Multiple Pathways of Putrescine Biosynthesis in Escherichia coli

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SUMMARY

Two routes of putrescine biosynthesis exist in Escherichia coli both emanating from the latter portion of the arginine-biosynthetic pathway. Isotope competition experiments indicate that both arginine and ornithine can give rise to putrescine. The formation of putrescine from ornithine is independent of the conversion of the latter to arginine.

The decarboxylation of ornithine to yield putrescine is catalyzed by cell-free preparations. This enzyme has been termed "biosynthetic" ornithine decarboxylase to distinguish it from the inducible degradative ornithine decarboxylase, from which it differs in many properties.

Cell-free extracts also carry out the conversion of arginine to putrescine. In this pathway, the arginine carboxyl group is first released as CO₂, and the amidine group is then removed as urea. The arginine decarboxylase has optimal activity at pH 8 and an absolute magnesium ion requirement.

Both of these characteristics distinguish it from the catabolic arginine decarboxylase. Agmatine ureohydrolase, the enzyme catalyzing the second step of this pathway, is also present in cell-free extracts.

Arginine biosynthesis in E. coli involves a branched pathway, the branch point to putrescine and arginine occurring at ornithine. The existence of two pathways of putrescine biosynthesis is interpreted in relation to control mechanisms of the arginine biosynthetic pathway.

The polyamines are found in almost all living cells. In many, they are a major low molecular weight component. Spermidine, 
\[ \text{NH}_2\text{(CH}_2\text{)}_3\text{NH(CH}_2\text{)}_2\text{NH}_2 \], is found in bacteria, fungi, and animals (2, 3). Spermine, 
\[ \text{NH}_2\text{(CH}_2\text{)}_3\text{N(CH}_2\text{)}_4\text{NH(CH}_2\text{)}_2\text{NCH}_2\text{NH}_2 \], has been reported in only one bacterial species (4), but is commonly found in animals and fungi (2, 3). Putrescine, 
\[ \text{NH}_2\text{(CH}_2\text{)}_4\text{NH}_2 \], a metabolic precursor of spermidine and spermine (5, 6), has been extracted from gram-negative bacteria and fungi (3). These compounds are presumed to play an important metabolic role because of their broad distribution in high concentration and because certain microorganisms require them for growth (7-9). Little is known of their physiological function, however.

To study the function of these compounds, one should be able to control their intracellular concentration. With this in mind, we have initiated a study of the biosynthesis of putrescine, the basic unit of the polyamines. In this paper, we will show that in Escherichia coli there are two major pathways of putrescine biosynthesis under nonspecialized culture conditions. The first route of putrescine biosynthesis is the decarboxylation of ornithine (1). The second pathway is from arginine to putrescine, independent of ornithine. This conversion appears to involve agmatine (decarboxylated arginine) as an intermediate.

EXPERIMENTAL PROCEDURE

Growth of Bacteria—These studies were performed with strain 3080-arg 4 of E. coli K-12 (obtained from Dr. W. Maas). This is an Hfr strain which requires arginine because of a block between N-acetylglutamic acid and N-acetylglutamic semialdehyde. This organism was routinely grown at 37° with vigorous shaking in minimal medium (Medium E, described by Vogel and Bonner (10)). The medium was supplemented with trace elements (11), thiamine (0.5 ug per ml), and arginine (20 ug per ml) or ornithine (20 ug per ml).

Extraction and Separation of Polyamines—The cells from logarithmic phase cultures (approximately 5 X 10⁸ cells per ml) were harvested by centrifugation and washed once with 0.9% NaCl at 0°. The cells were then extracted with 0.3 N trichloracetic acid as described by Dubin and Rosenthal (12). The protein precipitate was removed by centrifugation and was saved for analysis. After the trichloracetic acid supernatant solution was extracted three times with 2 volumes of ether, it was applied to a column (1.4 X 20 cm) of Bio-Rad AG 50W-X8 (100 to 200 mesh, hydrogen form). The first elution was by a linear gradient formed with 400 ml of water in the mixing chamber and 400 ml of 2.5 N HCl in the reservoir. A typical elution pattern of a mixture of putrescine and basic amino acids is shown in Fig. 1. Lysine was the only amino acid which cochromatographed with putrescine under these conditions. Agmatine was eluted after arginine. The neutral and acidic amino acids were eluted in the earlier fractions. After completion of the gradient elution, 4 N HCl was passed through the column to elute spermidine. The putrescine- and spermidine-containing fractions were located.
Putrescine Biosynthesis

