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Aspects of Light Production by *Photobacterium fischeri*

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Studies of luminescence in growing cultures of *Photobacterium fischeri* revealed the characteristic kinetics of light emission, including a minimal phase of bacterial light output. A dialyzable substance present in the nutrient broth medium caused this transient inhibition in light production, although this substance did not affect culture growth. Experiments were carried out to determine the mechanism of action and the chemical properties of the inhibitor. The results suggest that the inhibitor may be binding directly to the luciferase molecules.

Previous studies of luminescent bacteria (1) indicated that light production and growth are closely related. In these studies, luminescence was measured by use of relatively insensitive photoelectric detectors, such as cesium oxide. Baylor (Ph.D. Thesis, Princeton Univ., Princeton, N.J., 1949), Hastings (*personal communication*), and the present investigators, using the more sensitive photomultiplier, did not find a simple relationship between growth and light production. This investigation was undertaken to study the conditions required for differentiating between growth and light production in luminous bacterial cultures.

Materials and Methods

Cultures of *Photobacterium fischeri* (kindly supplied by J. W. Hastings) were maintained on nutrient agar slants containing 3% NaCl. Liquid subcultures were grown at 24 C in scintillation counter vials containing nutrient broth, 3% NaCl, and antifoam A agent (Dow Chemical Co., Midland, Mich.). These subcultures were aerated by bubbling filtered air through small bore steel tubes inserted through the plastic caps on the vials.

The cultures were monitored by use of a modified light-scattering apparatus (Phoenix Precision Instrument Co., Philadelphia, Pa.) having an RCA 1 P 21 photomultiplier tube operated at 700 v. The signal from the photomultiplier was passed through a linear-to-log amplifier to a chart recorder. Seven vials held in a movable carriage were passed sequentially between light source and photomultiplier. Appropriate shutters and filters permitted independent measurements of bacterial luminescence and optical density (OD) of the culture. Each parameter was measured every 6 min.

The enzyme measurements were performed in vitro, as described by Hastings, Riley, and Massa (2).

Results and Discussion

Light emission from a fresh broth subculture of *P. fisheri* is shown in Fig. 1. Initially, there was a period of variable light output during which the emission changed, within a factor of two, from the starting intensity. The duration of this period is inversely related to the cell density, and, in the experiment shown, this phase lasted 100 min. This "lag" was succeeded by an abrupt depression in light emission; light output then rose exponentially with a doubling time of 6 to 10 min. Bacterial luminescence finally became constant at about 10^3 quanta per second per cell, as the cultures become heavily populated. Eventually, when the cultures became overgrown, the light level declined (not shown in Fig. 1). If the cells were subcultured at any time during this process, the entire sequence recurred. Similar kinetics of light emission were obtained when the bacteria are cultured in a yeast extract medium.

The abrupt depression in light emission (at 135 min in Fig. 1) can be eliminated if the medium is "conditioned" by preliminary exposure to bacteria. Such an experiment is illustrated in Fig. 2. Two cultures of *P. fischeri* were grown for 164 min, until the exponential luminescence phase was reached (curves A and B). The bacteria were then removed by centrifugation and were discarded. The "conditioned" medium was then reinoculated to start two new cultures, one of which was supplemented with concentrated fresh

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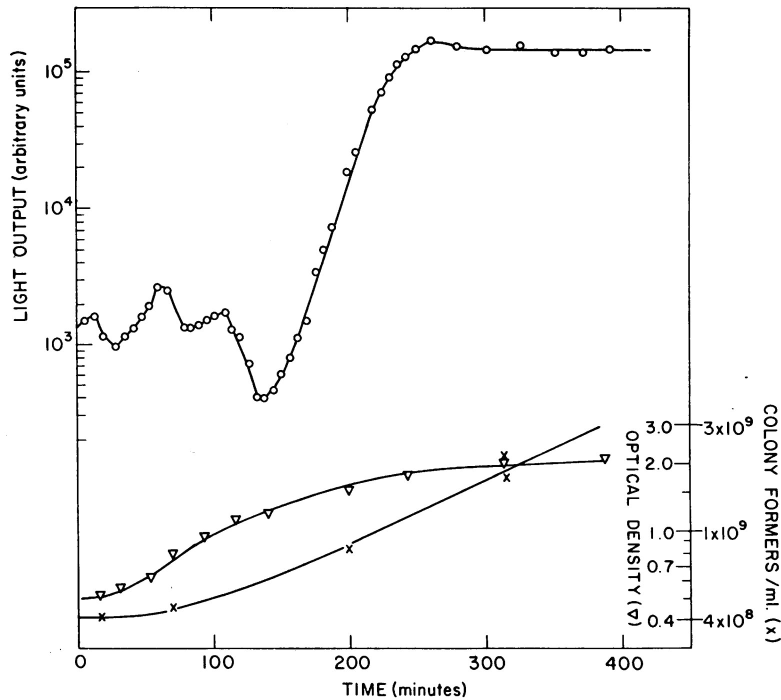

