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Poising of the Arginine Pool and Control of Bioluminescence in *Beneckea harveyi*

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Arginine dramatically stimulates bioluminescence in the marine bacterium *Beneckea harveyi* growing in minimal media, an effect that is due to increases in both the synthesis and expression of luciferase. To elucidate the mechanism of this phenomenon, studies were made of the transport and metabolism of arginine in *B. harveyi*. The transport of arginine and lysine involves two kinetically distinct transport systems for the uptake of arginine and lysine. In contrast, ornithine is transported only by a system common to all three amino acids. The internal amino acid pools were measured in mutants that do not show stimulation of bioluminescence by arginine and in wild-type cells that do. In minimal media, the internal arginine pools are undetectably low. Furthermore, exogenously added labeled arginine is rapidly transported and converted to citrulline and argininosuccinate. The results can be accommodated by a model in which the internal arginine is poised at a very low concentration; the stimulatory effect of exogenous arginine on luciferase biosynthesis occurs at the transcriptional level, and the actual mediator can be either arginine or argininyl transfer ribonucleic acid.

The synthesis of bacterial luciferase and the concomitant development of bioluminescence in *Beneckea harveyi* require the presence of an autoinducer and relative freedom from catabolite repression (13, 14). Autoinducer is a species-specific, low-molecular-weight molecule that the cells produce themselves (6a, 7, 12). When cells are grown in a minimal medium, there is, in addition, a stimulation by exogenously supplied arginine (19). The effect of arginine was first described by Coffey (6), who postulated that arginine was the inducer of bacterial bioluminescence. Nealson et al. (14) provided evidence that arginine potentiated the effect of the autoinducer rather than acted as the sole inducer; wild-type bacteria incubated in growth medium with arginine will not synthesize luciferase unless a sufficient amount of autoinducer is also present.

Mutants that are bright without added arginine, termed minimal brights or MB mutants, have been isolated and partially characterized (19). They do not appear to contain an expanded intracellular pool of arginine, nor are they overproducers of autoinducer. They exhibit catabolite repression, and their luciferases are immunologically indistinguishable from that of the wild type. Although there was no clue concerning the mechanism of action, the evidence suggested that arginine itself or a product of its metabolism exerts control on luciferase synthesis at the transcriptional level.

We have examined the metabolism of arginine in relation to the induction of luciferase in wild-type *Beneckea harveyi* beginning with a quantification of the arginine effect, measurement of the internal arginine pool, and examination of the arginine transport system. The fate of freshly transported arginine in terms of conversion to other products, both soluble and trichloroacetic acid precipitable, has also been determined. The data are used to construct a model to account for the effect of exogenously supplied arginine in stimulating the synthesis of luciferase in minimal media.

MATERIALS AND METHODS

Bacterial strains and cultivation. The bacteria used were *B. harveyi* strain MAV, designated strain B-392 by Reichelt and Baumann (16), and minimal bright (MB) mutants derived from it (19). *B. harveyi* is the same as *Lucibacterium harveyi* in *Bergey's Manual of Determinative Bacteriology*, 8th edition (1).

The bacteria were cultivated and maintained on HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered media. The HEPES-minimal medium contained: 20 g of NaCl, 12.35 g of $MgSO_4 \cdot 7H_2O$, 11.9 g of HEPES, 0.75 g of KCl, 1 g of NH_4Cl, 3 ml of glycerol, and 0.64 ml of 50% NaOH (to adjust the pH to 7.0) and 1.4 mg of ferric ammonium citrate per liter of distilled water. K_2HPO_4 to make the me-

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dium 2.5 mM in phosphate was added aseptically after autoclaving. HEPES-peptone medium had the same composition as that given above with the addition of 0.5% peptone (Difco Laboratories, Detroit, Mich.). The stock cultures were maintained on agar slants of HEPES-peptone medium to which 15 g of agar (Difco) and 0.1 g of $CaCO_3$ were added per liter of medium.

Some experiments were done with phosphate-buffered media: 30 g of NaCl, 7 g of Na_2HPO_4, 1 g of KH_2PO_4, 0.5 g of $(NH_4)_2HPO_4$, 0.1 g of $MgSO_4$, and 3 ml of glycerol per liter of distilled water.

During the study of the arginine effect, HEPES-minimal was developed in an effort to make a prototrophic marine minimal broth with an artificial buffer. Of the many different buffers and salt formations tried, HEPES-minimal supported more bioluminescence and a faster rate of growth than did other formulations. Additionally, HEPES-minimal more closely approximates the ionic conditions of seawater than does phosphate-minimal.

The bacteria were cultivated at 25°C in flasks incubated in a New Brunswick Aquatherm water bath reciprocating shaker at 150 rpm. Optical densities of the cultures were measured in tubes (16 by 125 mm) in a Coleman Jr. II spectrophotometer at 660 nm. The relationship between viable cell titer and total cell titer to optical density is linear from 0.05 to 0.7 Coleman optical density units. Cultures used in experiments were inoculated with cells growing exponentially. Addition of amino compounds to cells growing in minimal media were made from 0.1 M stock solutions sterilized by filtration (Millipore Corp.).

Bioluminescence and luciferase activity. Luminescence was measured in tubes or in scintillation vials with a calibrated photometer (8, 11) as light units per milliliter (1 light unit equals 7×10^{10} quanta per s). Luciferase activity in vitro was measured in 0.02 M phosphate buffer, pH 7.0, at 25°C containing microliter (as described below) amounts of aqueous 0.1% decanal suspensions prepared by sonication. The reaction was initiated by the injection of 1.0 ml of 5×10^{-5} M $FMNH_2$ solution made in 0.02 M phosphate buffer, pH 7.0, and reduced by bubbling hydrogen in the presence of platinized asbestos.

