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Sensitive, Rapid, and Specific Bioassay for the Determination of Antilipogenic Compounds

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A sensitive and rapid bioassay for the determination of the antilipogenic compounds cerulenin and CM-55 is described. The bioassay is based on the inhibitory effect of cerulenin and CM-55 on the in vivo luminescence of an aldehyde-requiring mutant of the marine bacterium *Beneckea harveyi*. A total quantity as low as 0.1 μg of cerulenin can be determined within 15 min with an error of ±2%. The bioassay, as presented, is specific for compounds that are known to inhibit fatty acid biosynthesis and, as such, it might be used as a general screening method for the detection of antilipogenic compounds.

Cerulenin is an antibiotic isolated from the culture filtrate of the fungus *Cephalosporium caerulens* (4) and has the structure (2S)(3R)-2,3-epoxy-4-oxo-7,10-dodecadienoyl amide (1). It is the first known antibiotic that inhibits total fatty acid synthesis as its primary action (2, 8, 10, 13). Because cerulenin possesses a number of remarkable biological activities that have never been found in other antibiotics, it was used as a biochemical tool for studies on fatty acid biosynthesis and structure and functions of membranes in diverse biological systems (11). However, the instability of the antibiotic in animals prevents its use in therapy as an antimicrobial and antifungal agent or as an antilipogenic agent (11). A study has been made to find more stable and active synthetic analogs of cerulenin, such as CM-55 (2,3-dodecenyl-4-oxo-dimethyl amide) (9).

The search for such analogs or for other antilipogenic compounds is difficult mainly because of two reasons: (i) these compounds are produced by various fungi in relatively low concentrations and usually together with other compounds such as steroidal antibiotics (9), and (ii) no sensitive, rapid, and specific method is available for the bioassay of antilipogenic compounds.

Assay procedures that use a bioluminescence system of marine luminous bacteria have been described previously. These assays are based on the determination of the luciferase activity, and components of the system such as reduced nicotinamide adenine dinucleotide and reduced flavine mononucleotide ($FMNH_2$) can be detected at very low concentrations. Changes in the bioluminescence in intact cells have been used to monitor the effects of ionizing radiation, air pollutants, and anesthetics (5).

The in vitro bacterial bioluminescence system involves a reaction between $FMNH_2$, a long-chain aliphatic aldehyde (such as dodecanal [RCHO]), oxygen, and the enzyme luciferase, as shown by the stoichiometry (7):

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

Ulitzur and Hastings (unpublished data) have recently shown that in several aldehyde-requiring mutants of *Beneckea harveyi* (such as M_{17}) exogenously added myristic acid replaced the requirement for an aldehyde for the initiation of the in vivo luminescence. In addition to myristic acid, another internal lipid factor is required to initiate the light emission in this group of mutants. This conclusion is based on the observation that cerulenin caused a decrease in the in vivo luminescence in the aldehyde-requiring mutant M_{17} in the presence of exogenous myristic acid, but not in the presence of exogenous decyl aldehyde. This observation serves as the basis for a rapid, specific, and sensitive bioassay for cerulenin and CM-55 as described in the present work. We suggest that this assay can be used as a general bioassay for the determination of various antilipogenic compounds.

MATERIALS AND METHODS

Materials. Decyl aldehyde, capric acid, lauric acid, myristic acid, palmitic acid, and stearic acid (all of these ≥99% pure) were obtained from Sigma Chemical Co., St. Louis, Mo. Decyl aldehyde (0.1%) was suspended in a solution of 0.1% Triton X-100. Fatty acids were in the form of K^+ salt solutions in

slightly alkaline aqueous ethanol (1:3, vol/vol). Cerulenin and helvolic acid (4) were purchased from Makor Chemicals Ltd., Jerusalem, Israel, and CM-55 (9) was a gift from S. Omura, Kitasato Institute, Tokyo, Japan. Fresh solutions of cerulenin and CM-55 were prepared in 10% ethanol. Helvolic acid (4) was disolved in a 10% ethanol solution in a slightly alkaline aqueous solution. The final concentration of ethanol was kept lower than 0.1%, and the same amount of ethanol was added to the control system.

