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Luciferase-Dependent Oxygen Consumption by Bioluminescent Vibrios

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Oxygen uptake due to luciferase in two luminous *Vibrio* species was estimated in vivo by utilizing inhibitors having specificities for luciferase (decanol) and cytochromes (cyanide). Cyanide titration of respiration revealed a component of oxygen uptake less sensitive to cyanide which was completely inhibitable by low concentrations of decanol. From this it was estimated that in vivo luciferase is responsible for less than 12% (*Vibrio harveyi*) or 20% (*Vibrio fischeri*) of the total respiration. From these data in vivo bioluminescent quantum yields are estimated to be not lower than 1.7 and 2.6%, respectively.

It is of considerable interest to know what the energy requirements for in vivo luminescence might be, especially in relation to the total energy budget for the organism. Although estimates are not available for most luminous organisms, there are several studies with luminescent bacteria. Based on inhibition of respiration by cyanide, Eymers and van Schouwenberg (4) estimated that about 20% of the total oxygen consumption was attributable to the luciferase system in *Photobacterium phosphoreum*. Watanabe et al. (20) reported that 0.045 photons were emitted per oxygen molecule consumed in *P. phosphoreum*; based on a bioluminescent quantum yield of 0.2 (15), this would mean that the luminescent system was responsible for 23% of the cellular oxygen uptake. More recently, and since the present work was completed, Dunlap (3) measured the oxygen consumption and light emission in *P. leiognathi* cells just after removal from the light organ of the fish that serves as the host. Based on a quantum yield of 0.2, he calculated that 3.4% of the oxygen consumption is due to luciferase. A much lower percentage (100-fold less) was found for cultured bacteria.

In the experiments reported here I used cultured cells of two luminous *Vibrio* species, one of which (*V. harveyi*) is not known to occur as a light organ symbiont; the other (*V. fischeri*) was isolated from *Monocentrus japonicus* light organs (7). Oxygen uptake rates after inhibition by cyanide were used to estimate the oxygen consumption attributable to luciferase. This residual (cyanide-insensitive) respiration

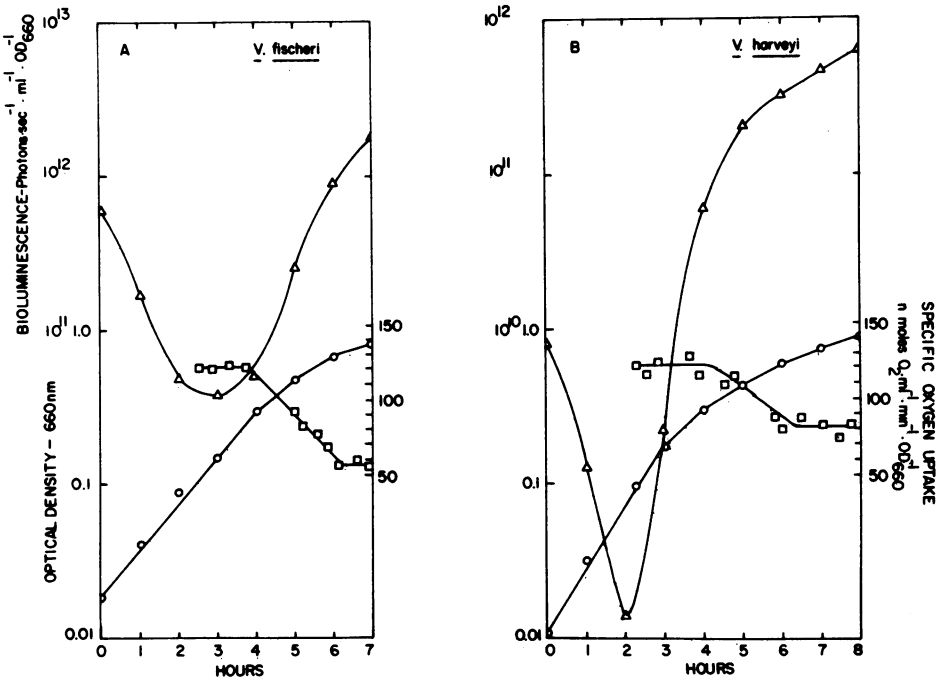

FIG. 1. Growth, bioluminescence, and oxygen uptake of *V. fischeri* (A) and *V. harveyi* (B) in HEPES-peptone. Note that the bioluminescence scale in A is 10× that of B; all the other scales are in registration. Shown are cell mass as optical density at 660 nm (○), specific bioluminescence (△), specific oxygen uptake (□), measured as given in the text.

