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Paula Allene Tower for the degree of Doctor of Philosophy in Microbiology presented on July 28, 1987.

Title: Homospermidine, Spermidine, and Putrescine: The Biosynthesis and Metabolism of Polyamines in Rhizobium meliloti

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Dr. Adolph J. Ferro

Rhizobium, in symbiotic association with leguminous plants, is able to fix atmospheric nitrogen after first forming root nodules. Since polyamines are associated with and found in high concentration in rapidly growing cells and are thought to be important for optimal cell growth, it is possible that these polycations are involved in the extensive cell proliferation characteristic of the nodulation process. As a first step towards elucidating the role(s) of polyamines in the rhizobial-legume interaction, I have characterized polyamine biosynthesis and metabolism in free-living Rhizobium meliloti.

In addition to the detection of putrescine and spermidine, the presence of a less common polyamine, homospermidine, was confirmed in Rhizobium meliloti, with homospermidine comprising 79 percent of the free polyamines in this procaryote.

The presence of an exogenous polyamine both affected the intracellular levels of each polyamine pool and inhibited the accumulation of a second amine. DL-α-Difluoromethylornithine (DFMO), an

irreversible inhibitor of ornithine decarboxylase, was found to: (1) inhibit the rhizobial enzyme both *in vitro* and *in vivo*; (2) increase the final optical density; and (3) create a shift in the dominant polyamine from homospermidine to spermidine.

A series of radiolabeled amino acids and polyamines were studied as polyamine precursors. Ornithine, arginine, aspartic acid, putrescine, and spermidine, but not methionine, resulted in the isolation of labeled putrescine, spermidine, and homospermidine.

Evidence is presented for the existence of an alternate pathway of spermidine synthesis in *R. meliloti*, one which is derived from aspartic acid and is unrelated to methionine and S-adenosyl-methionine.

Homospermidine synthase, the enzyme which synthesizes homospermidine from putrescine, was partially purified and characterized. A K_m with respect to putrescine and pH and temperature optima were determined. Spermidine was the most effective inhibitor of the analogs tested and exhibited competitive inhibition kinetics.

Homospermidine, Spermidine, and Putrescine: The Biosynthesis and Metabolism of Polyamines in *Rhizobium meliloti*

by

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CHAPTER I

Introduction

Polyamines, which are ubiquitous to the biological world, are associated with and found in high concentrations in rapidly proliferating cells. Although these naturally occurring compounds have been implicated in a number of physiological reactions, the precise role of polyamines *in vivo* has not been defined. (See refs. 1-4 for detailed reviews of synthesis and function.)

Synthesis of Polyamines

Putrescine and spermidine are the di- and polyamine most commonly found in procaryotic systems. The synthesis of these compounds in *Escherichia coli* is well established and is detailed in Figure I.1. Methionine combines with the adenosyl moiety of ATP to form S-adenosylmethionine (SAM), which is then decarboxylated by SAM decarboxylase to form S-5'-deoxyadenosyl-(5')-3-methylthiopropylamine (decarboxylated SAM). The aminopropyl group of the decarboxylated SAM is transferred to the diamine, putrescine, to form spermidine. Note that putrescine can be synthesized by either of two pathways. The first involves the decarboxylation of ornithine by ornithine decarboxylase. In the alternate pathway, arginine is

