BIOLUMINESCENCE FROM SINGLE BACTERIAL CELLS EXHIBITS NO OSCILLATION

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ABSTRACT Since the usual measurements of light emission from marine bacteria involve many (10^6–10^{10}) cells, the question has often been raised as to whether or not the individual cell's luminescence is truly continuous. To investigate this question, we assembled a sensitive photon-counting system with computerized data acquisition. Several luminous species were studied: *Beneckea harveyi, Photobacterium belozerskii, P. fischeri,* and *P. leiognathi.* Isolated single cells gave count rates ranging from 2 to 10 times the background, depending on the brightness of the strain and the state of induction. No flashes, bursts, or oscillations were evident from data collected in counting intervals of 100 ms, using both photon time-correlation and power spectral analysis. Our algorithms could detect an oscillating component with an intensity as low as 0.3% of the average, as determined by the analysis of reference light sources. That photons are emitted randomly was further shown by the fact that the count distribution from the living cell closely matched that of a reference light source attenuated to the same average count rate.

INTRODUCTION

In many luminous organisms light emission occurs as flashes of ~100 ms duration, and sometimes as trains or bursts of such pulses (1, 2). Such flashes are known to be of functional importance in some species, either offensively, defensively, or in communication (3). Cellular control mechanisms may be involved in turning on or off the biochemical systems (luciferin-luciferase) directly responsible for the light emission (4).

In contrast, there are a few organisms, such as luminous bacteria and fungi, in which light emission is apparently continuous (no bursts or flashes). Since the usual measurements of emission from bacteria involve many cells (10^6–10^{10}), the question has been raised as to whether or not the luminescence is truly continuous at the level of the individual cell (5). The single cell light output might occur in bursts or exhibit rhythmicity, reflecting perhaps some fluctuations or oscillations in the flow of electrons from substrate to oxygen; bacterial luciferase may be viewed as a shunt of this pathway. Hagins et al. (5) first investigated this question and concluded that the bacterial emission exhibited no oscillation. However, their experimental method was relatively insensitive; since many cells were used, only large amplitude oscillations could have been detected.

More recently, there have been reports of periodicity in light emission from bacteria (6, 7). In the former publication (6), light emission from several (5–10) cells was measured in a photon counter at a limiting resolution of 2 ms. By using time correlation, it was deduced that there was an oscillation with a fundamental frequency of 8 cycles/s, thus a peak about every

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0.125 s. In a subsequent publication from the same laboratory (7), light measurements were reported from single isolated cells, as verified by plating for colony counts. Light was reported to be emitted in discrete flashes, each ~0.1 s duration (decay rate, 0.3–0.5 s^{-1} at 20°), with several (5–10) flashes in a packet within a period of 5 to 10 s; the packets were repeated about every 50 s (0.02 Hz).

The rate of light emission from a single bacterium provides a unique tool for studying biochemical phenomena at the cellular level. It permits the continuous monitoring in vivo of one enzymatic reaction rate, without altering the cell or its environment. Moreover, the sensitivity is very high: the reaction of as few as 10,000 substrate molecules per second can be followed. Since the bioluminescence reaction is coupled to the pathway of electron transport in the cell (8), the existence of oscillations in the light output would have important implications for the energy metabolism and its control.

For all these reasons we undertook to investigate the light emission of single isolated cells and to evaluate the possibility of oscillations in this emission. For this purpose, we have assembled a sensitive detection system linked to a computerized data acquisition system. We studied several strains of bacteria and used a variety of techniques to acquire and analyze data. We were, however, unable to detect any oscillations, pulses, or any type of emission that differed beyond our experimental error from fluctuations of a continuous incoherent source.

MATERIALS AND METHODS

Bacteria, Media, and Growth of Cells

The following strains were used in this study: *Beneckea harveyi;* strain MAV, 392 (9); *Photobacterium fischeri;* strain MJ-1, (10); *Photobacterium leiognathi,* strains EGMB and 721, and, through the courtesy of Dr. Gitel'zon (L. V. Kirenskii Institute of Physics, Krasnoyarsk, U.S.S.R.), *Photobacterium belozerski* (6–8).

Cells were grown on a complex medium made up of artificial sea water (ASW) to which 0.3% glycerol and 0.5% casein amino acid (Difco Laboratories, Detroit, Mich.) were added. ASW contains (grams per liter): NaCl, 17.55; KCl, 0.75; $MgSO_4 \cdot 7H_2O$, 12.3; $CaCl_2 \cdot H_2O$, 1.45; and $K_2HPO_4 \cdot 3H_2O$, 0.075, buffered at pH 7.3 with 0.02 M 3-(*N*-morpholino) propanesulfonic acid. Stock cultures were maintained on a solid medium (1.5% agar) of the same composition. Growth was carried out in liquid culture with shaking at 28°C, except for *Photobacterium fischeri,* which was cultured at 22°C. Cell density was determined at 660 nm in a spectrophotometer (Coleman Systems, Irvine, Calif.; model 620). Before light measurement, cells were diluted in ASW with different additives such as glycerol, myristyl aldehyde, myristic acid, or NaCN, as indicated in each experiment.

