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The Aldehyde Content of Luminous Bacteria and of an "Aldehydeless" Dark Mutant

(*Achromobacter fischeri/Photobacterium phosphoreum*/luciferin/luciferase)

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ABSTRACT **Fatty aldehydes, present in the luminescent cells of *Photobacterium phosphoreum* and *Achromobacter fischeri*, and to a very slight extent in the cells of a visually dark, "aldehydeless" mutant of the latter species, were extracted, purified, and oxidized to the corresponding acids. The acids were analyzed by mass spectrometry. The results, in conjunction with various other lines of evidence, indicate that saturated fatty aldehydes, comprising mostly dodecanal, tetradecanal, and hexadecanal, function in the bioluminescent reaction in living cells of these luminous bacteria. The amount of these aldehydes in the cells was computed to be sufficient to sustain steady-state luminescence for a period of about 1 sec, and under such conditions the rate of oxidation of the aldehydes in the process of luminescence must be balanced by their rate of production.**

Cell-free extracts of luminous bacteria that produced an NADH-activated luminescence were reported in 1953 (1). Evidence soon followed that the system required FMN (2) and, in addition, a factor "KCF" obtained from kidney cortex (3), subsequently identified as hexadecanal (4). This aldehyde could be replaced by any one in a series of saturated, straight-chain aldehydes having chain lengths from C_7 to C_{16} with varying degrees of effectiveness (5). Until now, however, no evidence has been reported that such aldehydes are specifically required for luminescence of the bacterial system *in vivo*. In fact, the specific requirement of an aldehyde is questionable on the grounds that a terminal nitrite group can satisfactorily replace the terminal aldehyde group (6).

Recent evidence has shown that the above type of aldehydes of chain lengths from C_9 to C_{14} are oxidized to the corresponding acids by the bacterial luminescent system *in vitro* (7–9), with a quantum yield for each aldehyde amounting to 0.17 ± 0.01 photons per aldehyde molecule reacted (7). Suggestive evidence that aldehydes function in luminescence also *in vivo* has been available in the fact that certain dark mutants, which seem identical to the parent strain except for failing to emit visible luminescence, respond practically at once by emitting a bright glow of light when a suitable aldehyde is added to the dark cells (10). Moreover, an aldehydogenic compound in extracts of *Photobacterium phosphoreum* has been described (11).

The present study demonstrates the presence of saturated fatty aldehydes in luminescent bacterial cells of *Achromobacter fischeri* and of *P. phosphoreum*, and their presence in only negligible amounts in a dark mutant of the former species. No dark mutant of the latter species was available in this study, despite repeated attempts to obtain one with the aid of ultraviolet irradiation and by other means.

MATERIALS AND METHODS

Measurement of Luminescence and Assay for Aldehydes. Light emission was recorded by means of a photomultiplier-amplifier assembly that had been calibrated in absolute units for response per photon and for variation in sensitivity with wavelength of light.

During extraction and purification of aldehydes from the bacteria, assays were made, to monitor the procedure at each step, on the basis of the amount of light emitted with a bacterial luciferase preparation from *A. fischeri*. To a specimen containing aldehyde for assay, a combination of the luciferase preparation, FMN, and NADH was added, and the total luminescence was measured under conditions described (see explanation of Fig. 2, p. 2088, ref. 7).

Preparation of Bacterial Cells. Each species was cultivated on the surface of hard agar, consisting of 30 g of Difco Bacto-Nutrient Agar Dehydrated per liter of 3% (w/v) NaCl, plus 10 ml of glycerol, plus 5 g of powdered $CaCO_3$, autoclaved, and aseptically poured into sterile aluminum baking pans with sheet aluminum covers, or into sterile fiberglass-type pans with covers, to a depth of about 0.5 inch (1.3 cm) (the deeper than usual medium favors better growth and luminescence). A total surface area of 1.3 m^2 was inoculated for each extraction. The growth of *A. fischeri* was harvested after incubation for 24 hr at 25°, and that of *P. phosphoreum* after 48 hr at 15°, in each case by brushing up the cells in a total of 1800 ml of cold 3% NaCl. Plate counts revealed that such suspensions generally contained about 2×10^{10} viable cells per ml. Growth of the "aldehydeless" mutant of *A. fischeri* appeared to be very much the same as that of the parent strain except that no visible light was produced unless aldehyde was added, whereupon luminescence appeared immediately and lasted for some time. In fact, when dodecanal was used, the resulting luminescence was quantitatively the same as the normal luminescence of the parent strain at the same cell density.