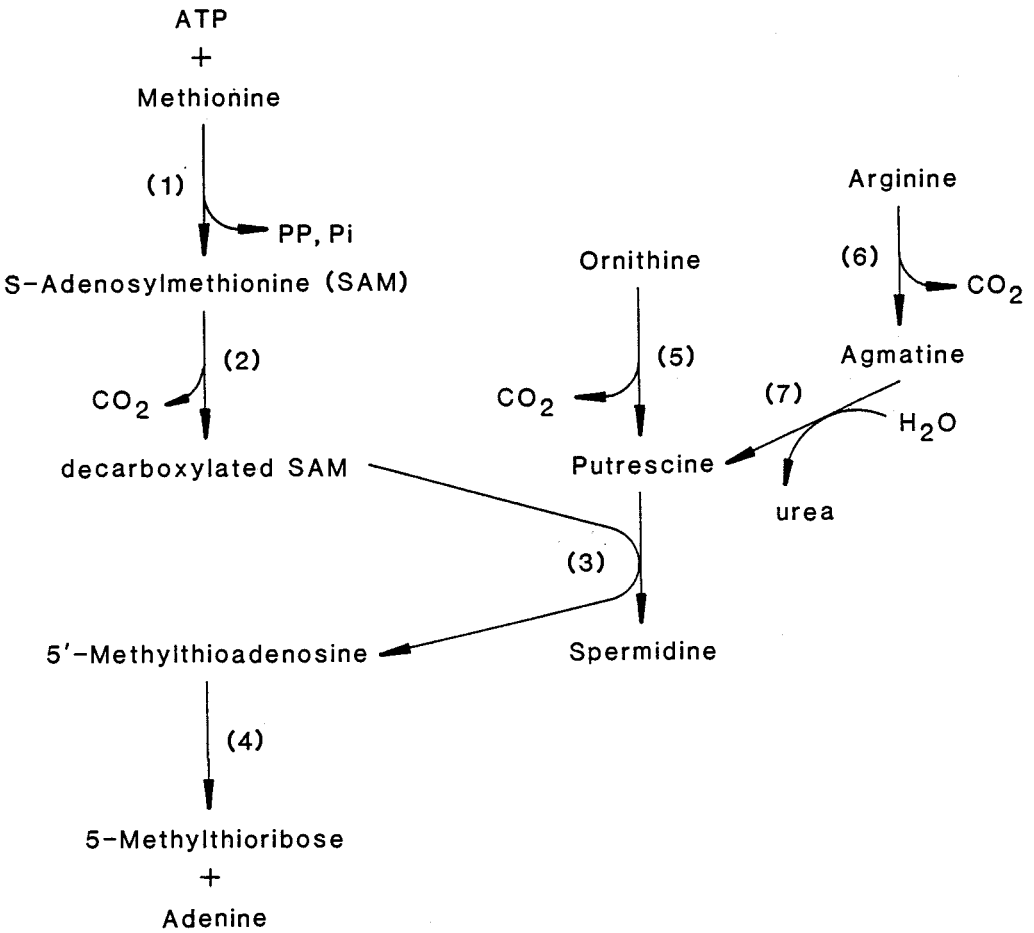

Figure I.1. Polyamine Biosynthesis in Escherichia coli. The reactions are catalyzed by the following enzymes: (1) S-adenosyl-methionine (SAM) synthetase; (2) SAM decarboxylase; (3) putrescine aminopropyltransferase; (4) methylthioadenosine nucleosidase; (5) ornithine decarboxylase; (6) arginine decarboxylase; and (7) agmatine ureohydrolase.

decarboxylated to agmatine which is subsequently converted to putrescine. The co-product of spermidine synthesis, 5'-deoxy-5'-methylthioadenosine (MTA), is degraded by the action of MTA nucleosidase to 5-methylthioribose and adenine.

In eucaryotes, an additional aminopropyl transfer occurs, with spermidine as the acceptor, forming spermine and a second molecule of MTA. Unlike mammalian cells which have only ornithine decarboxylase, plants can also utilize arginine in the synthesis of putrescine. Both arginine-related pathways in plants involve N-carbamoylputrescine as an intermediate.

Because of the complexity of polyamine synthesis with respect to the number of interacting pathways, the regulation of synthesis is not fully understood. The short half-life of some of the enzymes, the intracellular concentrations of ornithine and arginine, as well as the polyamine levels themselves are involved in control. Post-translational regulation of ornithine decarboxylase may include inhibition by "antizymes": proteins which bind noncovalently and reversibly with ornithine decarboxylase to inactivate the enzyme.

Biological Effects of Polyamines

Although these aliphatic cations have been implicated in a number of physiological processes, including protein synthesis, DNA replication, and membrane stability, and are required for optimal growth, the precise role of polyamines *in vivo* has not been established. Since growth rate is actually a measure of a number of

processes, polyamines likely function in more than one capacity.

In attempts to define the role(s) of polyamines, a number of strategies have been employed. E. coli mutants, deleted in the polyamine biosynthetic genes, still grow at one third the rate of the same cells grown in the presence of exogenous polyamines. Inhibitors of polyamine biosynthesis have also been used and include methylglyoxyl bis(guanylhydrazone), a reversible inhibitor of SAM decarboxylase, and a class of enzyme-activated inhibitors, of which DL-α-difluoromethylornithine is the most notable.