Measurements of Luminescence from Single Cells

In some species the intensity of the luminescence of bacteria depends upon the stage in the growth cycle; the luminescent system and luciferase are induced (8, 11–13). At its peak, luminescence in induced bacteria may be up to 10^5 times greater than in noninduced cells, and may be readily measured in single cells.

As reported by Booth and Nealson (14), single cells may be obtained by tracking sequential dilutions through repeated transfers from vial to vial. To do this, the contents of a vial are divided each time into two vials and light emission from both is measured. When a vial contains only a single cell, all the counts go to only one of the two vials in the next transfer. That there is one and only one cell in the vial is then confirmed by several more transfers in which light emission is found in only one of the vials. A typical flow chart of such a procedure is traced in Fig. 1 for *P. leiognathi.*

In all our experiments single cells were selected by this procedure and suspended in ASW without any

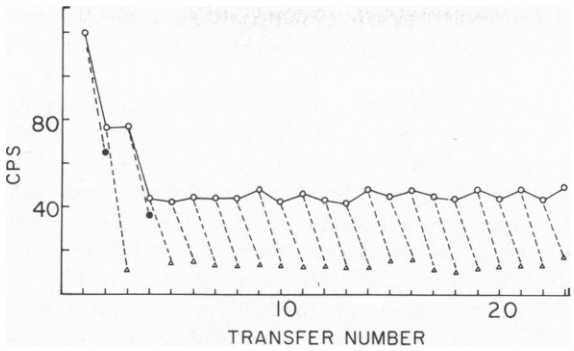

FIGURE 1 "Divide and measure" experiments. A culture of *P. leiognathi* (strain EGMB) was grown in liquid medium at 28°C. At a density of ~5 × 10^8 cells/ml the culture was diluted ~10^7-fold in ASW with 1 mM NaCN, 5 μM myristic acid, and 0.3% glycerol added. A 0.1-ml sample was measured for light output, which is the first point on the graph. Following that, another 0.1 ml of the same medium was added, the sample was divided into two tubes, and their luminescence was measured. Both samples appeared to contain light-emitting cells, so one of them was discarded (filled circle) and the other redivided for the next measurement. This procedure of "divide and measure" was continued until only one of the sample tubes contained an emitting cell (open circles). The measurement from the other tube (triangles) was the same as background. After this, several additional transfers were made to verify that only a single cell remained. In the experiment shown, only one cell appears to be present from the fourth transfer on.

nitrogen source, thus preventing further growth. The luminescence of cells suspended in such a medium (and also, in a growth medium) typically decreases by a factor of 10 or more over the period of 1–2 h (11, 15, 16). This can be largely prevented by the addition of substrate (0.3% glycerol), or 1 mM NaCN, or 5 μM myristyl acid or aldehyde. The glycerol provides a continuing source of electrons to maintain the level of reduced flavin. Exogenously added myristyl aldehyde or acid can enter the cell and react directly in the luminescence reaction, with the acid being first reduced to the aldehyde (16). The cyanide is believed to act by preventing the flow of electrons to oxygen via the cytochromes: a high intracellular reducing power is thus maintained, flavin remains in the reduced state, and the fatty acid produced by the luciferase reaction is reduced to the corresponding aldehyde (16, 17).

Instrumentation

The data collection system for the measurement of emission by a single cell was based on low noise photomultipliers operated as photon counters. We used two different detection systems: (1) A scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.; model 2003) using only one of the photomultipliers (EMI Electronics Ltd., Middlesex, England) and (2) a photomultiplier tube (ITT, Roanoke, Va.; model FW-130) with 0.5-cm-diam photocathode.

In system 1, a low-potassium glass tube 4 mm i.d., 50 mm long, selected for low background emission, was fastened to the side wall of a scintillation vial and used as the sample container. A mirror was used to reflect the light on the phototube.

In system 2 a thermoelectrically cooled, RF-shielded housing was used (Products for Research, Danvers, Mass., model TE-104 RF). The solution containing a single bacterium was placed in a spherical cell (3.5 mm i.d.), imaged with no magnification on the photocathode by a pair of identical aspheric lenses (Melles Griot, Arnhem, Holland; focal length, 33 mm; diameter, 52 mm). The second lens was also the inner window of the PMT-housing. Light collection efficiency was doubled by means of a spherical mirror whose focus was at the sample cell itself.

