Urea Production and Putrescine Biosynthesis by *Escherichia coli*

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Cultures of *Escherichia coli* B and K-12 produce urea during growth in minimal medium. Results of isotopic labeling experiments were consistent with the sole source of urea being from the conversion of arginine to putrescine. Since *E. coli* itself has no demonstrable urease activity, the rate of urea production is a measure of the flux through the arginine to putrescine pathway.

A previous report demonstrated the presence of two major pathways of putrescine biosynthesis in *Escherichia coli* grown in minimal medium (8). The first route of putrescine biosynthesis is the decarboxylation of ornithine.

$$NH_2(CH_2)_3\overset{\overset{\displaystyle NH_2}{|}}{C}HCOOH \quad (1)$$
$$\rightarrow \quad NH_2(CH_2)_4NH_2 \quad + \quad CO_2$$

The second pathway is from arginine to putrescine.

$$NH_2\overset{\overset{\displaystyle NH}{||}}{C}NH(CH_2)_3\overset{\overset{\displaystyle NH_2}{|}}{C}HCOOH \quad (2)$$
$$\rightarrow NH_2\overset{\overset{\displaystyle NH}{||}}{C}NH(CH_2)_4NH_2 + CO_2$$

$$NH_2\overset{\overset{\displaystyle NH}{||}}{C}NH(CH_2)_4NH_2 + H_2O \quad (3)$$
$$\rightarrow NH_2(CH_2)_4NH_2 + NH_2\overset{\overset{\displaystyle O}{||}}{C}NH_2$$

Considering the second pathway, one would predict that whole cells of *E. coli* produce 1 mole of urea per mole of putrescine synthesized via this route (reaction 3). Møller demonstrated that *E. coli*, grown under conditions of amino acid catabolism, carried out the conversion of agmatine to putrescine and urea (6). This experiment, however, was measuring the pathway of arginine degradation rather than a putrescine biosynthetic function (*see* 7 and 8). To test the above prediction for the biosynthetic arginine to putrescine pathway, we examined the production of urea in cultures of *E. coli* growing in

minimal medium. Urea synthesis was observed at a rate consistent with the above interpretation. In addition, isotopic labeling experiments suggested that the sole source of urea is from reaction 3. These results form the basis of a technique to measure the in vivo flux through the arginine to putrescine pathway without disturbing the metabolic balance of the cells.

MATERIALS AND METHODS

Growth of bacteria. These studies were performed with wild-type *E. coli* B and *E. coli* K-12, strains 3000 and 30SO-arg 4 (from Werner Maas). The arginine-requiring mutant, 30SO-arg 4, is blocked between *N*-acetylglutamic acid and *N*-acetylglutamic semialdehyde. Growth was routinely carried out with vigorous shaking at 37 C in M63 medium described by Cohen and Rickenberg (2). The medium was routinely supplemented with thiamine (0.5 µg/ml), trace elements (1), and dextrose (0.2%). In some experiments, arginine or an arginine precursor was supplied at a concentration of 50 µg/ml.

To measure urea production, samples of overnight cultures were centrifuged, washed, and resuspended in fresh experimental medium. The cultures were incubated until exponential growth occurred. Samples were then again centrifuged, washed, and resuspended in experimental medium at a level of 10^8 cells/ml. The cultures were then routinely grown into late exponential phase (10^9 cells/ml). After centrifugation, the cultures were analyzed for urea as described below.

Preparation of urea samples. To analyse urea in growth media, cultures were centrifuged and 0.26 ml of 70% perchloric acid was added to 20-ml samples of the supernatant culture fluid. The suspensions were filtered after standing overnight at 0 C. The filtrates were then passed through columns of Bio-Rad AG 50W-X8 (H⁺), and the urea-containing fractions were evaporated to dryness in a rotary evaporator. The residues were resuspended in 2 ml of 0.3 M potassium phosphate buffer, pH 7.0, and the solution was decolorized with activated charcoal.

To extract urea from cells, the pellet obtained from the above centrifugation was extracted with 0.3 N trichloroacetic acid as described by Dubin and Rosenthal (3). The protein precipitate was removed by centrifugation. The trichloroacetic acid supernatant solution was extracted three times with 1 volume of ether, and then passed over a column and concentrated as described above. The ether extraction resulted in the transfer of less than 1% of the urea into ether phase.

