(A9), $r_{Ca} = 1$; both classes of sites are saturated with Ca^{2+} to the same extent. At this stage, according to (A6), $r_{Ln} = 1/K$, and it is the distribution of the residual bound Ln^{3+} that depends on all the affinities.

For nonsaturating [Ln], the added Ca^{2+} will, according to (i)(a), bind preferentially to the high-affinity sites; this first causes redistribution of Ln^{3+} from the high- to the low-affinity sites, resulting in a temporary accumulation of Ln^{3+} at the low-affinity sites. As [Ca] increases, Ln^{3+} is eventually displaced from the protein into solution.

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Structural Identification of Autoinducer of *Photobacterium fischeri* Luciferase†

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ABSTRACT: Synthesis of bacterial luciferase in some strains of luminous bacteria requires a threshold concentration of an autoinducer synthesized by the bacteria and excreted into the medium. Autoinducer excreted by *Photobacterium fischeri* strain MJ-1 was isolated from the cell-free medium by extraction with ethyl acetate, evaporation of solvent, workup with ethanol–water mixtures, and silica gel chromatography, followed by normal-phase and then reverse-phase high-performance liquid chromatography. The final product was >99% pure. The structure of the autoinducer as determined by high-resolution ^1H nuclear magnetic resonance spectroscopy, infrared spectroscopy, and high-resolution mass spectrometry was *N*-(3-oxohexanoyl)-3-aminodihydro-2(3*H*)-furanone [or *N*-(β-ketocaproyl)homoserine lactone]. The formation of homoserine by hydrolysis of the autoinducer was consistent with this structure. Synthetic autoinducer, obtained as a racemate, was prepared by coupling homoserine lactone to the ethylene glycol ketal of sodium 3-oxohexanoate, followed by mildly acidic removal of the protecting group; this synthetic material showed the appropriate biological activity.

Bacterial luciferase, a mixed function oxidase, catalyzes the reaction

$$O_2 + RCHO + FMNH_2 \xrightarrow{\text{luciferase}} RCOOH + FMN + H_2O + h\nu$$

Both its synthesis and level of activity are regulated by a highly complex set of control mechanisms (Hastings & Nealson, 1977; Nealson & Hastings, 1979).

The synthesis of luciferase in *Photobacterium fischeri* can be turned on and off by the bacteria themselves in a unique way. Bacteria excrete an autoinducer into the medium that allows induction of luciferase synthesis when its concentration reaches a critical level (Nealson et al., 1970; Eberhard, 1972; Nealson, 1977). The *P. fischeri* autoinducer is species specific, inducing only other strains of *P. fischeri* and no other luminous species (Eberhard, 1972; Nealson, 1977). In addition, complex nutrient media contain an inhibitor of luciferase synthesis, so that when bacteria are inoculated into fresh medium, luciferase synthesis is temporarily repressed. The bacteria must remove the inhibitor metabolically in addition to excreting a sufficient amount of autoinducer before luciferase synthesis can begin (Kempner & Hanson, 1968; Eberhard, 1972). The inhibitor is not species specific (Eberhard, 1972) and can be removed by different bacterial species. Luciferase synthesis must thus be indirectly controlled by the levels of enzymes involved in the metabolism of the inhibitor and synthesis of the autoinducer.

In *P. fischeri* and other luminous species several additional factors can also affect luminescence. These include the types and levels of carbohydrates in the growth medium, oxygen tension, cyclic nucleotides, temperature, salt concentration,

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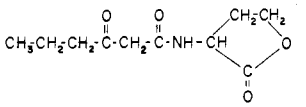
and specific amino acids. [See Nealson & Hastings (1979); also Bognar et al. (1978), Eberhard et al. (1979), Lin et al. (1976), and Makemson & Hastings (1979).] However, for most strains, the autoinducer–inhibitor system appears to be central to the control of luciferase synthesis, and much interest has thus been focused on the chemical nature of the autoinducer. Earlier work showed that both the inhibitor and autoinducer are low molecular weight organic molecules of reasonable stability (Eberhard, 1972). Classical methods of isolation failed, however, due to the rather small amounts of material involved and the complexity of the medium required for the growth of the bacteria. In this report, we describe the isolation of autoinducer from *P. fischeri* MJ-1 using high-performance liquid chromatography (LC),[1] its identification, and its synthesis. Autoinducer was identified as *N*-(3-oxohexanoyl)-3-aminodihydro-2(3*H*)-furanone (see structure I).



