JOURNAL OF BACTERIOLOGY, Nov. 1967, p. 1638-1647

Vol. 94, No. 5
Printed in U.S.A.

Inducible Synthesis of Bacterial Luciferase: Specificity and Kinetics of Induction

JOHN J. COFFEY[1]

Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218

Received for publication 4 August 1967

A nitrate-utilizing strain of marine bacteria was isolated in which luciferase was inducible by L-arginine. The induction was highly specific; structural analogues of arginine were ineffective, as were other natural amino acids. Several metabolites of arginine acted as weak inducers but did not affect the rate of induction in limiting concentrations of arginine. Hence, these compounds probably exerted a sparing effect on intracellular arginine. The kinetics of induction were followed by measurement of light output from intact cells, under conditions in which in vivo light output was determined only by the luciferase level. No enzyme appeared in the cells for 12 min after the addition of inducer, although the primary structure of the luciferase molecule was apparently synthesized within 2 to 4 min. It is proposed that during the remaining 8 to 10 min a precursor of luciferase was converted to active enzyme. The differential rate of synthesis rose during the first 5 min of induction, apparently as messenger ribonucleic acid accumulates in the cells; thereafter it remained constant for approximately 100 min.

Specific amino acid requirements for bacterial luminescence have been demonstrated by the studies of McElroy and Farghaly (12) on auxotrophic mutants of *Achromobacter fischeri*. Although the replacement of most metabolic deficiencies produces a parallel restoration of luminescence and growth, amino acids must be present at concentrations sufficient for fully normal growth before any appreciable increase in luminescence is seen. These requirements are also evident when amino acid deficiencies are produced by addition of amino acid analogues (3) or growth at elevated temperatures (1).

Recently, a mutant strain of *A. fischeri* has been isolated which, unlike the wild type, can grow on nitrate as the sole nitrogen source. Under these growth conditions, the cells do not luminesce significantly. When the nitrate-dark (ND) cells are grown in the presence of peptone, normal luminescence is restored. Although the drain on the reducing power of the cells might be expected (11, 15) to inhibit luciferase activity by depleting the supply of reduced flavin mononucleotide ($FMNH_2$), preliminary experiments showed that the low luminescence could be attributed to greatly reduced luciferase levels.

P. R. Elliott (*unpublished data*) demonstrated that the effect of peptone on ND luminescence was due to L-arginine. The purpose of this work is to show that arginine is a specific inducer of luciferase synthesis in ND cells, and to examine the kinetics of the inductive process by direct recording of enzyme levels in living cultures.

MATERIALS AND METHODS

Bacteria. The bacterial strain used in this study was isolated in our laboratory. The growth medium ("nitrate minimal") was modified from the minimal medium of Farghaly (4) by substituting 0.4 g of $NaNO_3$ per liter for ammonium phosphate. Liquid cultures were grown on a reciprocal shaker at 25 C. Growth temperature had a large effect on inducibility and thus was carefully controlled. Cell density was determined in a Klett-Summerson photoelectric colorimeter with a no. 42 blue filter. These densities are proportional to viable-cell count, obtained by plating cultures on an agar medium, up to a Klett reading of 300. A density of 100 Klett units corresponds to 6.7×10^8 cells/ml. The routine procedure was to dilute a heavy overnight culture with fresh medium to a Klett reading of 40 and to allow the cells to grow to a Klett reading of 90, a period of 4 to 6 hr depending on the density of the inoculum. Samples of 10 ml of the culture were used for light measurements as described below. Bacterial density measurements used for kinetic analysis were made at 20-min intervals on the induced cultures.

Measurement of in vivo light production. The apparatus used to measure light output of growing bacterial cultures consisted of a dark chamber, maintained at 27.5 C by means of a circulating water bath, with a 1-inch phototube window in the bottom. Within the

[1] Present address: Virus Laboratory, University of California, Berkeley, Calif. 94720.

chamber was a reciprocal shaker set at 120 cycles/min, with clamps for eight 125-ml Erlenmeyer flasks, any one of which could be rotated into position over the phototube. Stray light from the other cultures in the chamber was eliminated by painting the sides of the flasks black, leaving only a clear window in the bottom. An RCA 1P21 photomultiplier tube was used with a variable high voltage source, DC amplifier, and recording voltmeter. Intensities were measured at a constant amplification factor of 100, and the sensitivity of the instrument was varied by changing the phototube voltage. Light intensities are reported as output millivolts corrected to a phototube voltage of 1,000 v, calibrating the response at other voltages with a constant light source. Since the continuous records included frequent scale and sample changes, kinetic plots were constructed by taking readings from the recorder output at convenient time intervals.

Luciferase assays. Cells from 30 ml of culture were harvested and lysed in 1.5 ml of glass-distilled water, and the lysate was centrifuged at 125,000 × *g* for 30 min. Of this extract, 0.01 to 0.1 ml was used for each assay, together with 0.3 ml of a saturated aqueous solution of dodecanal and 1.0 ml of 0.1 M sodium phosphate buffer (*p*H 7.0). FMN was reduced by passing a stream of hydrogen through a 10^{-4} M solution in the presence of a small amount of platinized asbestos. The reaction was initiated by injecting 0.2 ml of the $FMNH_2$ solution into an assay tube positioned in front of an RCA 1P21 photomultiplier tube. It should be noted that light intensities measured in this apparatus are not directly comparable to those measured in vivo, since the geometry of the two systems is quite different. One unit of enzyme produces a peak light intensity of 1 mv with a phototube high voltage of 600 v, under the above conditions at room temperature.

