Induced Polypeptide Synthesis during the Development of Bacterial Bioluminescence*

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The dramatic increase in bioluminescence observed during the later exponential growth of *Beneckea harveyi* is due to the induction of luciferase activity. The mechanism by which luciferase activity is induced and the possible existence of other induced proteins was investigated in a double-labeling experiment: [4,5-H]leucine was incorporated into cellular proteins synthesized during the luminescence lag period in early growth; [14C]leucine was incorporated during the later period of luminescence induction. The proteins of the cell-free extract were extensively fractionated and luciferase was purified to homogeneity. Analysis of the radioactivity incorporated into the α and β subunits of luciferase showed a dramatic but equal decrease in the 3H/14C ratio for both subunits. This result proves that the synthesis of the α and β chains of luciferase is subject to similar controls and that the regulatory mechanism is operative at the level of gene transcription, or translation at the time of bioluminescence induction, or both. Several additional polypeptides have been found which also show a marked decrease in their 3H/14C ratio indicating that their synthesis is induced during the same period as luciferase. In addition, one polypeptide that is synthesized specifically in the bioluminescence lag period was also detected. The function and role of these new polypeptides with respect to the bioluminescent system is presently under investigation.

The development of light emission in exponentially growing cultures of luminous bacteria is delayed until considerable growth of the cells has occurred (1, 2). The light-emitting reaction is catalyzed by luciferase, a heteropolymeric protein, αβ (3), and involves the oxidation of reduced FMN and a long chain aliphatic aldehyde (4-11). During the luminescence lag period in early growth, the extractable luciferase activity remains constant but later increases at the same time and rate as in vivo luminescence (2). Previous work has shown that the addition of inhibitors of RNA or protein synthesis to bioluminescent bacteria prevented any further increase in extractable luciferase activity, suggesting that the control of luciferase activity is exerted at the level of gene transcription (2, 12). However, the bioluminescent system is highly sensitive to the metabolic and nutritional state of the cells (1, 13) and therefore other interpretations may be possible especially since many of these drugs are not only general metabolic poisons with more than one site of action, but also, prevent further exponential growth (1, 14, 15). Earlier work also showed that the development of luciferase activity is paralleled by an increase in antigenic material determined by the amount of luciferase antibody necessary to inhibit luciferase activity (2). Based on the assumption that a luciferase precursor would have the same antigenic properties as the native enzyme, it was concluded that the results showed no evidence for a luciferase precursor (2). Consequently, it may be possible that the observed lag in the development of bioluminescence may either reflect the synthesis of one or both polypeptides of luciferase in the form of an inactive precursor with different antigenic properties than the native enzyme or reflect the repression of synthesis of one or both of the subunits of luciferase at the level of gene transcription or translation.

There is now evidence to suggest that the activities of other enzymes may also be induced during the development of bioluminescence. Studies of the growth and bioluminescence of luminous bacteria (2) have indicated that the substrates of the luciferase reaction are not present in saturating levels at all stages of cell growth. In fact, a substrate limitation occurs specifically during the luminescence lag period and becomes progressively more acute just prior to the induction of bioluminescence. Presumably, this effect arises from a "diluting-out" of substrate-producing enzymes during the luminescence lag period. Coordinate control of substrate-producing enzymes and luciferase has also been suggested from a recent investigation of temperature-sensitive bioluminescent mutants (16).

In the present study, the cellular proteins were labeled with [4,5-H]leucine during the luminescence lag period and with [14C]leucine during the later luminescence induction period. This double-labeling procedure provides a direct approach for determining whether or not the polypeptide chains of luciferase are synthesized in concert with growth and if their synthesis is coordinately controlled. Furthermore a major advantage of this double-labeling technique is that it also permits us to investigate the possibility that the synthesis of other polypeptides is

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induced or perhaps repressed during the induction of bioluminescence without requiring prior knowledge of their functional activities.