I

This material was synthesized as a racemate, and the synthetic material was biologically active.

Experimental Procedures

Bacterial Strains and Growth Conditions. *P. fischeri* strain MJ-1 (Ruby & Nealson, 1976; Nealson, 1977) and strain B-61 (Allen & Baumann, 1971; Nealson, 1977) and *Beneckea harveyi* strain 392 [Reichelt & Baumann, 1973; formerly strain MAV (Hastings et al., 1969)] were used. The media have been described (Nealson, 1978). SWC medium was used for maximal production of autoinducer by strain MJ-1, and LM was used for the preparation of conditioned medium for the autoinducer assays (Nealson, 1978). Bacteria were grown at room temperature (22–25 °C) either in 16 × 125 mm screw-capped culture tubes containing 5 mL of medium or in 2L Erlenmeyer flasks containing 500 mL of medium. Cultures were aerated by gyratory shaking at 120–150 rpm. The optical density of bacterial cultures was measured in 16 × 125 mm screw cap culture tubes at 660 nm with a Coleman Jr. II spectrophotometer.

In Vivo Bioluminescence Activity. Light produced by living cultures was measured with a calibrated photomultiplier photometer (Mitchell & Hastings, 1971) for which 1 light unit (LU) equaled 2×10^{10} quanta/s (Hastings & Weber, 1963). Inducer assays involving low light levels were carried out with an ATP photometer (SAI Industries, Sorrento Valley, CA) using 1 mL of culture in a standard scintillation vial (Nealson, 1977).

Autoinducer Assay. The procedure of Nealson (1977) was followed by using medium conditioned by cells of *B. harveyi* strain 392 grown to OD 0.4 and sterilized by autoclaving after removal of the cells. The conditioned medium, which lacked inhibitor and autoinducer, was inoculated with a 1:100 dilution of a late log phase culture of *P. fischeri* strain B-61 (OD ~1). Autoinducer was added to dry scintillation vials as a dilute ethyl acetate solution, and the solvent was evaporated by using a stream of N_2 with gentle warming on a hot plate. A control vial containing 1 assay unit (AU) of autoinducer from a standard solution containing 2.5 AU/mL of ethyl acetate solution was included in each assay series. One assay unit is arbitrarily defined as the amount of extractable autoinducer contained in 0.1 mL of a certain culture of *P. fischeri* strain MJ-1 at OD 0.42 that was producing 450 LU/mL. A vial without autoinducer was also included. Each vial received 1 mL of the inoculated conditioned medium and was monitored for light production.

Purification of Autoinducer. A 500-mL culture of *P. fischeri* strain MJ-1 was grown to maximal brightness (more than 2000 LU/mL), and the cells were removed by centrifuging at 27000*g* for 10 min at 4 °C, followed by filtration through Gelman 0.2-μM filters. The filtrate showed no detectable light, and plating for viable counts showed no colonies. The filtrate was extracted twice by shaking for 5 min with 2 volumes of redistilled ethyl acetate containing 0.1 mL/L glacial acetic acid. The combined ethyl acetate extracts were dried briefly over Na_2SO_4 and evaporated to dryness in vacuo by using a water bath at ~40 °C. This precedure was repeated with 11 other cultures, giving the crude ethyl acetate extract from a total of 6 L of culture. This solid yellow-white material was extracted 3 times with 2 mL each of ethyl acetate. The residue from this extraction was treated with 1 mL of ethanol (95%), the ethanol solution reevaporated, and the residue again extracted 3 times with 2 mL of ethyl acetate. The six ethyl acetate extracts were combined and evaporated to dryness. The resulting material was treated with 1 mL of ethanol (95%), and then 10 mL of water was added, giving a cloudy suspension. This was centrifuged for 10 min at 12000*g* and filtered (0.2 μM), giving a clear solution which was evaporated to dryness. The resulting yellow oily material was extracted with ethyl acetate and the solution applied to a 1.5 × 60 cm column of 60–200 mesh silica gel. The column was eluted with ethyl acetate, and the active fractions were pooled, evaporated to dryness, then redissolved in 0.5 mL of ethyl acetate, filtered through a glass fiber filter, concentrated down to 0.1 mL, and applied to a 1 × 90 cm high-performance LC column of Lichrosorb S-1-100. The column was eluted with ethyl acetate, and the active fractions were pooled, evaporated to dryness, then taken up in 0.1 mL of 7:93 ethanol–water (v/v), applied to a 0.46 × 50 cm reverse-phase high-performance LC column of μC_{18}, and eluted with the same solvent. The active fractions were run again with the same reverse-phase high-performance LC column, but this time by using 5:95 ethanol–water (v/v). Evaporation of the active fractions gave a total of 0.0027 g of material to which we refer below as "natural autoinducer."