In both cases the phototube output was fed via a preamplifier into a photon counter (Ortec Inc., Oak Ridge, Tenn.; amplifier discriminator 9302, fast preamplifier 9301, and counter 9315).

The photon counter was controlled by a Nova 1200 computer with 32 k words of core and 2.5-M byte

disk (Data General Corp., Southboro, Mass.). All subsequent data analyses were done on this computer. The counts accumluated by the photon counter were either stored on the disk or processed between actual counting times.

When data were collected and stored, the length of counting time intervals was controlled by the internal clock of the photon counter, which issued a stop signal at the preset time. The scalar was then read by the computer program and the counter was reset and restarted by a signal generated by the program after 2.5 ms dead time. (This dead time was caused by the program.) When the system was operated in the on-line processing mode, the length of counting intervals was determined by the number of steps in the program which generated the stop, reset, and start signals with a dead time of a few microseconds. The length of the counting intervals was preset by the number of time points in the shift register, and the length of a dummy loop in the program. The time calibration of the counting intervals was tested by measuring in our system the time-correlation of a signal of a known frequency generated by a stroboscope. The lower limit for $\Delta\tau$ in this program was 0.5 ms, when only 16 channels were used and no dummy loops in the program were executed.

The efficiencies of the systems were estimated by measuring the light output of a freshly diluted bacterial suspension. The photon output of this suspension was determined with a standard photometer operated in a current mode (18), calibrated with a ^{14}C-hexadecane standard (19). The overall efficiencies of the systems under our working conditions were found to be 0.010 ± 0.005 counts/photon when the Packard counter phototube was used as a detector (system 1 above) and about one-third of that when the ITT tube (system 2) was used. The average background (including dark counts and luminescence from glassware, etc.) was 8–10 cps for the first system and 3–4 cps for the second. The corresponding noise equivalent signals were 800–1,000 photons/s for tube one and 900–1,200 for tube two. There were some points within the cell at which light trapping by total internal reflection was significant (20). In particular, this occurred for positions within approximately $d/10$ of the cell wall (where d is the cell diameter), and within a small angular range about the plane normal to the axis of the detection system. The reflective nature of the enclosure suppresses this effect and makes detailed calculations difficult, but variations of up to 10–20% in light collection efficiency may be possible. Such variations would be aperiodic and may contribute to the low frequency fluctuation spectrum.

We found it convenient to employ weak reference light sources activated by radioactivity to analyze the data and simulate a statistically nonoscillating light source. For each of the detection systems we therefore prepared a sealed sample tube with a dilute solution of H^3-toluene in toluene (with POP and POPOP as fluorescers), as previously described (19). These will hereafter be referred to as reference light sources. The photon counter was operated without an upper threshold limit, so that each multiphoton radioactive disintegration was counted as a single event.

Data Processing

To detect possible nonrandomness in the photon emission, we used three approaches: photon time-correlation (on- and off-line), power spectral analysis using the fast Fourier transform, and statistics of count rates.

I. Photon Time-Correlation

ON-LINE Computer storage capacity and computing time become limiting factors when frequencies >10 Hz are measured. To avoid this problem, we used a program that essentially computes, without storage, $C_k(\tau)$, the singly "clipped" photon counting time-correlation function $C_k(\tau) \equiv \langle n_k(0)n_k(\tau)\rangle\Delta\tau$ as follows. In each experiment the average count rate is first determined and a threshold k (which is equal to that average rounded up to the next integer) is set for the number of counts per time interval $\Delta\tau$.

At the end of time interval t_1, the number of counts $I(t_1)$ is read from the photon counter and compared with k. If $I(t_1)$ is greater than k, the corresponding input value $n_k(t_1)$ is given a value of zero. At the next counting time, t_2, the value $n_k(t_1)$ is entered into the first location of a software shift register (with up to 1,024 bins). Upon each subsequent reading at time t_i, a new value of $n_k(t_i)$ is determined in the same fashion and is entered into the shift register with all previous bins being shifted along by one

bin. For each time t_i, the value of $n_k(t_1)$ is also "anded" with the contents of each shift register bin, and the result in each case is added to the corresponding bin of a nonshifting memory register of equal length. The accumulated result is the "clipped" time-correlation function

$$C_k(\tau) = \frac{1}{N}\sum_{i-1}^{N} n_k(t_i)n_k(t_i + \tau)\Delta\tau.$$

OFF-LINE When frequencies of 5 Hz and lower were investigated, we used an alternative procedure; namely, we stored the data on the disk, and analyzed them later in a batch mode. Up to 10 h of data could easily be stored using time intervals ($\Delta\tau$) of 0.1 s. The time correlation $C(\tau)$ is computed as

$$C(\tau) = \frac{1}{N}\sum_{i-1}^{N} I(t_i)I(t_i + \tau)\Delta\tau,$$

where $I(t_i)$ and $I(t_i + \tau)$ are the number of counts recorded at times $t = i\Delta\tau$ and $t = i\Delta\ \tau + \tau$, respectively. N is the total number of time points recorded.