**EXPERIMENTAL PROCEDURE**

**Growth and Radioactive Labeling of Bacterial Cells**—A leucine auxotroph of *Beneckea harveyi*, originally obtained from Dr. K. Nealson and Dr. J. W. Hastings, Biological Laboratories, Harvard University, was grown at 27 °C in minimal medium (pH 7.3) containing 0.3% glycerol (17), supplemented with a 0.17% amino acid mixture limited in leucine content. Amino acids (Sigma) were mixed in the relative concentrations reported for casein (18), except that the leucine content was reduced to the lowest levels which did not adversely affect exponential cell growth nor the development of bioluminescence. This medium was chosen because (a) it produced good growth and high levels of bioluminescence, (b) it could be limited in leucine content, and (c) it was precisely defined.

The cells from an exponentially growing culture were inoculated into 250 ml of the above medium containing 5.0 mCi of [4,5H]leucine (New England Nuclear) to give an optical density of 0.01 at 660 nm. The final concentration of leucine was 8 μg/ml. The culture was then incubated at 27 °C in a 1-liter Erlenmeyer flask shaking at 100 cycles/min in a reciprocating water bath shaker (Precision Scientific, model 50). Prior to induction of luminescence, the cells were harvested by centrifugation, washed with minimal media, and then resuspended in 500 ml of fresh medium containing 30 μg/ml of unlabeled leucine. When the induction of bioluminescence was well established, 250 μCi of uniformly labeled [H]leucine (New England Nuclear, 311 Ci/mmol) were added and the cells grown until maximum luminescence was attained. The cells were harvested, washed repeatedly with minimal media to remove extracellular radioactivity, and mixed with a large excess of unlabeled carrier cells (=20 g) grown to the same optical density (A660 = 1.2). The cells were then harvested by centrifugation, frozen overnight, and lyed by resuspension in 200 ml of 10 M dithiothreitol/0.01 M EDTA, pH 7.0, at 4 °C. Cellular debris was removed by low speed centrifugation and the resulting supernatant centrifuged at 100,000 x g for 60 min to remove particulate matter.

**Enzyme Activity Measurements**—Light emission was measured with a photometer (19) calibrated in quanta per s using the standard of Hastings and Waber (20). In vivo bioluminescence measurement are reported as light units per ml of culture, where 1 light unit is defined to be 3.3 x 10⁶ quanta/s.

Luciferase activity was measured "in vitro" from the maximum light intensity obtained after injection of 1 ml of 5 x 10⁻⁷ M FMN (Sigma), (catalytically reduced with H₂ over platinumized asbestos) into 1.0 ml of 0.02 M phosphate/0.2% bovine serum albumin (Sigma), pH 7.0, containing luciferase and 10 μl of a 0.1% decanal (Aldrich) suspension dispersed in 0.1% Triton X-100. One unit of activity is defined to be the amount of enzyme that results in a decrease in light intensity of 1 light unit at 24 °C. FMN reductase activity was determined from the decrease in absorbance at 340 nm on addition of 10 μl of 5 x 10⁻⁷ M FMN (Sigma) to 1 ml of 0.01 M phosphate/0.1% bovine serum albumin, pH 7.0, containing enzyme and 10 μl of NaN₃ (Sigma). One unit of activity is defined to be the amount of enzyme that results in a decrease in absorbance of 0.01/min at 24 °C. All phosphate buffers, unless otherwise indicated, were prepared by mixing appropriate amounts of Na₂HPO₄ and K₂HPO₄.

**Radioactive Measurements**—Radioactive samples were mixed with 10 absorbance at 27 °C in a 1-liter Erlenmeyer flask shaking at 100 cycles/min in a Packard scintillation counter. Tritium measurements have been corrected for an 8% crossover of ³C counts per min into the ¹H channel. The counting efficiency for ¹H and ¹C was 8.0% and 44%, respectively.

**Isotope Ratios**—All ¹H and ¹C measurements have been reported as a percentage of their respective counts in the supernatant (%) ¹H and ¹C. Isotope ratios have been presented as %¹H/%¹C or %¹C/%¹H depending on whether a given sample has shown a relative increase in ¹H to ¹C or ¹C to ¹H, respectively, compared to the supernatant. This method of presentation provides equal weight to changes in the relative amount of either isotope for any given sample. For example, samples in which the ¹H/¹C ratio increases 3-fold relative to the supernatant (3 H/3 C = 3.0) would have the same value for the isotope ratio as samples in which the ¹C/¹H ratio had increased 3-fold relative to the supernatant (1.0 C/1.0 H = 3.0). Consequently, both induced and repressed peptides can be detected equally well. The reporting of experimental data simply as ¹H/¹C on a linear scale would have minimized the decreases in this ratio while emphasizing any increases.