Growth of the cells. *B. harveyi* strain M_{17}, an aldehyde-requiring mutant, was obtained from J. W. Hastings, Harvard University. This mutant is very dim unless long-chain aldehyde or myristic acid is added to the medium. Cells were grown at 30°C with shaking in a complex medium containing 0.3% yeast extract (Difco Laboratories, Detroit, Mich.), 0.5% peptone (Difco), 3% NaCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.1% KH_2PO_4, and 0.5% Na_2HPO_4. The pH of the medium was 7.2. The growth of cells was followed by measuring the density of the culture in a Klett-Summerson colorimeter with a no. 66 filter (100 Klett units is equal to 5×10^8 cells/ml). When the density of the cell suspension reached 180 to 220 Klett units, samples were taken for the bioassay.

C. caerulens KF-140 was used for the production of cerulenin. The cultivation of the organism was carried out as described by Iwai et al. (4) in a 10-liter New Brunswick Microferm fermentor at 27°C. Samples of fermentation broth were kindly provided by Makor Chemicals Ltd.

Measurement of bioluminescence. Portions of 0.1 to 1.0 ml of cell suspension were placed in sets of scintillation vials, and the light emission was measured with a photomultiplier photometer (6). Light intensities are expressed in light units; 1 light unit equals 2×10^7 quanta/s as established by a standard of Hastings and Weber (3).

RESULTS

Growth and luminescence of M_{17} cells. Figure 1 shows the kinetics of growth and development of the in vivo luminescence of an aldehyde-requiring mutant (M_{17}). The light emission reached a maximum at the end of the exponential phase of growth. After 4.5 h of growth, the light emission per cell was about 0.6 quanta/s. This residual light emission was stimulated several hundredfold by the addition of decyl aldehyde or myristic acid, at low concentrations (1×10^{-5} to 2×10^{-5} M), to the assay mixture. After 4.5 h of growth, the in vivo luminescence per cell was 380 quanta/s in the presence of decyl aldehyde and 160 quanta/s in the presence of myristic acid.

Inhibition of the in vivo luminescence by cerulenin. Cerulenin inhibited the light emission in M_{17} cells incubated in the presence or in the absence of myristic acid (Fig. 2). In the absence of myristic acid, cerulenin caused a rapid decay in the residual luminescence to undetectable levels within 25 to 30 min. When myristic acid was added during this period, less and less light was induced until it decayed to a level as low as 1/500 of the level of untreated cells. When cerulenin was added to a myristic acid-containing culture, the in vivo luminescence rapidly decreased. In all cases, the rate of the decay in luminescence caused by cerulenin was almost the same, having a half-life of about 4 min.

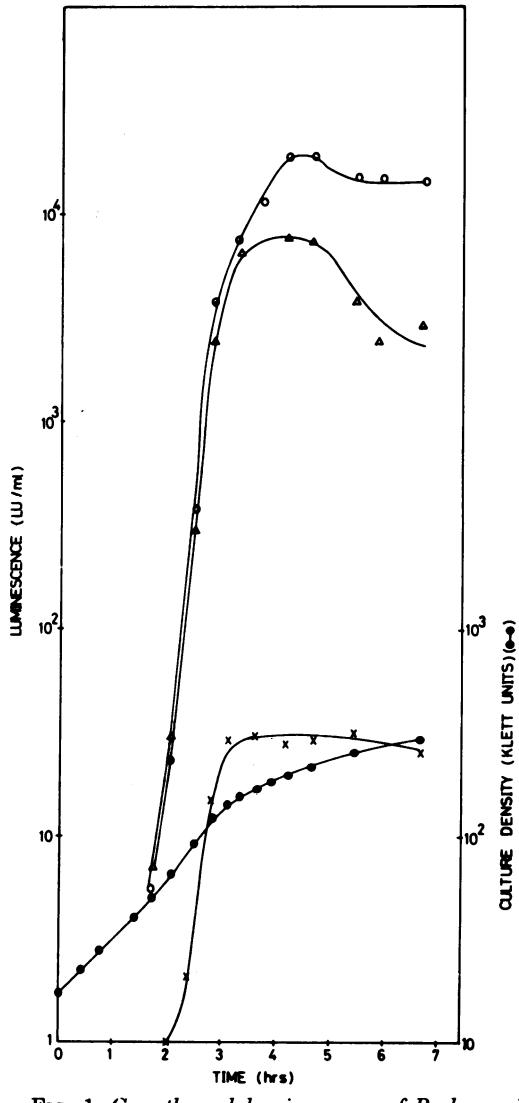

FIG. 1. *Growth and luminescence of B. harveyi M_{17}. M_{17} cells were grown in a liquid complex medium at 30°C with shaking. Samples of 1 ml were taken with time for luminescence determination in the absence (×) or presence of 2×10^{-5} M myristic acid (△) or 2×10^{-5} M decyl aldehyde (○). LU, Light units.*