II. Power Spectral Analysis

We have used a fast Fourier transform (FFT) (5, 21, 22) algorithm (provided by M. J. Ross, Genetech Inc., South San Francisco, Calif.) to compute the power spectrum of the output of the photon-counter recorded for a single bacterial cell and stored in the disk.

When measuring the light emission of a single bacterial cell we are faced with the task of detecting very low amplitude (possibly oscillating) components superimposed on a constant bacterial signal and electronic noise. Therefore it is essential to compare the power spectrum computed for the bacterial signal with the power spectrum of a constant light source which will include the same noise contribution from the measuring system as appears in the bacterial signal. Consequently, the protocol used for evaluating the statistics of bacterial light emission was applied to a reference light source, described above. The intensity of the reference source was masked with black tape to further attenuate it, so that it matched the average intensity of the light emitted by a bacterial cell. This measurement gives us the noise power spectrum for our experimental setup, and thus allows us to evaluate the significance of the amplitudes determined in the experiments with bacteria.

Statistics of Count Rates

If the light is emitted in a truly random fashion, then we can expect a Poisson distribution of the count rate with the low numbers of counts per time interval obtained with a single cell. Modulation of the rate of light emission, bursts, and/or flashes should cause deviation from the Poisson distribution. Hence one can detect even nonperiodic fluctuations in the rate of light emission by comparing the distribution of the number of counts per time interval with the expected Poisson distribution for the same average number of counts. We therefore calculate the histogram of the number of counts per time interval from the data recorded for single cells. Since the detector is not ideal, and there may be exogenous sources of noise, we compared the count rate distribution from the cell output with that obtained with the reference light source attenuated to the same average count rate in the same detection system. This comparison enables us to detect deviations from randomness in the photon output contributed by the living organism, even though the detection system itself is not ideal.

Test of Data Acquisition and Processing Methods I and II: Comparison with a Known Oscillating Light Source

To determine the lowest limit of detection of a possible oscillating component in the bacterial light emission, we have appoximated the intensity of a single cell using the reference light source combined with a known oscillating light component (Fig. 2). An example of such a signal, as detected by the photomultiplier, is given in Fig. 2 *A*. This periodic signal was attenuated by masking to an average level of only 0.06 cps and combined with the reference light source attenuated to an average intensity of 20