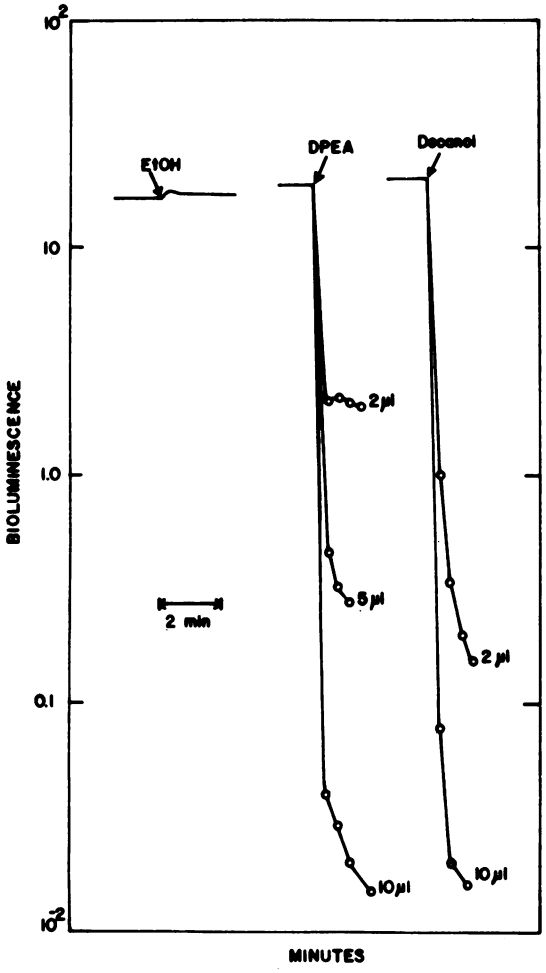

FIG. 2. Effect of DPEA and decanol on the bioluminescence of *V. harveyi*. Samples (1 ml) were removed from late log phase of a brightly luminescing culture. Different amounts of DPEA (0.1 M), decanol (5.1×10^{-6} M), or absolute ethanol were added at the times indicated by the arrows. One unit of bioluminescence is equal to 4.3×10^7 photons per s.

was completely blocked by decanol, an inhibitor specific for luciferase. This procedure has the advantage of including any and all oxygen consumed by the luciferase pathway whether or not light is emitted, and thus it is independent of the bioluminescent quantum yield.

MATERIALS AND METHODS

Cultures. Stock cultures of *V. harveyi* B-392 and *V. fischeri* MJ1 (1, 16) were maintained on slopes of Difco marine agar (Difco Laboratories, Detroit, Mich.) to which 0.1% (wt/vol) calcium carbonate and 0.3% (vol/vol) glycerol were added. Broth cultures were grown in a peptone-enriched HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)–minimal medium at pH 7.5 (11) that contained 5 g of Bacto-peptone (Difco), 20 g of NaCl, 11.9 g of HEPES, 6 g of $MgSO_4$, 0.75 g of KCl, 1 g of NH_4Cl, 0.21 g of alpha-glycerol phosphate, and 3 ml of glycerol per liter of deionized water. In one experiment the cultures were grown in HEPES medium in which 0.2% Difco Casamino Acids (vitamin free) replaced the peptone.