Amino acid pools. To extract the free amino acid pools, 0.5×10^{10} to 1.0×10^{10} cells were removed from the culture and collected on a 47-mm DA Millipore filter (0.6-μm pore size). The filter was washed with two volumes of ice-cold medium-salts and immediately plunged into boiling 80% ethanol and boiled for 5 min. Control experiments showed that labeled cells washed with ice-cold and 25°C medium-salts retained the same total counts; the ice-cold wash was used to lessen the possible enzymatic conversion of internal amino acid pools before immersion of the cells into the boiling 80% ethanol. The time from initiation of the filtration to immersion of the filter in boiling ethanol was less than 15 s. If the cells were left on the filter for longer periods, changes in the extractable pools were noted (see below). Control trials showed that there was no amino acid contamination of the filter from the medium and that the DA filters completely retained the cells. The ethanol extract was evaporated in a rotary evaporator, and the amino acids were taken up in 0.1 N HCl and analyzed on a Beckman 121M amino acid analyzer. Recovery of soluble amino acids from the filters was quantitative as shown by control experiments with [^{14}C]arginine added to filters containing boiled cells.

The amino acid peaks from the cell extracts were compared with known standards which included the usual 20 amino acids plus argininosuccinate, citrulline, and ornithine. All amino acids chromatograph as single peaks except for argininosuccinate, which under acidic conditions spontaneously forms an anhydride. Argininosuccinate forms two peaks which occurred near buffer changes and were labeled peaks B and C. Peak B seems to be argininosuccinate, whereas peak C is the anhydride(s), since this latter peak showed an enhanced 440-nm absorbance with the ninhydrin reaction indicative of the secondary amine character of the argininosuccinate anhydride(s). In the amino acid program used, citrulline chromatographs between glutamate and proline. The amounts of citrulline and proline had to be estimated by manually integrating the area under the curves. Because these peaks partially overlap, the reported concentrations contain significant error.

The limit of detection of this sampling-analysis procedure is 0.01 to 0.03 nmol/10^9 cells. For the purpose of calculation, the lower figure, 0.01 nmol, is used.

Amino acid transport and labeling of amino acid pools. The transport of arginine, lysine, and ornithine was characterized by the methods of Britton and McClure (2). Culture samples were removed and mixed with appropriate dilutions of either ^{14}C- or ^{3}H-amino acid, and this mixture was sampled for total incorporation and incorporation of label into trichloroacetic acid-precipitable material by simultaneously removing two 100-μl portions. One portion was immediately filtered on a (0.45-nm pore size) HA Millipore filter (25 mm) followed by 20 volumes of ice-cold medium salts. The other portion was immediately injected into 2 ml of ice-cold 5% trichloroacetic acid. The trichloroacetic acid precipitates were collected on 25-mm HA Millipore filters and rinsed with 20 volumes of ice-cold 5% trichloroacetic acid. The filters were counted in Aquasol (New England Nuclear Corp.) after thorough drying by evaporation.

Amino acid pools were labeled by incubating culture samples with dilutions of the appropriate ^{14}C- or ^{3}H-amino acid. The labeled pools were extracted as described above and analyzed by chromatography on the Beckman 121M amino acid analyzer, with the column effluent being collected by a Gilson fraction collector. Each fraction was counted in a Beckman scintillation counter after addition of 1.5 ml of Aquasol to the fraction (about 100 μl). The retention times were derived from a known L-amino acid-^{3}H-labeled mixture (New England Nuclear) containing amino acids at different concentrations. The identity of some experimental peaks was verified by co-chromatography with the authentic ^{14}C-amino acids. None of the three buffers used in the analyzer caused significant quenching if the volume of analyzer buffer was kept less than 10% of the Aquasol.

Cell volumes. The cell volumes were measured with a Coulter Counter Channelyzer after dilution of the bacteria growing in HEPES-minimal or peptone media into ultrafiltered seawater. The cell volumes

were calculated from calibration with standard latex beads diluted under the same conditions. The volumes were calculated on the peak mode and do not represent the distribution of sizes but rather represent the average cell volumes.

RESULTS

Arginine effect. The effect of arginine and ornithine on the stimulation of bioluminescence in HEPES-minimal is shown in Fig. 1. Arginine concentrations from 1 μM to 1 mM stimulated the development of bioluminescence at nearly identical rates. Arginine stimulation of bioluminescence could be due to an effect either on luciferase synthesis or on its expression. Both appear to be involved: in experiments in which arginine stimulated a 30-fold increase in bioluminescence, there was only a 10-fold stimulation of extractable luciferase activity. Arginine must stimulate other in vivo factors which increase luciferase expression as well as mediation of the transcription of the bioluminescent system (14, 19).

Ornithine also stimulated bioluminescence but not as effectively as arginine. The maximum stimulation was at a lower (100 μM) concentration, and higher (1 mM) levels of ornithine resulted in less than maximum bioluminescence. Citrulline and argininosuccinate are also less effective than arginine (Fig. 2), but unlike ornithine, the higher concentrations were not inhibitory. The effects of many compounds related to arginine have been tested for the specific stimulation of bioluminescence (Waters, Ph.D. thesis, Harvard University, Cambridge, Mass., 1975). These experiments demonstrated that, with the exception of proline, only arginine or its biosynthetic precursors cause a marked stimulation of bioluminescence. Some amino acids such as aspartate and glutamate increased the rate of the development of bioluminescence, but they also stimulated growth, eliciting little if any specific stimulation of bioluminescence.