The suspension was centrifuged for 5 min at $15,000 \times g$ at 0°, the supernatant was discarded, and the cells were resuspended in 600 ml of cold 3% NaCl. This suspension was centrifuged as before, yielding a cell paste of about 40 g wet weight after discarding the supernatant.

Extraction Procedures. All solvents, including distilled water, used in the extraction process and subsequent procedures, were redistilled in an all-glass still before use. Because preliminary experiments revealed that contact of organic solvents with plastic vessels often results in the leaching

of substances with aldehyde-like activity, plastics in general were avoided except for the Teflon stopcock of a separatory funnel.

The cell paste described above was mixed with 700 ml of acetone at −5–0° and stirred for 1 hr, followed by centrifugation. The supernatant was concentrated to 50–60 ml under reduced pressure and was then extracted with an ether–pentane mixture (3:1; hereafter called "ether–pentane"). The precipitate from centrifugation was first added to water and the mixture was then extracted with ether–pentane. The two ether–pentane extracts were combined, washed with water, and evaporated to remove nearly all solvent. The residue was dissolved in 3 ml of ether and added to 7 ml of methanol. Cloudiness that resulted from addition of methanol was removed by centrifugation, and the precipitate, which contained less than 10% of the total extracted aldehyde-activity, was discarded. The solvents of the supernatant were evaporated off and the residue was redissolved in 4 ml of methanol.

Purification of Aldehydes was carried out by the formation of $NaHSO_3$—addition compounds as follows. To 4 ml of the methanolic solution of aldehydes was added 4 ml of 30% (w/v) $NaHSO_3$, and the mixture was kept under an argon atmosphere for 2 hr at room temperature (25°) with occasional agitation. The mixture was then extracted with ether–pentane. Both the $NaHSO_3$ and the ether–pentane layers were saved. The latter, which contained considerable aldehyde-activity, was evaporated to remove the solvent and the residue was dissolved in 4 ml of methanol, followed by addition of 4 ml of 5% $NaHSO_3$. After the solution was left for 1 hr at room temperature, it was extracted with ether–pentane; again, both the aqueous and organic solvent layers were saved. The cycle of evaporation, addition of methanol followed by 5% $NaHSO_3$, and extraction with ether–pentane was repeated, as much as was found necessary, until the aldehyde-activity remaining in the final ether–pentane extract became negligible.

All $NaHSO_3$ layers were pooled and cooled to 0°. Under an argon atmosphere, the pooled solutions were made alkaline by addition of 1 M NaOH until the pH was between 12.5 and 13; they were then extracted with ether–pentane. The ether–pentane layer, containing purified aldehydes, was evaporated nearly to dryness, and the residue was dissolved in 2 ml of methanol.

Oxidation of Aldehyde to Acid. The purified aldehydes in methanol were oxidized to acids by treatment with an equal volume of Tollens' reagent, prepared according to Fieser (12). This reaction mixture was left at room temperature overnight in the dark. After repeated washing with pentane, the reaction mixture was acidified with H_2SO_4, and the product acids were finally extracted with ether–pentane.

Mass Spectrometry of Acid Products. The ether–pentane solution was washed with water, dried with anhydrous Na_2SO_4, and evaporated to dryness by an aspirator. The residue was used for mass spectrometric analysis at the Morgan-Schaffer Corp., Montreal. The product obtained from one batch of about 40 g of cell paste was used in each analysis. The sample was introduced into the instrument by the direct inlet system at room temperature, and the temperature was slowly raised while the spectrum was repeatedly scanned for the whole mass region involved at a fixed time interval. The scanning was continued until the sample was nearly exhausted, usually after five to seven scannings, and at a temperature of 60–70°.

Molecular peaks in each of several spectra thus obtained were first corrected for the contribution of the "plus one" isotope peak, whenever any peak was present one mass unit below the molecular peaks. The corrected peak heights were then separately integrated according to each mass number. Fragments for the peak one unit lower than the molecular peaks were interpreted as having the composition $C_{n+1}H_{2n+1}O_2$.

The content of each saturated fatty acid in the sample (mole percent, taking the total saturated fatty acid as 100%) was calculated from the integrated value of the molecular peaks (peak height/mol) obtained from a mass spectral measurement of a standard mixture containing known amounts of authentic samples of acids. The ratio of the values of (peak height)/mol for lauric acid/myristic acid/palmitic acid/stearic acid was 0.37/0.71/1.0/0.89. For acids having an odd number of carbons (C_{13}, C_{15}, and C_{17}), the values used were obtained by averaging the values for acids containing one carbon more and one carbon less.