Chromatography of ^{14}C-labeled cell extracts. A 10-ml culture of cells was incubated for 10 min with 0.27 μmoles of ^{14}C-arginine (37 μc/μmole). The cells were collected by centrifugation, and 1 ml of ice-cold trichloroacetic acid was added. After 15 min, the acid-insoluble material was removed by membrane filtration (Millipore Corp., Bedford, Mass.). The acid supernatant fluid was extracted five times with 10-ml portions of ether to remove the trichloroacetic acid, and the aqueous phase was neutralized with a small amount of dilute NaOH. The supernatant fluid was then applied to a 0.8 × 11 cm column of Bio-Rad AG 11A8 ion retardation resin and eluted with glass-distilled water, and 1-ml fractions were collected. Tubes comprising the single radioactive peak were pooled and evaporated to dryness at 40 C under vacuum. The residue was redissolved in 0.05 ml of water with 50 μg of unlabeled arginine and applied to Whatman 3 MM filter paper. The chromatogram was developed with 88% aqueous phenol and air-dried, and the residual phenol was removed by washing with ether. Radioactivity of the paper strip was measured in a Packard radiochromatogram scanner, and amino acids were located by dipping in 0.25% ninhydrin in acetone.

RESULTS

The effect of adding 4.8 × 10^{-4} M L-arginine to a culture of ND cells at a density of 90 Klett units is shown in Fig. 1. After a 12-min lag period (which appears more clearly in later figures), luminescence rose rapidly, reaching a level of 3,300 mv after 2 hr, and declining slowly thereafter. At cell densities below 90 Klett units, the increase was more difficult to observe, though it occurred as early as luminescence was detectable in the untreated cells (50 to 60 Klett units). Beyond a Klett reading of 175 to 200, the cells showed little or no response to arginine. Thus, all subsequent experiments were performed on cells at a Klett density of 90, the heaviest cultures which would remain responsive to arginine for a few hours. Studies on cell growth have shown no change in the division time of ND cells upon addition of arginine.

Figure 1 shows a highly inducible culture; many cultures were less so. Despite every effort to grow and induce the cells under controlled conditions, the light levels observed were highly variable from day to day, with a range of approximately a factor of 10. Thus, it was necessary to carry out almost all experiments on parallel samples of a single culture, which never varied by more than a few per cent. All experiments were repeated on cultures of widely varying inducibility; time dependence and relative effects of inducers and inhibitors on luminescence were the same in every case.

As the cell-free enzyme assays of Fig. 1 indicate, the increase in luminescence may be directly ascribed to an increase in bacterial luciferase. This was clearly due to de novo protein synthesis; puromycin, chloramphenicol, and *p*-fluorophenylalanine all prevented the appearance of enzyme when added together with arginine

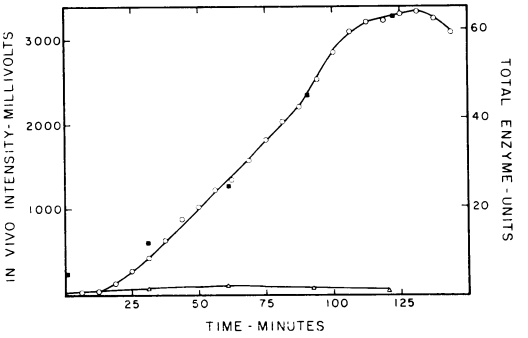

FIG. 1. *Induction of luciferase by 4.8 × 10^{-4} M arginine, added at zero-time. Cells were grown on nitrate-minimal medium to a Klett reading of 90. Under these conditions, the doubling time is 115 min. Symbols: ○, in vivo light output; ■, total enzyme, in vitro assay; and △, uninduced control, in vivo.*

TABLE 1. *Effect of inhibitors on luciferase induction by 4.8 × 10⁻⁴ M arginine*[a]

Inhibitor	Concn (μg/ml)	Δmv at 60 min
None		83
Puromycin	5	−2
Chloramphenicol	5	−2
p-Fluorophenylalanine	100	−2
5-Fluorouracil	20	−1

[a] Conditions as described in the legend to Fig. 1, with inhibitors added at zero-time.

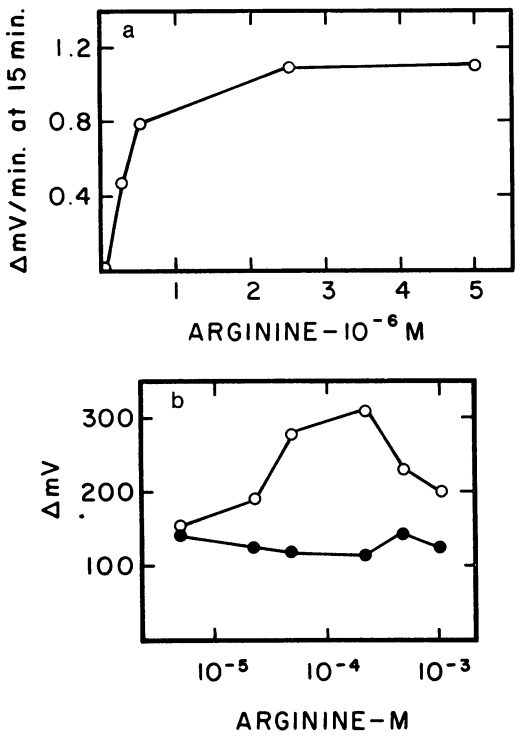

FIG. 2. *(a) Dependence of initial rate of induction on arginine concentration. Rate of induction determined as Δmv/min at 15 min. (b) Dependence of extent of induction on arginine concentration. Symbols: ●, increase in intensity at 30 min; ○, Increase in intensity at 75 min.*

(Table 1). The effect of *p*-fluorophenylalanine and similar effects of 7-azatryptophan, ethionine, 2-methylalanine, and ε-chloroacetyllysine were reversible by the appropriate normal amino acids. 5-Fluorouracil was also a potent inhibitor of the stimulation of luciferase synthesis (Table 1). Apparently, then, messenger RNA as well as protein was synthesized in response to arginine, and the stimulation of luciferase synthesis may be described as a true enzyme induction.