FIG. 1. Ion exchange chromatography of putrescine and basic amino acids. A mixture of 1 mg each of 14C-putrescine-2HCl (200,000 cpm), 14C-ornithine-HCl (480,000 cpm), 14C-arginine-HCl (350,000 cpm), 14C-lysine-HCl (100,000 cpm), and 14C-diaminopimelic acid (220,000 cpm) was applied to a column of Bio-Rad AG 50W and eluted as described in the text. Samples of approximately 5 ml each were collected, and 0.1-ml aliquots were counted. The radioactive peaks were identified by thin layer chromatography. Peak 1 contained ornithine and diaminopimelic acid, Peak 2 contained putrescine and lysine, and Peak 3 was composed solely of arginine.

either by radioactivity measurements or by amine analysis. The pooled putrescine and spermidine fractions were concentrated on a rotary evaporator and were decolorized with Darco G-60. These pooled fractions were then analyzed for radioactivity and amine content.

Estimation of Amines—Aliquots of the putrescine- and spermidine-containing column fractions were evaporated under reduced pressure over NaOH. The amine content of these fractions was then determined by formation of their dinitrophenyl derivatives (13).

Protein Analysis—The precipitates from the trichloracetic acid extractions were dissolved in 0.5 N NaOH to give a protein concentration of approximately 3 to 5 mg per ml. An aliquot of this solution was then diluted in 0.5 N NaOH and was analyzed for protein content by the method of Lowry et al. (14). The remainder of the NaOH solution was saved for radioactivity analysis.

Thin Layer Chromatography—Thin layer chromatography of putrescine and spermidine was carried out on cellulose layers 0.25 mm thick. A solvent consisting of 1-butanol, acetic acid, pyridine, and water (4:1:1:2) was used for development. After development, the plates were dried at room temperature, sprayed with 0.5% ninhydrin in 70% ethanol, and heated at 100° for 5 min. With the use of this solvent system, a separation of arginine, ornithine, agmatine, putrescine, and spermidine was effected.

14C Determinations—For the determination of radioactivity present in the HCl effluents from the ion exchange columns, aliquots were placed in scintillation vials and evaporated under reduced pressure over NaOH. The residues were redissolved in 0.1 ml of water, and 15 ml of Bray's scintillation fluid (15) were added to each vial. The samples were counted in a Packard scintillation counter.

The amount of radioactivity incorporated into protein was measured by counting 5 μl of the NaOH solution of the trichloracetic acid precipitate. This quantity of 0.5 N NaOH in 15 ml of scintillation fluid did not quench.

For the determination of radioactivity on thin layer plates, the radioactive spots were excised and placed in scintillation vials with 15 ml of Bray's solution (15). The observed counts were corrected for 30% quenching.

Radioactive CO2 was trapped and counted as previously described (1).

Cell-Free Extracts and Enzymatic Assays—The preparation, dialysis, and assay of ornithine decarboxylase have been previously described (1). A unit of ornithine decarboxylase activity is defined as that quantity of enzyme which catalyzes the formation of 1 μmole of CO2 per min under the standard assay conditions.

For the preparation of extracts capable of catalyzing the conversion of arginine to putrescine, the cells were ground for seven minutes in a mortar with alumina (approximately 2.5 times bacterial weight). The mixture was suspended in 0.05 M Tris HCl, pH 7.6, containing 10-3 M EDTA and 10-4 M dithiothreitol it was centrifuged twice for 15 min at 18,000 × g. The supernatant solution was used as a source of enzymes. When it was necessary to remove low molecular weight cofactors, 2 ml of the supernatant solution were passed through a column (1.4 × 13 cm) of coarse Sephadex G-25 which had been equilibrated with the extraction buffer.