**Sodium Dodecyl Sulfate Gel Electrophoresis**—Sodium dodecyl sulfate-polyacrylamide gels (85 x 5 mm) contained a final concentration of 10% (w/v) acrylamide (Eastman), 0.1% sodium dodecyl sulfate (Mann, ultrapure), 0.06% ammonium persulfate (Fisher), 0.05% (w/v) N,N,N',N'-tetramethylethylenediamine, and 0.1 M sodium phosphate, pH 7.2. During polymerization, each gel was overlaid with a solution of 0.1% sodium dodecyl sulfate, 0.06% ammonium persulfate, and 0.05% N,N,N',N'-tetramethylethylenediamine. When polymerization was completed (30 min) the gels were rinsed and overlaid with 0.1% sodium dodecyl sulfate/0.1 M sodium phosphate, pH 7.2 containing 0.1% (w/v) thiglycolic acid, and allowed to stand for at least 60 min.

Protein samples (10 to 200 μg) in 0.01 M sodium phosphate, pH 7.2, were prepared for electrophoresis by incubating for 5 min at 10°C with 1% sodium dodecyl sulfate and 0.2% β-mercaptoethanol. Glycerol (10% v/v) and a tracking dye, pyronin Y (0.1 μg in 5 to 10 μl), were added to the sample prior to application to the gel surface. The protein sample was then overlaid with the electrophoresis buffer consisting of 0.1% sodium dodecyl sulfate/0.2% thiglycolic acid/0.1 M sodium phosphate, pH 7.2. Electrophoresis was conducted at room temperature with an applied current of 8 mA/tube. After the tracking dye had migrated about 8 cm, the gels were removed and the protein fixed and stained overnight in a solution of 25% isopropyl alcohol, 10% acetic acid, and 0.025% Coomassie blue (21).

Gels were destained by repeated washing with 10% isopropyl alcohol/10% acetic acid. The distribution of ³H and ¹C counts in the destained polyacrylamide gels was analyzed following manual slicing or fractionation using a Gilson gel fractionator. Excess water associated with the gels fractionated mechanically was removed by evaporation at 50°C. Each gel fraction was then completely digested by incubation overnight at 50°C with 0.2 ml of 30% H₂O₂ (Fisher) in sealed glass scintillation vials. The gel fractions were then cooled to 4°C before the addition of Aquasol. Gel digestion by the above procedure shows very little, if any, preferential loss of ¹H or ¹C label as judged from a comparison of the isotope ratio for the total counts in the gel to that of the applied sample. If any difference was observed then the isotope

\[ \text{O.D. at 660 nm} \]

FIG. 1. The specific radioactive labeling of proteins synthesized during induction of bacterial bioluminescence. A leucine auxotroph was grown in the presence of [4,5-H]leucine during the bioluminescence lag period, and then transferred into fresh medium containing unlabeled leucine. After the start of induction, [¹C]leucine was added as indicated and the cells grown until maximum luminescence. The "in vivo" bioluminescence in light units (LU/ml) is plotted against the optical density at 660 nm. Additional details are given under "Experimental Procedure."
FIG. 2 (left). Sodium dodecyl sulfate electrophoresis of the cell-free soluble extract. The gel was fractionated mechanically into 1-mm fractions and the isotope ratio of each determined as described under "Experimental Procedure." The isotope ratios are presented as an increasing 3H/14C (> 1) above the abscissa and as an increasing 14C/3H (> 1) below the abscissa.

ratios of the gel fractions were multiplied by an appropriate correction factor such that the ratio of the total 3H/14C counts in each gel was equal to that of the applied sample. Repeated analysis of the same sample showed that this procedure gave reproducible results. All samples were counted for at least 30 min and those fractions which were low in radioactivity were counted for longer periods. Any isotope ratio with a coefficient of variation greater than 20% due to low counts was rejected. The isotope ratios have been plotted (under "Results") as a function of electrophoretic mobility (RF), where RF is the distance of the gel slice from the top of the gel divided by the distance migrated by pyronin Y. Since no significant counts or protein staining were detected on any gel with an electrophoretic mobility greater than 0.8, gel photographs and experimental data in this region have not been presented.