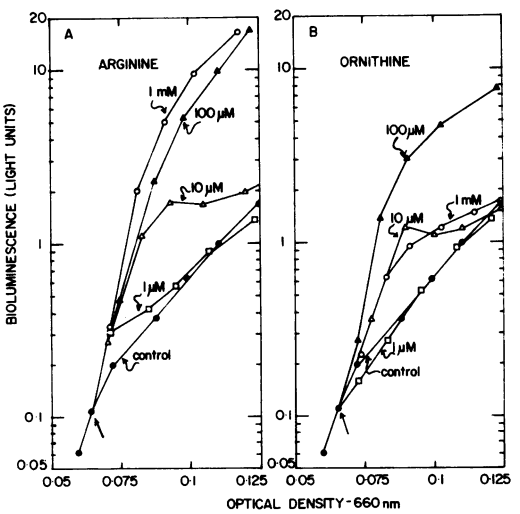

FIG. 1. *Effect of arginine and ornithine on the production of bioluminescence of B. harveyi growing in HEPES-minimal broth. The culture (100 ml) was inoculated at a low optical density at 660 nm (about 0.0002), and after the onset of autoinduction, at the arrow, the culture was split into 3.0-ml portions in screw-capped test tubes (16 by 125 mm), each containing one of the concentrations of arginine or ornithine shown in the figure. The control has no additions.*

Amino acid pools. In attempting to understand the arginine effect, the intracellular pools of arginine and other amino acids were determined to see whether there were significant differences between pre- and post-autoinduction cells (Table 1). During autoinduction, in cells grown in HEPES-minimal, the pool sizes of tyrosine, phenylalanine, and ammonia were increased, whereas those of threonine and glutamate were lowered. The amino acid pools of cells grown in HEPES-peptone varied during the growth curve. These variations were not similar to those of cells grown in HEPES-minimal media. These variations of pool sizes during peptone growth probably represent the differential utilization of different amino acids from the peptone at the different times.

In cells grown in HEPES-minimal, the intracellular arginine pool was very low. In 31 analyses of extracts from minimal (phosphate or HEPES)-grown cells, arginine was detected only once as a trace of <0.03 nmol/10^9 cells. The absolute limit of detection of the method used was 0.01 nmol/10^9 cells; based on this value, there are less than 6,000 molecules of arginine per cell. However, cells growing in peptone media consistently had 2 to 4 nmol of arginine per 10^9 cells. The arginine pool of these cells, however, is a mobile one; cells growing in HEPES-peptone medium that were rapidly transferred to HEPES-minimal on Millipore filters rapidly lost their intracellular pools of arginine (Table 2), whereas intracellular pools of other amino acids (e.g., glutamate, threonine, citrulline, and argininosuccinate) were maintained. This rapid change of the arginine pool suggests that our estimate of the arginine pools in these cells may be low.

There is a class of mutants, termed MB mutants, that are bright when grown in minimal medium without exogenously supplied arginine (19). This does not appear to be explained by an increased arginine pool or the alteration of internal pools of other free amino acids. In cells grown in both minimal and peptone media, the arginine and other amino acid pool sizes of MB

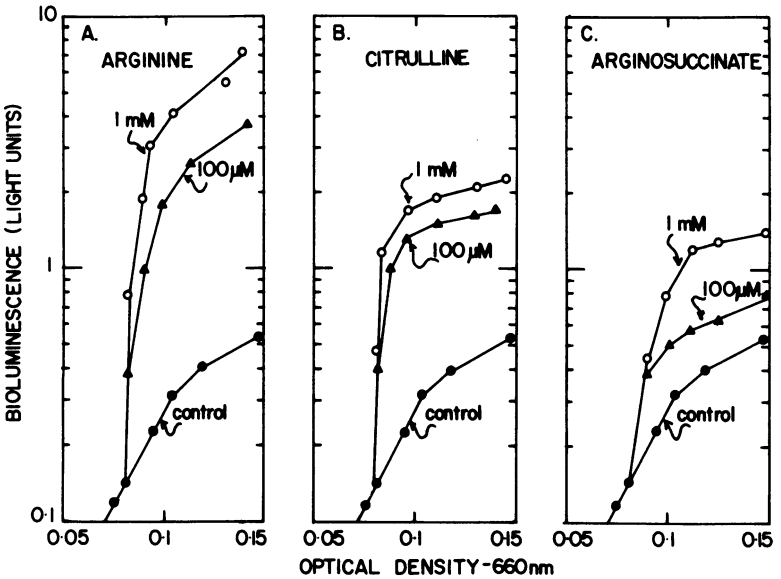

FIG. 2. *Effect of arginine, citrulline, and argininosuccinate on the production of bioluminescence of B. harveyi growing in HEPES-minimal broth. The culture and other conditions were as those described in the legend to Fig. 1.*

cells were similar to those found in wild-type cells. In minimal media there was no detectable arginine, and in peptone there was about 2 to 4 nmol of arginine per 10^9 cells (data not shown). The mutants analyzed were MB-1, MB-3, MB-4, MB-20, and MB-41, and all the analyses were from cells (including wild-type cultures) grown in phosphate-minimal and phosphate-peptone media.

The effect of the addition of certain amino acids during induction of luciferase synthesis on the amino acid pools of wild-type cells grown in minimal media is shown in Table 3. Exogenous arginine resulted in an internal arginine pool of the same size (2 to 4 nmol of arginine per 10^9 cells) as that in HEPES-peptone-grown cells. In other experiments carried out in a phosphate-minimal medium, arginine supplementation for periods as long as 40 min did not increase the internal arginine pool further (data not shown). Addition of citrulline or ornithine (but not argininosuccinate) resulted in significantly expanded pools of these amino acids on the order of 10 nmol/10^9 cells in HEPES-minimal. Forty-minute incubation with ornithine in phosphate-minimal resulted in an ornithine pool size of 165 nmol/10^9 cells. Addition of ornithine, citrulline, or argininosuccinate, however, resulted in no augmentation of the arginine pool to detectable levels or appreciable increases in the pool size of other amino acids. The argininosuccinate pool size increased when the cells were incubated with arginine, citrulline, and ornithine but not with argininosuccinate. Apparently argininosuccinate did not enter the cells as fast as arginine because the increase in argininosuccinate pool did not occur when it was supplied exogenously compared to the increase in argininosuccinate (2 to 4×) with exogenous arginine, citrulline, and ornithine. Longer incubations in phosphate-minimal, however, showed that argininosuccinate can enter the cells, but the pool was less than when the cells were incubated with arginine. The citrulline pool increased when the cells were incubated with arginine, citrulline, or ornithine, but the ornithine pool only increased when the cells were incubated with arginine and ornithine (not citrulline).

Cell volumes. *B. harveyi* growing in HEPES-minimal broth had an average volume during logarithmic phase of growth of 0.37 μm^3, whereas cells growing in HEPES-peptone had an average volume of 1.0 μm^3.

