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# Spermidine Biosynthesis During Germination and Subsequent Vegetative Growth of *Bacillus megaterium* Spores

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Spermidine biosynthesis was extremely low early in germination of *Bacillus megaterium* spores and the spermidine level remained constant. Rapid synthesis began after 130 min and thereafter accounted for the increase in spermidine level which began at this time. Biosynthesis was greatly (>84%) diminished by exogenous spermine or spermidine. Arginine and ornithine were both converted efficiently into spermidine, but arginine was the more immediate precursor as shown by isotope competition studies and by the absence of ornithine decarboxylase and the presence of arginine decarboxylase. Exogenous putrescine was not incorporated into spermidine, although it was taken up rapidly and degraded.

Spermidine is the only polyamine found in *Bacillus megaterium* (10). This is in contrast to organisms such as *Escherichia coli* which contain mainly putrescine and smaller amounts of spermidine (6). Putrescine appears to be an intermediate in all biosynthetic pathways for spermidine (reaction 4, Fig. 1), and two different pathways for putrescine synthesis have been identified in *Escherichia coli:* (i) decarboxylation of ornithine (reaction 1, Fig. 1); and (ii) decarboxylation of arginine and hydrolysis of the agmatine produced (reactions 2 and 3, Fig. 1) (4, 5).

Although data are available on the mechanism and control of putrescine and spermidine biosynthesis in *E. coli* (4, 5, 15), little is known about polyamine biosynthesis in *Bacillus* species other than the observation that carbon number 2 of methionine is incorporated into spermidine in *B. subtilis* (1). During germination of dormant spores and subsequent vegetative growth of *B. megaterium* QM B1551 the spermidine content remains constant for the first 130 min and then rises rapidly (10). This synchronous initiation of spermidine accumulation appeared to be an ideal system to investigate the mechanism and control of spermidine biosynthesis in *Bacillus* species.

## MATERIALS AND METHODS

**Chemical reagents.** Putrescine, spermine, and spermidine hydrochlorides, and L-arginine, L-ornithine, pyridoxal phosphate, chloramphenicol, and agmatine sulfate were purchased from Sigma Chemical Co. The agmatine sulfate was contaminated with 5 to 10% putrescine. Ninhydrin was obtained from the Pierce Chemical Co.; [1,4-$^{14}$C]putrescine, L-[3-$^{3}$H]arginine, and DL-[1-$^{14}$C]ornithine were purchased from New England Nuclear. DL-[1-$^{14}$C]arginine and L-[$^{3}$H]ornithine were obtained from Schartz/Mann.

**Isolation and germination of spores.** *B. megaterium* QM B1551 was originally obtained from Hillel Levinson (U.S. Army Natick Lab., Natick, Mass.). Spores were produced at 30 C in supplemented nutrient broth, harvested, cleaned, and stored as previously described (11). The spores were >95% refractile and were free of vegetative cells and cell debris as judged in the phase contrast microscope.

Standard conditions for spore germination were as follows unless otherwise noted: spores (20 mg [dry weight]/ml) were heat shocked for 10 min at 60 C, cooled, and germinated at 30 C in Spizizen medium (12) without added Casamino acids at a spore concentration of 500 $\mu$g (dry weight)/ml. Initiation of germination was >95% complete by 15 min as observed in a phase contrast microscope; germination was considered to end and vegetative growth to begin after 130 min as previously described (10).

**Extraction and quantitation of spermidine.** Samples (30 ml) of germinating spores were centrifuged (1.5 min at 18,000 $\times$ *g*), extracted for 45 min with 2 ml of cold (4 C) 5% trichloroacetic acid, and centrifuged. Trichloroacetic acid was removed from the supernatant fluid by five extractions with diethyl ether with addition of a few drops of concentrated hydrochloric acid after each extraction.

Spermidine and putrescine were separated from other ninhydrin positive compounds by electrophoresis (105 min at 30 V/cm) on Whatman 3MM paper in 0.1 M sodium citrate (pH 4.3) (9), and polyamines were quantitated on the dried electropherogram as previously described (10). To determine the amount of labeled precursor incorporated into spermidine, two samples from the same extract were run in parallel as described above: one was used for spermidine determinations, and the spermidine spot in the second sample was cut out and counted in a scintillation counter.