Metabolism of Polyamines

The oxidative degradation of polyamines by bacterial enzymes differs from that found in mammaliam systems. Instead of oxidizing the terminal primary amino groups, cleavage occurs adjacent to the secondary amine. The action of the spermidine oxidase from Pseudomonas results in the formation of both putrescine (which potentially can be recycled) and aminopropionaldehyde. In contrast, oxidation by the spermidine dehydrogenase from Serratia yields 1,3-diaminopropane and 4-aminobutyraldehyde, which spontaneously cyclizes to Δ'-pyrroline.

Summary of Objectives

Rhizobium, as the procaryotic partner in the symbiotic association with leguminous plants, is able to fix atmospheric nitrogen following infection of the plant roots and nodule forma-

tion. This nodulation-nitrogen fixation process is a series of complex interactions of both plant host and bacterial factors (6). In addition to the multiplication of the invading bacterium, one step of this process involves the stimulation of root cortical cell division, initiating nodule formation (7). Given the relationship previously found between polyamines and rapid growth processes (1-4), it is possible that polyamines play a role in the extensive cell proliferation characteristic of the nodulation process. In addition, polyamines have anti-senescing properties and act more like second messengers in plants (5), both of which may be involved in this system.

The long-term goal of this project has been to determine any role(s) polyamines may play in the rhizobial-legume interaction. In order to understand any physiological responses in the symbiotic association, it was first necessary to gain a basic understanding of each individual system. Towards that objective, I have characterized polyamine biosynthesis and metabolism in free-living Rhizobium meliloti under a variety of physiological conditions and initiated studies on the same with the bacteria in symbiotic association with alfalfa.

The work presented in this dissertation focuses on three main areas: the biosynthesis of (a) putrescine; (b) spermidine; and (c) homospermidine in free-living Rhizobium meliloti.

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CHAPTER II

Polyamine Analysis of *Rhizobium meliloti*

Authors: Paula Allene Tower and Adolph J. Ferro

SUMMARY

The profiles and characteristics of polyamines in Rhizobium meliloti 41 were examined under a variety of physiological conditions. High performance liquid chromatography (HPLC) analyses revealed the presence of homospermidine as the dominant polyamine (79 percent of the total polyamine pool), in addition to spermidine and putrescine. Polyamine levels fluctuated in response to exogenously supplied polyamines, with the addition of putrescine or homospermidine resulting in a dramatic reduction of the spermidine pool. DL-α-Difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase, was found to: (1) inhibit the rhizobial enzyme both in vitro and in vivo; (2) increase the final optical density; and (3) create a shift in the dominant polyamine from homospermidine to spermidine. The accumulation of [1,4-^{14}C]-putrescine and [^{14}C]-spermidine occurred at equivalent rates and was inhibited greater than 80 percent by excess homospermidine and putrescine, respectively. [1,4-^{14}C]-Putrescine was incorporated into both homospermidine and spermidine, although at a faster rate into the former. Likewise, [^{14}C]-spermidine was distributed with time into the three amine pools. Preliminary findings on the polyamine content of alfalfa nodules are also reported.

INTRODUCTION

Putrescine and spermidine are the di- and polyamines most commonly found in procaryotic systems and are thought to be important for optimal bacterial growth (1-3). Putrescine can be synthesized by either of two pathways. The first involves the decarboxylation of ornithine to putrescine by ornithine decarboxylase. In the alternate pathway, arginine is decarboxylated to agmatine which is subsequently converted to the aliphatic diamine. The transfer of an aminopropyl group to putrescine results in the synthesis of spermidine.

Enzyme-activated irreversible inhibitors of these polyamine biosynthetic enzymes are commonly used in studies to elucidate the biological role of these aliphatic cations. DL-α-Difluoromethylornithine (DFMO), one of these substrate analogs, specifically and irreversibly inhibits ornithine decarboxylase (4). Ornithine decarboxylase from Pseudomonas aeruginosa is sensitive to this inhibitor in vitro, whereas the enzyme from Escherichia coli is not (5). However, DFMO has no effect on the growth of either organism.

Less common polyamines have also been detected in a variety of organisms. One such polyamine, homospermidine (H_2N-$[CH_2]_4$-NH-$[CH_2]_4$-NH_2), was first reported as a naturally occurring compound in sandal leaves by Kuttan et al. (6). This polyamine has since been found in a number of plant, animal, procaryotic, and archaebacterial systems (7-16, 28). Tait (10) has shown that, in Rhodopseudomonas,

homospermidine is synthesized from two molecules of putrescine via a NAD-requiring enzyme, with 4-aminobutyraldehyde as the postulated intermediate (10, 17).