Synthesis of N-(3-Oxohexanoyl)-3-aminodihydro-2-(3H)-furanone [*(N-(β-Ketocaproyl)homoserine Lactone*]. The ethylene glycol ketal of ethyl 3-oxohexanoate [Salmi, 1938; bp 115 °C (10 mm)] was hydrolyzed by heating at reflux for 3 h in water with a slight excess of NaOH. To 0.002 mol of the dried ethylene glycol ketal of sodium 3-oxohexanoate were added 5 mL of CH_3CN and 0.002 mol of *N*-ethyl-5-phenylisoxazolium-3′-sulfonate (Woodward et al., 1966), and the mixture was stirred at room temperature until it was homogeneous (~5 h). Then 0.002 mol of homoserine lactone hydrobromide and 0.002 mol of $(C_2H_5)_3N$ were added, and the mixture was stirred for 2 days at room temperature. After the addition of 100 mL of CH_3CN, the precipitate was removed by centrifugation, and the supernatant was evaporated to dryness. The residue was extracted with 3 × 10 mL of ethyl acetate, and the combined extracts were evaporated to dryness. The resulting syrupy material was purified by chromatography in water through 1 × 15 cm columns of Dowex 50-W-X4–Na^+ and Dowex 1-X4–Cl^-. The protecting group was removed by heating at reflux in 0.1 M HCl for 1 h. Evaporation of the hydrolysis mixture gave a syrup which was extracted with 3 × 0.5 mL of ethyl acetate. After evaporation of the solvent,

[1] Abbreviations used: SWC, sea water complete medium; LM, liquid medium; LC, liquid chromatography; NMR, nuclear magnetic resonance; LU, light unit; AU, autoinducer unit.

the compound was taken up in water and again chromatographed through the same two Dowex columns. Removal of the water gave 0.097 g (23% yield from the ethylene glycol ketal of ethyl 3-oxohexanoate) of the synthetic autoinducer as a syrup which crystallized slowly upon standing.

Amino Acid Analysis. A 0.5-mg sample of the natural autoinducer was hydrolyzed in vacuo in 6 M HCl for 20 h at 110 °C. After evaporation to dryness, 0.5 mL of 1.3 M NH_3 was added, and the mixture was heated in a boiling water bath for 10 min, then evaporated to dryness, and analyzed with a Beckman model 116 amino acid analyzer.

Spectra. The complete high-resolution mass spectrum of the autoinducer was determined by using a direct inlet probe with a modified Kratos/AEI MS9 mass spectrometer on line to a Xerox Sigma 7/Logos II computer system (Meili et al., 1979). Scanning was at 8 s/decade with an ion source temperature of ~250 °C, at 70 eV, with a mass resolution $M/\Delta M$ = 10000. NMR spectra were obtained at 360 MHz with a Bruker HXS-360 NMR spectrometer at the Stanford Magnetic Resonance Laboratory. Chemical shifts were measured from internal standards by using tetramethylsilane when deuterated dimethyl sulfoxide was the solvent and by using 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$ when deuterium oxide was the solvent. Infrared spectra were obtained with a Perkin Elmer Model 137 spectrophotometer using NaCl plates.