Extremely low concentrations of arginine will initiate the induction of bacterial luciferase (Fig. 2), with an effect clearly visible at 0.5 × 10⁻⁶ M, and a maximal initial rate reached at 2.5 × 10⁻⁶ M. It is probable that these are not the true concentrations sufficient to induce; most likely, the cells are capable of concentrating arginine, maintaining a high internal concentration, and clearing the medium when only small amounts are added.

One of the complications in the analysis of this inducible system is the fact that arginine is both incorporated into protein and broken down in a fairly complex metabolic pathway. Thus, the internal level of inducer cannot be assumed to be constant. One consequence of this fact is shown in Fig. 2b. Although at very high concentrations of arginine the initial rate of induction, as measured by the increase at 30 min, was constant, a concentration of 5 × 10⁻⁶ M did not sustain induction beyond that point, and successively higher concentrations (at least up to 2.5 × 10⁻⁴ M) maintained the synthesis for longer times, as seen in the 75-min points (Fig. 2).

Of the natural amino acids, only arginine showed an appreciable inductive effect; except for aspartic acid and proline (to be discussed below), no amino acid showed more than 1% of the activity of arginine. Structural analogues of L-arginine, including variants in the optical configuration, the guanido group, the α-amino and carboxyl groups, and the nature and length of

TABLE 2. *Effects of analogues and metabolites of L-arginine on luciferase synthesis*

Compound	Concn	Induction
	M	%
L-Arginine	4.8 × 10⁻⁴	100
D-Arginine	4.8 × 10⁻⁴	0.0
ω-Nitro-L-arginine	4.9 × 10⁻⁴	−1.4
Agmatine	4.4 × 10⁻⁴	0.0
Argininic acid	5.8 × 10⁻⁴	0.0
L-Canavanine	3.7 × 10⁻⁴	−10.6
L-2-Amino-3-guanidopropionic acid	5.5 × 10⁻⁴	0.0
L-α-Amino-γ-guanidobutyric acid	4.7 × 10⁻⁴	0.0
Urea	5.0 × 10⁻⁴	−0.2
NH_4Cl	5.0 × 10⁻⁴	−0.3
L-Ornithine	5.0 × 10⁻⁴	11.4
Carbamyl phosphate	5.0 × 10⁻⁴	−2.8
L-Citrulline	5.0 × 10⁻⁴	37.3
L-Aspartic acid	5.0-×-10⁻⁴	2.5
L-Argininosuccinic acid	5.0 × 10⁻⁴	26.5
Fumaric acid	5.0-×-10⁻⁴	−0.1
L-Glutamic acid	5.0 × 10⁻⁴	−1.6
L-Proline	5.0-×-10⁻⁴	6.1
Putrescine	5.0 × 10⁻⁴	−1.4

the carbon chain were assayed for inducing activity, and none was observed (Table 2).

Particularly in view of the fact that no induction seemed to take place for 12 min after the addition of arginine, the possibility exists that the true intracellular inducer was a metabolic produce of arginine. The acidic amino acids, proline, fumarate, carbamyl phosphate, and the urea cycle intermediates were examined for inducing activity. None was a better inducer than arginine (Table 2); indeed, several did not induce at all. The lack of induction by ammonium ion was of particular interest, since cells grown in the minimal medium of Farghaly (8) produced measurably more enzyme than those grown on the nitrate minimal medium. Nonetheless, no concentration of ammonium chloride between 1.8×10^{-4} M and 25×10^{-4} M stimulated any increase in luciferase over a 60-min period.

Amino acids other than arginine could, however, affect induction in several ways. Glutamate and proline might stimulate induction at low arginine levels either by generating arginine within the cell or by reducing the metabolic utilization of that which is added to the medium. On the other hand, methionine and lysine, which seem to stimulate luminescence in *A. fischeri* (12), might well show an independent effect on luciferase synthesis. The effects of these amino acids, together with that of leucine, which should show no effect, were examined at concentrations of arginine from 0.5 to 2.5 μM. At 0.5 μM, effects on initial rate of induction were examined (Table 3). Even the arginine-related amino acids did not augment the initial rate of induction in limiting arginine concentrations. Thus, it is unlikely that these compounds were themselves weak inducers. The results on extent of induction in 2.5 μM arginine contrasted sharply with the rate studies. The increase in luciferase between 30 and 45 min, which was increased approximately fourfold by an additional 2.5 μmoles of arginine per liter, was doubled by the arginine-related amino acids (Table 4). There was, then, a sparing effect not apparent during the initiation of enzyme synthesis, but evident at later times.

TABLE 3. *Effects of amino acids on rate of induction by 0.5×10^{-6} M arginine*

Supplement	Concn	Δmv/min at 15 min
	M	
None		.20
Proline	4.4×10^{-6}	.16
Glutamic acid	3.4×10^{-6}	.12
Methionine	3.4×10^{-6}	.04
Lysine	2.7×10^{-6}	.12
Leucine	3.8×-10^{-6}	.20

TABLE 4. *Effect of amino acids on extent of induction by 2.5×10^{-6} M arginine*

Supplement	Concn	$mv_{45} - mv_{30}$[a]
	M	
None		100
Arginine	2.5×10^{-6}	384
Proline	4.4×10^{-6}	225
Glutamic acid	3.4×10^{-6}	225
Methionine	3.4×10^{-6}	112
Lysine	2.7×10^{-6}	107
Leucine	3.8×10^{-6}	50

[a] Subscripts refer to time, in minutes, when assay, was made. Results are expressed as percentage of the control.