FIG. 1. *Kinetics of light emission, OD, and colony-former population of a culture of Photobacterium fischeri grown in nutrient broth containing 3% NaCl. The abscissa refers to time after subculturing.*

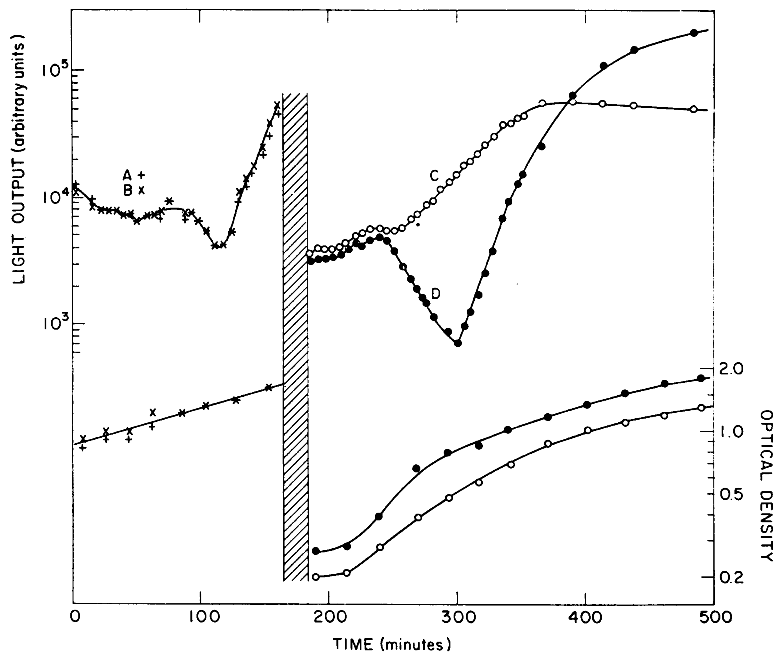

FIG. 2. *"Conditioned" medium experiment. Light output (upper curves) and OD (lower curves) of two identical cultures are indicated by + and ×. Cultures were harvested at 164 min and the cells were discarded. The medium was used to start two new cultures (C and D) at 183 min with 3 × 10⁷ cells/ml. Culture D (●) was supplemented with concentrated fresh broth.*

broth (0.1 volume of 10 × concentrated broth). The cells in the conditioned medium without supplement did not show the characteristic light minimum (curve C), but, when the medium was replenished with fresh broth, the minimum appeared (curve D). The more rapid rise in light in the supplemented culture may be due to the addition of fresh metabolites to the "used" medium. Replenishment with dialyzed broth did not affect light kinetics; however, the minimum was observed when the dialysate was added.

The only difference in the treatment of these two cultures was the addition of fresh broth to one culture. Therefore, the pronounced minimum in light production was due to a dialyzable factor in the fresh broth which inhibited light emission. The conditioned medium experiment indicated that the medium was exhausted of inhibitor by the time the rapid light increase occurred.

Since inhibited cells eventually showed an exponential light increase, the inhibitor must have been internally destroyed, presumably by metabolic action of the bacteria themselves.

Mechanism of action of the inhibitor. It is possible that the inhibitor acts by altering the general bacterial metabolism. The usual index of general metabolism is cell growth. As measured by changes in colony-forming cell population and by OD (Fig. 1 and 2), bacterial growth curves do not reflect the kinetics of light emission. When inhibitor (as concentrated dialysate of broth) was added to a culture in the rapid phase of light increase, the growth rate was unaffected (trace F of Fig. 3), whereas the light output was drastically curtailed (trace B of Fig. 3). These effects were independent of the time during the culture cycle at which inhibitor was added.