Criteria for Defining a Polypeptide Whose Synthesis Is Induced or Repressed—The detection of an altered isotope ratio in a particular sample can reflect either a random deviation from the "average" isotope ratio\(^1\) (1.0) or indicates the existence of a polypeptide which is synthesized specifically or predominantly during the lag period (3H/14C > 1) or the bioluminescence induction period (14C/3H > 1).

We have defined those polypeptides whose isotope ratio is observed reproducibly with a value greater than 2.5 as either induced or repressed during the development of bioluminescence. This criterion was used so that it would be highly improbable, even upon analysis of a large number of samples (≈1000), that a noninduced polypeptide would be observed with such a large deviation due to random variation\(^1\) from the "average" isotope ratio.

\(^1\)A standard deviation \(\pm 0.34\) was calculated for the isotope ratios of the noninduced polypeptides assuming that the isotope ratios of these

FIG. 3 (right). Sodium dodecyl sulfate gel electrophoresis of the 40 to 75% ammonium sulfate precipitate of the 0.35 M eluate. A plot of the isotope ratio against electrophoretic mobility (RF) was obtained by fractionating the gel mechanically into 1-mm slices and determining the isotope ratio of each slice as described under "Experimental Procedure."

RESULTS

In order to determine whether the induction of bacterial luciferase activity is regulated at the level of gene transcription-translation or by post-translational processes, a leucine auxotroph of *B. harveyi* was incubated in the presence of \(\left[4,5-^3\text{H}\right]\)leucine during the luminescence lag period in early cell growth \(\left(A_{660} < 0.2\right)\) and \(\left[^1\text{C}\right]\)leucine during the induction of bioluminescence \(\left(A_{660} \approx 0.6 \text{ to } 1.2\right)\) (see Fig. 1). The soluble proteins were then extracted from the doubly labeled cells and the isotope ratio of the component polypeptides analyzed after sodium dodecyl sulfate gel electrophoresis (Fig. 2). Since the majority of proteins should be synthesized in concert with growth, very little change in isotope ratio is expected. In fact, the observed change in isotope ratio between 1.0 and 1.4 simply represents the normal experimental variation. It is therefore clear that a more extensive fractionation is necessary if polypeptides with a significantly altered isotope ratio are to be detected.

polypeptides have a Poisson distribution centered on the average isotope ratio of 1.0 and using Chauvenet’s criterion (22) to reject data not representative of this distribution (i.e., indicative of a repressed or induced polypeptide). The chance of observing a noninduced polypeptide with an isotope ratio greater than 2.5 (= 4 S.D., from the average) is thus less than 0.001% for a single reading and less than 1% on analysis of 1000 samples.
Since our initial goal was to determine whether luciferase is synthesized specifically during the induction period, the soluble proteins were fractionated according to the purification procedure for bacterial luciferase (23) by adsorption onto DEAE-cellulose and then stepwise elution with 0.10 M, 0.15 M, and 0.35 M phosphate buffers (Table I). This initial step did not result in a change in the isotope ratio of the four eluted fractions since each still contains a large variety of proteins, the majority of which are synthesized in concert with growth. In addition, sodium dodecyl sulfate gel electrophoresis of each eluate did not produce sufficient resolution so that a significant change in the isotope ratio could be observed.

The activities of two enzymes, luciferase and FMN reductase, were followed during the separation of the soluble proteins (Table I). Although the initial fractionation of DEAE-cellulose resulted in the separation of luciferase and FMN reductase into the 0.35 and 0.15 M eluates, respectively, both activities can still be detected to various degrees in all four fractions. This result shows that although the eluates (Table I) are enriched in certain proteins, each may contain at least a small amount of most of the proteins originally present. Consequently, the same polypeptides with altered isotope ratios might be detected in different eluates depending on their relative concentration and how well they can be resolved from the noninduced polypeptides.