The reaction mixture for the enzymatic conversion of arginine to putrescine contained 30 μmoles of Tris-HCl buffer (pH 7.5) 2.22 μmoles of arginine-U-14C (7.75 × 10^6 cpm per μmole 0.012 μmole of pyridoxal phosphate, and 0.60 μmole of magnesium sulfate, in a total volume of 0.30 ml. The reaction was stopped, after incubation at 37°, by the addition of 0.02 ml of 100% (w/v) trichloroacetic acid.

For the determination of agmatine ureohydrolase activity, cell-free extracts were prepared as described above for the conversion of arginine to putrescine. The assay mixture contained 30 μmoles of Tris-HCl, pH 7.5, and 2.2 μmoles of agmatine sulfate, in a total volume of 0.30 ml. The reaction was started by the addition of enzyme. After incubation at 37° for 10 min 0.10 ml of 7% perchloric acid was added. The protein precipitate was removed by centrifugation. To the supernatant solution were then added 0.10 ml of 3 N sodium acetate, pH 5, and 0.25 ml of 2,3-butanedione reagent (Hycel blood urea nitrogen reagent). After they were heated in a boiling water bath for 2 min, the tubes were placed on ice for 3 min. To each tube were added 0.5 ml of water, and the absorbance at 470 μm was determined. The amount of urea produced was determined by comparison with a standard curve. The range of this assay was between 0.2 and 0.8 μmole of urea. A unit of agmatine ureohydrolase activity is defined as that amount of enzyme which catalyzes the formation of 1 μmole of urea in 1 min under the standard assay conditions.

Materials—Ornithine-5-14C, arginine-5-14C, and lysine-U-14C were obtained from Calbiochem. Arginine-U-14C was purchased from Volk. Ornithine-U-14C was synthesized as previously described (1) by the method of Rivard and Carter (16). Putrescine-1,4-14C and diaminopimelic acid-2,6-14C were purchased from New England Nuclear. Merck supplied the glucose-U-14C refer to the uniformly labeled compounds.

1 Arginine-U-14C, lysine-U-14C, ornithine-U-14C, and glucose U-14C refer to the uniformly labeled compounds.

2 This assay is a modification of the agmatine assay recommended by Worthington.
RESULTS

Conversion of Labeled Precursors to Putrescine—Studies by Tabor, Rosenthal, and Tabor demonstrated that 14C-ornithine is incorporated into the putrescine residue of spermidine in intact E. coli (6). These results indicate that putrescine arises from an intermediate in the arginine-biosynthetic pathway. Table I confirms and extends the experiments of these workers. The first two experiments show that both ornithine and arginine are converted to putrescine with equal efficiency, the specific activities of putrescine and spermidine in both cases being equal to that of the intracellular arginine pool, measured by the radioactivity of the arginine in cell protein.

Since arginine is converted to putrescine in Experiment 2, two possibilities exist for the conversion of ornithine to putrescine (Experiment 1): (a) conversion to arginine and then to putrescine or (b) direct decarboxylation to yield putrescine. The last two experiments in Table I were designed to distinguish between these two possibilities. If ornithine gave rise to putrescine solely by its conversion to arginine, the specific activity of putrescine and, therefore, spermidine would be expected to be equal to that of the arginine pool under all growth conditions. This was not the case. When the cells were grown in the presence of 14C-ornithine and unlabeled arginine (Experiment 3), the specific activities of the polyamines were much greater than that of the arginine pool. The low radioactivity content of the arginine pool in this experiment is due to the repression of the arginine biosynthetic enzymes by the arginine present in the culture medium (18-20). In the inverse experiment (No. 4), with cells grown on 14C-arginine and unlabeled ornithine, the specific activities of putrescine and spermidine were lower than that of the arginine pool. These experiments clearly show that E. coli has a mechanism for the conversion of ornithine to putrescine which does not involve arginine as an intermediate.