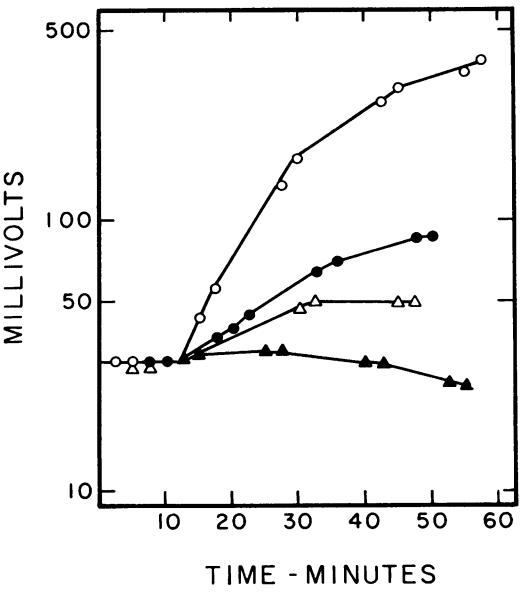

FIG. 3. *Induction by urea cycle intermediates. Symbols: ○, arginine, 4.8×10^{-4}M; ●, citrulline, 5.8×10^{-4} M; △, ornithine, 5.9×10^{-4} M; and ▲, no addition.*

That the inductive effects of arginine metabolites were due to the intracellular production or sparing of arginine is most strongly suggested by the relative kinetics of induction with arginine cycle intermediates. As Fig. 3 demonstrates, the low rates of induction by ornithine and citrulline were accompanied by an early cessation of synthesis; thus, it is most likely that a small excess of arginine was present in the cells for a brief time.

Kinetics of luciferase induction. As has been noted already, and as Fig. 3 makes particularly clear, no synthesis of luciferase occurred for some time after the addition of arginine. Unlike the

enzyme levels observed during induction, the lag period was highly reproducible, ranging from 11 to 12.5 min. Within this range, parallel samples of the same culture showed the same lag to within 30 sec. The earliest point at which it is reasonable to expect a delay in the inductive sequence of events—the entry of inducer into the cell—is ruled out as a contributing factor in this case. Figure 4a shows that 30 or 60 min of preincubation at 5 C in 4.8 $\times$ 10^{-3} M arginine had no effect on the lag. All the cultures shown in Fig. 4a were incubated at 5 C for 60 min, arginine being added during the incubation at the appropriate times. After warming the cells to 27.5 C, one of the two control cultures was induced; the lag period persisted in both cases. There was, in fact, a small but reproducible increase in the lag period with cold-treated cells. The results of a preinduction experiment also argue against the role of a permease in the lag. Preinduced cells were prepared, by use of a concentration of arginine which is exhausted soon after the beginning of luciferase synthesis. Figure 4b shows the result of reinduction; at 20 min, just as the primary induction began to slow down, a secondary addition of arginine was made, and simultaneously a culture without arginine was induced. The secondary addition of arginine to a culture already induced had the same effect, after the same lag, as the primary addition to an uninduced culture. Thus, there is no evidence for a prior inductive event; the response of the cell to arginine was the same whether or not arginine was already functioning as an inducer.

The persistence of the lag in preinduced cells shows that some metabolic response to the newly added arginine was necessary before it could function as an inducer. Even though none of the obvious metabolites of arginine was a better inducer than arginine itself, and all showed the same lag (see, for example, Fig. 3), the true inducer might be a metabolic product of arginine, accumulating in the cells over a 12-min period. However, chromatography of the acid-soluble fraction of cells labeled with ^{14}C-arginine for 10 min, as described in Materials and Methods, shows a single radioactive peak, which co-chromatographs with added unlabeled arginine. Apparently, arginine was itself the intracellular inducer, and the lag involved steps in the inductive process itself.

Incubation of the cells for 15 min with inducer and 7-azatryptophan, an inhibitor of protein synthesis (14), was followed by resuspension in medium containing inducer and a large excess of tryptophan. Although the lag was shorter in preincubated cells than in the control culture (Fig. 5), it was by no means eliminated. A large part of