Our initial studies concentrated on the further resolution of the 0.35 M eluate which contains luciferase with the highest specific activity. Ammonium sulfate fractionation of the 0.35 M eluate resulted in the majority of the radioactivity and luciferase activity being recovered in the 40 to 75% ammonium sulfate precipitate. Separation of this fraction by sodium dodecyl sulfate gel electrophoresis (Fig. 3) and analysis of the isotope ratios of the gel fractions shows that a large increase in $^{14}\text{C}/^{3}\text{H}$ occurs only in the region of the luciferase subunits ($R_{c}=0.25$ and 0.29). The two remaining ammonium sulfate fractions (0 to 40% precipitate, 75% supernatant) were also examined; however no additional polypeptides with altered isotope ratios were detected even after sodium dodecyl sulfate gel electrophoresis. Since these latter fractions were low in radioactivity and protein content, no further analysis was done.

The proteins of the 40 to 75% ammonium sulfate precipitate were further resolved by DEAE-Sephadex chromatography as shown in Fig. 4. Luciferase activity is associated with a peak of radioactivity (tube 61) that corresponds directly to a large increase in $^{14}\text{C}/^{3}\text{H}$. No significant change in isotope ratio is observed at any other position on this column. The fractions were combined into six pools as indicated on the abscissa and further resolved by sodium dodecyl sulfate gel electrophoresis. The pool of luciferase activity (1) gives only two major polypeptide bands on electrophoresis (Fig. 5) corresponding in mobility to the luciferase subunits ($R_{c}=0.25, R_{c} = 0.29$). The $^{14}\text{C}/^{3}\text{H}$ increases dramatically to $\frac{1}{3}$ for both polypeptide chains of luciferase proving that the lag in the development of bioluminescence is due to the repression of synthesis of the $\alpha$ and $\beta$ subunits of luciferase. Furthermore, if the synthesis of other proteins is induced along with luciferase during the development of bacterial bioluminescence, then it should be possible to detect these proteins as well, providing sufficient resolution from noninduced polypeptides can be attained.

Sodium dodecyl sulfate gel electrophoresis of the five remaining pools (Fig. 4) indeed indicates that one other pool (II) contains induced polypeptides (Fig. 5). Three polypeptides with mobilities of 0.09, 0.15, and 0.32, show a substantial increase in $^{14}\text{C}/^{3}\text{H}$, ranging from 2.5 to 3.6. Although the change in isotope ratio is less than that previously observed for the luciferase subunits ($^{14}\text{C}/^{3}\text{H} = 7$), the incomplete resolution from the noninduced polypeptides of this pool could

![Fig. 4. DEAE-Sephadex chromatography of the 0.35 M eluate after precipitation with 40 to 75% ammonium sulfate. The ammonium sulfate precipitate was dissolved in 2 ml of 0.25 M phosphate/10$^{-4}$ M dithiothreitol, and then dialyzed exhaustively against the same buffer. The sample was applied to a DEAE-Sephadex A-50 column (2.5 x 30 cm) pre-equilibrated with 0.35 M phosphate/10$^{-4}$ M dithiothreitol, pH 7.0, and then eluted with 700 ml of the same buffer at 4°C. Fractions (7 ml) were assayed for $^{14}\text{C}$, $^{3}\text{H}$, and luciferase activity. A plot is given of the isotope ratio versus fraction number. The fractions were combined into six pools as indicated on the abscissa and then further resolved by sodium dodecyl sulfate gel electrophoresis. The shaded areas indicate pooled fractions in which polypeptides with altered isotope ratios were detected after electrophoresis. LU, light units.]
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FIG. 5. Sodium dodecyl sulfate gel electrophoresis of Pools I and II from the DEAE-Sephadex chromatography in Fig. 4. A plot of the isotope ratio versus electrophoretic mobility ($R_f$) is given.