Rhizobium, in symbiotic association with leguminous plants, is able to fix atmospheric nitrogen upon forming root nodules. Given the relationship previously found between polyamines and rapid growth processes (36-39), it is possible that polyamines play a role in the rapid cell proliferation characteristic of this nodulation process (40). Therefore, we have initiated experiments to elucidate any role that polyamines may play in the rhizobial-legume interaction.

In this report, we have characterized polyamine biosynthesis and metabolism in free-living Rhizobium meliloti, as compared to E. coli, and initiated studies on the same with the bacteria in symbiotic association with alfalfa.

MATERIALS AND METHODS

Chemicals

Putrescine (Sigma Chemical Co.), ornithine (Sigma), spermidine (Calbiochem), spermine (Calbiochem), diaminopropane (Sigma), and cadaverine (Aldrich Chemical Co.) were obtained as hydrochlorides. Authentic homospermidine trihydrochloride used as a standard was a gift from Dr. Seymour S. Cohen (SUNY, Stony Brook, NY); DL-α-difluoromethylornithine (MDL 71,782A) was a gift from Dr. Peter P. McCann (Merrell Dow Research Institute, Cincinnati, OH). Homospermidine hydrochloride was synthesized from the free base of putrescine (Sigma) and 4-bromobutylphthalimide (Aldrich) (2:1; mmol/mmol) according to the procedure of Okada et al. (18). L-[1-^{14}C]-Ornithine (59 mCi/mmol), [1,4-^{14}C]-putrescine (118 mCi/mmol), and ^{14}C-spermidine (N-(3-aminopropyl)-[1,4-^{14}C]-tetramethylene-1,4-diamine) (111 mCi/mmol) were purchased from Amersham Corp. L-[U-^{14}C]-Arginine (300 mCi/mmol) was obtained from Research Products International Corp. All other compounds were commercially available.

Bacterial Growth

The defined medium, based on Bergersen (19) and Saedi (20), contained (per liter): 0.1 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.5 g NH_4Cl, 1.1 g Na glutamate, 10.0 g D-mannitol, 0.23 g KH_2PO_4, 0.23 g Na_2HPO_4, 10 ml vitamin mixture (below), 10 ml trace elements solution (below), and was adjusted to pH 7.0. The vitamin mixture was

composed of 20 mg (each) of riboflavin, p-amino-benzoic acid, nicotinic acid, biotin, thiamine·HCl, pyridoxine·HCl, calcium pantothenate, and inositol per liter of 0.01 M-Na_2HPO_4. The trace elements solution contained (per liter): 5.0 g $CaCl_2$, 145 mg H_3BO_3, 125 mg $FeSO_4 \cdot 7H_2O$, 70 mg $CoSO_4 \cdot 7H_2O$, 5 mg $CuSO_4 \cdot 5H_2O$, 4.3 mg $MnCl_2 \cdot 4H_2O$, 108 mg $ZnSO_4 \cdot 7H_2O$, 146.25 mg $Na_2MoO_4 \cdot 2H_2O$, and 239 mg ferric ethylene diamine di(o-hydroxyphenylacetate).

Yeast extract-mannitol broth (21) was modified to include 3 g yeast extract at pH 6.8.

Rhizobium meliloti strain 41 was a gift from Dr. Lyle R. Brown (Oregon State University, Corvallis, OR). Growth studies were carried out in defined medium with or without supplementation at 30°C with vigorous aeration, and optical density was measured with a Klett-Summerson photoelectric colorimeter using a 530-640 nm filter.

Enzyme Extracts

Cells were harvested by centrifugation (10,600 x g), washed twice, and resuspended in extract buffer (50 mM-potassium phosphate, 10% glycerol, and 1 mM-dithiothreitol, pH 6.8). After passing the suspension through a French pressure cell (18,000 psi) three times, cellular debris was removed by centrifugation (20,000 x g) for 30 min at 4°C, and the supernatant was dialyzed against the extract buffer before freezing at -20°C. Protein concentration was determined as described by Bradford (22), using bovine serum albumin as the standard.