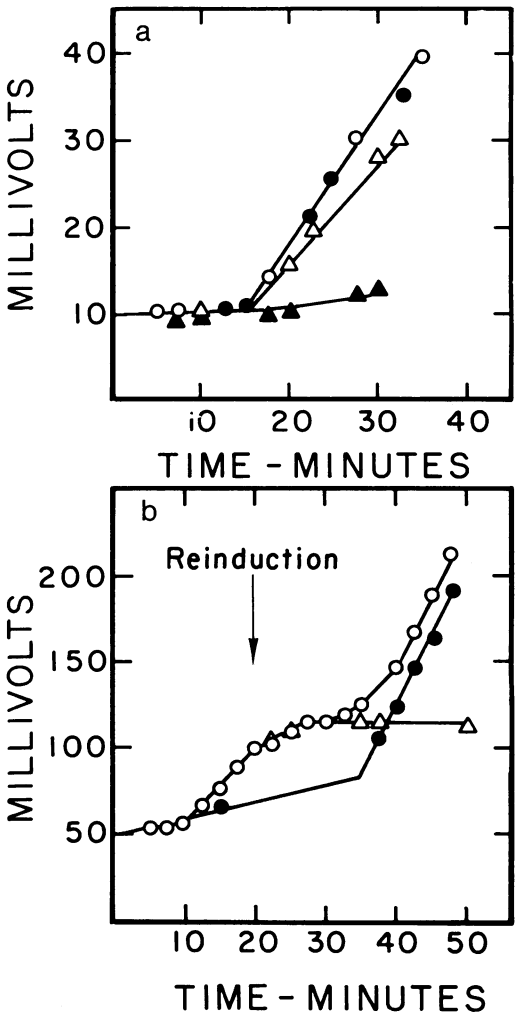

FIG. 4. *Lack of effect of preinduction on the initial kinetics of luciferase induction. (a) Effect of preincubation at 5 C with 4.8 $\times$ 10^{-3} M arginine upon the lag period for luciferase synthesis at 27.5 C. The low inducibility and increased lag are characteristic of cold-treated cells. Symbols: ○, no preincubation; ●, 30 min; △, 60 min; and ▲, uninduced. (b) Reinduction of a partially induced culture. At zero-time, 5 $\times$ 10^{-7} M arginine was added to experimental culture. At 20 min, 5 $\times$ 10^{-4} M arginine was added to experimental and control. Symbols: ○, preinduced, reinduced at 20 min; △, preinduced, not reinduced; and ●, not preinduced, induced at 20 min.*

the time during which no enzyme appeared must then be required for processes at or after the translational level. Furthermore, the rate of enzyme synthesis decreased by 65% in the presence of 0.5 μg of chloramphenicol per ml without effect on the lag; hence, most likely, the delay was in some process occurring after the completion of the

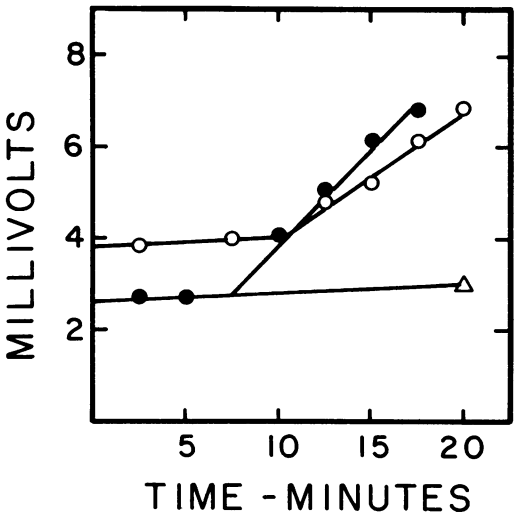

FIG. 5. *Effect of blocking protein synthesis on the lag period. Cells were incubated for 15 min with 10 μg of 7-azatryptophan per ml and 4.8 × 10⁻⁴ M arginine (●) or with no addition (○). Cultures were then centrifuged and resuspended in nitrate minimal medium containing 100 μg of tryptophan per ml and 4.8 × 10⁻⁴ M arginine. The control culture (△) remained in inducer plus 7-azatryptophan, and shows the complete blockage of induction by the analogue.*

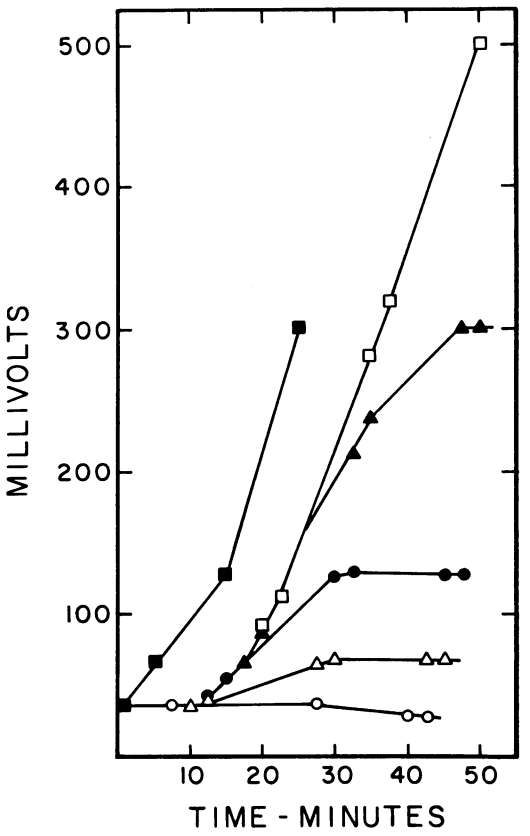

FIG. 6. *Effect of puromycin on the early induction of luciferase by arginine; 4.8 × 10⁻⁴ M arginine added at zero-time; 5 μg of puromycin per ml was added at times shown below. Symbols: ○, 0 min; △, 5 min; ●, 15 min; ▲, 25 min; □, control, no puromycin; and ■, final light level as a function of time of puromycin addition.*

primary structure of the enzyme. The most direct evidence against a role of protein synthesis in the lag is presented in Fig. 6. Addition of puromycin to induced cultures stopped the synthesis of new protein. When the final level of light output, representing the total amount of enzymes synthesized, is plotted against the time at which puromycin is added, the resulting curve parallels the induction curve directly determined from the light output of a control culture, except for a displacement on the time axis. If the puromycin curve represents the true amount of luciferase present at any given time, then there is an immediate initiation of enzyme synthesis upon the addition of inducer, and the apparent lag is due to the fact that the total luciferase activity at any time is proportional to the amount of enzyme whose primary structure was completed 20 min before.