Although the above experiments show that putrescine can arise from intermediates of the arginine-biosynthetic pathway, it was necessary to exclude the possibility of an additional route to putrescine which is independent of arginine biosynthesis. To investigate this possibility, the incorporation of 14C-glucose into putrescine was studied with the use of the arginine-requiring mutant (Table II). In Experiments 1 and 2, the incorporation of 14C-glucose into polyamines was examined in cells grown in the presence of arginine and ornithine, respectively. In Experiment 3, cells were incubated with 14C-glucose during arginine starvation. The incorporation into putrescine was quite low in Experiments 1 and 2, being 3 and 1.5%, respectively, of the theoretical value. The small amount of radioactivity which did appear in the putrescine fraction was probably due to lysine, which cochromatographs with putrescine on ion exchange chromatography (Fig. 1). The radioactivity of the spermidine in these experiments was at the level expected for labeling of the 3-carbon unit which arises from methionine (6). It appears, therefore, that the sole source of putrescine in E. coli is from the pathway of arginine biosynthesis.

Cell-free Conversion of Ornithine to Putrescine—Since the labeling data in vivo indicated a direct conversion of ornithine to putrescine, ornithine decarboxylase activity was sought in cell-free extracts of E. coli (1). The liberation of CO2 from ornithine, catalyzed by a dialyzed crude extract, is shown in Fig. 2. The rate of CO2 evolution was proportional to the concentration of enzyme, and the reaction had an absolute requirement for pyridoxal phosphate. The K_m for ornithine in this reaction is approximately 1 X 10^-4 m.

To show that putrescine, in addition to CO2, was a product of

TABLE I

Incorporation of labeled precursors into putrescine in ornithine-requiring mutant of E. coli

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Specific activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine pool</td>
<td>Putrescine</td>
</tr>
<tr>
<td>1. 14C-Ornithine</td>
<td>3.28</td>
</tr>
<tr>
<td>2. 14C-Arginine</td>
<td>3.25</td>
</tr>
<tr>
<td>3. 14C-Ornithine + arginine</td>
<td>1.53</td>
</tr>
<tr>
<td>4. 14C-Arginine + ornithine</td>
<td>2.03</td>
</tr>
</tbody>
</table>

TABLE II

Incorporation of 14C-glucose into putrescine in ornithine-requiring mutant of E. coli

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Specific activities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Putrescine</td>
</tr>
<tr>
<td>1. 14C-Glucose + arginine</td>
<td>0.21</td>
</tr>
<tr>
<td>2. 14C-Glucose + ornithine</td>
<td>0.11</td>
</tr>
<tr>
<td>3. 14C-Glucose</td>
<td>--</td>
</tr>
</tbody>
</table>

* Putrescine content of the cells was below the limits of detection after arginine starvation.
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Fig. 2. Enzymatic decarboxylation of ornithine. The decarboxylation of ornithine was measured under the standard assay conditions. The curves represent C02 evolution from ornithine in the presence of 0.10 mg of a dialyzed crude extract of 30S0-arg 4 (●—●), in the absence of pyridoxal phosphate (△—△), in the absence of enzyme (○—○), and in the presence of 0.20 mg of the dialyzed extract (▲—▲).

Table III

Stoichiometry of ornithine decarboxylase reaction

The standard ornithine decarboxylase reaction mixture was incubated with 0.74 mg of a crude extract of 30S0-arg 4 for 60 min. The reaction was stopped with trichloroacetic acid, the C02 was trapped and counted, and the protein precipitate was removed by centrifugation. The supernatant solution was extracted three times with equal volumes of ether. Ornithine and putrescine were separated by thin layer chromatography, and the radioactive spots were removed from the plate and counted.

<table>
<thead>
<tr>
<th>Compound determined</th>
<th>+Enzyme</th>
<th>−Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Putrescine</td>
<td>+1.3</td>
<td>0.0</td>
</tr>
<tr>
<td>C02</td>
<td>+1.5</td>
<td>0.0</td>
</tr>
<tr>
<td>Ornithine</td>
<td>−1.5</td>
<td>0.0</td>
</tr>
</tbody>
</table>

the reaction, ornithine-U14C was incubated with a crude extract, and thin layer chromatography was performed on the reaction mixture. Putrescine was the sole nonvolatile ninhydrin-positive product of the reaction. It was found that 1 µmole each of C02 and putrescine formed per µmole of ornithine which disappeared (Table III).