Results

Initial experiments showed that crude autoinducer was readily soluble in water, ethanol, methanol, acetone, ethyl acetate, acetonitrile, and methylene chloride, but not in hexane. It was best extracted from aqueous media with ethyl acetate. Methylene chloride was almost as effective while diethyl ether was much less so. Crude autoinducer preparations lost only some activity upon treatment for 1 h at room temperature with either 3% H_2O_2 or H_2/Pt or upon heating at 140 °C for 10 min as a dry thin film in air. Thin films of crude autoinducer lost no activity upon standing at room temperature for several days either in air or in vacuo. Autoinducer appeared not to be sufficiently volatile for purification by gas–liquid chromatography. It was retained by neither anion- nor cation-exchange resins. Autoinducer appeared to be stable to heating with glacial acetic acid at 50 °C/h and to heating in aqueous solution at pH 1.57 at 100 °C/10 min, but it was totally inactivated at pH 12 at room temperature in 10 min.

These initial results indicated that the autoinducer should be stable enough to isolate. Medium which had been conditioned by the growth of *P. fischeri* strain MJ-1 was extracted with ethyl acetate, and then the extracts were purified by solvent extractions, silica gel column chromatography, and normal-phase high-performance LC. The refractive index tracing of the high-performance LC separation showed several peaks, but only one peak gave activity in the bioassay with strain B-61. The material in this active peak was then subjected to two reverse-phase high-performance LC separations by using slightly different solvent systems. In each case only one peak appeared on the refractive index tracing, and this peak coincided with the activity in the B-61 assay. It appeared, therefore, that the compound had been purified to homogeneity. The 6 L of conditioned medium resulted in the isolation in pure form of 2.7 mg of natural autoinducer.

The ^{1}H NMR spectrum of the natural autoinducer (Figure 1) shows the presence of a 1-propyl group (0.89 ppm, 3, t; 1.58 ppm, 2, sextet; 2.63 ppm, 2, t) which is presumably attached to a carbonyl functionality. By use of homonuclear spin decoupling, the other resonances were found to represent a $-NH-\alpha-CH-\beta-CH_2-\gamma-CH_2$ moiety: α-CH, 4.700 ppm (dd, J = 10.6 and 9.3 Hz); β-CH_2, 2.657 ($J_{\alpha-\beta}$ = 10.6 Hz, ^2J = −12.6 Hz, $J_{\beta-\gamma}$ = 1.9 and 6.7 Hz), 2.369 ppm ($J_{\alpha-\beta}$ = 9.3 Hz, $J_{\beta-\gamma}$ = 9.2 and 10.8 Hz); γ-CH_2, 4.570 ppm (^2J = −9.2 Hz, $J_{\beta-\gamma}$ = 1.9 and 9.2 Hz), 4.407 ppm ($J_{\beta-\gamma}$ = 10.8 and 6.6 Hz). The chemical shift of the γ-CH_2 resonance is consistent with the presence of an electron-withdrawing heteroatom, i.e., oxygen. The chemical shift of the α-CH is typical for that of an α-amino acid in a peptide. Analysis of the coupling constants indicates that the α, β, and γ protons do not show a random distribution of rotamer populations that would be expected for an acyclic molecule. The spectrum is, however, consistent with the presence of a five-membered ring. With $(CD_3)_2SO$ as the solvent, there is an additional resonance at 8.6 ppm (1, d, J = 7.9 Hz); typical of that of an NH in an α-amino acid. Also, there is a singlet (2) at 3.32 ppm, but this was partially obscured by contaminating water. Two minor resonances at 8.41 and 5.00 ppm are consistent with the presence of a small amount (10–15%) of the enol form of the autoinducer. The resonances at 3.32, 5.00, 8.41, and 8.6 ppm all disappeared when D_2O was the solvent, a result of the exchange of these hydrogens with deuterium. The lack of any other observable peaks in the NMR spectrum indicated a purity >99%. Synthetic autoinducer gave an NMR spectrum obtained at a similar concentration that was identical (within 0.005 ppm) with that of the natural autoinducer.

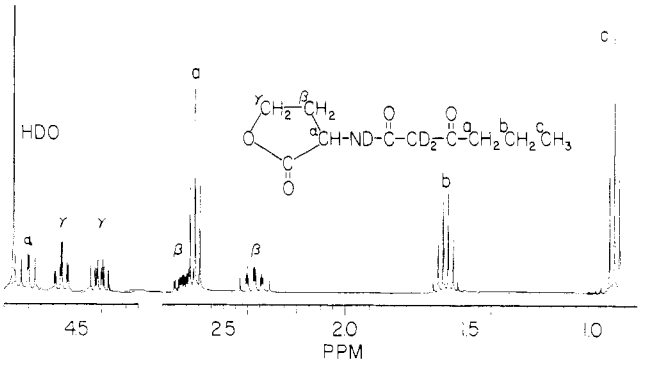


FIGURE 1: High-resolution NMR spectrum of 0.4 mg of natural autoinducer in 0.4 mL of D_2O.