Urea determination: Assay I. Samples prepared as above were incubated with and without urease (0.8 mg of crude urease per ml) for 30 min at 25 C. Color was developed by the procedure of Gerhart and Pardee (4) for carbamyl groups and the number of micromoles of urease-sensitive material was calculated. *Assay II.* Samples were incubated with and without urease as in assay I. The ammonia produced was distilled and trapped in Conway microdiffusion dishes and analyzed with Nessler's reagent (5).

Incorporation of radioactive precursors into urea. Exponentially growing cultures of 30SO-arg 4 were centrifuged, washed, and resuspended in M63 medium containing 0.05% dextrose plus the indicated amino acid. The specific activity of the guanidino-^{14}C-arginine was 161,000 counts per min per μmole, and the specific activity of the uniformly labeled ^{14}C-glucose was 105,000 counts per min per μg atom of carbon. Cultures of 10 ml each were grown in the presence of the radioactive precursor from an initial density of 5 $\times$ 10^7 cells/ml to a final density of 5 $\times$ 10^8 cells/ml. These cultures were centrifuged and urea samples were prepared as described above. Samples were incubated with and without urease as in assay I. After incubation, trichloroacetic acid was added to a final concentration of 5%, and the radioactive CO_2 was trapped and counted as previously described (7). The samples were then analyzed for ammonia as in assay II, and the amount of urea was determined. The specific activity of the urea was then calculated from the radioactivity in CO_2 and the amount of urea.

Determination of urea hydrolysis by E. coli. For determination of urea hydrolysis by whole cells, exponential cultures of strain 3000 (3 $\times$ 10^8 cells/ml) were centrifuged, washed, and resuspended in fresh medium containing 10^{-3} M ^{14}C-urea (7.4 $\times$ 10^5 counts per min per μmole). The cultures were incubated for 1 hr at 37 C. After incubation, they were diluted to 10% trichloroacetic acid and the radioactive CO_2 was trapped and counted (7).

Materials. Uniformly labeled ^{14}C-D-glucose was obtained from New England Nuclear Corp., Boston, Mass. Guanidino-^{14}C-arginine and ^{14}C-urea were obtained from Volk Radiochemical Corp., Burbank, Calif. Before use, ^{14}C-urea was made slightly acid and bubbled with nitrogen to remove contaminating radioactive CO_2. Urease (crude grade) was purchased from Worthington Biochemical Corp., Freehold, N.J.

RESULTS

Production of urea by E. coli. Samples of media from growing cultures of wild-type *E. coli* K-12 and B were examined for urea. The results in Table 1 show that both strains of *E. coli* produced

TABLE 1. *Production of urea by strains of Escherichia coli*

Strain	Culture medium	Urea[a]	
		Assay I	Assay II
3000	Minimal	10	11
3000	Minimal + arginine	38	41
B	Minimal	3	—
B	Minimal + arginine	65	—

[a] Expressed as nanomoles/10^9 cells.

urea. The agreement between assays I and II demonstrates that there were 2 moles of ammonia produced per mole of urease-sensitive carbamyl groups. This definitely establishes the identity of the compound produced as urea. Urea production was stimulated by supplementation of the culture medium with arginine.

The urea determinations in Table 1 were performed on centrifuged culture media. Cells in various growth stages were examined for the presence of intracellular urea. No urea was found by an assay which could have detected as little as 0.5 nmoles of intracellular urea per 10^9 cells. Therefore, essentially all of the urea produced was excreted into the medium.

Figure 1 illustrates the time course of urea production by *E. coli* K-12 growing in the presence and absence of arginine. The rate of urea synthesis is approximately fourfold higher in the arginine-supplemented culture, in agreement with the results in Table 1.