Optical densities of the cultures, measured at 660 nm in a Bausch & Lomb Spectronic 20 spectrometer, were used as a measure of cell number and cell biomass. One optical density unit represents 10^9 cells per ml. Bioluminescence was measured with a calibrated photomultiplier photometer (8, 13) at 25°C and was expressed as photons per second per milliliter. Broth cultures were incubated with shaking (200 rpm) at 25°C.

Oxygen consumption. Measurements of oxygen consumption were made at 25°C with a temperature-controlled Rank oxygen electrode calibrated to deionized water saturated with air. Because some rates of oxygen uptake were low, it was essential in those experiments to check the electrode calibration between every sample. Specific oxygen uptake rates are expressed as nanomoles of O_2 per milliliter per minute per unit of optical density, which is equivalent to the oxygen consumption rate of 10^9 cells.

Inhibitor studies. Samples (1.0 or 1.5 ml) were removed from growing cultures at or near maximal bioluminescence (late log phase) and placed in the oxygen electrode assembly or photometer. The inhibitors were added in 2- to 20-µl amounts from concentrated stock solutions after the uninhibited rate of oxygen uptake or bioluminescence was measured on a strip chart recorder.

Chemicals. 2,3 Dichloro-(6-penolphenoxy)-ethylamine (DPEA) was a gift from Eli Lilly and Co. (Indianapolis, Ind.), decanol and the long-chain alcohols were from Aldrich

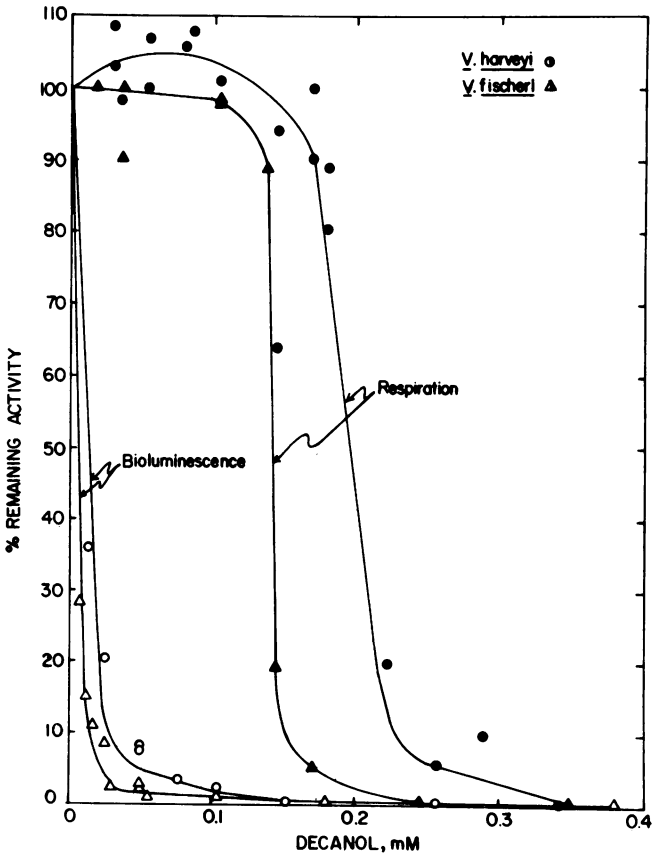

FIG. 3. Effect of different concentrations (final) of decanol on bioluminescence (○, △) and respiration (●, ▲) of *V. harveyi* (○, ●) and *V. fischeri* (△, ▲) measured 1 min after decanol addition. Cells were taken from bright, late-log-phase cultures grown in HEPES-Casamino Acids at 25°C.