The infrared spectrum of a sample of the natural autoinducer (major bands at 1780, 1710, 1640, and 1550 cm^{-1}) was suggestive of the presence of a five-membered ring lactone (consistent with the base sensitivity of the compound) and of a ketone and an amide group.

The nominal mass plot of the high-resolution mass spectrum of the natural autoinducer is shown in Figure 2. The corresponding assigned elemental compositions are presented in Table I. The molecular ion had m/z = 213.10030, consistent with the formula $C_{10}H_{15}NO_4$ (calcd m/z = 213.10011). The molecular ion displayed losses of the C_2H_4, C_3H_7, and C_4H_6O moieties. Taken together with the intense fragments C_4H_7O and $C_6H_9O_2$, these data establish the presence of a 3-oxohexanoyl group, while the additional rearrangement fragment $C_6H_{11}NO_2$ suggests its attachment to an amino function. A major fragment $C_4H_7NO_2$ represents a rearrangement loss of $C_6H_8O_2$, the 3-oxohexanoyl moiety. Losses of both CO and CO_2 from the molecular ion suggest the presence of an ester function.

These results left little doubt that the material that had been isolated from the conditioned medium had structure I. Amino acid analysis of the hydrolyzed natural autoinducer gave only one peak with a retention time identical with that of an authentic sample of homoserine, thus confirming structure I. Synthesis of this compound gave biologically active material with infrared, ^{1}H NMR, and mass spectra identical with those

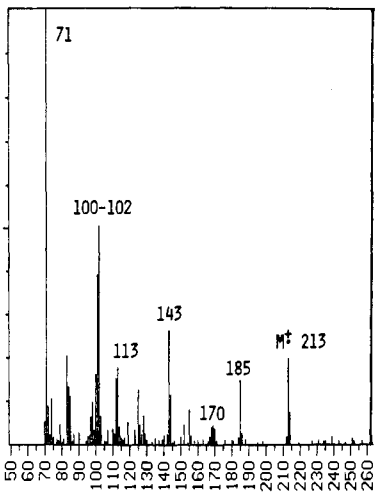


FIGURE 2: Nominal mass plot of natural autoinducer.

Table I: Mass Spectrum of Natural Autoinducer

m/z	elemental composition	error (in ppm)
71.0500	C_4H_7O	4.79
101.04749	$C_4H_7O_2N$	−1.83
102.05551	$C_4H_8O_2N$	0.11
113.06096	$C_6H_9O_2$	6.23
129.07467	$C_6H_{11}O_2N$	1.27
143.05890	$C_6H_9O_3N$	4.78
169.11053	$C_9H_{15}O_2N$	1.47
170.04577 (2)[a]	$C_7H_8O_4N$	2.56
170.11268 (1)[a]	$C_8{}^{13}CH_{15}O_2N$	−5.58
171.05266	$C_7H_9O_4N$	−2.92
185.06921 (7)[a]	$C_8H_{11}O_4N$	2.19
185.10400 (1)[a]	$C_9H_{15}O_3N$	−6.43
195.08940	$C_{10}H_{13}O_3N$	−0.75
198.07634	$C_9H_{12}O_4N$	−1.49
213.10030	$C_{10}H_{15}O_4N$	0.92

[a] Approximate relative abundances.

of the natural material, showing that structure I was indeed the structure of the autoinducer. When equal concentrations of natural and synthetic autoinducer were tested with strain B-61, the natural material gave a slightly higher response than the synthetic compound (Figures 3 and 4). The dose–response curve for synthetic autoinducer compared to that of natural autoinducer was shifted to higher concentrations by a factor of 2–3 (Figure 4). Maximal amounts of luminescence were produced by strain B-61 with concentrations of autoinducer between 0.3 and 3 μg/mL while higher concentrations resulted in less light production. *P. fischeri* MJ-1, the strain from which the autoinducer was isolated, was also tested for its response to synthetic autoinducer. A dilute culture (OD 0.0001 in fresh SWC) responded to 0.2 μg/mL autoinducer with immediate and increasing light production while a control without autoinducer showed the usual initial lag. After 9 h, the culture with autoinducer gave 250 times as much light as the control.