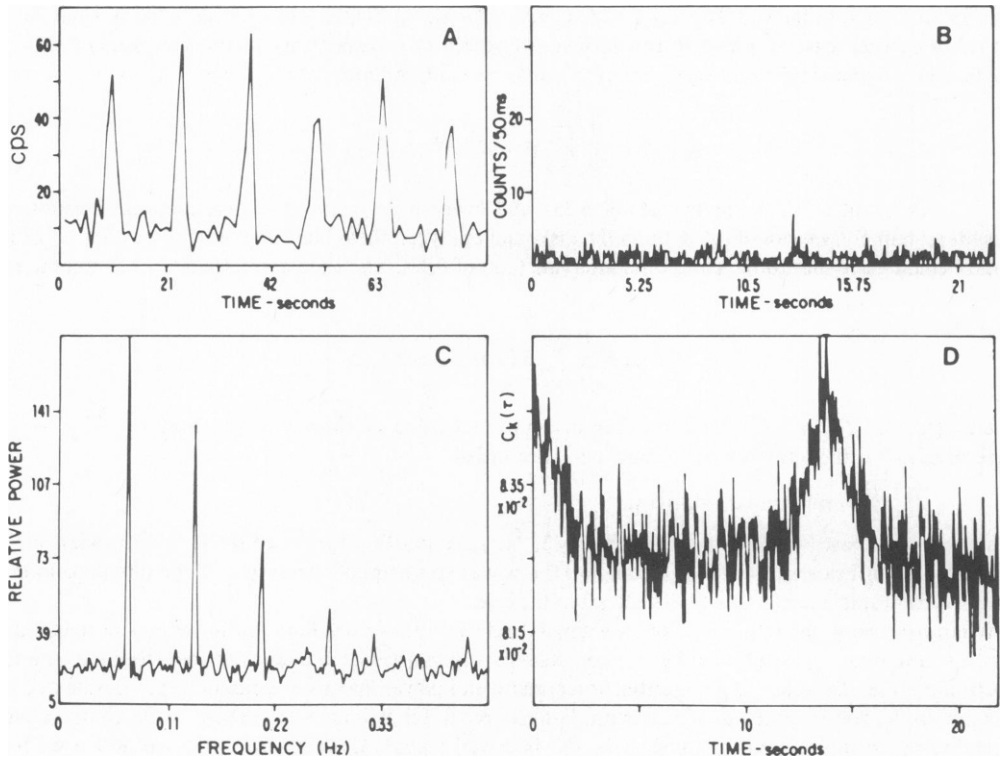

FIGURE 2 Measurements and analyses of a known oscillating light source. (*A*) A photodiode placed in the sample compartment and controlled by a function generator provided light pulses of ~2.6 s duration (width at half height) at a period of 13.8 s (frequency 0.072 Hz). The resulting waveform of the light output is shown as detected. (*B*) The reference light source was placed in the sample compartment next to the photodiode, and both were attenuated by masking so that 0.3% of the total number of counts per unit time was contributed by the oscillating source. By direct inspection of the resulting counter output, which averaged ~20 counts s^{-1}, it is not possible to differentiate the light oscillations from the noise. (*C*) The output *B* was used as input for the FFT algorithm. The result of analysis of 1.76 h of recording is presented. The fundamental frequency obtained is 0.072 Hz, as expected. (*D*) The same oscillating light source was used as input for the on-line time-correlation program, at a threshold level of $k = 1$, and sampling at 50-ms intervals. Averaging for 3 h by this procedure resulted in the period of 13.9 s, as shown. Ordinate, clipped time correlation, $C_k(\tau)$; abscissa, correlation time, τ (in seconds).

cps (including the dark counts). These count rates were determined by averaging over an extended time period (~30 min) both with and without the oscillating light source. The resulting signal (Fig. 2 *B*) was recorded and stored on the disk and then used as input for the FFT algorithm, whose output is traced in Fig. 2 *C*.

From this figure, it is evident that an oscillating component contributing 0.3% of the light intensity can be detected. However, reducing the oscillating light source to 0.1% of the average intensity renders this component undetectable (data not shown). Fig. 2 *D* shows that the clipped time-correlation program can also detect a 0.3% oscillating component.

Thus the methods can detect a perfect oscillating intensity component contributing as little as 0.3% of the total light intensity. However, any oscillating component that may exist in a bacterium's light emission is unlikely to behave as a perfect oscillator for the duration of the experiment (2–3 h). Consequently, the practical threshold for detection of an oscillating component in the bacterial emission will be higher.