Crucial to this interpretation is the assumption that the effect of puromycin is immediate. This appears likely from the data in Fig. 7. The incorporation of ^{14}C-arginine into protein was partially inhibited as early as 1 min after the addition of puromycin, and incorporation ceased altogether at a level equivalent to about 4 min of incorporation in the uninhibited cells. The estimate of 4 min of luciferase synthesis in the presence of puromycin, however, represents a generous upper limit; since puromycin interrupts growing peptide chains (13), it is likely that the production of peptides large enough to be enzymatically active is appreciably more sensitive than the production of those merely large enough to be acid-insoluble. In any case, all incorporation ceased after 5 to 6 min, whereas luciferase activity continued to rise for 20 min. The azatryptophan and puromycin experiments, then, suggest that 2 to 4 min of the lag were involved in the process of completing polypeptide chains, and that the remainder represented the conversion of a protein precursor, synthesized de novo, into active enzyme.

Once the lag period has been accounted for, the kinetics of enzyme synthesis are amenable to analysis. Figure 8a shows the data of Fig. 1 replotted as a function of bacterial density. Since

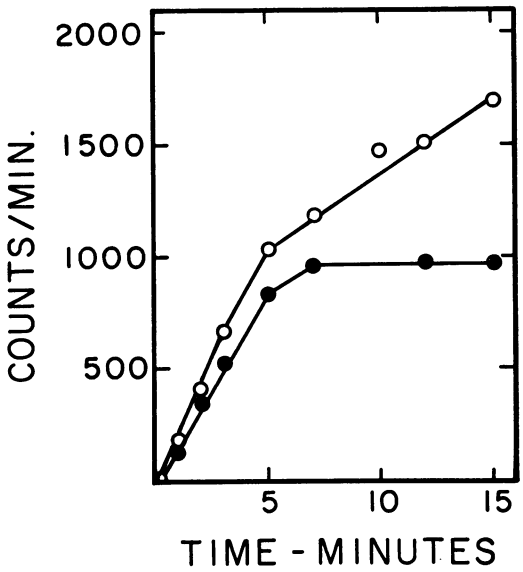

FIG. 7. *Effect of puromycin added at zero-time on acid-insoluble incorporation of ^{14}C-arginine by ND cells. At a concentration of 4.8 × 10^{-4} M, ^{14}C-arginine (0.4 μc/μmole) was added to each culture at zero-time, and 2-ml samples were poured onto membrane-filters at the times indicated. Filters were immersed in ice-cold 10% trichloroacetic acid containing 10 mg of unlabeled arginine per ml for 30 min, and in 5% trichloroacetic acid at 90 C for 15 min, then glued to planchets, dried, and counted. Symbols:* ○, *control;* ●, *puromycin.*

Klett readings were made on the induced culture at 20-min intervals, the points are calculated from interpolated values on the growth curve. For this purpose, the assumption was made that enzyme synthesis begins immediately upon the addition of inducer. Since only 2 to 4 min of the lag were unaccounted for by precursor activation, the effect of this assumption on the plotting of Fig. 8 was extremely small; however, the early kinetics showed no lag due to the completion of polypeptide chains, since the zero point represented the first apperarance of complete enzyme.

At early times, the differential rate of synthesis (defined as the slope of the mv versus Klett curve) gradually rose to its final constant value (Fig. 8). By extrapolation of the linear portion of the curve to the X-axis, and noting that the points in Fig. 8 represent 2.5-min intervals of time, it is apparent that this period is of approximately 5 min duration. If the differential rate of synthesis is considered to depend on the level of messenger ribonucleic acid (RNA) specific for luciferase in each cell at a given time, the transient period may be explained in terms of a gradual accumulation of messenger, from the level present in uninduced cells to the fully induced level. It can, in fact, be shown that the time course of messenger accumulation and that of the increase in differential rate of synthesis are identical.

As has been noted already, the addition of 5-fluorouracil to induced cultures blocks the synthesis of luciferase, presumably by becoming incorporated into a messenger which directs the synthesis of an inactive protein (5). Thus, the total amount of enzyme produced by a culture to which fluorouracil has been added at a given time may be taken as a measure of the total amount of messenger present at that time. This figure divided by the bacterial density at the time of fluorouracil addition yields a number proportional to the amount of messenger per cell, assuming that there is no lag in fluorouracil action and that the average number of enzyme molecules synthesized under the direction of a molecule of messenger is constant. 5-Fluorouracil was added to samples of an induced culture at various times, and the final level of light measured after induction had ceased.

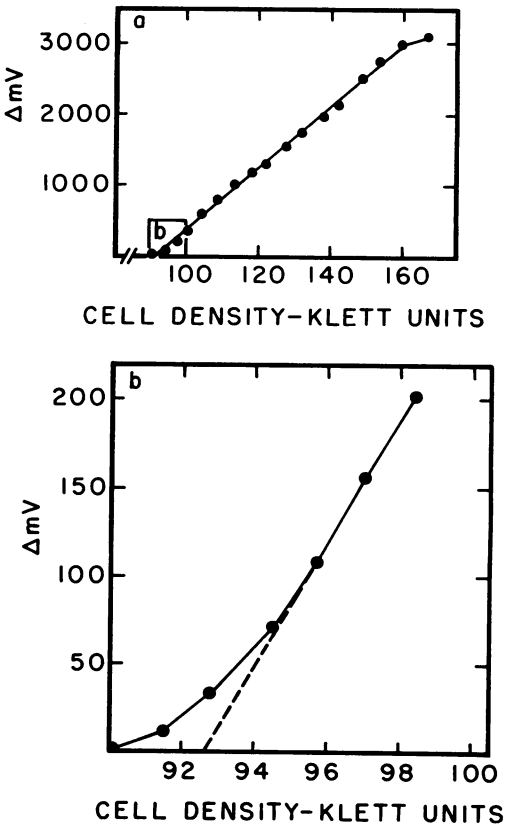

FIG. 8. (*a*) *The induction curve of Fig. 1 plotted as a function of bacterial density.* (*b*) *An expansion of Fig. 8a, showing the initial 15 min of induction. A density of 100 Klett units corresponds to 6.7 × 10^8 cells/ml.*