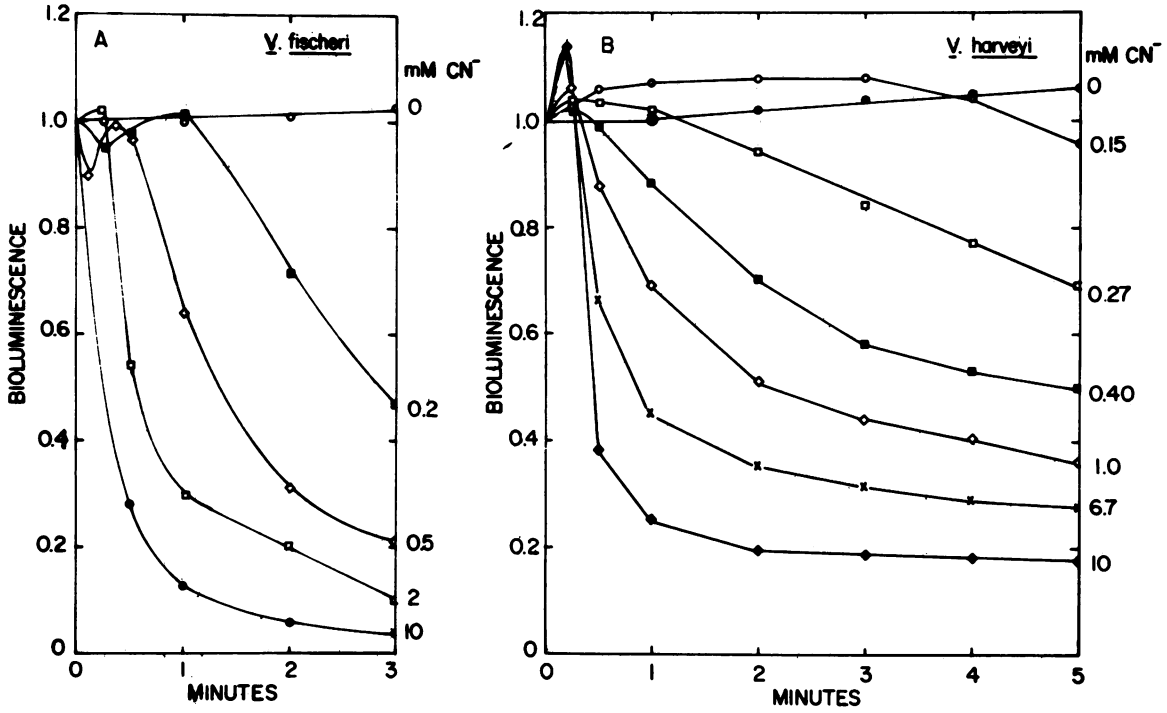

FIG. 4. Effect of different concentrations of cyanide on the bioluminescence of *V. fischeri* (A) and *V. harveyi* (B). Different amounts of 1 M cyanide (microliters as noted) were added. The luminescence of 1-ml samples of the culture just before the additions was set equal to 1.

Chemical Co., Inc. (Milwaukee, Wis.), and all other reagents were of analytical grade. Stock solutions were prepared fresh in distilled water (KCN, DPEA) or in absolute ethanol (decanol) (10 μl of long-chain alchohol to 10 ml of ethanol).

RESULTS

Oxygen uptake during growth. In both *V. fischeri* and *V. harveyi* the specific oxygen uptake rate (absolute rate divided by the cell biomass) was highest and constant during