These results show that cell-free extracts of an ornithine-requiring mutant of E. coli K-12 are capable of carrying out the conversion of ornithine to putrescine. Approximately the same levels of ornithine decarboxylase activity have been found in cells of wild type E. coli K-12 and E. coli B which have been grown in minimal medium (3.5 × 10⁻² and 3.2 × 10⁻² unit per mg of protein, respectively, as compared with 3.5 × 10⁻² unit per mg for 30S0-arg 4). These results show that this mode of putrescine biosynthesis is not peculiar to the E. coli mutant used in these studies.

Cell-free Conversion of Arginine to Putrescine—The labeling studies with intact cells reported in this paper showed that E. coli possesses a mechanism for the conversion of arginine to putrescine. Crude extracts of strain 30S0-arg 4 were examined for the ability to release C02 from arginine, a reaction which must take place regardless of the route of arginine conversion to putrescine. Fig. 3 shows that crude extracts do indeed catalyze the formation of C02 from arginine-U14C. The reaction, when carried out with Sephadex-treated extracts, showed an absolute requirement for magnesium and a partial pyridoxal phosphate requirement. As can also be seen from this figure, the kinetics of C02 evolution was not altered when unlabeled ornithine was included in the reaction mixture, suggesting that the latter compound is not an intermediate in this reaction. The reaction had optimal activity at pH 8 and a Kₘ for arginine of about 1 × 10⁻³ M.

In order to identify the products of this reaction, arginine-U14C was incubated with a cell-free extract, and the product was separated by ion exchange chromatography. The elution profile of radioactivity from the column is shown in Fig. 4. Putrescine and unreacted arginine were identified by thin layer chromatography and by their elution volumes from the column. Urea was identified by its stoichiometric degradation to CO2 and NH3 during incubation with urease (Table IV). The first peak of radioactivity from the column was an impurity present in the C14-arginine. The stoichiometry of the conversion of arginine-U14C to putrescine, urea, and CO2 is shown in Table V. It was found that 1 µmole each of putrescine, urea, and C02 formed per µmole of arginine which disappeared. The specific activities of the putrescine, urea, and recovered arginine were approximately equal to the theoretical values.

These results show that cell-free extracts of E. coli, strain 30S0-arg 4, are capable of converting arginine to putrescine. In this reaction, the carboxyl group of arginine is released as C02 and the amidine group is converted to urea. Putrescine and unreacted arginine were identified by thin layer chromatography and by their elution volumes from the column. A second peak of radioactivity from the column was an impurity present in the C14-arginine. The stoichiometry of the conversion of arginine-U14C to putrescine, urea, and CO2 is shown in Table V. It was found that 1 µmole each of putrescine, urea, and C02 formed per µmole of arginine which disappeared. The specific activities of the putrescine, urea, and recovered arginine were approximately equal to the theoretical values.

FIG. 3. Enzymatic liberation of C02 from arginine. The evolution of C14C02 was measured under the standard conditions for enzymatic conversion of arginine to putrescine. All reactions were run with 0.40 mg of Sephadex-treated crude extract of 30S0-arg 4. Curve 1 (O—O) represents C02 evolution in the complete reaction mixture, and Curve 2 (△—△) was measured in the presence of 2.22 µmoles of unlabeled ornithine. Curves (▲—▲) and 4 (●—●) were measured in the absence of pyridoxal phosphate and magnesium, respectively.
Degradation of enzymatically produced urea by urease

After ion exchange chromatography of the products of the arginine to putrescine conversion (Fig. 4), the urea-containing fractions were evaporated to dryness on a rotary evaporator and redissolved in water. Samples containing 0.8 μmole of urea (determined by the procedure outlined in "Experimental Procedure" for agmatine ureohydrolase activity) were incubated at room temperature with 100 μmoles of potassium phosphate buffer, pH 7.5, and 0.02 mg of urease, in a total volume of 0.3 ml. The reactions were stopped at the indicated times by the addition of 0.02 ml of 100% (w/v) trichloracetic acid. The 14CO₂ was trapped and counted. The protein was removed by centrifugation, and the supernatant solution was subjected to ion exchange chromatography by the method described in "Experimental Procedure." Fractions of 0.3 ml each were collected.