Origin of urea. To determine whether the sole source of urea was from arginine, incorporation of labeled precursors into urea was studied by use of an arginine-requiring mutant of *E. coli* K-12. The results are summarized in Table 2. When the cells were grown in the presence of guanidino-^{14}C-arginine, label was incorporated into urea with high efficiency. Cells grown in media supplemented with radioactive glucose and unlabeled arginine incorporated no radioactivity into urea. These results indicate that far less than 1% of the urea could be produced from a precursor other than arginine. The high level of incorporation with cells grown in ^{14}C-glucose and unlabeled ornithine is due to the production of the carbamyl group of arginine from carbamyl phosphate. The low level of incorporation in the presence of citrulline could be due to small amounts of contamination of citrulline by ornithine, equilibration of intracellular ornithine and citrulline pools, or low level functioning of an alternate route of urea synthesis which does not function in the presence of arginine. The
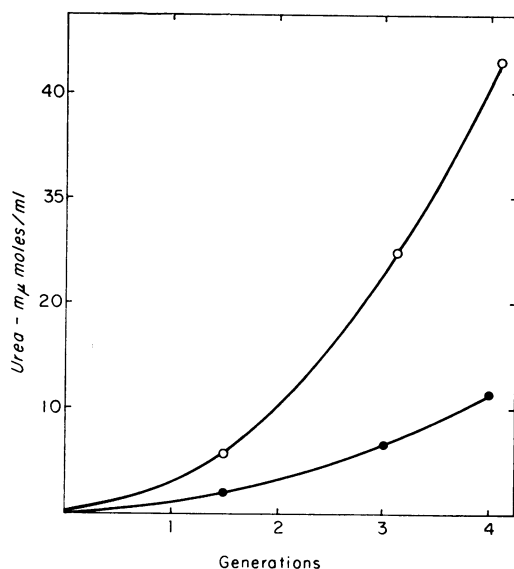
Fig. 1. *Time course of urea formation by Escherichia coli K-12, strain 3000. Symbols:* ●, *unsupplemented medium;* ○, *arginine-supplemented. Cultures were started at an initial density of 10^8 cells/ml and grew with a generation time of 1 hr.*

TABLE 2. *Incorporation of radioactive precursors into urea*

Culture conditions	Urea specific activity (counts per min per μmole)
^{14}C-Arginine...............	147,000
^{14}C-Glucose + ornithine.....	45,200
^{14}C-Glucose + citrulline.....	1,090
^{14}C-Glucose + arginine......	0

present results do not distinguish among these possibilities.

Urease in E. coli. To understand fully urea metabolism in *E. coli*, cells were examined for capacity to hydrolyze urea. The test for urea hydrolysis was a sensitive one which involved the measurement of radioactive CO_2 production from ^{14}C-urea. No hydrolysis of urea was observed in cultures growing in the presence of 10^{-3} M urea. This result was found in cultures with and without arginine supplementation. The test could have detected the hydrolysis of 0.2 nmoles of urea in 1 hr by 1 ml of a culture containing 5×10^8 cells. Cell-free extracts of cells grown with and without arginine were also examined for urease activity. Under conditions where a specific activity of 0.02 nmoles per mg of protein per hour could have been detected, no urea hydrolysis was observed. From these results, it may be concluded that, if *E. coli* hy-

drolyzes urea, the rate is insignificant compared to the rate of production.

DISCUSSION

As predicted from in vitro studies, whole cells of *E. coli* produce urea. A great proportion of the urea produced is excreted into the medium, leaving an insignificant intracellular pool. The major source of urea is the guanidino group of arginine. From studies on incorporation of uniformly labeled glucose and guanidino-labeled arginine into urea, it can be concluded that in cells growing with arginine supplementation, more than 99% of the urea is formed from arginine. This does not rule out the possibility of an alternate pathway in cultures growing in the absence of arginine. However, this is highly unlikely since the presence or absence of arginine would not be expected to affect urea production by an unrelated pathway.

The lack of urease activity in both whole cells and in cell-free extracts indicates that there is not significant urea degradation by *E. coli*. This fact, and the evidence suggesting that *E. coli* contains no major pathway of urea production other than from arginine, indicates that the amount of urea produced is a measure of the number of moles of putrescine produced via the arginine to putrescine pathway (reactions 2 and 3). In support of this, the amount of urea produced by *E. coli* K-12 growing in the presence of arginine and the amount of putrescine synthesized under these same conditions (D. R. Morris and K. L. Koffron, *unpublished data*) are identical (40 nmoles per 10^9 cells). These considerations suggest a method for determining the relative in vivo rates through the two pathways of putrescine synthesis. Since the rate of urea production reflects the rate of functioning of the arginine to putrescine pathway (reactions 2 and 3), the difference between this rate and the total rate of putrescine production gives one the rate of functioning of the ornithine to putrescine pathway (reaction 1). By this technique, one can study the regulation of these two pathways in vivo. These studies are presently being carried out.

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