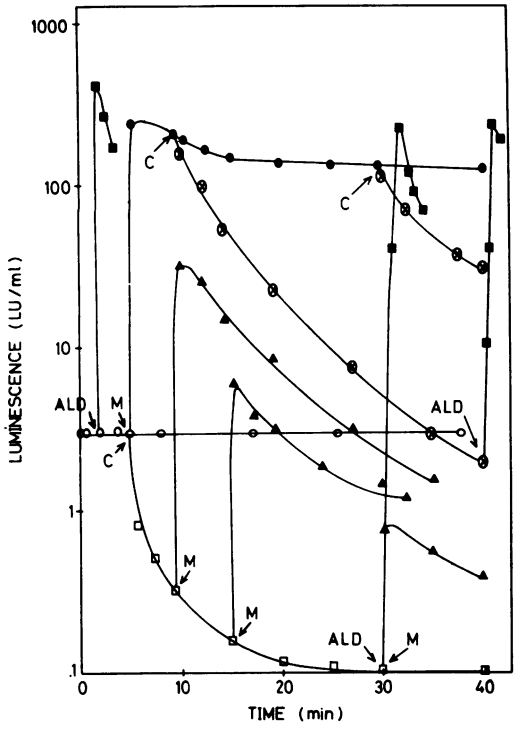

FIG. 2. *Effect of cerulenin on M_{17} luminescence. M_{17} cells were grown in a liquid complex medium at 30°C with shaking to a cell density of 220 Klett units. Samples of 0.5 ml were placed in a set of vials that were preincubated for 5 min at 20°C. One vial (○) served as a control. To other vials (□), cerulenin (C; 50 μg/ml) was added, whereas myristic acid (M; 20 nmol/ml) was added to a third set of vials (●). All of the vials were incubated with shaking at 20 to 22°C. At the specified times, decyl aldehyde (ALD; ■) was added to some vials to a final concentration of 20 nmol/ml. To other vials, myristic acid (▲) to a final concentration of 20 nmol/ml or cerulenin to a final concentration of 50 μg/ml (⊗) was added. All changes in luminescence were followed with time. LU, Light units.*

Unlike myristic acid, the addition of decyl aldehyde to a cerulenin-containing culture with or without myristic acid restored very rapidly and almost completely the in vivo luminescence as compared with a control system containing aldehyde but not cerulenin (Fig. 2).

The in vivo luminescence system of *B. harveyi* M_{17} was much more sensitive to cerulenin than was cellular growth. The rate of growth of M_{17} cells was almost not affected, for at least 2 to 3 h, by a cerulenin concentration of up to 25 μg/ml. During this time the in vivo luminescence was inhibited to a large extent by relatively low concentrations of cerulenin (about 1 μg/ml; Fig. 3).

Luminescence assay of cerulenin. Based on the results shown in Fig. 2, the following method was developed for the quantitative determination of cerulenin.

(i) A volume of M_{17} culture (any desired volume between 0.1 and 1 ml) was placed in sets of scintillation vials.

(ii) Various amounts of cerulenin were added to the vials. When the volume of the added cerulenin exceeded 25% of the culture volume, the cerulenin was dissolved in 3% NaCl solution.

(iii) Vials were incubated for 30 min at 30°C with shaking (180 rpm).

(iv) The maximal in vivo luminescence was determined after the addition of myristic acid (1.5×10^{-5} M final concentration) to each vial.

Figure 3 shows the application of this method for the determination of cerulenin. This assay was sensitive, and 50% of the in vivo luminescence was inhibited by 0.18 μg of cerulenin in the assay mixture (total volume of 0.1 ml). A linear relationship on a semilogarithmic scale was obtained between the degree of inhibition of luminescence and cerulenin quantities of 0.05 to 0.75 μg in the assay mixtures. The approximate error for two standard deviations (95% confidence level) was ±2%.