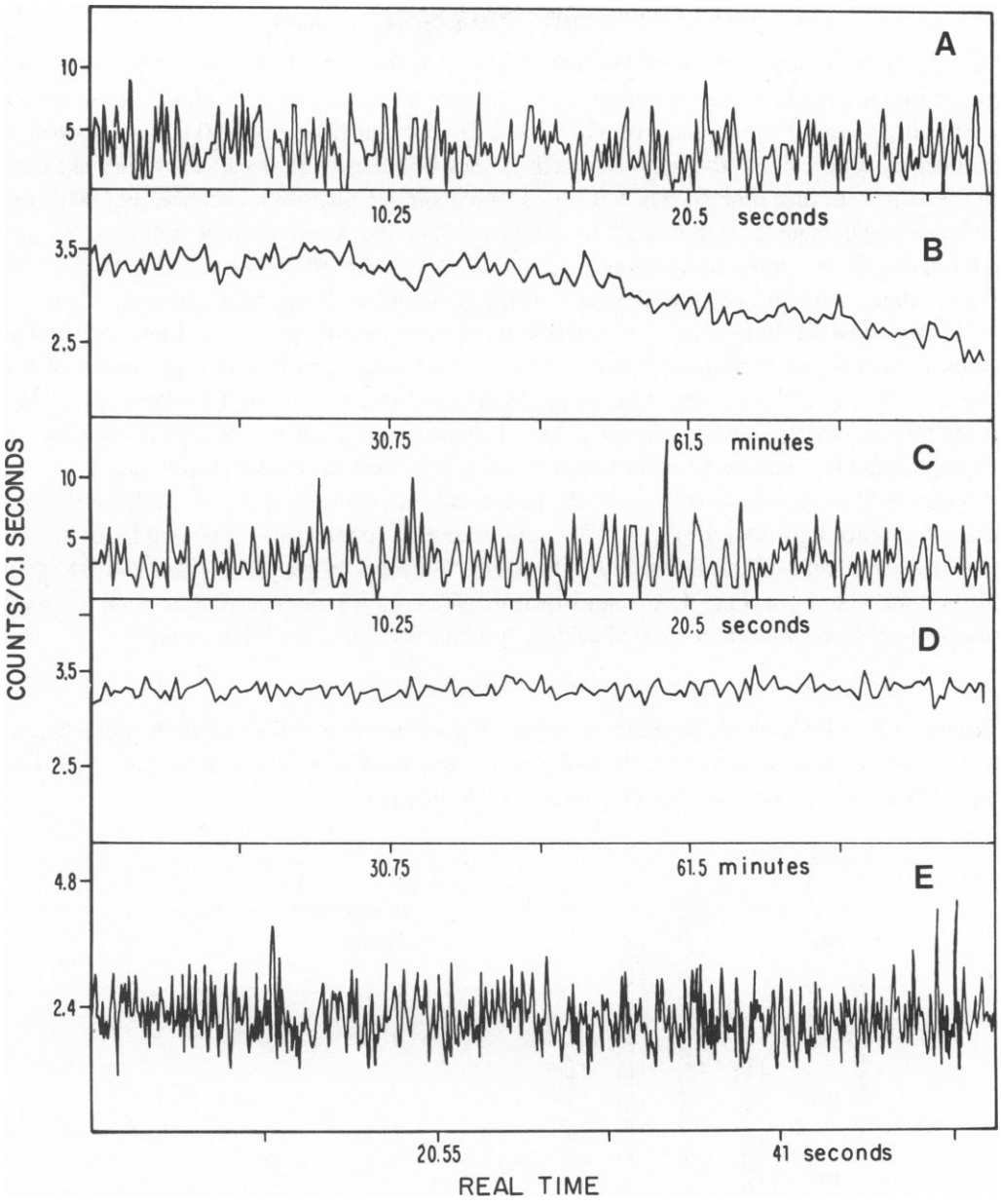

FIGURE 3 Light output of single cells compared with that of a constant light source. Cells were selected by the "divide and measure" procedure, and light emission from single cells was recorded directly from the output of the photon counter. In these experiments the output counts over 0.1-s intervals were stored in the disk, with a 2.5-ms dead time between measurements. (*A*) The light output over successive 0.1-s intervals of a single *Photobacterium fischeri* (MJ-1) cell suspended in ASW. (*B*) The same experiment is presented over a 90-min period. Each point here is the average of 300 points, which amounts to 0.5 min plus 0.75 s cumulative dead time. Background is ~600 cpm. (*C*) The light emission of the reference light source attenuated to the same level as the single bacterial cell, presented as in *A*. (*D*) The same experiment as in *C*, presented as in *B*. (*E*) Light output obtained from a single *Photobacterium belozerski* cell; each point is the average of 10 intervals of 0.1 s each.

RESULTS

Fig. 3 illustrates measurements of the rate of light emission from single isolated bacterial cells grown and maintained under conditions giving optimal luminescence. In Fig. 3 *A* the counts acquired during 0.1-s intervals are plotted as a function of time up to 30 s. The integrated number of counts over 30-s periods, which is shown in Fig. 3 *B*, decreases somewhat over the 90-min counting time shown. For comparison, similar measurements obtained with the reference light source (attenuated to approximately the same average intensity as the bacterial cell) are presented in Figs. 3 *C* and *D*. No difference between the bacterial and the reference emissions could be detected by direct inspection of any segment of the data.

We searched for flashes by two methods: by directly recording the real times at which a count occurred that exceeded the average by a preset value, and by batch processing of the recorded data at the end of the experiment. In no case, with any of the strains or media used, could we observe any bursts or flashes of light differing from the characteristic fluctuations in an appropriately attenuated reference light source with the same detection system.

This result is also demonstrated by the histograms of count rates of a single bacterial cell and a reference light source (Fig. 4). The reference light source was attenuated to match the average count rate of the living cell. Although a chi-square test (23) shows that the two histograms shown in Fig. 4 are statistically different, we do not regard such a small discrepancy as meaningful in view of evident systematic effects (see Discussion).

Search for Periodicity in Light Emission by Single Cells

Because we couldn't detect large bursts or flashes by direct observations, or in the pulse height histograms, we used time-correlation and power spectral analysis to search for low amplitude periodic oscillating components in the light emitted by single cells.