Fig. 6. DEAE-Sephadex chromatography of the 0.15 M eluate. The proteins in the eluate were precipitated$^a$ (>99% cpm) by addition of ammonium sulfate to 85% saturation, and then redissolved by dialysis into 0.10 M phosphate/10$^{-1}$ M dithiothreitol, pH 7.0 at 4°. The sample (15 ml) was applied to a DEAE-Sephadex A-50 column (2.5 x 40 cm) pre-equilibrated with the same buffer. Fractions (7.0 ml) were eluted at 4° with a linear gradient of 350 ml of 0.10 M phosphate, 10$^{-1}$ M dithiothreitol, pH 7.0, versus 350 ml of 0.60 M phosphate, 10$^{-1}$ M dithiothreitol, pH 7.0. The fractions were then assayed for FMN reductase activity, $^3$H, and $^{14}$C, and the isotope ratio calculated. The easily account for this result. Co-electrophoresis of Pool II with pure luciferase also showed that these induced polypeptides have mobilities distinct from those of the subunits of luciferase.

Since the above results showed that extensive fractionation of proteins is a prerequisite for the detection of induced polypeptides, subsequent studies concentrated on the further resolution of the three remaining eluates from DEAE-cellulose (Table I). The filtrate, 0.10 M eluate, and 0.15 M eluate proteins were precipitated$^a$ by the addition of ammonium sulfate to 85% saturation and then chromatographed on DEAE-Sephadex.

Fig. 6 gives the DEAE-Sephadex chromatography profile of the 0.15 M eluate which contains the majority of the FMN reductase activity. The isotope ratio changes significantly only in the region of Fraction 78. However, examination of the pooled fractions from the column after sodium dodecyl sulfate gel electrophoresis revealed the presence of induced polypeptides in three of the seven pools (Fig. 7; I, II, and IV). Pool I, which contains luciferase activity, has two induced polypeptides with mobilities ($R_f$) of 0.25 and 0.29, corresponding to the $\alpha$ and $\beta$ subunits of luciferase. These results reconfirm the previous findings for luciferase in Pool I from the 0.35 M eluate (see Figs. 4 and 5) and consequently this pool is designated in an identical manner. Similarly, Pool II of this column (Fig. 7) contains the same induced polypeptides that were detected previously in Pool II of the 0.35 M eluate (see Fig. 5). However, Pool IV, contains an induced polypeptide with a mobility of 0.13 that has not been detected previously. Although present in small $^a$Less than 1% of the radioactivity remained in the supernatant after addition of ammonium sulfate to 85% saturation.
quantities, it has been well resolved by gel electrophoresis from any noninduced proteins as indicated by the large change in isotope ratio (\(^{14}\text{C}/^{3}\text{H} = 6\)).

In contrast, sodium dodecyl sulfate gel electrophoresis of Pool III containing the majority of the FMN reductase activity gave no indication of any polypeptides with an increase in \(^{14}\text{C}/^{3}\text{H}\) (Fig. 8). Although this enzyme was not purified further, the apparent absence of induced polypeptides in this pool would indicate that the FMN reductase is probably not specifically synthesized during the induction of bioluminescence. However, this pool does contain a component with a mobility (\(R_f\)) of 0.48 that has a large increase in \(^{3}\text{H}/^{14}\text{C}\). This was the only polypeptide detected during the analysis of the bacterial proteins which is apparently synthesized specifically during the luminescence lag and repressed later in growth during the induction of bioluminescence.

The fractionation of the 0.10 M eluate (Table I) by DEAE-Sephadex chromatography is given in Fig. 9. Although no significant change can be observed in the isotope ratio for any fraction on this column, one induced polypeptide could be detected in two adjacent pools (Fig. 9, IVa and IVb) after sodium dodecyl sulfate gel electrophoresis (Fig. 10). This polypeptide has the same mobility (\(R_f = 0.14\)) as the induced polypeptide detected previously in Pool IV of the 0.15 M eluate (see Figs. 6 and 7). The results given in Fig. 10 for Pools IVa and IVb also show that the isotope ratio is independent of the staining intensity of the peptide band.