Table IV

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>CO₂</th>
<th>NH₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.44</td>
<td>0.84</td>
</tr>
<tr>
<td>20</td>
<td>0.59</td>
<td>1.17</td>
</tr>
</tbody>
</table>

Stoichiometry of conversion of arginine to putrescine

The combined column fractions from the experiment described in Fig. 4, in which 2.0 μmoles of 14C-arginine were supplied, were concentrated, and total radioactivity was determined. Urea was determined as in Table IV, and putrescine and arginine were estimated by the formation of the dinitrophenol derivatives (13). The specific activity of the 14CO₂ was assumed to be one-sixth that of the arginine-U-14C, for the calculation of the amount of CO₂ produced.

Table V

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount (μmoles)</th>
<th>Specific activity (cpm/g Atom C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO₂</td>
<td>14.2</td>
<td></td>
</tr>
<tr>
<td>Urea</td>
<td>14.4</td>
<td>13,800</td>
</tr>
<tr>
<td>Putrescine</td>
<td>13.2</td>
<td>10,300</td>
</tr>
<tr>
<td>Arginine remaining</td>
<td>3.6</td>
<td>12,050</td>
</tr>
</tbody>
</table>

Effect of agmatine on conversion of 14C-arginine to putrescine

A crude extract of 3080-arg 4 (9.7 mg of protein) was incubated for 30 min as described in Fig. 4, with and without the addition of 39 μmoles of unlabeled agmatine sulfate. Analysis of 14CO₂ and ion exchange chromatography were carried out as in Fig. 4. The counts in urea and agmatine were corrected for the 14C-urea (2000 cpm) and 14C-agmatine (1300 cpm) present in a zero time control. The results are expressed as micromoles of the indicated product arising from arginine-U-14C. This was calculated from the specific activity of the arginine-U-14C (7.75 × 10⁴ cpm per μmole).

Table VI

<table>
<thead>
<tr>
<th>Compound determined</th>
<th>From 14C-arginine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No addition</td>
</tr>
<tr>
<td>CO₂</td>
<td>2.90</td>
</tr>
<tr>
<td>Urea</td>
<td>2.04</td>
</tr>
<tr>
<td>Putrescine</td>
<td>1.96</td>
</tr>
<tr>
<td>Agmatine</td>
<td>1.52</td>
</tr>
</tbody>
</table>

Fig. 5. Agmatine ureohydrolase activity in E. coli extracts. Reaction mixtures containing 30 μmoles of Tris-HCl buffer (pH 7.5), 2.2 μmoles of agmatine, and 1.1 mg of a crude extract of 3080-arg 4 in a total volume of 0.3 ml were incubated at 37°C. The reactions were stopped at the indicated times by the addition of 0.02 ml of 100% (w/v) trichloracetic acid. After the removal of the protein precipitate by centrifugation, the reaction mixtures were analyzed for urea (Curve 1, △−△) by the method of Koritz and Cohen (23) and for the guanidino group of agmatine (Curve 2, ○−○) by the procedure of Conn and Davis (24). Reaction mixtures incubated in the absence of enzyme were also analyzed for urea (△−△) and agmatine (○−○).

Opposed to 1.3 × 10⁻² for 3080-arg 4). Therefore, this reaction is not a specific property of the mutant strain used in these studies.