An alternative method can be used for the determination of cerulenin, in which the cells are incubated with myristic acid, cerulenin is added, and the decay in the in vivo lumines-

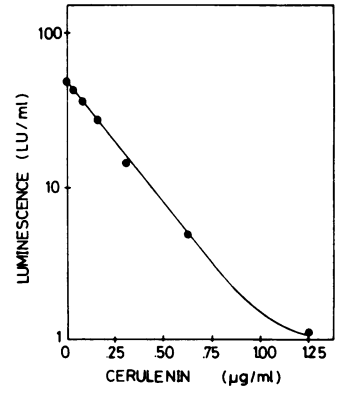

FIG. 3. *Inhibition of the in vivo luminescence of B. harveyi M_{17} by different concentrations of cerulenin added prior to myristic acid. M_{17} cells were grown in a complex medium at 30°C with shaking to a cell density of 180 Klett units. Samples of 0.2 ml were placed in sets of vials containing different amounts of cerulenin. The vials were incubated for 30 min at 30°C with shaking, and the in vivo luminescence was determined within 30 s after the addition of myristic acid (1.5×10^{-5} M final concentration) to each vial. LU, Light units.*

cence is determined with time. As this method was found to be less sensitive an assay than the former method (50% inhibition in the in vivo luminescence was obtained with 0.75 μg of cerulenin in the assay mixture [total volume of 0.1 ml]), we used the method described for Fig. 3 in further experiments.

Factors affecting the bioassay. (i) pH. The assay described for Fig. 3 was performed at pH 7.2. The inhibition of luminescence by cerulenin was maximal between pH 7 and 8 (Fig. 4). The sensitivity of the assay progressively decreased at lower or higher pH values.

(ii) Temperature. The bioassay could be performed at any temperature in the range of 20 to 37°C. As the temperature in this range increased, the preincubation period with cerulenin could be shortened. Thus, at 37°C a preincubation period of 15 min was sufficient.

(iii) Fatty acids. The requirement for myristic acid for the induction of light emission in M_{17} cells could not be replaced by other saturated fatty acids, such as capric, lauric, palmitic, or stearic acid. The presence of these acids (up to a final concentration of 1×10^{-5} M) in the assay mixtures had no effect on the stimulation of light caused by myristic acid.

Determination of cerulenin during the fermentation of *C. caerulens* KF-140. To study whether the bioassay developed can be used as a specific assay for cerulenin, we determined the production of this antibiotic in the course of the cultivation of the fungus *C. caerulens* (Fig. 5). The amount of cerulenin attained a maximum after about 36 h. These results were similar to those obtained by Iwai et al. (4), who used a different assay system for the determination of cerulenin. External cerulenin, when added to the supernatant solution obtained from a 14-h sample of the fermentation medium, was fully expressed in the assay. When added to supernatant solutions obtained from later stages during fermentation, the effect of external cerulenin was additive to the activity of cerulenin that was present in the medium.

Effect of CM-55 and helvolic acid on light emission in *B. harveyi* M_{17}. CM-55 is a synthetic analog of cerulenin, having antilipogenic activity (9). Its mechanism of action is quite different from that of cerulenin (9). Figure 6 shows that CM-55 could be determined by the bioassay similar to the determination of cerulenin. However, cerulenin was more effective than CM-55 as an inhibitor of light emission in M_{17} cells. The addition of decyl aldehyde to a CM-55-containing culture completely restored the in vivo luminescence.

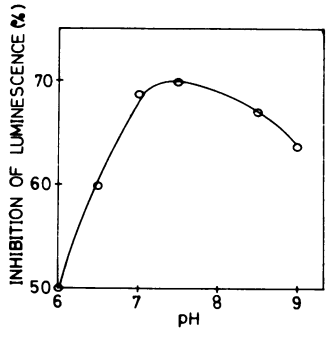

FIG. 4. *Effect of pH on the degree of inhibition of the in vivo luminescence of B. harveyi M_{17} caused by cerulenin. M_{17} cells were grown as described in the legend to Fig. 3. Samples of 0.9 ml containing 8 × 10^8 cells were placed in sets of vials that contained 0.1 ml of 0.5 M imidazole-hydrochloride buffer at different pH's with or without cerulenin (final concentration, 5 μg/ml). The vials were incubated for 30 min at 30°C with shaking. The in vivo luminescence was determined within 30 s after the addition of myristic acid (1.5×10^{-5} M final concentration) to each vial. The percent inhibition of luminescence was calculated for each cerulenin-containing culture from the luminescence of the control system at the same pH but without cerulenin.*