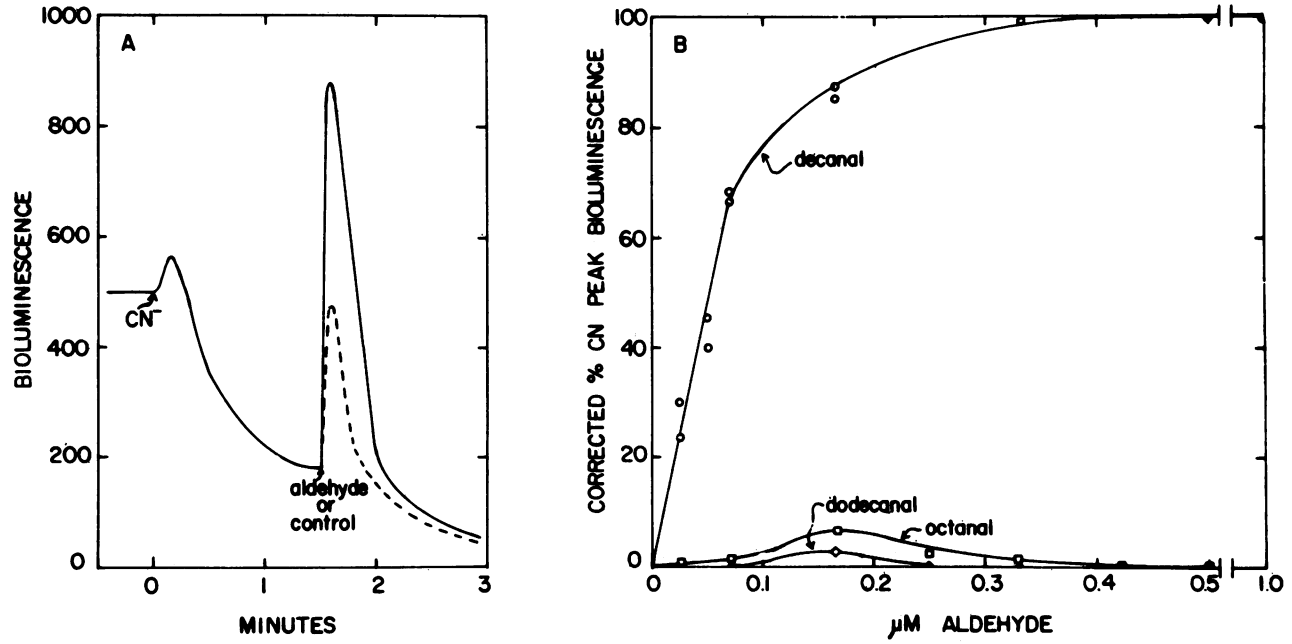

FIG. 5. Aldehyde reversal of cyanide inhibition of *V. harveyi* bioluminescence. A, Time course of luminescence after cyanide addition (10 mM final concentration), followed by aldehyde (10^{-5} M decanol in 20% ethanol, final concentrations), or 20% ethanol alone (-----) as a control for optical effects of the injection (see reference 12). B, Comparison of aldehyde reversal of cyanide inhibition of three aldehydes: octanal, decanal, and dodecanal. The percent CN peak bioluminescence was corrected for the control (no aldehyde) injection. Culture samples were 1 ml, and all injections were 1 ml.