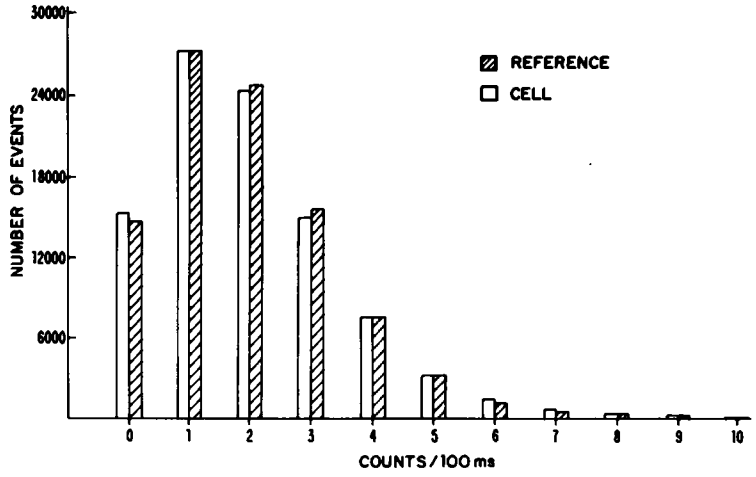

FIGURE 4 Histograms of the number of counts accumulated by the photon counter in 0.1-s intervals, both from a living cell and a reference light source. A cell of *P. fischeri* MJ-1 was suspended in ASW + 0.3% glycerol and the count output was recorded for 2.6 h. An average of 1.92 counts per 0.1 s was obtained (background was 1 count per 0.1 s). The same experiment was repeated and the output of the photon counter was recorded under the same conditions, measuring the light emitted by the reference light source, attenuated to give an average of 1.91 counts/0.1 s.

In the in vitro luciferase reaction the intermediates have decay times at 20°C of longer than 1 s. On the assumption that similar kinetics pertain in vivo, we searched for oscillations mainly in the frequency range of 0.01 to 10 Hz. The reaction in vivo may be faster, so we also used shorter time intervals, down to 1 ms. We have carried out many such experiments using different species and strains and under different medium conditions (see Materials and Methods). There was no difference, in any experiment, between the light emitted by a single cell and that emitted by the reference light source. The length of the experiments (2–3 h) was limited by the spontaneous decrease of the cellular light emission, which sets a limit on the sensitivity of the measurement. Although this could in principle be overcome to some extent by superimposing several replicate experiments with single cells, such a procedure would introduce new uncertainties due to possible differences in the actual frequencies of the individual cells. Therefore, each experiment was based on one cell only.

Fig. 5 *A* shows an example of the time correlation of the light emission of an isolated single *P. fischeri* cell (strain MJ-1) calculated on-line by the "clipped" time-correlation program. The threshold level, k, was adjusted to be equal to the average number of counts (rounded up to the next integer) per time interval. The time intervals were 50 ms; by using 1,024 time points, periodic changes in the rate of light emission with period times of up to 50 s (40 s in the plot shown) would have been detected. As in many other experiments performed with the several species studied, and also with the addition of myristic acid or myristyl aldehyde, no periodicity was detected. The results obtained with the living cell cannot be distinguished from those obtained with the reference light source (Fig. 5 *B*).

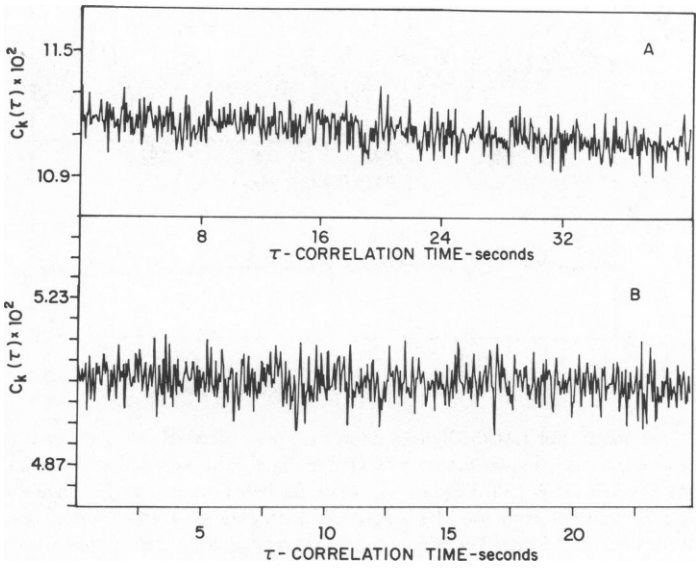

FIGURE 5 On-line time correlation. Ordinate, clipped time correlation, $C_k(\tau)$; abscissas, correlation time, τ (in seconds). (*A*) Time correlation of the light output of a single *P. fischeri* cell (strain MJ-1) in ASW with 0.3% glycerol. The light emission of the cell gradually decreased during the time of the experiment, so the result plotted is a superposition of two consecutive 50-min long observations with the same cell, which differed only in the threshold level, k, which was adjusted to be equal to the average output. (*B*) For comparison, a measurement by the same procedure with the reference light source.

Fig. 6 *A* shows the result of an experiment in which counts from a single *P. fischeri* (strain MJ-1) cell, accumulated over 100-ms time intervals and recorded over a total period of 2 h, were stored on the disk, and subsequently used as input for power spectral analysis using the FFT program. The program was written with 1,024 data points and, by integrating input data points over longer intervals, lower frequency ranges were computed. However, in no experiment performed with any of the species or strains could we detect any amplitude that was significantly different from the reference light source (Fig. 6 *B*). When intervals of 5 to 10 s were used as the time input, a significant amplitude was obtained at the very low frequency end, reflecting the gradual decrease of light emission.