Enzyme Assays

Ornithine decarboxylase (EC 4.1.1.17) activity was measured as a function of $^{14}CO_2$ released from L-[1-^{14}C]-ornithine. The reaction mixture consisted of (final concentrations): 0.08 mM-pyridoxal phosphate; 0.06 mM-EDTA; 50 mM-Tris·HCl (pH 7.55); 2.5 mM-dithiothreitol; 5 mM-L-[1-^{14}C]-ornithine (0.5-1.0 μCi); and up to 2 mg bacterial protein in a total volume of 0.5 ml. Each 25 ml flask was sealed with a rubber stopper holding a polypropylene center well containing 0.3 ml methylbenzethonium hydroxide (1 M in methanol) and incubated for 60 min at 37°C in a shaking water bath (23). The reaction was stopped by injecting 0.1 ml 1.8 M-trichloroacetic acid into the flask, and $^{14}CO_2$ was collected for an additional 30 min. The center well and its contents were transferred to a vial containing a scintillation fluid of 10 ml toluene (with 9 g PPO and 0.2 g POPOP per liter) and 5 ml 95% ethanol and shaken vigorously.

Arginine decarboxylase (EC 4.1.1.19) activity was measured as a function of $^{14}CO_2$ released from L-[U-^{14}C]-arginine. The reaction mixture consisted of (final concentrations): 50 mM-Tris·HCl (pH 7.55); 0.04 mM-pyridoxal phosphate; 0.12 mM-EDTA; 2 mM-$MgSO_4$; 2.5 mM-dithiothreitol; 8.3 mM-L-[U-^{14}C]-arginine (1.25 μCi); and up to 1 mg bacterial protein in a total volume of 0.5 ml. The assay itself was carried out as for ornithine decarboxylase (above).

Inhibition of Ornithine Decarboxylase by DFMO

The time-dependent, in vitro inhibition of ornithine decarbox-

ylase by DFMO was determined using extracts of R. meliloti 41 which had been cultured in yeast extract-mannitol broth until early-mid log phase. The procedure of Kallio and McCann (5) was modified such that pyridoxal phosphate was added to the preincubation mixture at 0.1 mM final concentration. Aliquots of 80 µl were removed at various times and assayed as above, using 1 µCi L-[1-^{14}C]-ornithine.

R. meliloti was grown in 100 ml defined medium for 24 h, harvested, washed twice with and resuspended in defined salts (per liter: 0.1 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.5 g NH_4Cl, 0.23 g KH_2PO_4, 0.23 g Na_2PO_4, pH 7.0) with or without 5 mM-DFMO (pH adjusted to 7.0) to study DFMO inhibition in vivo. Suspensions were shaken for one hour at 30°C prior to harvesting, preparing extracts, and assaying ornithine decarboxylase activity. Similarly, 5 mM-DFMO was added to Rhizobium in defined medium, and the cells were harvested at 10 hours, extracted, and assayed.

Polyamine Analysis

Cultures of R. meliloti were harvested by centrifugation (10,600 x g), washed twice, and the cell pellets were frozen at -20°C. Pellets were extracted with 5% (v/v) perchloric acid for 2 h on ice. After deproteinization, the supernatant was neutralized with KOH and centrifuged to remove salts. Bacterial extracts and di- and polyamine standards were derivatized with benzoyl chloride as previously described (24, 25). The benzoyl amines were analyzed by high performance liquid chromatography (HPLC) using a Waters

Model M-6000A system equipped with a Model U6K injector, a reverse phase μBondapak C18 column (3.9 mm I.D. x 30 cm; 10-μm particle size), Model 440 UV absorbance detector (254 nm), Model 740 data module, plus a Co:Pell ODS guard column (2.1 mm I.D. x 7 cm; Whatman). The mobile phase consisted of methanol/water (55:45; v/v) at a flow rate of 1.0 ml/min, unless stated otherwise.

Perchloric acid extracts and standards were also derivatized with dansyl chloride (5-dimethylamino-1-naphthalene sulphonyl chloride) according to the method of Seiler and Weichmann (26) as modified by Flores and Galston (25). Thin-layer chromatography (TLC) was carried out on two systems: (A) silica gel 60 F_{254} in cyclohexane/ethylacetate (3:2, v/v); (B) aluminum oxide F_{254}, neutral, Type T, on aluminum in chloroform/n-butanol (49:1, v/v).

Mass spectra of the benzoylated polyamines were obtained with a Finnigan 4023 mass spectrometer and 4500 source at 70 eV-ionizing voltage.