The Filtrate (Table I) was the last fraction to be resolved by DEAE-Sephadex chromatography (Fig. 11). No significant change in isotope ratio can be observed at any position on the column. The fractions were combined into 10 pools as indicated on the abscissa and analyzed further following sodium dodecyl sulfate gel electrophoresis. Some of the induced polypeptides previously detected were observed in the corresponding pools (e.g. I and II). However, the change in isotope ratio was less than previously observed due to the presence of a larger proportion of noninduced protein that co-electrophoreses with the induced polypeptides. Only one induced polypeptide...
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Fig. 9. DEAE-Sephadex chromatography of the 0.10 M eluate from Table I. The 0.10 M eluate proteins were precipitated by the addition of ammonium sulfate to 85% saturation, and then redissolved by dialysis against 0.05 M phosphate/10^{-4} M dithiothreitol, pH 7.0. The sample (11 ml) was then applied to a DEAE-Sephadex A-50 column (2.6 x 40 cm) pre-equilibrated with 0.05 M phosphate/10^{-4} M dithiothreitol, pH 7.0, and eluted at 4° with a linear gradient of 350 ml of 0.05 M phosphate/10^{-4} M dithiothreitol, pH 7.0, versus 350 ml of 0.50 M phosphate/10^{-4} M dithiothreitol, pH 7.0. The fractions were then analyzed for ^14C and ^1H, and the isotope ratio calculated as described under "Experimental Procedure." The fractions were then combined into nine pools as indicated on the abscissa and further resolved by sodium dodecyl sulfate gel electrophoresis. The shaded areas indicate the pooled fractions in which a polypeptide with an altered isotope ratio was detected after electrophoresis.

not observed previously was detected following resolution of the filtrate. Sodium dodecyl sulfate gel electrophoresis of Pool V (Fig. 12) shows the presence of a polypeptide with an altered isotope ratio (%14C/%3H = 4) having a mobility (R_f) of 0.29.

A summary of the polypeptides that have been detected with substantially altered isotope ratios is given in Table II. The experiments have shown that the synthesis of both subunits of luciferase (Pool I) is induced during the development of bacterial bioluminescence. Furthermore, only a limited number of additional polypeptides (Pools II, IV, and V) have been detected which are synthesized specifically in this same period as well as a single polypeptide (Pool III) that is synthesized specifically during the bioluminescent lag period.

DISCUSSION

By labeling the cells of B. harveyi with [4,5-^3H]leucine during the luminescence lag period and [^14C]leucine during luminescence induction, it has been possible to show that a differential rate of synthesis occurs for a limited number of specific polypeptides during exponential growth of the bacteria. This conclusion was based on the premise that peptides isolated with a high %^3H/%^14C (>2.5) were synthesized specifically or predominately during the bioluminescence induction period whereas peptides with a high %^3H/%^14C (>2.5) were synthesized predominantly during the bioluminescence lag period. Extensive fractionation of the proteins was essential before polypeptides with significantly altered isotope ratios could be distinguished from the vast majority of proteins having isotope ratios ranging from 1.0 to 1.4.

A total of seven polypeptides with markedly increased %^14C/%^3H ratios were detected during the course of this investigation indicating that their synthesis is induced during the development of bioluminescence (Table II). Furthermore, these changes in isotope ratio are not the result of degradation of the older or ^3H-labeled protein since introduction of the labels (^3H and ^14C) into two separate cultures, harvesting both cultures immediately after their respective growth periods, and mixing the two cultures, resulted in the identification of the same polypeptides (data not given) as listed in Table II. Two of these polypeptides correspond to the α and β subunits of
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1.5 \% / \text{pc} 

FIG. 11. DEAE-Sephadex chromatography of the filtrate (see Table I). The filtrate proteins were precipitated by the addition of ammonium sulfate to 85 \% saturation and then dissolved by dialysis against 0.01 M phosphate/0.1 M dithiothreitol, pH 7.0. The sample (35 ml) was applied to a DEAE-Sephadex A-50 column (2.5 x 60 cm) pre-equilibrated with 0.01 M phosphate/0.1 M dithiothreitol, pH 7.0, and then eluted with a 700-ml linear gradient from 0.01 to 0.20 M phosphate/0.1 M dithiothreitol, pH 7.0, followed by a 700-ml linear gradient of 0.20 to 0.60 M phosphate/0.1 M dithiothreitol, pH 7.0. Fractions of 7 ml were collected and analyzed for \(^{3}H\) and \(^{14}C\). The isotope ratios were calculated as described under “Experimental Procedure.” The fractions were combined into 10 pools as indicated on the abscissa. The shaded areas indicate the pooled fractions in which a polypeptide with a significantly altered isotope ratio was detected after electrophoresis.