The bacterial densities at 0 and 25 min were determined, and values for densities at the time of fluorouracil addition were interpolated, assuming growth to be linear over the 25-min (approximately 0.2 generation time) course of the experiment. Figure 9 presents the final levels of enzyme divided by bacterial density (Δ mv/K) as a function of time of fluorouracil addition. It is noteworthy that the apparent lag in fluorouracil action is quite short, since the curve extrapolates to zero enzyme synthesis at less than 1 min to the left of the origin.

Along with the ratio of enzyme increase to bacterial density, Fig. 9 presents measurements of the differential rate of synthesis, determined in samples of the same culture in the following manner: puromycin was added at various times, and a plot similar to Fig. 6 was constructed. Slopes of line segments between each pair of points (Δmv/Δt) were measured and converted to functions of bacterial density by assuming that growth is approximately linear over the 25-min course of the experiment, determining the rate of increase in Klett reading (ΔK/Δt) from the values at 0 and 25 min, and calculating Δmv/ΔK as Δmv/Δt/ΔK/Δt. These are presented in Fig. 9 as a function of the midpoints of the time intervals employed.

The level of messenger in the cells, as indicated by the "fluorouracil points," and the differential rate of synthesis, as indicated by the "puromycin points," both rose for the first five min and leveled off thereafter. Although it cannot be shown that other processes did not accelerate during this time, the simple supposition that the differential rate rises as messenger accumulates to its steady-state level is sufficient to explain the kinetic measurements.

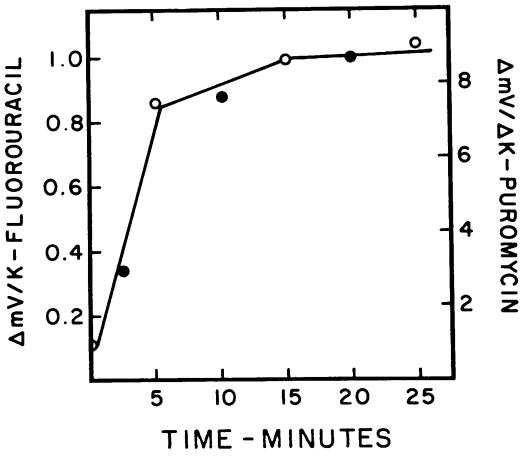

FIG. 9. *Kinetics of early induction in the presence of inhibitors. Experiment as described in the text. Symbols: ○, addition of 5-fluorouracil, 20 μg/ml; ●, addition of puromycin, 5 μg/ml.*

DISCUSSION

In ND cells under the conditions employed, it may be concluded that arginine is the sole inducer of luciferase. The only other compounds with any measurable inducing activity are metabolically related to arginine, and all show weak induction for limited periods of time. Arginine-related amino acids are without effect on the rate of induction in limiting concentrations of arginine, although they prolong synthesis somewhat by sparing intracellular arginine. Intracellularly as well, arginine appears to be the true inducer of luciferase; arginine added to the medium is unchanged when induction is well underway. Yet, no enzyme appears for 12 min after the effective entry of inducer. What processes must occur in the cell during that time? Arginine must be continuously present, or the cells immediately react as if they had never previously been induced. Moreover the cell must be capable of synthesizing protein. The preinduction experiment, however, demonstrates that, if any protein other than luciferase is required for the expiration of the lag, it is uncommonly unstable, since there is no indication that cells recently induced contain any component which affects the response to a subsequent addition of arginine. If, then, only the single inductive pathway leading to luciferase is of any significance, it follows that luciferase must itself be synthesized before the lag is apparent owing to the fact that the rate of protein synthesis may be varied widely without effect on the lag.

The increase in enzyme after puromycin inhibition is also indicative of a slow step occurring after completion of the primary structure. However, some caution must be exercised in interpreting these data. If synthesis of luciferase should be less susceptible to puromycin than is that of most proteins, the effective lag in puromycin action could be far greater than the incorporation measurements suggest, and the small amount of luciferase appearing after blockage correspondingly less.

There are several types of precursor-enzyme conversions which could explain the duration of the lag. A few observations, however, may narrow the range of possibilities. The most important is that the same lag is observed in the increase of luciferase assayed in vitro. Thus, no release of inhibition is involved unless the inhibitor is quite tightly bound to the enzyme. In fact, since all cell-free extracts were assayed together at the end of a complete induction experiment, the presence of the lag in vitro indicates that the activation

step can only be observed in the intact cell. However, the fact that the early enzyme assay points are consistently slightly high may indicate a limited amount of activation under these conditions. The constant time interval between the puromycin addition curve and the induction curve (Fig. 6) demonstrates that the amount of active enzyme (and presumably of precursor) present in the cells does not affect the rate of the activation process. Although these observations do not rule out the possibility of subunit association, and though bacterial luciferase is reported to contain subunits (9), they suggest that the most fruitful approaches to the activation problem are along other lines.