Discussion

Although autoinduction has been described for several species of luminous bacteria (Nealson et al., 1970; Eberhard, 1972; Nealson, 1977; Greenberg et al., 1979; Rosson & Nealson, 1980), the chemical identity of any of the autoinducer compounds remained unknown. This was in part due to the low levels of autoinducer produced during growth, the chemical complexity of the media needed to grow the bacteria to maximal light production, and the difficulty in performing the bioassay for autoinducer. In the studies reported here, isolation

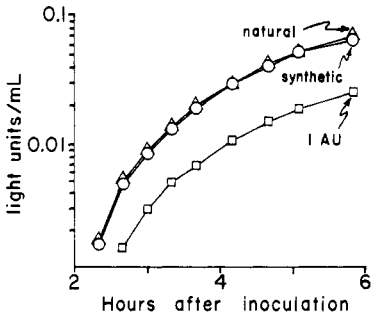


FIGURE 3: Assay of 1 μg of natural and 1 μg of synthetic autoinducer and of 1 AU of natural autoinducer (see Experimental Procedures for definition of AU and for assay procedure). With no autoinducer added, <0.001 LU/mL was produced throughout the experiment.

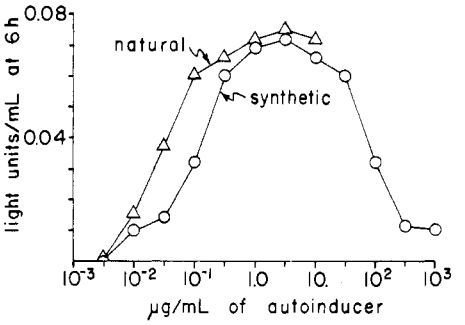


FIGURE 4: Dose–response curves for natural and synthetic autoinducers.

was facilitated by the availability of a superbright strain, MJ-1, which produces unusually large amounts of autoinducer (Ruby & Nealson, 1976) and a sensitive assay system using a natural isolate, B-61, that produces extremely low levels of autoinducer itself but responds normally to autoinducer from other strains (Nealson, 1977).

Purification of the autoinducer was possible by avoiding any exposure to basic conditions to which the lactone ring is sensitive. Silica gel column chromatography removed much of the lipid material that coextracted in ethyl acetate, and high-performance LC allowed purification to homogeneity. Indeed, the use of high-performance LC was critical, since the compound was not sufficiently volatile for gas chromatography and since a complex mixture of compounds was involved.

Identification of autoinducer as structure I was made by high-resolution ^1H NMR spectroscopy, high-resolution mass spectrometry, and infrared spectroscopy. Proof of the structure was obtained by hydrolysis of natural autoinducer to yield homoserine and finally by sythesis of biologically active material with structure I.

The assay of autoinducer showed that the natural and synthetic materials both stimulated light production in strain B-61 (Figure 3). The dose–response curve for synthetic autoinducer compared to that of natural autoinducer (Figure 4) was shifted to higher concentrations by a factor of 2–3. Inactivity of one of the enantiomers in the racemic synthetic material presumably can account for this difference. The amount of light produced increased over the range 0.003–3 μg/mL and then decreased at higher concentrations (Figure 4). This inhibition at high concentration can create problems during the preparation of antoinducer extracts: care must be taken always to dilute the material sufficiently before assay so that fractions containing large amounts of autoinducer do not show low or zero activity.

In the cultures of *P. fischeri* strain MJ-1 from which the autoinducer was isolated and which were producing ~2000 LU/mL, the autoinducer concentration was $\sim 5 \times 10^{-6}$ M (1