RESULTS AND DISCUSSION

The steps for purification and oxidation were designed so that only carboxylic acids that were produced by oxidation of isolated aldehydes would be obtained as the final products. A pilot experiment, using authentic dodecanal, showed that the oxidation method used produced very close to the quantitative equivalent of lauric acid.

In the course of extraction and purification, the presence of strong inhibitors of the light-emitting process became evident. Therefore, in making each assay, only a very small amount of the test specimen was used, i.e., 0.01–0.02% of any one batch of sample from 40 g of bacterial paste. It should be interesting to investigate the significance of these inhibitors in the luminescence of live bacteria.

In mass spectral analysis, the sample was introduced by the direct inlet method because the small amount of sample was thought to be insufficient for the indirect introduction method, and because of the easier confirmation of components due to fractionation.

Fig. 1. illustrates one of a series of sequentially recorded mass spectra of a sample obtained from *P. phosphoreum*. Large molecular peaks are evident for palmitic acid (molecular weight 256) and myristic acid (molecular weight 228), with traces of stearic acid (molecular weight 284), and pentadecenoic acid (molecular weight 242). Peaks at m/e 241, 227, 213, 199, 185, 171, and 157 are all, or practically all, fragments of these saturated fatty acids.

Peaks at m/e 250 and 236 are, respectively, assigned as fragments of m/e 268 and 254 molecular peaks, judged by the presence of metastable peaks at m/e 233.2 and 219.3. Thus, the compounds having molecular weights 268 and 254 are most likely *cis*-2-heptadecenoic acid and *cis*-2-hexadecenoic acid, respectively, although a confirmation as well as quantitation could not be made due to unavailability of authentic samples of these unsaturated acids. Peaks at m/e 222, 208, and 194 are likewise judged to indicate the presence of *cis*-2-pentadecenoic acid, *cis*-2-tetradecenoic acid, and *cis*-2-tridecenoic acid, respectively. Mass spectra of a sample obtained from *A. fischeri* indicated the presence of a much greater amount of *cis*-2-hexadecenoic acid. Because 2-decenal is a

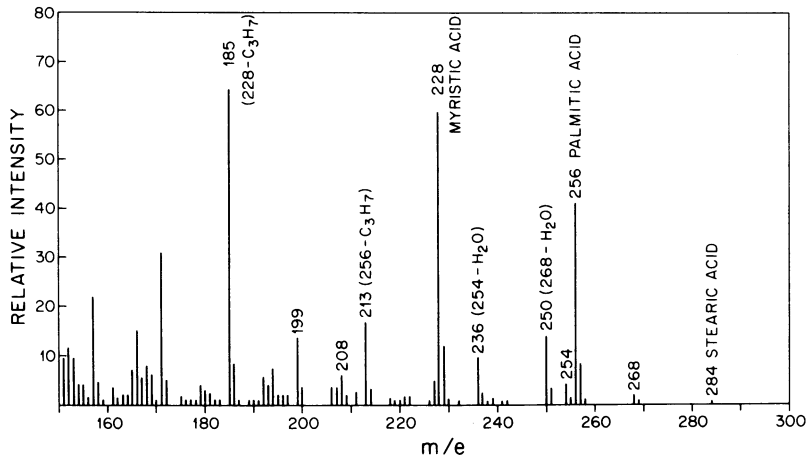

FIG. 1. Mass spectrum (70 eV) of a mixture of acids resulting from oxidation of aldehydes extracted and purified from cells of *P. phosphoreum*. Temperature: 40°.

strong inhibitor of bacterial luminescence (13), presumably all 2-unsaturated fatty aldehydes prior to oxidation to the corresponding unsaturated acids are potentially inhibitory. Thus, the strongly inhibitory substance(s) found in extracts, as mentioned above, should consist, at least in part, of 2-unsaturated aldehydes.

No significant amount of any compound, other than saturated fatty acids and 2-unsaturated fatty acids, were detected in any of the mass spectra obtained in this study, thus indicating that the purification method used was successful.