Arginine, lysine, and ornithine transport. To study the transport and incorporation of exogenously added amino acids, radioactively labeled compounds were employed. With 1 μM exogenous arginine, the label was rapidly transported, and the labeled arginine pool was rapidly depleted and incorporated into trichloroacetic acid-precipitable material (Fig. 3).

Ornithine and lysine compete to some extent for uptake of labeled arginine but not as effectively as unlabeled arginine (Fig. 4). Citrulline, argininosuccinate, or D-arginine (up to 1 mM) did not compete with the uptake of 1 μM labeled

TABLE 1. *Amino acid pools of B. harveyi grown in HEPES-minimal media*

Amino acid	Amt (nmol/10^9 cells)[a]				
	Pre-autoinduction[b]		Autoinduction[b]		
	0.051[c]	0.067	0.111	0.145	0.192
Aspartate	+	+	0.4	0.5	+
Threonine	17.2	13.5	12.2	11.2	8.3
Serine	−	−	−	−	−
Glutamate	104.5	89.3	80.1	76.6	81.6
Citrulline	3.3	2.7	2.6	1.2	0.4
Proline	+	+	+	+	+
Glycine	0.4	0.3	0.4	0.4	0.6
Alanine	2.5	1.7	1.9	2.1	3.1
Cystine	−	−	−	−	−
Valine	−	−	−	−	−
Argininosuccinate	0.1	0.1	0.2	0.2	0.1
Methionine	+	+	+	+	+
Isoleucine	1.1	1.0	1.0	1.0	1.0
Leucine	0.1	+	0.1	+	+
Tyrosine	0.4	0.3	0.1	1.0	0.6
Phenylalanine	0.1	0.1	0.1	2.4	1.4
Histidine	0.2	0.1	0.1	0.1	0.1
Ornithine	0.1	0.1	0.1	0.1	0.4
Lysine	0.2	0.1	0.1	0.1	0.1
Ammonium	7.0	8.2	6.1	18.7	19.1
Arginine	−	−	−	−	−
Total (excluding NH_4^+)	130.2	109.3	101.3	96.9	97.7

[a] +, Trace; −, undetectable.
[b] State of bioluminescence induction.
[c] Culture optical densities at 660 nm.

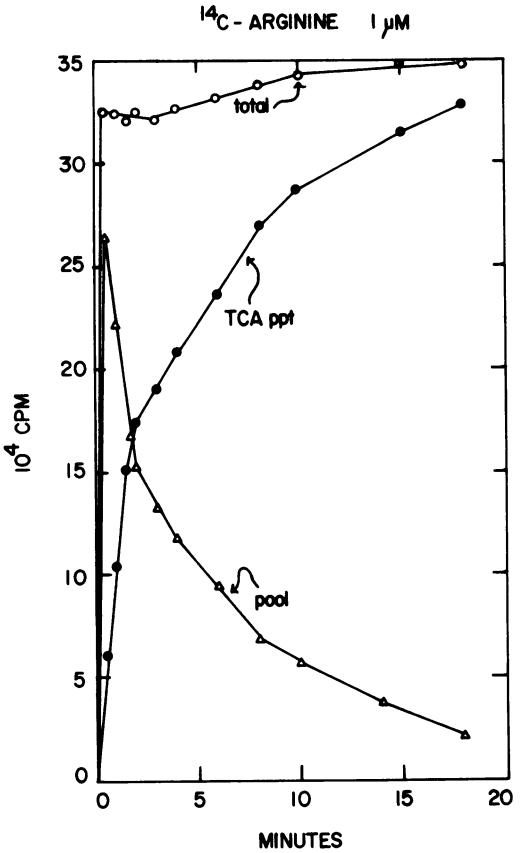

FIG. 3. *Uptake of labeled arginine into B. harveyi growing in phosphate-minimal. The culture was tested in mid-autoinduction and contained 4 × 10^8 cells per ml producing 1.2 × 10^9 quanta·s^{-1}·ml^{-1}. The [^{14}C]arginine was diluted so that at time zero 17 μl of diluted label (uniformly labeled [^{14}C]arginine) was mixed with 2.6 ml of culture, resulting in a final arginine concentration of 1 μM. Duplicate 100-μl portions were sampled at the times indicated. The arginine pool is the calculated difference between the total incorporation and the incorporation into trichloroacetic acid precipitate (TCAppt).*

L-arginine (data not shown). The transport of arginine appeared to be mediated by two kinetically distinct systems: a high-affinity system (Fig. 5A) and a low-affinity system (Fig. 5B).

Since transport of arginine in *Escherichia coli* may occur via a system also capable of lysine uptake (5), the uptake of labeled lysine (Fig. 6) and ornithine (Fig. 8) and their incorporation into trichloroacetic acid-precipitable material were examined. Uptake of lysine occurred without a lag (Fig. 6), and competition by ornithine, arginine, and unlabeled lysine with labeled lysine showed that ornithine and arginine were only partially effective competitors compared with unlabeled lysine (Fig. 7). The uptake of labeled ornithine occurred as rapidly as arginine and lysine uptake, but its incorporation into trichloroacetic acid-precipitable material involved a lag of 2 min (Fig. 8). Arginine was a more effective competitor of labeled ornithine uptake than was unlabeled ornithine (data not shown). Kinetic studies of the uptake of lysine suggested two kinetically distinct systems for lysine uptake, but only ornithine uptake appeared to be taken up by only one system (Table 4).