μg/mL). This value was calculated from the yield during purification by assuming an ~50% recovery (6 L of culture yielded 2.7 mg of pure autoinducer; thus the original culture contained ~1 mg/L, which, with a molecular weight of 213, is $\sim 5 \times 10^{-6}$ M). This value is also near the optimum for light production in the B-61 assay (Figure 4). The inhibition seen in the assay at higher concentrations may be related to the lowered rate of luciferase synthesis in stationary phase cultures (Nealson et al., 1970). Comparison of Figure 3 with Figure 4 shows that 1 AU is approximately equal to 0.02 μg of pure autoinducer. Since this was derived from 0.1 mL of a culture, the concentration of autoinducer in this culture, which was emitting ~450 LU/mL, was $\sim 10^{-6}$ M. Emission of light by the MJ-1 cultures is thus roughly proportional to the autoinducer concentration (Nealson, 1977) but reaches a maximum at $\sim 5 \times 10^{-6}$ M. Since MJ-1 cultures can show induction of luciferase synthesis while producing at least 10^6-fold less light than the maximum of 2000 LU/mL, they are presumably capable of responding to 10^6-fold lower concentrations of autoinducer, or $\sim 5 \times 10^{-12}$ M. However, the B-61 assay was not responsive to such low autoinducer concentrations; the lowest level which could be detected with this assay was 3×10^{-10} M.

The autoinducer molecule combines homoserine lactone, an intermediate in amino acid metabolism, with β-ketocaproic acid, related to intermediates in fatty acid metabolism. Neither of these components is a substrate or otherwise directly involved in the luciferase reaction itself. One might speculate that the autoinducer, being a condensate of intermediates in these two major biosynthetic pathways, could be a signal of nutritional viability communicated to other bacteria. The signal may be a direct one, involving chemotaxis toward the autoinducer. In any case, the autoinducer may be considered to be a bacterial pheromone (Eberhard, 1972) and may act as a population-sensing device. Autoinducer is made constitutively (Nealson, 1977), but otherwise nothing is known about either its biosynthesis or mode of action. Specifically, it is not known whether it acts as such or must be further metabolized or whether it interacts with another small molecule (corepressor?), protein, nucleic acid, or membrane component. Further experiments with radioactively labeled autoinducer and with various structural analogues are being undertaken to address these questions.

The autoinducer of *P. fischeri* luciferase is thought to be species specific (Eberhard, 1972; Nealson, 1977). The inducer preparation from *P. fischeri* turns on luciferase synthesis in all other strains of *P. fischeri* tested (Nealson, 1977) but has no effect when added to other species of *Photobacterium* or to other genera of luminous bacteria. Some strains of all species of luminous bacteria are now known to display autoinduction (Rosson & Nealson, 1980), but whether or not these autoinducers are all species specific remains to be proven. Greenberg et al. (1979) have reported that the autoinducers in the genus *Beneckea*, while apparently specific for that genus, interact among several species. They also reported that nonluminous species of *Beneckea* produce substantial amounts of autoinducer. Some strains of luminous bacteria have been reported not to be subject to autoinduction. That is, they apparently have constitutive patterns of synthesis for bacterial luciferase (Katznelson & Ulitzur, 1977; Watanabe et al., 1975). This has been confirmed by using chemostat studies for strains of *Photobacterium phosphoreum* and *Photobacterium leiognathi* (Rosson & Nealson, 1980). Nevertheless, autoinducer may still be involved in those strains if, for instance, it is produced but not excreted. Alternatively, the operator may be constitutive in these strains. Further work on the nature and biochemistry of the autoinducers will be required before the autoinduction system can be correlated with the ecology of the luminous bacteria and the expression of bioluminescence in the natural environment.

In summary, the autoinducer of *P. fischeri* luciferase is the first molecule of its kind to be identified structurally. This small molecule is a specific genetic regulator that is unrelated to at least one of the enzyme systems that it induces, and it acts after excretion and accumulation in the extracellular medium. Its role in the natural environment remains unknown.

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Metabolism in the Cytosol of Intact Isolated Cattle Rod Outer Segments as Indicator for Cytosolic Calcium and Magnesium Ions†