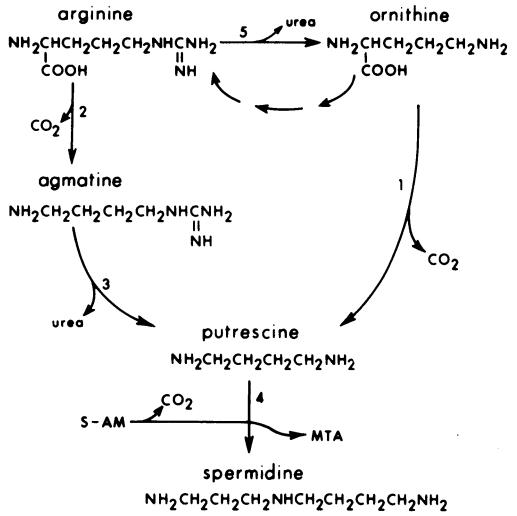


FIG. 1. *Possible pathways for arginine and ornithine interconversion and polyamine synthesis. The abbreviations used are: S-AM, S-adenosylmethionine; MTA, 5'-methylthioadenosine.*

Uptake of spermidine was measured as previously described (10). Putrescine uptake was measured similarly by collecting and washing spores on a membrane filter; the spores were then extracted with trichloroacetic acid, and labeled putrescine was separated and quantitated as described above.

**Enzyme assays.** Unless otherwise noted, enzyme assays utilized germinated spores made permeable by freezing and thawing (14). Germinated spore suspensions were centrifuged (1.5 min at 18,000 × *g*) and the pellets were frozen in ethanol-dry ice. The frozen spores were suspended in 67 mM $KPO_4$ buffer (pH 7.4) containing 100 μg of chloramphenicol per ml, and the suspension was frozen and thawed five times.

Ornithine decarboxylase and arginine decarboxylase were assayed by measuring $CO_2$ evolution using a modification of the procedure of Russell (8, 9). The reaction mixture contained 50 μmol of tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.0), 50 nmol of pyridoxal phosphate, 2.5 μmol of L-arginine or L-ornithine, 5 μCi of DL-[1-$^{14}$C]arginine or DL-[1-$^{14}$C]ornithine, spores, and water in a final volume of 0.5 ml. The assay was carried out in 20-ml glass scintillation vials with a strip (4 by 1 cm) of Whatman no. 1 filter paper taped in the vial cap. The paper strip was soaked with 40 μliters of a 2:1 mixture of methyl cellosolve and ethanolamine. After reaction for 30 min at 30 C with gentle agitation, 0.5 ml of 6 M $H_2SO_4$ was added, the vials were shaken for an additional 30 min, and the paper strip was removed and counted in a scintillation counter. Although DL-[1-$^{14}$C]amino acids were used in these assays, they measured only L-amino acid decarboxylation since a 20-fold increase in cold L-arginine resulted in a 15-fold decrease in [$^{14}$C]$CO_2$ produced. This is approximately the result expected if 10 mM L-[$^{14}$C]arginine is saturating for L-arginine decarboxylase, and if L-arginine is not a good inhibitor of any D-arginine decarboxylase present.

## RESULTS

**Spermidine synthesis during spore germination.** Previous work had shown that the spermidine level remained constant during the first 130 min of spore germination and then rose during subsequent vegetative growth (Fig. 2) (10). As measured with exogenous arginine as a precursor, there was also almost no spermidine synthesis in the first 130 min (Fig. 2). However, at this time rapid synthesis began, which thereafter quantitatively accounted for the increase in the spermidine level (Fig. 2). Identical results were obtained using ornithine as a precursor. In contrast exogenous putrescine was an extremely poor precursor (Fig. 2, and see below).