By using higher concentrations of arginine, the calculated "arginine pool" (differences between the total arginine label incorporation and incorporation into trichloroacetic acid-precipitable material) is very large (Table 5). This value differed from the direct chemical measurement

TABLE 2. *Amino acid pools of B. harveyi grown in HEPES-peptone to midautoinduction and transferred to minimal media*[a]

Amino acid	Amt (nmol/10^9 cells)					
	Expt 1		Expt 2			
	Peptone cells at zero time	Minimal at 1.8 min	Peptone cells at zero time	Minimal media		
				1 Min	2 Min	4 Min
Aspartate	1.5	–	1.7	–	–	–
Threonine	7.8	6.9	8.8	16.3	8.9	12.0
Serine	+	+	+	+	+	+
Glutamate	94.2	79.6	91.1	101.6	95.7	132.4
Citrulline	1.5	0.3	1.5	0.5	0.3	0.3
Proline	+	+	+	+	+	+
Glycine	16.5	0.4	19.4	7.5	6.4	2.1
Alanine	92.9	55.3	109.5	84.5	80.5	100.2
Cystine	–	–	–	–	–	–
Valine	3.8	–	4.9	–	–	–
Argininosuccinate	3.0	1.2	2.2	1.0	0.5	2.0
Methionine	7.2	3.2	7.3	+	–	–
Isoleucine	3.3	1.1	4.2	1.2	1.0	1.0
Leucine	7.3	–	8.8	2.4	2.5	1.8
Tyrosine	+	–	+	+	–	–
Phenylalanine	2.0	–	2.6	+	–	–
Histidine	0.5	+	0.6	0.9	0.4	–
Ornithine	+	+	–	–	–	–
Lysine	1.9	+	2.3	0.4	0.5	–
Arginine	3.3	–	4.4	+	+	–
Total (excluding NH_4^+)	246.7	148	269.6	216.3	196.7	251.4

[a] Approximately 5×10^9 cells were filtered from HEPES-peptone medium and were then either (i) immediately washed with ice-cold salts as described in the text (zero time) or (ii) washed with 25°C fresh, sterile HEPES-minimal medium and allowed to incubate for the times indicated at which ice-cold salts were washed over the cells as described in the text.

of the arginine pool (Table 3) and suggested that after arginine is transported into the cell, it is converted into other soluble products which eventually become incorporated into trichloroacetic acid-precipitable material.

Labeling of intracellular amino acid pools. Labeled arginine and ornithine were added to cultures; in different experiments, the cells were removed by rapid Millipore filtration, and the amino acid pools were chromatographed on the Beckman 121M analyzer. Fractions were collected directly from the column, mixed with Aquasol, and counted.

The fate of added (1 μM) labeled arginine was followed for 1 min (Fig. 9). Most of the label was found initially in the arginine peak but was rapidly converted to citrulline and argininosuccinate. Roughly five times more label was found in citrulline than in argininosuccinate in this labeling experiment (with 1 μM arginine). Labeling with 100 μM arginine resulted in more even distribution of the label in the citrulline and argininosuccinate (data not shown). A pulse-chase experiment in which a 1 μM labeled arginine pulse was followed by a 1 mM unlabeled arginine chase showed that the label accumulated first in citrulline and was found later in the argininosuccinate pool as the citrulline label decreased (Fig. 10). The quantity of the arginine chase was large enough to maintain an arginine pool and trap some of the label in that pool.

The addition of a 1 μM [^{14}C]ornithine resulted in labeling the citrulline, argininosuccinate, and an unknown peak (Fig. 11). A small portion of the label was found in the glutamate peak, but none of the label appeared at the location of

TABLE 3. *Amino acid pools of B. harveyi in HEPES-minimal supplemented with amino acids*[a]

Amino acid	Amt (nmol/10^9 cells)				
	Control	Arginine	Citrulline	Ornithine	Argininosuccinate
Aspartate	0.8	1.1	–	2.1	1.0
Threonine	28.4	20.9	19.2	19.0	25.1
Serine	–	–	–	–	–
Glutamate	68.1	69.0	68.5	76.6	62.7
Citrulline	0.5	23.5	12.9	3.3	+
Proline	–	–	–	–	–
Glycine	0.9	0.8	0.9	1.2	0.9
Alanine	9.7	9.4	9.8	10.9	9.7
Cystine	–	–	–	–	–
Valine	–	–	–	–	–
Argininosuccinate	2.3	5.9	5.5	10.9	2.3
Methionine	+	+	+	+	+
Isoleucine	1.2	1.2	0.9	1.0	1.2
Leucine	+	+	+	+	+
Tyrosine	+	+	+	+	+
Phenylalanine	+	+	+	+	+
Histidine	+	+	+	+	+
Ornithine	0.9	5.4	+	36.2	0.9
Lysine	+	+	+	+	+
Ammonium	54.1	25.1	13.1	22.6	54.1
Arginine	–	3.0	–	–	–

[a] The cells were grown to mid-autoinduction in HEPES-minimal and split into 10-ml portions each incubated for 10 min with (or without) the L-amino acid at a final concentration of 1 mM.

arginine, presumably due to the low level of the arginine pool of the cells in minimal media.

DISCUSSION

Arginine effect. Exogenously added arginine causes an increase in bioluminescence (6, 14, 19); the maximum initial stimulation occurs at a concentration of 1 μM. Higher concentrations of arginine result in higher final levels because the effect continues for a longer time.

Ornithine also stimulates bioluminescence but less so than does arginine. In fact, at higher levels ornithine may inhibit the development of luminescence. Other arginine biosynthetic precursors, citrulline and argininosuccinate, also stimulate bioluminescence but, again, less effectively than arginine. The decreased amount of stimulation may be due to a slower transport rate, slow conversion to the molecule which is the actual stimulator, or both. In any event, bioluminescence stimulation can be ascribed to arginine and its precursors; except for proline,

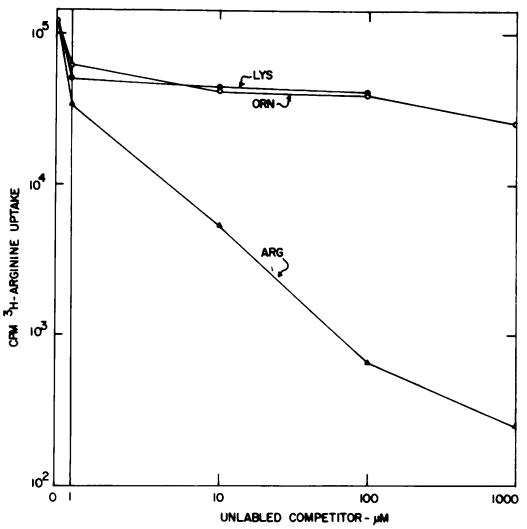

FIG. 4. *Competition for [^{3}H]arginine uptake by unlabeled arginine, lysine, and ornithine. Aliquots of culture incubated for 30 s with a mixture of 1 μM [3-^{3}H]arginine with and without different concentrations of the unlabeled competitors in HEPES-minimal broth, 25°C. At 30 s, 100 μl of culture was Millipore filtered and counted as described in the text for total uptake.*

which stimulates only slightly (19), other amino acids lack the ability to stimulate bioluminescence.