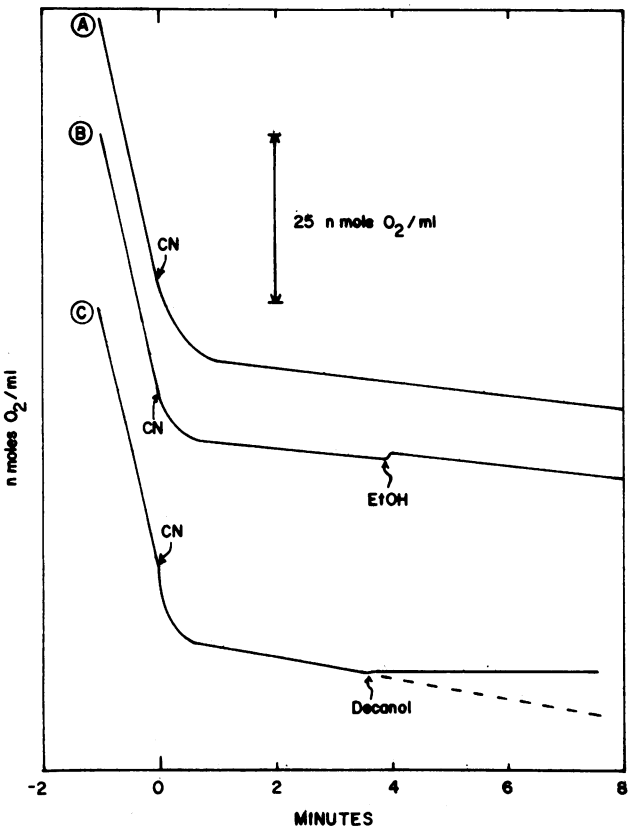

FIG. 6. Effect of cyanide on the oxygen uptake rate of *V. harveyi*. Samples (1.5 ml) of bacteria growing in HEPES-peptone were placed in the oxygen electrode vessel. After the initial uninhibited rate (here, 37.4 nmol of O_2 per ml per min) was established, cyanide was added at the arrows (10 mM final concentration; 0 time) with 40 to 50% oxygen saturation remaining. The curves are separated in this plot. Other additions were (A) none, (B) 1.5 μl of absolute alcohol, and (C) 1.5 μl of 0.5 M decanol in absolute alcohol. The dashed line in (C) is taken from curve A representing the cyanide-resistant respiration, 2.8 nmol of O_2 per ml per min without decanol. In these experiments, the respiration rates did not lower the oxygen concentration below 20 to 30% saturation.

the early logarithmic phase of growth, but then decreased to a lower plateau during the late-log and early-stationary phases (Fig. 1). During this transition period, as a result of autoinduction (15), the luminescence of the bacteria increased dramatically, attaining a maximal level of 650 to 1,000 photons per s per cell for *V. harveyi* and 2,000 to 3,000 photons per s per cell for *V. fischeri* in HEPES-peptone medium.

Luciferase inhibitors. The specific inhibition of luciferase might allow one to determine the fraction of oxygen uptake attributable to the luminescent pathway. Long-chain alcohols and DPEA are two such inhibitors whose inhibitory action has been attributed to blockage of the aldehyde-binding site (2, 14, 17, 21). For *V. harveyi*, decanol was more effective than the other chain lengths tested. The percent inhibition (after 1 min) of luminescence of 1 ml of a bright culture (1.2×10^{10} photons per s per ml) was as follows (after the addition of 10 μl of the long-chain alchohol solution or ethanol): for octanol, 74%; for nonanol, 87%; for decanol, 98%; for undecanol, 94%; for dodecanol, 74%; for ethanol, 0%. Inhibition of bioluminescence by long-chain alcohols and DPEA was rapid (Fig. 2). Similar effects were found with *V. fischeri*. At the concentrations of decanol required to block bioluminescence, there was no decrease, but rather a slight stimulation, of the rate of oxygen consumption (Fig. 3). Inhibition of respiration required considerably higher concentrations of decanol. Compared with decanol, much higher concentrations of DPEA were required to inhibit bioluminescence and respiration, and the DPEA concentrations that completely inhibited bioluminescence also inhibited respiration (data not shown). It is of particular importance to note that the lowest decanol concentrations that completely inhibit bioluminescence had no inhibitory effect on respiration.

How could it be that decanol inhibition of luciferase, an oxygen-requiring enzyme, resulted in no decrease in the rate of oxygen consumption? One possibility is that the fraction of the oxygen uptake due to luciferase was small compared with that due to the cytochrome pathway. Another is that under these conditions the cytochrome pathway was not operating at saturation, and the electron flow via luciferase, especially if small, could have been diverted to and absorbed by the cytochrome pathway.

Cytochrome inhibitors. Azide and cyanide were tested to ascertain whether cytochrome respiration could be inhibited without inhibiting bioluminescence. Sodium azide, up to 10 mM, did not inhibit respiration. Cyanide inhibited oxygen uptake almost completely, but it also inhibited bioluminescence and in an interesting way in *V. harveyi* (Fig. 4). After the addition of cyanide, there was an initial but transient stimulation of bioluminescence, which was greater at higher concentrations. This stimulation may be interpreted as due to an increased level of reduced flavin as a consequence of the blockage of electron flow via the cytochromes. A secondary inhibition of luminescence then occurred which was also greater at higher cyanide concentrations. *V. fischeri* bioluminescence (Fig. 4) appeared to be somewhat more sensitive to inhibition by cyanide than that of *V. harveyi*.