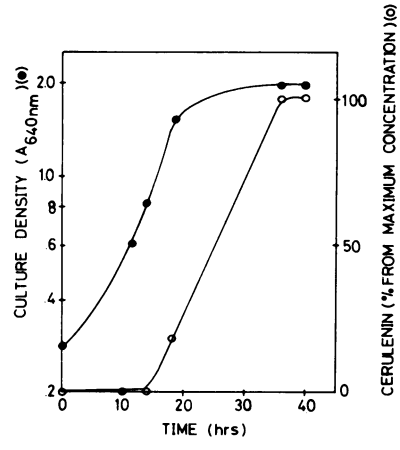

FIG. 5. *Time course of cellular growth and cerulenin production of C. caerulens KF-140. Mycelia were grown as described in the text. At different times during growth, samples (10 ml) of cell suspensions were centrifuged at 10,000 × g for 10 min. Samples (2 to 100 μl) were taken from the clear supernatant fermentation broth and added to sets of vials containing complex medium to a total volume of 0.2 ml. M_{17} cells (1.8 × 10^8 cells in 0.2 ml) were added to the vials, which were then incubated at 30°C for 30 min with shaking. The in vivo luminescence was determined within 30 s after the addition of myristic acid (1.5×10^{-5} M final concentration) to each vial. The concentration of cerulenin in each sample was calculated from a standard curve as shown in Fig. 3. A_{640nm}, Absorbance at 640 nm.*

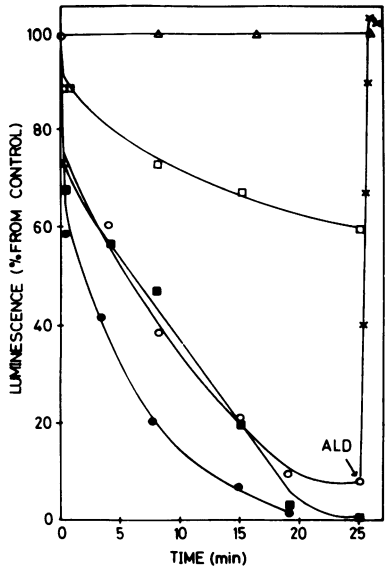

FIG. 6. *Effects of cerulenin, CM-55, and helvolic acid on the in vivo luminescence of B. harveyi* M_{17}. M_{17} *cells were grown as described in the legend to Fig. 3. Samples containing* 2×10^8 *cells were placed in sets of vials that contained a total volume of 0.2 ml, myristic acid* (1×10^{-5} *M final concentration), 2 μg (■) and 4 μg (●) of cerulenin, 2 μg (□) and 4 μg (○) of CM-55, or 50 μg of helvolic acid (△). The vials were incubated at 20°C with shaking, and the in vivo luminescence was determined with time. Decyl aldehyde (ALD) (*1×10^{-5} *M final concentration) was added after 25 min to a CM-55-containing vial (×).*

The antibacterial protolanostan-type antibiotic helvolic acid (9) is produced by the same cerulenin-producing strain (4). Although this compound inhibited the growth of different bacteria (4), no effect of the antibiotic was observed on the in vivo luminescence up to 50 μg/ml (Fig. 6). In addition, when present together, helvolic acid (50 μg/ml) had no effect on the inhibition of luminescence by cerulenin (4 μg).

DISCUSSION

Based on the antimicrobial and antifungal activities of cerulenin, two alternative methods are currently used for the determination of this antibiotic. In the first one, cerulenin is determined by an agar dilution technique, and its activity is expressed as minimal inhibitory concentration (9). The test organisms are different strains of bacteria, yeasts, and fungi. In the second method, cerulenin is determined in a bioassay based on measuring the inhibition zone around a paper disk, using *Candida albicans* KF-1 as the test organism (4).

These methods have four major disadvantages. (i) No internal control is included to assure that the growth of the test organism was not inhibited by other antibiotics (such as helvolic acid) or by a nonspecific inhibitor (such as metals, organic acids, or other compounds), but by the antilipogenic activity of the antibiotics. (ii) As both methods used a yeast as the test organism, it is not possible to know whether growth was inhibited as a result of a primary inhibition of fatty acid or steroid synthesis. This is of importance since steroidal antibiotics are usually produced together with the antilipogenic antibiotics during fermentation (4). (iii) In both methods the incubation period required is between 24 and 72 h. Because of the long incubation period, the determination of antilipogenic compounds during a fermentation process is not possible. (iv) The lowest concentration of cerulenin that could be determined by the paper disk method is about 25 μg/ml (4) whereas the first method is to some extent more sensitive.