Once luciferase has begun to appear, the analysis of the rate of its appearance is quite straightforward. Except for a brief period at the beginning of induction, the differential rate of synthesis of luciferase is constant until the end of the induction process. The 5-min period during which messenger RNA rises to its steady-state level is comparable to the 3-min lag period in the induction of β-galactosidase (2, 7, 8, 14), both in duration and in the processes which have been implicated. As Heinmets (6) has pointed out in some detail, the internal derepression assumed to initiate enzyme induction may occur at any one of several steps involving repressor synthesis or function. Depending on the steps affected, the initial kinetics will differ markedly. Although the data at hand are not sufficient for a complete analysis, there is evidence that the derepression process itself is quite rapid. A plot of specific activity of luciferase against $1 - e^{-kt} - kte^{-kt}$, where k is the exponential constant in the bacterial growth equation, is linear over the first 5 min, as would be the case if the differential rate of synthesis depended upon the concentration of an intermediate—e.g., messenger RNA—which was itself synthesized at a constant differential rate (10). More simply stated, there appears to be no lag in the differential rate of messenger synthesis; however, the number of data points was small, the exponential function was insensitive to small changes in t, and it is uncertain how large a transient effect could have occurred unobserved. If it is true that the differential rate of synthesis of messenger is constant, the derepression process itself is not significant in the transient phase and is probably complete within 2 min of the addition of inducer.

The time required to complete new polypeptide chains, although not represented in Fig. 8, may be estimated from the inhibitor data. The 2-min decrease in the lag period evident after preincubation with azatryptophan and inducer provides a lower limit, whereas the upper limit may be represented by the estimate of 4 min as the maximal time during which protein synthesis may continue in puromycin, i.e., the maximal portion of the 12-min lag not attributable to precursor activation. The estimate of 2 to 4 min is somewhat greater than the 1-min interval determined more directly by Kepes (7, 8) for β-galactosidase induction, but may not be unrealistic in view of the longer division time of the ND cells.

Tentatively, then, the following sequence of events may be proposed. At 0 to 2 min, arginine enters the cells at a concentration sufficient to induce. The process of derepression is completed, and messenger RNA begins to be synthesized at a constant differential rate. At 2 to 4 min, the first luciferase polypeptide chains are completed. At 5 min, messenger RNA reaches its steady-state level. Luciferase polypeptide chains (precursor) are now synthesized at constant differential rate. After 12 min, first precursors complete the 8- to 10-min activation process. Active luciferase makes first appearance, and there is steady-state induction.

It seems likely that the effect of arginine on luciferase synthesis in ND cells is comparable to the amino acid requirements observed in *A. fischeri*. The apparent specificity for arginine in this case may be due merely to a metabolic state of nitrate-grown cells in which arginine is in short supply in the intracellular pool, while other amino acids are in excess.

ACKNOWLEDGMENTS

Chloramphenicol was the gift of H. E. Machamer of Parke, Davis & Co., Detroit, Mich. I am indebted to W. D. McElroy, in whose laboratory this work was carried out, for his advice and guidance.

During this study, the author held a National Science Foundation Graduate Fellowship.

LITERATURE CITED

1. ANDERSON, R. E. 1948. The growth requirements of luminous bacteria at various temperatures. J. Cellular Comp. Physiol. **32**:97–100.
2. BOEZI, J. A., AND D. B. COWIE. 1961. Kinetic studies of β-galactosidase induction. Biophys. J. **1**:639–647.
3. DANIEL, E. E. 1950. The reversal of norleucine inhibition in luminous bacteria. J. Cellular Comp. Physiol. **36**:41–57.
4. FARGHALY, A. H. 1950. Factors influencing the growth and light production of luminous bacteria. J. Cellular Comp. Physiol. **36**:165–183.
5. HEIDELBERGER, C. 1965. Fluorinated pyrimidines. Progr. Nucleic Acid Res. Mol. Biol. **4**:1–50.
6. HEINMETS, F. 1964. Elucidation of induction and repression mechanisms in enzyme synthesis by analysis of model systems with the analog computer, p. 415–479. *In* B. Pullman [ed.], Electronic aspects of biochemistry. Academic Press, Inc., New York.

7. KEPES, A. 1963. Kinetics of induced enzyme synthesis. Determination of the mean life of galactosidase-specific messenger RNA. Biochim. Biophys. Acta **76**:293–309.
8. KEPES, A. 1963. Kinetic analysis of the early events in induced enzyme synthesis. Cold Spring Harbor Symp. Quant. Biol. **28**:325–327.
9. KUWABARA, S., M. J. CORMIER, L. S. DURE, P. KREISS, AND P. PFUDERER. 1965. Crystalline bacterial luciferase from *Photobacterium fischeri* Proc. Natl. Acad. Sci. U.S. **53**:822–828.
10. MARR, A. G., AND S. MARCUS. 1962. Kinetics of induction of mannitol dehydrogenase in *Azotobacter agilis*. Biochim. Biophys. Acta **64**:65–82.
11. MCELROY, W. D. 1961. Bacterial luminescence, p. 479–508. *In* I. C. Gunsalus and R. Y. Stanier [ed.], The bacteria, vol. 2. Academic Press, Inc., New York.
12. MCELROY, W. D., AND A. H. FARGHALY, 1948. Biochemical mutants affecting the growth and light production in luminous bacteria. Arch. Biochem. Biophys. **17**:379–390.
13. NATHANS, D. 1964. Puromycin inhibition of protein synthesis: Incorporation of puromycin into peptide chains. Proc. Natl. Acad. Sci. U.S. **51**:585–593.
14. PARDEE, A. B., AND L. S. PRESTIDGE. 1961. The initial kinetics of enzyme induction. Biochim. Biophys. Acta **49**:77–88.
15. SADANA, J. C., AND W. D. MCELROY. 1957. Nitrate reductase from *Achromobacter fischeri*. Purification and properties: function of flavines and cytochrome. Arch. Biochem. Biophys. **67**:16–34.