Amino acid pools. The amino acid pools of *B. harveyi* do not appear significantly different from amino acid pools of other gram-negative bacteria (15, 18). Glutamate represents by far the major amino acid in the total amino acid pools. With *B. harveyi* cells grown in minimal media, glutamate constitutes about 82% of the free amino acids, whereas in complex media the glutamate pool is about 30% of the total. However, in complex media the total amino acid pool was roughly 260 nmol/10^9 cells (average, peptone-grown cells, Table 2) compared with minimal media in which the pool size was about 110 nmol/10^9 cells. By taking cell volume into consideration, the total pool sizes are roughly equivalent because the cells growing in minimal media are smaller than those growing in peptone media. Bacteria are known to utilize the amino acids glutamate, proline, and gamma-aminobutyrate for osmoregulation (10). The decrease in the glutamate pool of cells in complex media compared with minimal media probably represents a regulatory change in response to the overall increase in the levels of other amino acids, thereby providing a balance in the total internal solute concentration. In *B. harveyi* the pool level of total free amino acids is different

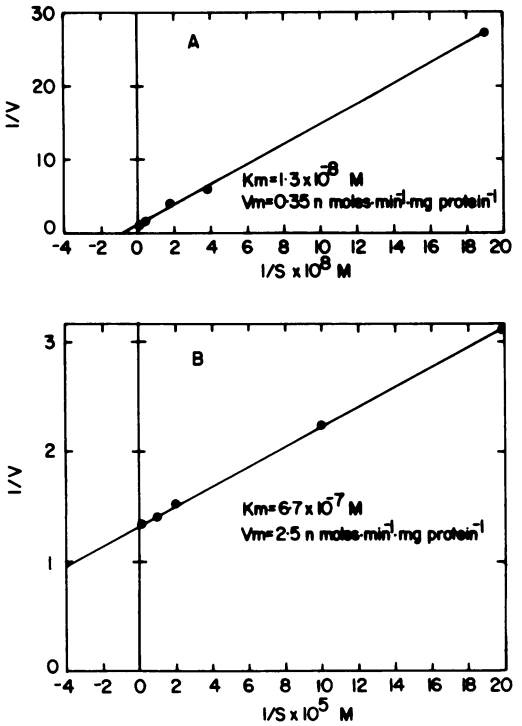

FIG. 5. *Lineweaver-Burk plots of the kinetics of arginine uptake in B. harveyi in HEPES-minimal broth. (A) The high-affinity system. (B) The low-affinity system. Substrate concentration in molar, velocity in picomoles of substrate taken up in 30 s. Experiments were done as described in the text.*

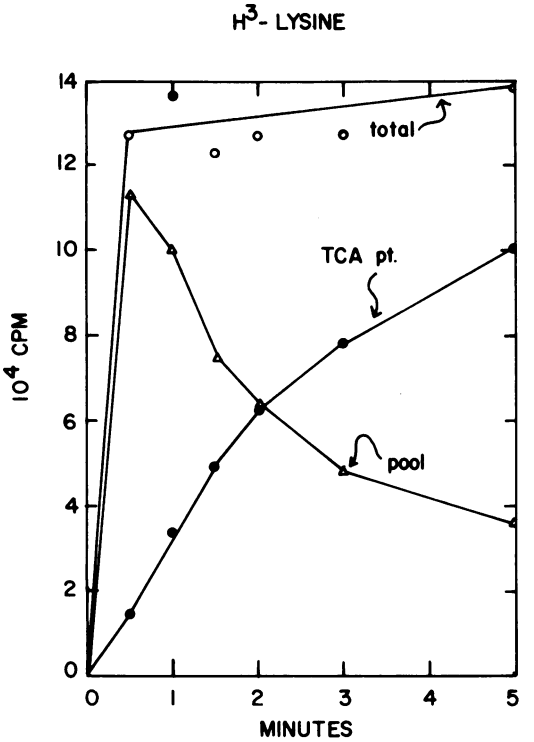

FIG. 6. *Uptake of labeled lysine into B. harveyi growing in HEPES-minimal. The culture was tested in mid-autoinduction, 2.6 × 10⁸ cells per ml, 3.4 × 10⁸ quanta·s⁻¹·ml⁻¹. The final [4,5-³H]lysine concentration was 0.16 μM. Duplicate 100-μl portions were sampled at the times indicated as described in the text. The lysine pool is the calculated difference between the total incorporation and the incorporation into trichloroacetic acid-(TCA) precipitable material.*

depending on external NaCl concentration; this involves primarily a change in the glutamate pool size (9).

Transport of basic amino acids. In *E. coli* there are four distinct transport systems for basic amino acids: one each for arginine, lysine, and ornithine, and a high-affinity system capable of transporting all three basic amino acids (5, 17). Experimental evidence for these systems is based on biphasic kinetics of transport and on competitive inhibition of transport in cells of the wild type and transport-defective mutants (3–5, 17). In *B. harveyi* there appear to be two transport systems for lysine and two for arginine, but only one for ornithine. The system common to all three (lysine-arginine-ornithine general permease system [LAO system]) is probably the high-affinity system, since the transport of lysine and arginine is inhibited at low concentrations, but the inhibition is not increased by high concentrations of competitors. Ornithine transport seems to occur only through the LAO system, since it can be completely inhibited by arginine and lysine. This might also be explained by a lack of ornithine-specific binding protein(s) and having ornithine transport via two permeases obligatorily coupled through an LAO binding protein. However, because the ornithine transport system binds arginine and lysine more tightly than ornithine, this suggests, but does not prove, that ornithine transport occurs through the LAO system.