The present work describes a simple bioassay for the quantitative determination of compounds that inhibit fatty acid and hence lipid synthesis. The technique is based on the measurement of the decrease in light emission, caused by these compounds, in an aldehyde-requiring mutant (M_{17}) of *B. harveyi*.

This bioassay could be performed within a relatively short period of time. At 37°C the period during which the cells were incubated with the inhibitory substance was 15 min, whereas at lower temperatures the incubation periods were more prolonged (up to 30 min). This relatively short period of time enables the determination of antilipogenic compounds during the course of the cultivation of the producer organism, and a rapid feedback control on the fermentation process is feasible.

One of the advantages of this method is its simplicity. The test organism can be grown within 3 to 4 h, starting from a loopful of cells taken from an agar plate, to a culture density of 180 to 220 Klett units (Fig. 1). Samples of cell suspension are incubated with different concentrations of the inhibitor at any temperature between 20 and 37°C; myristic acid is added, and the light emission is recorded. The luminescence developed can be measured by a photomultiplier apparatus measuring light in the range of 450 to 550 nm with a sensitivity to detect at least 5×10^7 quanta/s. This measurement can be achieved also by the use of a scintillation counter photometer (12), but as this instrument is very sensitive to light, fewer cells (approximately 10^7 cells/ml) are used in the assay mixtures, while the total volume should be increased to 5 ml.

Results obtained with this bioassay were con-

sistent and reproducible within an error limit of ±2%. A linear relation on a semilogarithmic scale was obtained between the extent of inhibition of the in vivo luminescence and cerulenin concentrations up to 0.75 μg in the assay mixture; 50% inhibition was obtained with 0.18 μg of cerulenin. The high sensitivity of this method to cerulenin (or CM-55, Fig. 6) can be explained by the fact that in this mutant the emission of light is dependent on the presence of a very low concentration of lipid components. From the results shown in Fig. 2 it can be speculated that a lipid factor is required, in addition to myristic acid, to enhance light emission in this bacterium. The synthesis of this lipid factor is specifically inhibited by cerulenin, and as a result of its rapid turnover the level of this lipid factor is decreasing in the cells. Thus, the extent of luminescence caused by the addition of myristic acid to a cerulenin-containing culture is directly proportional to the residual concentration of the unknown lipid factor in the cells. It might also be speculated that both the lipid factor and myristic acid are the precursors for the internal natural aldehyde, as the addition of decyl aldehyde to a cell suspension restored almost completely the luminescence in the presence of cerulenin.

Finally, the method employed seems to be specific for compounds inhibiting fatty acid biosynthesis because of the following reasons. (i) As the test organism is a bacterium that does not involve or produce any steroids, the inhibition of luminescence by the antilipogenic compounds is not caused as a result of their inhibitory effect on steroid synthesis, but on fatty acid synthesis (4, 11). Cerulenin has been shown to specifically inhibit condensing enzyme of the fatty acid synthetase (11), whereas CM-55 inhibited profoundly the acetyl-coenzyme A carboxylase, the rate-limiting enzyme of overall fatty acid synthesis (4). The difference in the sensitivity of the in vivo luminescence to these antibiotics might be due either to a difference in their permeation properties to the cell or to differences in the K_i values of the different enzymes (4). Helvolic acid, an antibacterial antibiotic that is produced simultaneously with cerulenin by *C. caerulens*, had no effect on the in vivo luminescence of *B. harveyi* M_{17} under the assay conditions used (Fig. 6).

(ii) The complete reversion of light emission obtained when decyl aldehyde was added to CM-55- or cerulenin-containing cultures can serve as a good criterion for the specificity of this assay. Antibiotics or compounds affecting protein or nucleic acid synthesis should not affect the luminescence of M_{17} cells. When added to the bioassay, M_{17} cells have already synthesized their maximal content of luciferase while the culture enters the stationary phase of growth, and almost no further cell division occurs. However, to avoid the possibility that other factors will influence the bioluminescence, aldehyde should be added before and after the addition of the antilipogenic compound. Thus, the assay for the tested compound is reliable only when almost complete reversion of luminescence by aldehyde is observed.

The speed, simplicity, sensitivity, and specificity of the present method indicate that it can be applied on a routine basis through screening, diagnostic production, and purification procedures of antilipogenic compounds.

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