Since, as indicated above, ornithine appeared not to be an intermediate, agmatine (decarboxylated arginine) was tested for a role in this conversion. The reaction was carried out in the presence and absence of a large excess of unlabeled agmatine (Table VI). With agmatine present, all of the counts were trapped in the agmatine pool as shown by little or no activity in urea and putrescine and by the equimolar amounts of CO₂ and agmatine produced from 14C-arginine. In the absence of unlabeled agmatine, a significant labeled agmatine pool appeared. This did not enter into the stoichiometry of the previous experiment (Table V) because it represented only 10% of the total arginine converted under the previous conditions. The 70% inhibition of CO₂ evolution in the presence of agmatine was probably due to product inhibition of the arginine decarboxylase.

Agmatine Ureohydrolase Activity in Cell-free Extracts—The
hydrolysis of agmatine to putrescine and urea was demonstrated in intact E. coli by Møller (22). Since agmatine appears to be an intermediate in the cell-free conversion of arginine to putrescine, extracts of 38S0-arg 4 were examined for agmatine ureohydrolase activity. These experiments are shown in Fig. 5. When agmatine was incubated with a cell-free extract, there was a rapid disappearance of the agmatine guanidino group (Curve 2). Concomitant with the disappearance of agmatine was the formation of urea (Curve 1). The reaction had no requirement for a divalent cation, as was the case with an analogous enzyme from mycobacteria (25). This enzymatic activity was present at a level of 0.08 unit per mg of protein. It exhibited a \( K_m \) of 2.5 \( \times 10^{-4} \) M for agmatine. Extracts of wild-type E. coli B and K-12 also contained comparable levels of this enzyme (specific activities of about 0.04 unit per mg in extracts of both strains).

**DISCUSSION**

In this paper, evidence is presented for the coexistence of two pathways of putrescine biosynthesis in cultures of E. coli grown in neutral minimal media. The existence of these pathways has been established both by radioactive labeling experiments in vivo and by the demonstration in vitro of the reactions in crude extracts. The most direct route of putrescine biosynthesis is the decarboxylation of ornithine (Equation 1).

\[
\text{NH}_3 + \text{NH}_4 \text{(CH}_3\text{)}_2\text{CHCOOH} \rightarrow \text{NH}_4 \text{(CH}_3\text{)}_2\text{CH}_{\text{COOH}} + \text{H}_2\text{O} \quad (I)
\]

The conversion of arginine to putrescine is the other route found under nonspecialized culture conditions. When this reaction is catalyzed by crude extracts of E. coli, the carboxyl group of arginine is converted to CO\(_2\), and the amidine group appears as urea. Therefore, the conversion of arginine to putrescine probably involves two reactions: a decarboxylation and a hydrolysis of the guanidino group. It is necessary to establish the order in which these two reactions take place. Arginine could first be converted to ornithine and urea, followed by formation of putrescine by the ornithine decarboxylase described above, or, alternatively, arginine could first be decarboxylated to arginine, with subsequent cleavage of arginine to urea and putrescine. Three pieces of evidence argue for the second mechanism. First, ornithine ureohydrolase, the enzyme which would be required to convert arginine to ornithine and urea, has been shown in cell-free extracts. Second, if the first mechanism were true and ornithine were an intermediate in the conversion of arginine to putrescine, the inclusion of unlabeled ornithine in the reaction mixture should cause a marked lag in the evolution of \(^{14}\text{CO}_2\) from arginine-U-\(^{14}\text{C}\). When this experiment was performed (Fig. 3, Curve 2), the kinetics of \(^{14}\text{CO}_2\) production was not changed. This result therefore indicates that ornithine is not a free intermediate in putrescine production from arginine. Third, it was possible to trap the intermediate radioactive arginine in an unlabeled pool of arginine (Table VI). Therefore, the transformation of arginine to putrescine appears to involve decarboxylation of arginine (Equation 2) followed by hydrolysis of arginine (Equation 3).

\[
\text{NH} \quad \text{NH}_2 \quad \text{NH} \quad \text{NH}
\]

\[
\text{NH}_2\text{CNH(CH}_3\text{)}_2\text{CHCOOH} \rightarrow \text{NH}_4\text{CNH(CH}_3\text{)}_2\text{NH}_2 + \text{CO}_2
\]

\[
\text{NH}_2 \quad \text{NH}_2
\]

\[
\text{NH}_2\text{CNH(CH}_3\text{)}_2\text{NH}_2 + \text{H}_2\text{O} \rightarrow \text{NH}_4\text{(CH}_3\text{)}_2\text{NH}_{\text{COOH}} \quad (3)
\]