TABLE 1. *Content of saturated fatty aldehydes in luminous bacteria*

		P. phosphoreum	*A. fischeri*	*A. fischeri* "aldehydeless" mutant
Luminescence intensity of bacterial suspension (about 1 g/liter) at 20° (photons/sec per ml)		6×10^{12}	1.1×10^{12}	1×10^{8}
Total amount of saturated fatty aldehydes extracted and purified from about 40 g of cell paste* (nmol)		600	90	7
Saturated fatty acids formed by the oxidation of extracted aldehydes† (nmol)	C_{10}	—	—	—
	C_{11}	—	—	—
	C_{12}	30 (5%)	32 (36%)	1.5 (22%)
	C_{13}	6 (1%)	2 (2%)	—
	C_{14}	380 (63%)	29 (32%)	0.9 (13%)
	C_{15}	6 (1%)	6 (7%)	0.5 (7%)
	C_{16}	180 (30%)	18 (20%)	3.8 (54%)
	C_{17}	—	2 (2%)	0.14 (2%)
	C_{18}	—	—	0.07 (1%)

* Calculated from light-emitting capacity on the basis that the quantum yield of each aldehyde is 0.16 (see *text*).

† Calculated from the mol-percent composition of acids obtained by mass spectrometry (shown in parentheses) and the total amount of acids that resulted from oxidation of the aldehydes, assuming that the oxidation is quantitative, in accordance with the results of control experiments as described in the *text*. Dashes indicate less than 1%.

Table 1 summarizes the data concerning the content of saturated fatty aldehydes in the luminous bacteria and mutant studied. From the same amount of bacterial cells, *P. phosphoreum* afforded aldehydes in amounts close to 6 times that of *A. fischeri;* approximately the same ratio characterizes the intensity of luminescence in these two species.

The aldehydes found in the dark mutant of *A. fischeri* amounted to less than 1/10 that of the luminescent parent strain. The luminescence intensity in the mutant, however, amounted to only about 1/10,000 that of the parent strain. Two possible reasons for this circumstance are: most of the aldehydes extracted from the dark mutant might be derived from cell constituents not related to luminescence, and such aldehydes might originate in an "aldehydogenic" compound.

The amount of purified aldehyde obtained from the initial crude extract was sufficient to account for more than 80% of the light-emitting capacity of the same initial extract. No significant activity was found in the residue. It follows that no significant amounts of compounds other than these aldehydes are involved in the luminescence of the living bacteria. Because of these and other facts mentioned in the text, there seems to be no reasonable interpretation other than that the aldehydes obtained from *P. phosphoreum* and *A. fischeri* function in the intracellular luminescence of both species.

The composition of the acids that were derived from the aldehydes extracted from *P. phosphoreum* and *A. fischeri* indicates that in each luminous species the aldehydes consist mostly of dodecanal, tetradecanal, and hexadecanal. Tetradecanal predominates in *P. phosphoreum*, whereas the amounts of the three aldehydes are more nearly the same in *A. fischeri*. Further evidence was obtained by use of the indirect introduction method, which required larger amounts of sample but should give better quantitative values. Results essentially similar to those listed in Table 1 were obtained by this method with a sample consisting of the combined products from two batches of *P. phosphoreum;* the only notable difference was a slightly lower content of palmitic acid according to the indirect introduction method.

In each instance of *P. phosphoreum*, *A. fischeri*, and the "aldehydeless" mutant of the latter species, plate counts indicated that 1 g wet weight of cells collected by centrifugation contained about 1×10^{12} viable bacterial cells. Thus, in con-

junction with the data of Table 1, each bacterium of *P. phosphoreum* and of *A. fischeri* emits, under the conditions involved, an average of about 6000 photons and 1100 photons, respectively, per sec. Under conditions that spontaneous luminescence is being emitted at a constant brightness, it is reasonable to assume a steady-state rate of production of aldehydes equal to the rate of their oxidation in the luminescent process. In such event, one bacterial cell of *P. phosphoreum* produces and consumes an average of 37,000 aldehyde molecules per sec, and one cell of *A. fischeri* an average of 7000 aldehyde molecules per sec, based on the actually measured, i.e., uncorrected for absorption of emitted light by FMN in the reaction mixture, quantum yield of 0.16 for all saturated fatty aldehydes of C_{12} to C_{14} (7); presumably, the yields with C_{15} and C_{16} would be the same as with C_{14}.

If the overall yield of aldehydes in the extraction and purification is assumed to be 65–70%, it follows that the amounts of aldehydes shown in Table 1 can support luminescence for only 0.3–0.4 sec in the cells of either *P. phosphoreum* or *A. fischeri* from which the aldehydes were obtained. When the extraction was carried out on a much smaller scale, starting with about 1/100 the amount used for the results in Table 1, the aldehydes obtained would be sufficient to support luminescence for as much as a full second rather than the fractional values just given, computed on the samea bsis. The difference is probably due to the much shorter time required for harvesting and centrifuging the bacterial cells prior to the addition of acetone and, thus, markedly decreasing the time during which chemical alteration of the aldehydes could take place before extraction.

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