Cyanide inhibition of in vivo bioluminescence could be overcome by the addition of exogenous aldehyde (Fig. 5); the reversal was dependent on the concentration and the chain length of the aldehyde. This suggests that the inhibition of luminescence involves either inhibition of aldehyde synthesis, as recently suggested by Grogan (5), or reaction of cyanide with aldehyde to form cyanohydrins (Makemson and Hastings, unpublished data). Since the luciferase-catalyzed oxidation of reduced FMN can proceed in the absence of aldehyde (the "dark"pathway), possibly at a slower rate, this inhibition of luminescence by cyanide would not completely block luciferase oxygen uptake.

The kinetics of inhibition of oxygen uptake by cyanide are shown in Fig. 6. Additions were made after establishing the uninhibited rate; a new and much lower steady-state rate of oxygen uptake was arrived at in less than 1 min. The postulation that this remaining oxygen uptake (at high cyanide concentrations) is due to luciferase was tested by adding decanol; respiration was then completely abolished (Fig. 6).

If this residual cyanide-insensitive respiration is really due to luciferase, then it should be absent in bacteria possessing very low amounts of luciferase. With cells harvested at 2 h (Fig. 1), more than 99% of the oxygen uptake was inhibited by 3 mM cyanide.

Inhibition of respiration by cyanide was biphasic as related to cyanide concentration (Fig. 7A). The major decline, which occurs at the lowest cyanide concentrations (less than 0.2 mM), is attributed to cytochrome respiration. The second and less pronounced effect is attributed to inhibition of