A second possibility is that the inhibitor affects the light-producing reaction itself, by altering the level of active enzyme present or by suppressing the synthesis of a specific reactant. The data in Fig. 3 illustrate an experiment which was used to test these possibilities. Two cultures were started with very large inocula (to 10^9 cells per ml). Samples taken at various times were used to determine the level of light-producing enzyme (luciferase) in cell extracts (2). The light output of the cultures exhibited the characteristic kinetics already described. The cellular enzyme level increased slightly during the first 100 min, and, during the rapid increase in light output, there was a simultaneous increase in enzyme concentration. After 110 min, concentrated dialysate from fresh broth medium, containing the inhibitor, was added to one culture (curve B of Fig. 3). Bacterial luminescence was immediately de-

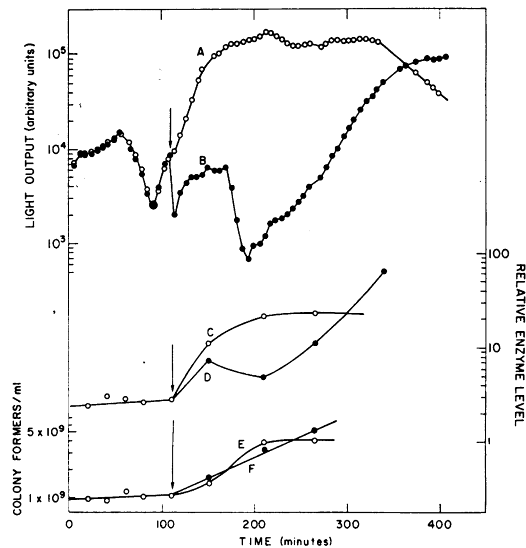

FIG. 3. *Measurements of culture-light output (A, B), luciferase assay (C, D), and colony-forming population (E, F). Symbol:* ●, *culture receiving 0.1 volume of 90× concentrated broth dialysate at 110 min (see text).*

pressed; this depression appeared to be an osmotic effect resulting from the sudden addition of highly concentrated dialysate. The light output returned to the earlier level and then decreased to a minimum at 195 min; the extractable luciferase level was also depressed. Eventually, as the culture increased in luminosity, the luciferase level rose again. If the inhibitor only interfered with the synthesis of reduced flavin mononucleotide or other reactants, less of these substrate materials would be available in the presence of inhibitor. Binding of reactants would cause the same limitation. Since the extracts were used only as a source of enzyme (all other reactants being supplied), the assay would show no difference between control and experimental cultures. Therefore, the depression in the enzyme level of the cells exposed to the inhibitor implies that the inhibitor affects luciferase directly.

The inhibitor could be interfering with the synthesis of luciferase, or, alternatively, with the activity of this enzyme. To distinguish between these possibilities, chloramphenicol was used to determine if the inhibitor can act in the absence of enzyme synthesis. The antibiotic, which specifically blocks protein synthesis, was added to cultures to a final concentration of 10 μg/ml. Growth, as measured by OD, was quickly shifted to a linear function of time. Within a few minutes, the antibiotic curtailed increases in light production; for several hours thereafter, the light

intensity of these cultures slowly decreased (Fig. 4). This decrease is ascribed to a gradual decline in the luciferase level which existed previous to the addition of chloramphenicol. If a 0.1 volume of 10× concentrated broth was added during this slow decline in light output (during linear growth), the light soon decreased sharply and then rapidly regained its former level (Fig. 4). Protein synthesis and, therefore, enzyme synthesis should have ceased by the time the broth was added. Since the inhibitor still curtailed light production, we conclude that its mechanism of action is independent of luciferase synthesis. The inhibitor does not irreversibly inhibit the enzyme, since the light level recovers completely after about 40 min.

In view of the finding that the inhibitor can act independently of luciferase synthesis, it is possible that binding of the inhibitor to the enzyme molecules causes the lowered activity found in cell extracts. Support for this hypothesis was obtained in another experiment (Table 1). Crude luciferase was prepared from cells and tested for activity; successively larger amounts of concentrated inhibitor were added to the luciferase assay reaction. Purified luciferase (supplied by J. W. Hastings) was also studied. The results demonstrated that both crude cell extracts and purified enzyme are inhibited by broth dialysate. This experiment should be interpreted cautiously since broth dialysate is a complex mixture of many different substances, any of which may interfere with a sensitive enzyme assay.

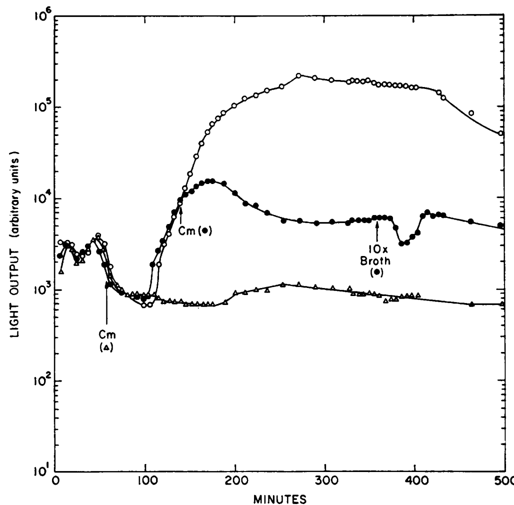

FIG. 4. *Light output of three cultures of Photobacterium fischeri. All cultures growing exponentially when chloramphenicol was added (to a concentration of 10 μg/ml) at 59 min (△) or 138 min (●). Thereafter, growth was linear, and OD increased by 30% in 4 hr. At 360 min, 0.1 volume of 10× concentrated broth was added to one culture (●). Controls (○) had no additions.*