Luciferase proving that the synthesis of both polypeptide chains is induced during the development of bioluminescence and is coordinately controlled. The detection of five additional polypeptides with significantly increased \(^{14}C/\(^{3}H\) ratios provides direct evidence that proteins other than luciferase are synthesized specifically in the bioluminescence induction period.

Although it can not be definitely concluded that these proteins are involved directly in the bioluminescence system, Cline and Hastings (16) have provided evidence from the study of temperature-sensitive bioluminescence mutants that there are at least two enzymes whose activities may be induced and involved in the biosynthesis of the aldehyde substrate for the luciferase reaction. In addition, one (or more) of the above polypeptides could be involved in synthesis of FMNH\(_2\) for the luminescent reaction. It should be noted however that sodium dodecyl sulfate gel electrophoresis of the major DEAE-Sephadex fraction containing FMN reductase activity (Fig. 8) provided no evidence for the presence of any induced polypeptides. This result is in agreement with evidence showing that FMN reductase activity parallels growth of the bioluminescent bacteria (24). However, since this enzyme was not fully purified it is still possible that its component polypeptides may not have been sufficiently resolved to permit detection of a change in isotope ratio.

Recent evidence in this laboratory has also suggested a relationship of one of the induced polypeptides to the bioluminescent system. An enzyme involved in the oxidation of long chain aliphatic aldehydes through a nonluminescent pathway has been found which chromatographs on DEAE-Sephadex and migrates on sodium dodecyl sulfate gels in a similar manner to the induced polypeptide observed in Pool IV (see “Results”). In addition, the activity of this enzyme is induced at the same time as luciferase. Consequently, it would seem probable that at least five of the seven polypeptides are directly involved in the bioluminescent system; two of these have been shown to be the subunits of luciferase, one may be

**Table II**

| Pool designation | No. of peptides with altered isotope ratios | Morbilities on sodium dodecyl sulfate gels (RF) | Isotope ratio | Functional activity |
|------------------|------------------------------------------|-----------------------------------------------|---------------|---------------------|
| I                | 2                                        | 0.25, 0.29                                    | 7             | Luciferase          |
| II               | 3a                                       | 0.09, 0.15, 0.32                              | 3             | Unknown            |
| III              | 1                                        | 0.48                                          | 3             | Unknown            |
| IV               | 1                                        | 0.14                                          | 6             | Unknown            |
| V                | 1                                        | 0.29                                          | 4             | Unknown            |

*An additional induced polypeptide with an \(R_f\) of 0.06 may also be present in Pool II (see Fig. 5).

Recent experiments have shown that a long chain aldehyde dehydrogenase is present in this fraction whose activity increases upon induction of bioluminescence.

Recent evidence in this laboratory has also suggested a relationship of one of the induced polypeptides to the bioluminescent system. An enzyme involved in the oxidation of long chain aliphatic aldehydes through a nonluminescent pathway has been found which chromatographs on DEAE-Sephadex and migrates on sodium dodecyl sulfate gels in a similar manner to the induced polypeptide observed in Pool IV (see “Results”). In addition, the activity of this enzyme is induced at the same time as luciferase. Consequently, it would seem probable that at least five of the seven polypeptides are directly involved in the bioluminescent system; two of these have been shown to be the subunits of luciferase, one may be
involved in the oxidation of aldehyde, and at least two more should be involved in aldehyde synthesis.

An additional and perhaps surprising finding was the detection of a single polypeptide synthesized in the bioluminescence lag period and apparently repressed during the induction of bioluminescence (Pool III, Table II). It is difficult to propose a potential function for this polypeptide although it perhaps could be involved in the repression of synthesis of the enzymes involved in the bioluminescent system. Only by further work investigating the possible functions of this polypeptide as well as the induced polypeptides will it be possible to directly relate them to the luminescent system. The detection of a limited number of polypeptides with altered isotope ratios has, however, provided the incentive to further investigate the possible functions of these proteins. It is hoped that the elucidation of the function of these proteins will provide greater understanding of the regulation of the development and induction of bioluminescence.

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