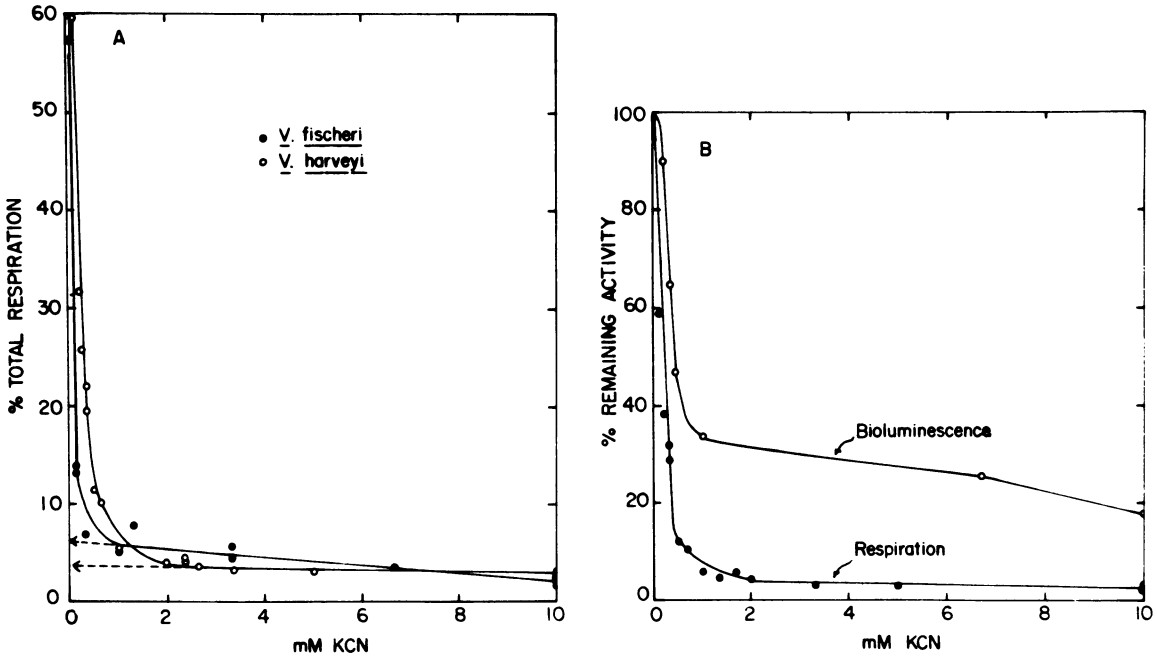

FIG. 7. Cyanide titration of oxygen uptake and bioluminescence. A, Cyanide titration of oxygen uptake of samples (1.5 ml) of *V. fischeri* (●) and *V. harveyi* (○). After the initial uninhibited rate was obtained, microliter quantities of 1 M fresh KCN were added, and the cyanide-inhibited rate of oxygen uptake was measured and expressed as a percentage of the uninhibited oxygen uptake rate. B, Comparison of cyanide inhibition of oxygen uptake and bioluminescence of *V. harveyi*. Inhibition of bioluminescence was measured as described in the legend to Fig. 3 for decanol inhibition.

the luminescent system, because this remaining oxygen uptake was completely inhibitable by decanol. By extrapolation of the cyanide-resistant respiration curves back to zero cyanide concentration, estimates can be made for the in vivo oxygen uptake by luciferase in the presence of cyanide. Because luminescent oxygen uptake is partly cyanide sensitive (assuming 70% inhibition in the case of *V. harveyi*), the luminescent oxygen uptake in the presence of cyanide is calculated to be 12% [3.6% × (1/0.3)] for *V. harveyi* and 20.7% [6.2% × (1/0.3)] for *V. fischeri*. The standard error of oxygen uptake measurement in the presence of cyanide was 38% of these values.

Figure 7B compares the effect of cyanide on respiration and bioluminescence of *V. harveyi*. Similar data were obtained for *V. fischeri*; respiration was more sensitive than bioluminescence to cyanide inhibition.

DISCUSSION

Previous measurements of total oxygen consumption rates of luminous bacterial cultures have been expressed in a variety of different units (4, 6, 8–10, 18–20). To make comparisons, all of the data have been converted to nanomoles of oxygen consumed per minute per 10^9 cells regardless of temperature (20 to 30°C). The data of Karl and Nealson (9) range from 10 to 15 nmol of O_2 per min per 10^9 cells, whereas in other reports the values range from 120 to 300 nmol of O_2 per min per 10^9 cells (4, 6, 10, 18–20). Luminous bacterial symbionts (*Photobacterium leiognathi*) taken directly from ponyfish light organs have been reported to consume 150 ± 70 nmol of O_2 per min per 10^9 cells (3). The values obtained in the present work range from 50 to 120 for *V. fischeri* and 80 to 120 for *V. harveyi*, close to the values of Dunlap (3) and most earlier reports.

Several of the recent estimates of the oxygen consumption attributable to luciferase have been made by calculations based upon meansurements of the light emission and overall oxygen consumption rate (4, 9, 20). Such estimates are subject to errors from at least two sources: (i) assumption of the in vivo quantum yield of the luminescent pathway and (ii) the possible existence of nonluminescent luciferase-mediated oxygen consumption.

The estimates from the present experiments of the in vivo oxygen consumption attributable to luciferase are derived instead from extrapolations of the cyanide-resistant respiration (at cyanide concentrations above 2 mM) to the uninhibited level and are not subject to the two errors mentioned above. These values are for fully induced cultures possessing maximal (or nearly so) levels of luciferase. From this the fraction of oxygen uptake by these bacteria through luciferase appears to be somewhat lower than some of the previously reported values (4, 9, 20).

Based on the uninhibited level of bioluminescence the culture, the in vivo quantum yield can also be estimated from these data. For *V. harveyi* in HEPES-peptone medium, the bacteria consume 80 nmol of O_2 per min per 10^9 cells (Fig. 1). Of this, luciferase consumes 9.6 nmol of O_2 per min or 5.6×10^{15} molecules of oxygen per min. Based upon 1,000 photons per s per cell, 10^9 cells emit 6×10^{13} photons per min. A bioluminescence quantum yield in vivo (photons emitted per O_2 molecule taken up by luciferase) of 0.017 can then be calculated for *V. harveyi*. A similar calculation for *V. fischeri* gives a value of 0.026, based on 3,000 photons per s for 10^9 cells (Fig. 1).

ACKNOWLEDGMENTS

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