+ \text{NH}_2\text{CNH}_2

In 1940, Gale reported the presence of arginine and ornithine decarboxylases in cultures of E. coli grown under specialized conditions (26). The biosynthetic decarboxylases described in the present paper appear to be distinct from the inducible enzymes found by Gale. A previous report from this laboratory showed that the biosynthetic and induced ornithine decarboxylases differ in their pH optima, heat stabilities, and conditions for formation (1). The biosynthetic arginine decarboxylase, postulated to be involved in the arginine to putrescine transformation, appears to differ from the arginine decarboxylase described by Gale in its pH optimum (8 as opposed to 5) and its high degree of stimulation by magnesium ion (Fig. 4). Both catabolic decarboxylases are inducible and require special conditions for formation (26, 29), whereas both biosynthetic enzymes are present in cells grown in neutral minimal medium. It seems, therefore, that E. coli possesses the genetic information for two arginine and two ornithine decarboxylases.

The formation of putrescine from arginine in E. coli appears to take place by a mechanism identical with the pathway of arginine degradation by mycobacteria (25). Plantes also seem to follow a similar route (30, 31). However, they hydrolyze arginine to N-carbamylputrescine with subsequent conversion to putrescine (32), rather than directly to putrescine and urea as in the bacterial cases. Only in E. coli has a second pathway of ornithine to putrescine, been reported.

Putrescine and spermidine are present in E. coli at a combined concentration of approximately 0.1 \( \mu \) mole per mg of protein under the culture conditions described here. This means that in one generation time (approximately 60 min in minimal media), 1 \( \mu \)g of cell protein must synthesize at least 0.1 \( \mu \) mole of putrescine. The rates in vivo of putrescine synthesis observed in cell-free extracts are compatible with this requirement. A examination of the specific activities of the three enzymes reported in this paper shows that the optimal assay conditions differ for each pathway of putrescine synthesis. Ornithine decarboxylase has a specific activity of approximately 2 \( \mu \) moles/min per mg protein; arginine decarboxylase and ornithine ureohydrolase have specific activities of 0.8 and 5 \( \mu \) moles/min per mg protein, respectively.

The specific activities of the enzymes of the two putrescine biosynthetic pathways, measured under conditions of specific activity, cannot be extrapolated to their activities in vivo. However, it is possible to obtain the flux through the two pathways from the isotope competition experiments reported in this paper. From the data presented in Table I, approximately 40% of the cellular putrescine was formed via the ornithine route, and 60% came from arginine when surplus precursors were present.
is not surprising that the relative flux in vivo through the two pathways deviates from that predicted from the specific activities of the enzymes in crude extracts. One would expect that the activities of the enzymes in vivo would be modulated by the levels of substrates, cofactors, and inhibitors, while, under the optimal conditions of the assay in vitro, these would not be factors.

The existence of the two pathways of putrescine biosynthesis appears to provide a unique answer to the regulatory problems encountered in branched biosynthetic pathways. As can be seen from Fig. 6, the pathway of arginine biosynthesis in \textit{E. coli} K-12 is indeed branched, the branch point occurring at ornithine. The most economical route of putrescine synthesis, from the standpoint of energy conservation, is the direct decarboxylation of ornithine. However, when repressible strains of \textit{E. coli} are grown in the presence of arginine, the synthesis of the arginine-biosynthetic enzymes is inhibited (18-20). Since the formation of ornithine is blocked during growth on arginine, the organism can no longer synthesize putrescine from this source, but uses the now energetically favorable route from preformed arginine. Consequently, under these conditions, the conversion of arginine to putrescine is the only route of polyamine biosynthesis. This provides a reasonable explanation for the existence of the two pathways of putrescine biosynthesis.

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Multiple Pathways of Putrescine Biosynthesis in *Escherichia coli*

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