**Arginine not ornithine is the more immediate spermidine precursor.** Although both arginine and ornithine were incorporated into spermidine, it was of interest to determine if both amino acids served as independent precursors as has been observed in *E. coli* (4), or if one amino acid acted only as a precursor to the other (Fig. 1). The latter was a strong possibility since spores of *Bacillus* species are known to have high levels of arginase (reaction 5, Fig. 1) (2) (P. Setlow, unpublished data). However, the results strongly suggested that arginine was the more immediate precursor of spermidine, with ornithine acting only as a precursor of arginine. Incorporation of [$^3$H]arginine into spermidine was not inhibited by an excess of unlabeled ornithine (Table 1), whereas low levels of unla-

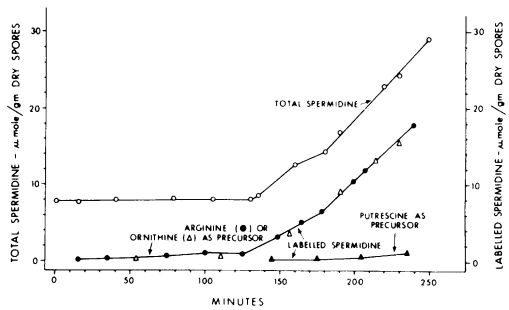


FIG. 2. *Spermidine biosynthesis during spore germination and subsequent growth. Spores were germinated under standard conditions with [$^3$H]arginine (0.7 mM, 410 counts per min per nmol), [$^3$H]ornithine (1 mM, 520 counts per min per nmol), or [$^{14}$C]putrescine (0.5 mM, 200 counts per min per nmol) present from zero time. Samples were harvested and analyzed for spermidine as described in Materials and Methods. Calculations of the amount of labeled spermidine assumed that there was no dilution of the specific radioactivity of the large amount of exogenous labeled precursor added.*

beled arginine completely blocked incorporation of labeled ornithine into spermidine. Similarly, unlabeled agmatine abolished synthesis of spermidine from either labeled arginine or ornithine, whereas putrescine had no inhibitory effect. The lack of inhibition of arginine incorporation by ornithine was due in part to the absence of ornithine transcarbamylase in the dormant spore (2), and the repression by arginine of its synthesis during germination (P. Setlow, unpublished data).

It is significant that none of these precursors had any significant effect on the spermidine level during germination (Table 1), suggesting that spermidine synthesis is closely regulated. This was further suggested by the strong inhibition of spermidine synthesis by both exogenous spermidine and spermine. High levels of these polyamines are taken up by *B. megaterium*, and spermine is able to completely replace spermidine (10). These effects of exogenous polyamine were not due to inhibition of amino acid uptake since incorporation of either exogenous arginine or ornithine into protein was not significantly inhibited by exogenous agmatine, spermine, or spermidine (P. Setlow, unpublished data).

**Levels of arginine and ornithine decarboxylase.** The results of the precursor studies reported above suggested that arginine decarboxylation (reaction 2, Fig. 1) was the first step in spermidine synthesis with little or no synthesis via decarboxylation of ornithine. This suggestion was strengthened by the absence of detectable ornithine decarboxylase through at least the first 240 min of incubation, and the presence of arginine decarboxylase (Table 2). The arginine decarboxylase activity showed very strong product inhibition by agmatine (Table 3), which probably is the cause of the efficient inhibition by agmatine of spermidine synthesis (Table 1). Spermidine and spermine, however, did not inhibit the arginine decarboxylase (Table 3), in contrast to results obtained in *E. coli* (5).

TABLE 2. *Levels of arginine and ornithine decarboxylase during germination and subsequent growth*[a]

| Time of Incubation (min) | ($\mu$mol of $^{14}CO_2$ per 30 min per g [dry weight]) | |
|---|---|---|
| | Ornithine decarboxylase[b] | Arginine decarboxylase |
| 15 | <0.05 | 4.8 |
| 120 | <0.05 | 10.5 |
| 200 | <0.05[c] | 21.3 |

[a] Spores were germinated under standard conditions with additions as noted. Samples (40 ml) were centrifuged at the indicated times, and treated and assayed.

[b] Under these assay conditions an *E. coli* extract had a specific activity of 110 nmole [$^{14}$C]$CO_2$ produced per 30 min per mg of protein.

[c] Similar results were obtained with an extract prepared by sonication.

