity is almost recovered. The electronic flux which originates by catabolic processes, enters the luciferase pathway only when the respiratory chain is fully supplied.

*P. phosphoreum* is an ideal tool for studying the regulation of bioluminescence by oxygen concentration.

In steady-state conditions, respiratory substrates, especially lactate and malate, perform better. This seems to be paradoxical as they are less reduced than glycerol; this observation is contrary to our hypothesis suggesting that luminescence intensity should be directly related to the availability of reducing equivalents. The enhancing effect of malate on luminescence in *P. phosphoreum* was first observed by Makigushi *et al.* (1980).

## Contribution of the Luciferase Pathway to the Total $O_2$ Consumption

The value we found, which is the same for the three bacteria, corresponds to earlier measurements made with *V. harveyi* (12%), but not with other bacteria (20%) (Hastings, 1978; Makemson, 1986). A slight underestimation of the contribution of the luciferase pathway in our methodology is not excluded, since it was reported *in vitro* (Makemson, 1990) that cyanide at millimolar concentrations could form with free aldehyde an inhibitory complex of luciferase. This might explain the decrease of ≈30% of the maximum luminescence intensity observed when bacteria are incubated on cyanide. If we suppose that the oxygen consumption by luciferase was similarly reduced by 30% , the contribution of luciferase to the total oxygen consumption can be adjusted to ≈17%.

## $P_{50}$ Determination of Bacterial Luciferase and Cytochrome Oxidase

Previous attempts to determine the $[O_2]$ giving half-maximal light emission were made by extrapolation, as an appropriate technology of low-level oxygen essays was inexistent, and the results obtained were, therefore, controversial (Oshino *et al.*, 1972; Lloyd *et al.*, 1985). To our knowledge, direct evaluation of both parameters has presently been achieved for the first time. In optimal working conditions, the HPLC system used here is able to detect dissolved oxygen down to a concentration of ≈1 nM, which is 100-fold more sensitive than conventional Clark electrodes. The disadvantage of this new method is that a continuous monitoring is not yet possible. When this technical improvement becomes available, the pattern of the curve represented on Fig. 3B, should appear sigmoidal, as it has already been observed when more dots are available (1998).

Another conclusion is that the concept of "anaerobiosis," (as used in general biology), should be more accurately defined. Indeed, it is possible to prove, by the presence of bacterial luminescence, that oxygen is still present in a biological environment usually considered anaerobic. In microbiology, for example, paraffin is often layered on top of a culture medium to avoid gas exchanges between the medium and the atmosphere; when bioluminescent bacteria are present in the medium, a luminescent zone stays at the interface between the paraffin and the

medium, clearly indicating that oxygen diffuses through paraffin.

### Metabolic Regulation of Bacterial Bioluminescence (Partitioning)

Under aerobic conditions, reducing equivalents provided by $FMNH_2$ are consumed first by the respiratory chain; when this system nears saturation, the luciferase pathway is activated. This confirms an earlier hypothesis suggesting that bacterial luciferase is principally a reducing equivalents-scavenging enzyme (Makemson and Hastings, 1986). Within the cell economy, it dissipates the energy contained in an overflow of electronic flux. Usually microorganisms convert energy produced by catabolic processes by enhancing anabolic ones (biomass production). Here, considering the ecological niche of bioluminescent bacteria (mostly symbiotic organisms living in confined environments), biomass increase is rapidly limited when cells are still in an exponential growth phase. Normally, catabolic reactions should slow down by feedback autoregulation. This seems not to occur in bioluminescent bacteria, where catabolic processes continue while anabolic ones are blocked. The energy liberated by catabolism, but not used by anabolism, is dissipated in the form of light. This is a consequence of the phenomenon called "autoinduction," presently renamed "quorum sensing." Autoinduction allows bioluminescent bacteria to sense their elevated density in the light organ of the host and express the luminescence system there, where it is required for the symbiosis, but not in seawater, where luminescence, which it is energetically expensive, would be frivolous (Greenberg, 1997).

The entire photogenic system of bioluminescent bacteria scavenges not only reducing equivalents (luciferase), but also ATP and NADPH (fatty acid reductase complex) generated by catabolic processes. It appears to work as a free-energy dissipating safety valve or, in other words, as a well of an excess of reducing power. Indeed, when biomass production is not impaired, that is to say, when the bacterial cells are free in seawater where their density is as low as $\approx$5 per milliliter, the energy expended by bioluminescence represents only 0.007% of the total catabolic energy output (Makemson and Gordon, 1989) instead of 17%.

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