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ABSTRACT: The metabolism of the chromophore of rhodopsin in the cytosol compartment of isolated intact cattle rod outer segments was used as an indicator for changes of the cytosolic Mg^{2+} and Ca^{2+} concentration upon changes of the external Mg^{2+} and Ca^{2+} concentration. The reduction of retinal to retinol upon photolysis of rhodopsin in situ in intact rod outer segments was critically dependent on the availability of cytosolic Mg^{2+}. The latter is necessary as chelator of endogenous adenosine 5′-triphosphate (ATP). Lowering the cytosolic Ca^{2+} concentration beneath 10^{-7} M resulted in an inhibition of the rate of retinol formation. This is presumably due to a light-activated process, which competes with retinol formation for the supply of high-energy phosphate from a common pool. These results led to the following conclusions. Changes of the external Mg^{2+} concentration are only followed by substantial changes of the cytosolic Mg^{2+} concentration when the ionophore A23187 is present. Changes of the external Ca^{2+} concentration are followed by parallel changes of the cytosolic Ca^{2+} concentration either when external Na^+ is present or in the presence of A23187. Li^+ and K^+ could not substitute for Na^+ in the former case, but K^+ diminished the effectivity of Na^+ at low Na^+ concentrations and enhanced it at high Na^+ concentrations. It is concluded that the control of the cytosolic Ca^{2+} concentration in isolated intact rod outer segments is predominantly provided for by Na–Ca exchange, i.e., by coupled fluxes.

Changes of the extracellular Ca^{2+} concentration in retinal rod photoreceptor cells result in changes of the membrane voltage and the membrane current in the dark but not in bright light (Yoshikami & Hagins, 1973; Hagins & Yoshikami, 1974; Brown & Pinto, 1974; Lipton et al., 1977; Bastian & Fain, 1979). These results were explained by the assumption that changes of the extracellular Ca^{2+} concentration are followed in these cells by concomitant changes of the cytosolic Ca^{2+} concentration in the same direction and that raising the cytosolic Ca^{2+} concentration subsequently results in a progressive blocking of ionic channels in the plasma membrane, which are open in the dark and are closed by bright light. This suggestion was strengthened by experiments in which the cytosolic Ca^{2+} concentration was manipulated more directly either by the introduction of Ca^{2+}-chelating substances into the cytosol (Brown et al., 1977; Hagins & Yoshikami, 1977) or by the application of Ca^{2+} ionophores (Hagins & Yoshikami, 1974; Bastian & Fain, 1979). In one of these studies (Hagins & Yoshikami, 1977) it was suggested that changes of the extracellular Mg^{2+} concentration likewise are followed by concomitant changes of the cytosolic Mg^{2+} concentration. In a study using isolated intact cattle rod outer segments, it was demonstrated that efflux of internal Ca^{2+} from these rod outer segments is selectively stimulated by Na^+ ions, most likely by a Na–Ca exchange mechanism (Schnetkamp, 1980). However, most of the internal Ca^{2+} is stored within disks, and it was difficult to assess the contribution of cytosolic Ca^{2+} (Schnetkamp, 1979). Therefore, in this study an attempt has been made to use metabolism confined to the aqueous cytosol compartment as an indicator for the cytosolic Ca^{2+} and Mg^{2+} concentrations.

In the vertebrate retina (Baumann, 1972; Baumann & Bender, 1973; Brin & Ripps, 1977) as well as in isolated intact rod outer segments (Bridges, 1962; Paulsen et al., 1975; Kaplan & Liebman, 1977; Schnetkamp et al., 1979) the final photoproduct, formed upon photolysis of rhodopsin, is *all-trans*-retinol (λ_{max} = 330 nm). From the various slow photoproducts, *all-trans*-retinal, metarhodopsin II (both λ_{max} = 380 nm), and metarhodopsin III (λ_{max} = 455–470 nm), the chromophore is reduced to *all-trans*-retinol by an intrinsic retinol dehydrogenase and by NADPH[1] (Futterman, 1963). In the rod outer segment cytosol this reaction is rate limited by the recycling of NADPH (Schnetkamp et al., 1979). The latter occurs in a series of reactions, which are most likely identical with the pentose phosphate pathway and fueled by MgATP (Futterman et al., 1970; Schnetkamp & Daemen, 1981). Thus, the reduction of the chromophore appears to be dependent on the availability of cytosolic Mg^{2+} and may serve as an indicator for the latter.

Exposure to Ca^{2+}-deficient media has been reported to increase the cGMP content of mouse retinas by 10-fold (Cohen et al., 1978) and to reduce the ATP and GTP content of

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[1] Abbreviations used: ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; cGMP, guanosine cyclic 3′,5′-monophosphate; OAc, acetate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; NADP, β-nicotinamide adenine dinucleotide phosphate; NADPH, β-nicotinamide adenine dinucleotide phosphate, reduced form; Tris, tris(hydroxymethyl)aminomethane; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone.

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