Arginine appears to be transported by two systems, both of which are stereospecific: a low-affinity, high-velocity system specific for arginine and a high-affinity, low-velocity general system also capable of transporting lysine and ornithine. All systems are synthesized in cells growing in minimal media and therefore appear constitutive. Peptone-grown cells transport arginine with similar kinetics (data not shown).

Arginine metabolism. A scheme depicting the pathways postulated for arginine metabolism in *B. harveyi* is presented in Fig. 12. Argi-

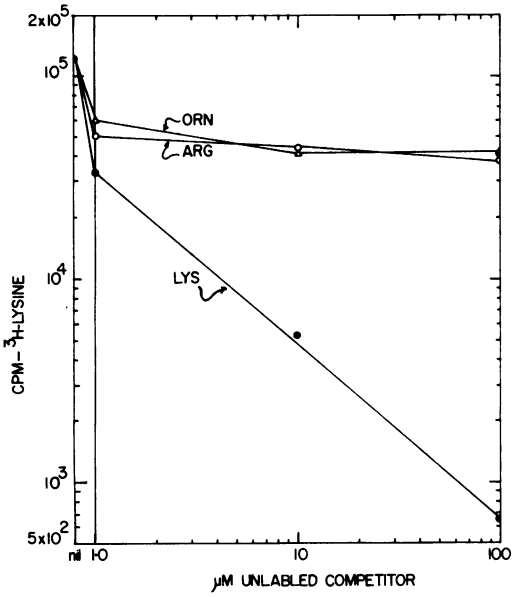

FIG. 7. *Competition of [^{3}H]lysine uptake by unlabeled lysine, arginine, and ornithine. The experiment was done as described in the legend to Fig. 4; the concentration of [4,5-^{3}H]lysine was 0.16 μM.*

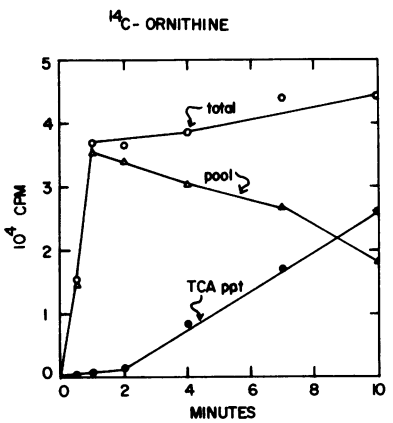

FIG. 8. *Uptake of uniformly labeled [^{14}C]ornithine into B. harveyi growing in HEPES-minimal. The culture contained 4 × 10^8 cells per ml and produced 2.8 × 10^8 quanta·s^{-1}·ml^{-1} and was in mid-autoinduction of the bioluminescent system. The experiment was done as described in the legend to Fig. 3. TCA, trichloroacetic acid.*

nine is probably synthesized from glutamate since a pulse of labeled ornithine will label the citrulline and argininosuccinate pools. Arginine biosynthetic precursors are detectable in the extracted amino acid pools (ornithine, citrulline, and argininosuccinate), and they are present in substantial and measurable quantities in cells grown on both minimal and peptone media. Therefore, the precursors of arginine (glutamate, ornithine, citrulline, and argininosuccinate) are probably not mediators of the arginine effect because they are present in substantial concentrations in cells grown in minimal media or peptone media.

TABLE 4. *Apparent K_m, V_m, and inhibitors of basic amino acid transport in B. harveyi*

Amino acid	K_m (M)	V_m (nmol transported·min^{-1}·mg of cell protein^{-1})	Inhibitor
Arginine			
System 1	0.7×10^{-8}–1.3×10^{-8}	0.35	Lysine, ornithine
System 2	3.3×10^{-7}–6.7×10^{-7}	2.5	
Lysine			
System 1	1.1×10^{-8}	0.54	Arginine, ornithine
System 2	5.2×10^{-7}	11.5	
Ornithine			
System 1	3.0×10^{-7}	2.15	Arginine, lysine

TABLE 5. *Arginine pool of B. harveyi*[a]

Labeled arginine added (μM)	Maximum pool size (nmol of arginine/10^9 cells)
100	100
500	145
1000	299
5000	676

[a] Calculated by the method of Britton and McClure (2). The maximum pool size was reached within 10 min; the samples from each [^{14}C]arginine concentration used were taken at 0.5, 1, 2, 5, 10, and 15 min after addition of the label. Pool size was calculated based on the difference of counts between the total uptake and those in the trichloroacetic acid precipitate and taking into account the specific activity of the label.

B. harveyi cells clearly maintain a very low internal level of arginine: in minimal media the arginine cannot be detected and must therefore exist as a free pool at a level lower than 0.01 nmol/10^9 cells. This must involve keeping the rate of cleavage of argininosuccinate to arginine and fumarate very low. It also must involve the conversion of any excess arginine to citrulline (arginine deiminase). That this occurs is indicated by the rapid intracellular conversion of labeled arginine to citrulline. Pulse-chase studies showed that the citrulline so formed is eventually converted to argininosuccinate and finally into trichloroacetic acid-precipitable material.