TABLE 1. *Effects on spermidine biosynthesis during germination and subsequent growth*[a]

| Precursor | Competitor | Spermidine ($\mu$mol/g of dry spores) | Specific radioactivity of spermidine |
|---|---|---|---|
| [$^{14}$C]ornithine (0.5 mM) | None | 18.5 | 1.0[b] |
| | Arginine (0.25 mM) | 19 | 0.03 |
| | Agmatine (1 mM) | 18 | 0.07 |
| | Spermidine (1 mM) | | 0.14[b] |
| [$^{3}$H]arginine (0.4 mM) | None | 18 | 1.0[a] |
| | Ornithine (2.4 mM) | 17.5 | 0.97 |
| | Agmatine (0.4 mM) | 19 | 0.15 |
| | Putrescine (2.8 mM) | 18 | 1.0 |
| | Spermidine (0.8 mM) | | 0.16[c] |
| | Spermine (0.8 mM) | | 0.09[c] |
| [$^{14}$C]putrescine (0.5 mM) | None | 18.5 | 0.03[d] |
| | Casamino Acids (0.1%) | 19.5 | <0.005[d] |

[a] Spores were germinated under standard conditions with labeled precursor and unlabeled competitor present from zero time. After 200 min, samples (30 ml) were centrifuged, the pellets were frozen, and radioactivity and total spermidine were determined.

[b] Specific radioactivity of spermidine arbitrarily set at 1.0 in the absence of competitor.

[c] These low values were not simply due to elevation of the endogenous polyamine level by the exogenous pool. They are true measurements of spermidine biosynthesis under these conditions, since values were calculated from the radioactivity in spermidine and assuming 18.5 $\mu$mol spermidine per g of dry spores.

[d] Calculated by setting the specific radioactivity of added putrescine at 1.0.

**Fate of exogenous putrescine during spore germination.** All pathways elucidated for spermidine synthesis include putrescine as an intermediate (Fig. 1). Therefore, it was surprising that exogenous putrescine was such a poor precursor for spermidine synthesis in *B. megaterium* (Fig. 2). Even the small amount of incorporation of putrescine into spermidine (Fig. 2) could be completely abolished by inclusion of Casamino acids in the growth medium (Table 1).

The inability of putrescine to serve as a precursor to spermidine was not due to a permeability barrier, since exogenous putrescine was rapidly taken up by germinating spores, as shown by its rapid intracellular degradation in germinating cultures (Table 4, Fig. 3). This rapid degradative activity (>290 μmol per 30 min per g of spores) was inactive against spermidine (Fig. 3). An active diamine oxidase has, in fact, been reported in *B. cereus* (3). The putrescine degradation in *B. megaterium* appeared to be wholly intracellular since supernatant fluid (but not the washed spores) from cultures rapidly degrading putrescine catalyzed no putrescine degradation (Table 4). This certainly suggests that putrescine can be taken up by germinating spores, however, it is possible that the putrescine degradative enzyme(s) is located at or near the cell surface. It should be noted that in cultures germinating in the presence of 1 mM putrescine, significant amounts of putrescine remained when rapid spermidine biosynthesis was occurring (compare Fig. 2 and 3). However, the endogenous putrescine level was at all times below the limits of detection (Table 5), although it is possible that these values were low because of putrescine degradation during isolation of spores before analysis.

TABLE 3. *Inhibition of arginine decarboxylase by spermidine, spermine, and agmatine*[a]

| Addition to assay | Activity (%) |
|---|---|
| None[b] | 100[c] |
| Spermidine (4 mM) | 97 |
| Spermine (4 mM) | 101 |
| Agmatine (1.2 mM) | 7 |
| Agmatine (0.5 mM) | 17 |
| Agmatine (0.20 mM) | 30 |

[a] Spores were germinated under standard conditions, harvested at 120 min of germination, and assayed. Similar results were obtained with frozen-thawed cells or with an extract prepared by sonication.

[b] Arginine present in assay at 1 mM.

[c] Activity in absence of inhibitor set at 100%; the specific activity was 6.7 μmol $^{14}CO_2$ per 30 min per g (dry weight).

TABLE 4. *Lack of putrescine degradation by exudate from germinating spores*[a]

| Time after [$^{14}$C]putrescine addition (min) | [$^{14}$C]putrescine remaining (%) | |
|---|---|---|
| | Exudate | Washed spores |
| 0 | 100[b] | 100[b] |
| 10 | 101 | 88 |
| 35 | 96 | 17 |
| 100 | 101 | <7 |

[a] Spores (10 ml) were germinated under standard conditions with unlabeled putrescine present at 100 μM from zero time. After 150 min, the sample was centrifuged (10 min at 15,000 × *g*) and the supernatant fluid (exudate) was saved. The pellet was washed once and then resuspended in 10 ml of fresh medium (washed spores). Both the exudate and washed spores were then made to 50 μM in [$^{14}$C]putrescine (310 counts per min per nmol) and incubated at 30 C with gentle agitation. Samples were analyzed for [$^{14}$C]putrescine.