Fig. 7 shows a representative experiment in which the time correlation was computed in the batch mode (off-line). Since the program is relatively slow on our computer, input time intervals of 300 ms (+15 ms dead time) were used. As was shown also with the on-line time correlation experiments, no periodicity of 60 s or shorter was found.

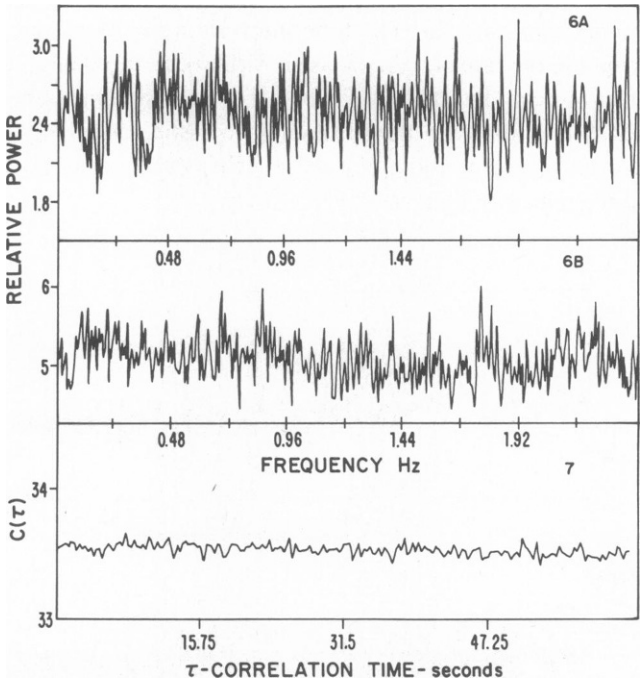

FIGURE 6 Power-spectra for (*A*) the light output of a single bacterial cell and (*B*) the reference light source. (*A*) In an experiment similar to that presented in Fig. 5, data were collected for 2.5 h from a single *P. fischeri* (MJ-1) cell in ASW (+0.3% glycerol). The data were used as input for calculation of the power spectrum of the light output using the FFT algorithm. Each data point was a summation of two 100-ms time intervals (dead time, 2.5 ms). The 506 output points plotted cover the frequency range 0.05–2.4 Hz. Different frequency ranges were analyzed by using different levels of summation of the time data points. Similar results were obtained over the wider frequency range from 0.01 to 10 Hz.

FIGURE 7 The time correlation of the light output of a single *P. fischeri* (MJ-1) cell. Data were collected at 50-ms intervals (2.5 ms dead time) for 2.5 h and stored on the disk. The time correlation was subsequently computed using the off-line routine (see Methods). Each input time point was the summation of six 50-ms intervals. Ordinate, time correlation, $C(\tau)$; abscissa, correlation time, τ.

DISCUSSION

In our experiments, bacterial bioluminescence appeared altogether continuous. We employed a variety of experimental conditions and highly sensitive instrumentation, but found no evidence for oscillations or bursts of any kind in photon emission within the limits of the methods and instrumentation employed, i.e., in the frequency range 0.01–10 Hz and an oscillating component between 1 and 2% of the average intensity. In a sense this is surprising. As noted by Hagins et al. (5), any control mechanism on the rate of luminescence or electron flow, or any pulsed or abrupt energy expenditures, could result in some bunching of the emitted photons. With regard to possible fluctuations in the level of reduced FMN, for example, the present results imply that there are no fluctuations (in the frequency ranges examined) in its intracellular concentration, at least down to the concentration level corresponding to its K_m for luciferase. Possible low frequency oscillations (<0.01 Hz) should be further investigated.

In analyzing the distribution of the number of counts per time interval (Fig. 4) some extra nonperiodic bursts or photon bunching were apparently observed, but these contributed <0.4% of the total light intensity in the range of 3 to 5 times the distribution average. This small difference between the histograms of the reference light source and of the cell luminescence should be interpreted with caution. It could well arise from the ~1% difference in the average intensity of the living cell as compared with the attenuated reference light source, and also from the slow drift with time of the average intensity of the living cell during the experiment. Both of these could give rise to errors of magnitude comparable to those differences seen in Fig. 4.

The earlier reports (6, 7) of periodicity and pulses in bacterial light emission are difficult to evaluate. Different oscillatory phenomena were reported in two publications; the same bacterial strain and apparently the same instrumentation were used. In our experiments we certainly would have detected "the deep modulation of the light signal of the bacteria (approximately 40–50%)" reported in reference 6.

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