TABLE 1. *Inhibition of luciferase by broth dialyzate*[a]

Addition (1 ml)	Purified enzyme		Crude lysate	
	Quanta	Per cent	Quanta	Per cent
Distilled water...	1.9×10^{10}	100	1.6×10^{11}	100
Broth dialysate				
0.9 ×...	1.1×10^{10}	58	1.3×10^{11}	81
4.5 ×...	6.5×10^{9}	34	6.5×10^{10}	41
18 ×...	1.8×10^{9}	9.5	1.5×10^{10}	9.4
90 ×...	Not detectable	0	$8. \times 10^{8}$	0.5

[a] The luminous bacterial enzyme system (2) emits a flash of light when the reagents are combined. These flashes were integrated electronically, the number of photons per flash was computed, and this number was corrected for changes in OD resulting from additions. The inhibitor was diluted to 1 ml with distilled water and then was added to the 2.3-ml reaction mixture.

A mechanism of action which is consistent with all of the above data is that the inhibitor binds to luciferase, thereby reducing its activity. At present, this hypothesis is inferential and therefore tentative. Several unanswered questions remain. For example, the long delay between the introduction of inhibitor to a bacterial culture and the occurrence of the light medium has not been explained. This phenomenon may reflect a permeability problem or may indicate that compounds in broth must be metabolized to an active form before inhibition can take place.

Attempts to isolate the inhibitor. Attempts to identify the inhibitor were not successful. Some inhibitory activity was found in each of the components of nutrient broth (peptone and beef extract). The use of anion, cation, and Sephadex columns was not successful in separating an active fraction. The inhibitory activity of broth dialysate was not destroyed by boiling at *p*H 1 or at *p*H 13; in addition, this inhibitory activity was not extractable with chloroform or precipitable with silver nitrate. It was, however, destroyed by ashing.

Attempts to mimic the inhibitory activity. A trace metal ion may be expected to have the chemical properties listed above. Each metal present in the broth (3) was added to *P. fischeri*

cultures at ten times the normal concentration. The metals, either individually or collectively, did not reproduce the kinetics of light inhibition found with broth dialysate.

Other chemicals have been known to effect bacterial luminescence (Baylor, Ph.D. Thesis, Princeton Univ., Princeton, N.J., 1949). Our cultures required 10^{-3} M KCN to achieve the same effects that Baylor reported with KCN at 10^{-6} M. Cyanide action depends on time of addition, whereas the inhibitor in broth is independent of time.

In normal cultures or in cultures containing inhibitor, *p*H is not a factor in the fluctuations in light intensity. Normal light curves were obtained from cultures buffered at *p*H values between 6 and 8. Outside this range, the light was extinguished either immediately or at the minimum. The addition of glucose at 1 mg per ml had little effect on the light output of a growing culture when the medium was buffered. In unbuffered glucose medium, light production was normal until the minimum was reached; subsequently, the *p*H dropped and the light was irreversibly extinguished.

NaCl concentration may be varied from 2 to 5% without altering the kinetics of light production. Other compounds tested in cultures of *P. fischeri* include gluconate, Casamino Acids, individual amino acids, purines, pyrimidines, ethyl alcohol, urea, tryptophan, various buffers, ethylenediaminetetraacetic acid, flavin mononucleotide, glycerol, vitamins, and distilled water. These substances either showed no inhibitory effects or showed inhibitory effects which differed from those exhibited by the broth.

One of the first studies of the kinetics of luminous bacteria which utilized a photomultiplier detector was that of Baylor (Ph.D. Thesis, Princeton Univ., Princeton, N. J., 1949). He reported several distinct phases in the time curves of bacterial light output, including the minimum which we have studied. It is now clear that this decline in light production is not an intrinsic property of the bacteria, but is due to a dialyzable inhibitor present in growth medium. This inhibitor curtails bacterial light production without affecting growth. By the time of rapid increase in brightness, the inhibitor has been destroyed or removed from the medium by the bacteria. The mechanism of action of the inhibitor is not related to general cellular metabolism, synthesis of specific reactants, or binding to these reactants. Its action is independent of protein synthesis. Several experiments suggest that the inhibitor may be binding directly to the luciferase molecules.

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