[b] Zero time value set at 100%.

## DISCUSSION

It appears likely that spermidine biosynthesis in *B. megaterium* proceeds by decarboxylation of arginine with formation of agmatine. This is shown by the absence of detectable ornithine decarboxylase in germinating spores (Table 2), the strong inhibition of incorporation of labeled ornithine into spermidine by unlabeled arginine (Table 1), and the strong inhibition of incorpo-

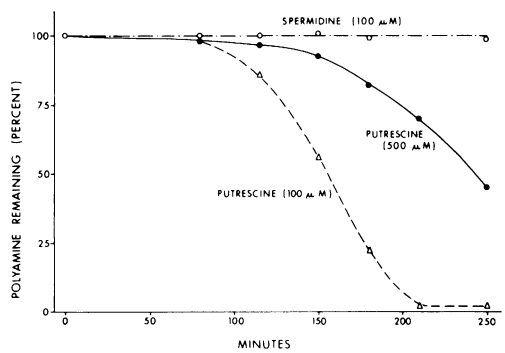


FIG. 3. *Polyamine degradation during spore germination. Samples were germinated under standard conditions with [$^{14}$C]putrescine (200 counts per min per nmol) or [$^{14}$C]spermidine (about 1000 counts per min per nmol) present from zero time at the indicated concentrations. Samples of 50 μliters were diluted 1:1 with 2 N HCl and centrifuged. The supernatant fluids were flash-evaporated, and the residue dissolved in 50 μliters of water. Samples of 20 μliters were run on electrophoresis, and the putrescine or spermidine regions of the paper counted in a scintillation counter.*

TABLE 5. *Uptake levels of exogenous polyamines by germinating spores and growing cells*[a]

| Time of incubation (min) | Exogenous polyamine taken up (μmol/g of dry spores at zero time) | |
|---|---|---|
| | Spermidine | Putrescine |
| 100 | 4 | <0.4[b] |
| 150 | 19 | <0.4[b] |
| 200 | 46 | <0.2 |
| 250 | 68 | <0.2 |

[a] Spores were germinated under standard conditions with 0.5 mM [$^{14}$C]putrescine (500 counts per min per nmol) or 0.1 mM [$^{14}$C]spermidine (1,000 counts per min per nmol) present from zero time. Polyamine uptake was determined.

[b] There were some counts in the putrescine region of the electropherogram, however, they did not co-electrophorese with the putrescine marker.

ration of labeled arginine into spermidine by unlabeled agmatine. Further steps in spermidine biosynthesis in *B. megaterium* remain unclear, since I have been unable as yet to detect agmatine ureohydrolase (Reaction 3, Fig. 1) in germinating spores or vegetative cells (P. Setlow, unpublished data). This negative finding does not, of course, rule out the presence of active enzyme in vivo.

The absence of conversion of exogenous putrescine to spermidine by germinating spores (Table 1, Fig. 2) was surprising in view of the ability of spores to take up and metabolize exogenous putrescine (Table 4) (Fig. 3). However, the endogenous level remained extremely low (Table 5). One possible explanation for the absence of spermidine biosynthesis from exogenous putrescine is of course that spermidine biosynthesis in *B. megaterium* proceeds by some novel pathway without putrescine as an intermediate. Although putrescine is not found in a number of different *Bacillus* species (10, 13), this explanation appears unlikely, since in all other organisms studied, both prokaryotes and eukaryotes, putrescine is an intermediate in spermidine biosynthesis. A more likely explanation may be that free putrescine itself does not participate in spermidine biosynthesis in *B. megaterium*, but rather that putrescine remains enzyme bound or compartmentalized during spermidine synthesis, and that this bound putrescine is not exchangeable with the exogenous pool. The rapidity with which germinating spores and growing cells degrade putrescine would certainly suggest a reason for such a compartmentalization of endogenous putrescine.

## ACKNOWLEDGMENTS

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