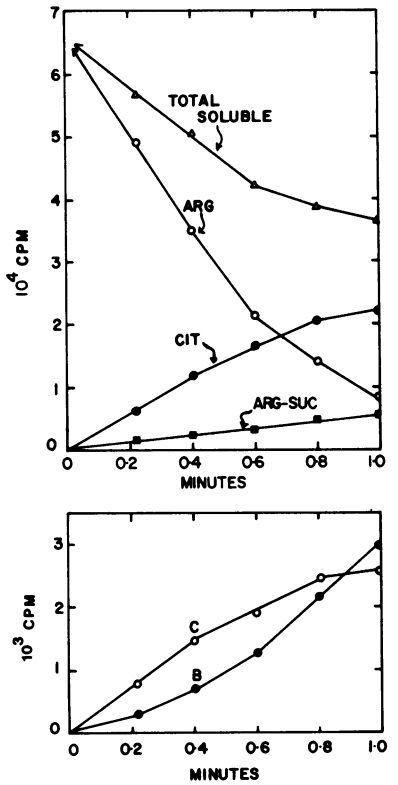

FIG. 9. *Labeling of intracellular amino acid pools by exogenously added [^{3}H]arginine. [3-^{3}H]arginine was 1 μM. Samples (100 μl) were removed, rapidly filtered, washed, and immediately immersed into boiling 80% ethanol at the times indicated; processing time was 10 to 15 s/sample. The analysis of the samples was done as described in the text. The culture was grown in HEPES-minimal and contained 3.9 × 10^8 cells per ml and was producing 1.6 × 10^9 quanta·s^{-1}·ml^{-1}.*

This implies that the charging of arginyl tRNA must be able to operate efficiently at very low internal arginine concentrations. Even with the very low level of arginine in cells grown in minimal media, these cells are not arginine auxotrophs nor is their growth in minimal medium stimulated by exogenous arginine.

The arginine effect (stimulation of bioluminescence and luciferase synthesis) seems to be ascribable to arginine. Cells growing in peptone media do not display the arginine effect and contain a substantial (2 to 4 nmol/10^9 cells) arginine pool. Cells growing in minimal media appear to maintain a very small arginine pool (up to now below the limits of detection) by converting excess arginine immediately to citrulline and then to argininosuccinate. Consequently, a low, unmeasurable arginine pool is maintained.

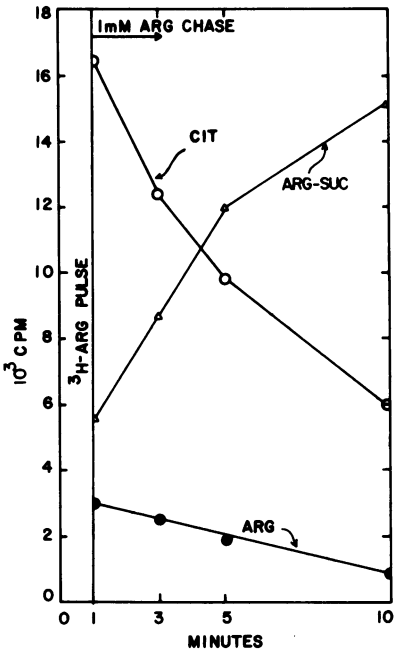

FIG. 10. *Pulse-chase experiment. B. harveyi growing in HEPES-minimal were pulsed with 1 μM [3-^{3}H]arginine at time zero and chased at 1.0 min with 1 mM cold arginine. Samples were taken at the times indicated and treated as described in the legend to Fig. 9. The culture contained 4.2 × 10^8 cells per ml which were producing 1.6 × 10^9 quanta·s^{-1}·ml^{-1}.*

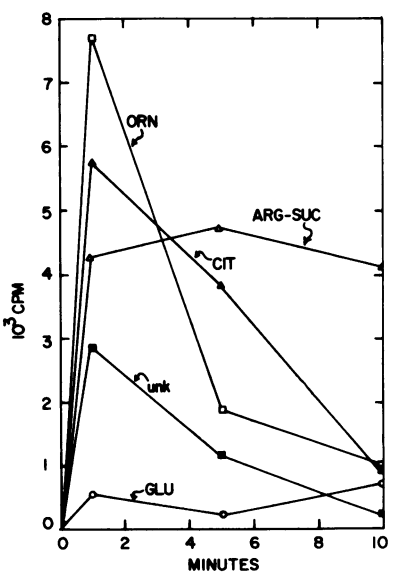

FIG. 11. *Labeling of intracellular amino acid pools with uniformly labeled [^{14}C]ornithine. Samples were treated as described in the legend to Fig. 9. B. harveyi was grown in HEPES-minimal; the culture contained 4.9 × 10^8 cells·ml^{-1} producing 6.7 × 10^8 quanta·s^{-1}·ml^{-1}.*

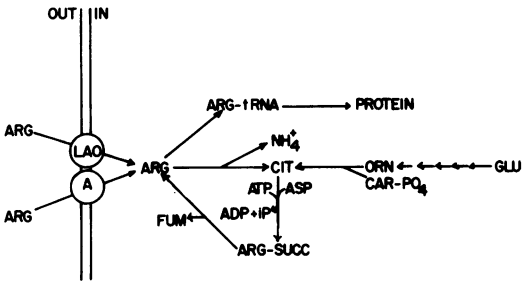

FIG. 12. *Model of arginine metabolism in B. harveyi. GLU, glutamate; ORN, ornithine; CAR-PO_4, carbamyl phosphate; CIT, citrulline; ATP, adenosine triphosphate; ASP, aspartate; ADP, adenosine diphosphate; ARG-SUCC, argininosuccinate; FUM, fumarate; ARG, arginine; A, arginine-specific permease; ARG-tRNA, arginyl tRNA.*

If arginine (or arginyl tRNA) is the mediator of the arginine effect, the cells growing prototrophically appear able to respond to the presence of exogenous arginine by maintaining a small arginine pool. Unlike other amino acid pools, the arginine pool remains relatively small (2 to 4 nmol/10^9 cells) even when the cells are supplied exogenously with high levels of the amino acid. In contrast, when the cells are supplied with ornithine or citrulline, these amino acids are substantially accumulated.

The maintenance of the low arginine pool and the concomitant arginine effect may be of some function as a sensory mechanism for nutritional (organic nitrogen) abundance. Even though *B. harveyi* can grow prototrophically, it does so slowly and its growth is greatly enhanced by complex organic nitrogen sources. Not only is the growth enhanced, but also the synthesis of luciferase and consequent production of bioluminescence is greatly enhanced by organic nitrogen sources. Arginine appears to be the amino acid responsible for the stimulation of both luciferase synthesis and the in vivo bioluminescence. The actual mediator, at the transcriptional level, could be arginine or arginyl tRNA; with the data presently available, we cannot distinguish between these possibilities.

ACKNOWLEDGMENTS

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