Accumulation/Incorporation of ^{14}C-Polyamines

A culture of R. meliloti 41 was grown in defined medium, harvested, washed twice, and resuspended in defined salts. After preincubating 10 ml suspension at 30°C for 15 min, either [1,4-^{14}C]-putrescine (0.5 μCi/ml suspension; 1.4 μM final concentration) or [^{14}C]-spermidine (0.16 μCi/ml suspension; 1.4 μM final concentration) was added. To monitor accumulation, aliquots of 0.1 ml were removed at various times and applied onto 0.2 μm cellulose

triacetate filters under vacuum filtration. After washing with 10 ml ice cold buffer (10 mM-potassium phosphate, 1 M-KCl, pH 6.8), the filters were dried and the radioactivity quantified by liquid scintillation spectrometry. Two ml aliquots were removed at various intervals, added directly into centrifuge tubes containing ice cold buffer, centrifuged (9,500 x g, 10 min, 4°C), and washed twice. The cell pellets were analyzed for polyamines by HPLC using a methanol/water (51:49, v/v) solvent system. One ml fractions were collected directly into scintillation vials and the radioactivity determined.

Polyamine Accumulation in the Presence of Competing Amine

Polyamine accumulation using radiolabeled substrates in the presence of a second exogenous amine in excess was carried out and monitored as described above. [^{14}C]-Spermidine (2.8 μCi; 5 μM) without and with 500 μM-putrescine or [1,4-^{14}C]-putrescine (5.9 μCi; 5 μM) without and with 500 μM-homospermidine was added to suspensions of R. meliloti 41 at a density of 1.3 x 10^9 cells/ml.

Plant Material

Seeds of two alfalfa cultivars (Medicago sativa L., cv Anchor; cv Vernal) were surface sterilized with 20% (v/v) commercial bleach and thoroughly rinsed with sterile water before germinating on agar plates (1.5%, w/v). Seedlings were grown on slants of seedling agar (27), supplemented with trace elements, in a controlled environment at 28°C with a 16 h light photoperiod. Three

days after sowing, each plant was inoculated with 1 x 10^9 cells *R. meliloti* 41 which had been cultured in defined medium, washed, and resuspended in 50 mM-potassium phosphate buffer (pH 6.8). Nitrogen-supplied controls were supplemented with 0.05% (w/v) KNO_3.

Nodules were harvested six weeks after inoculation by excision along the plane of the root, weighed, and ground to a powder in liquid nitrogen with a mortar and pestle. The powder was then extracted with 5% (v/v) perchloric acid. The neutralized supernatant was derivatized with benzoyl chloride and the polyamines analyzed by HPLC.

RESULTS

Polyamine Levels and Identification

In addition to putrescine and spermidine which are commonly found in procaryotes, the presence of homospermidine also was observed in extracts of free-living Rhizobium meliloti 41. The identity of homospermidine was established by high performance liquid chromatography (HPLC) of the benzoyl derivatives (Figure II.1) and on two thin-layer chromatography (TLC) systems using dansylated extracts. Identification of the molecule as homospermidine was verified by mass spectroscopy: the benzoyl derivative of homospermidine, derived from R. meliloti 41 and isolated by HPLC, produced a fragmentation profile in agreement with the report of Kuttan et al. (6) and with our benzoylated standard.

Homospermidine, the dominant polyamine, exhibited a 4.1-fold higher concentration than spermidine and a 38.8-fold difference from putrescine, which represented only two percent of the total polyamine pool (Table II.1). Spermine was absent from cultures maintained on defined medium, but a peak corresponding to the tetraamine appeared on HPLC chromatograms representing cultures grown on either yeast extract-mannitol broth or defined medium supplemented with spermine (data not shown).

Putrescine Biosynthesis In Vitro

The putrescine biosynthetic enzymes ornithine decarboxylase and

Figure II.1. HPLC analyses of benzoyl derivatives of di- and polyamines in Rhizobium meliloti. Perchloric acid extracts of R. meliloti cultures were derivatized with benzoyl chloride and separated by HPLC. (A) HPLC chromatogram of benzoylated standards, 1 nmol of each standard: putrescine (PUT), diaminopropane (DAP), cadaverine (CAD), spermidine (SPD), homospermidine (HOMO-SPD), spermine (SPM). (B) Representative HPLC chromatogram of polyamines in R. meliloti (equivalent to 3.5×10^9 cells).

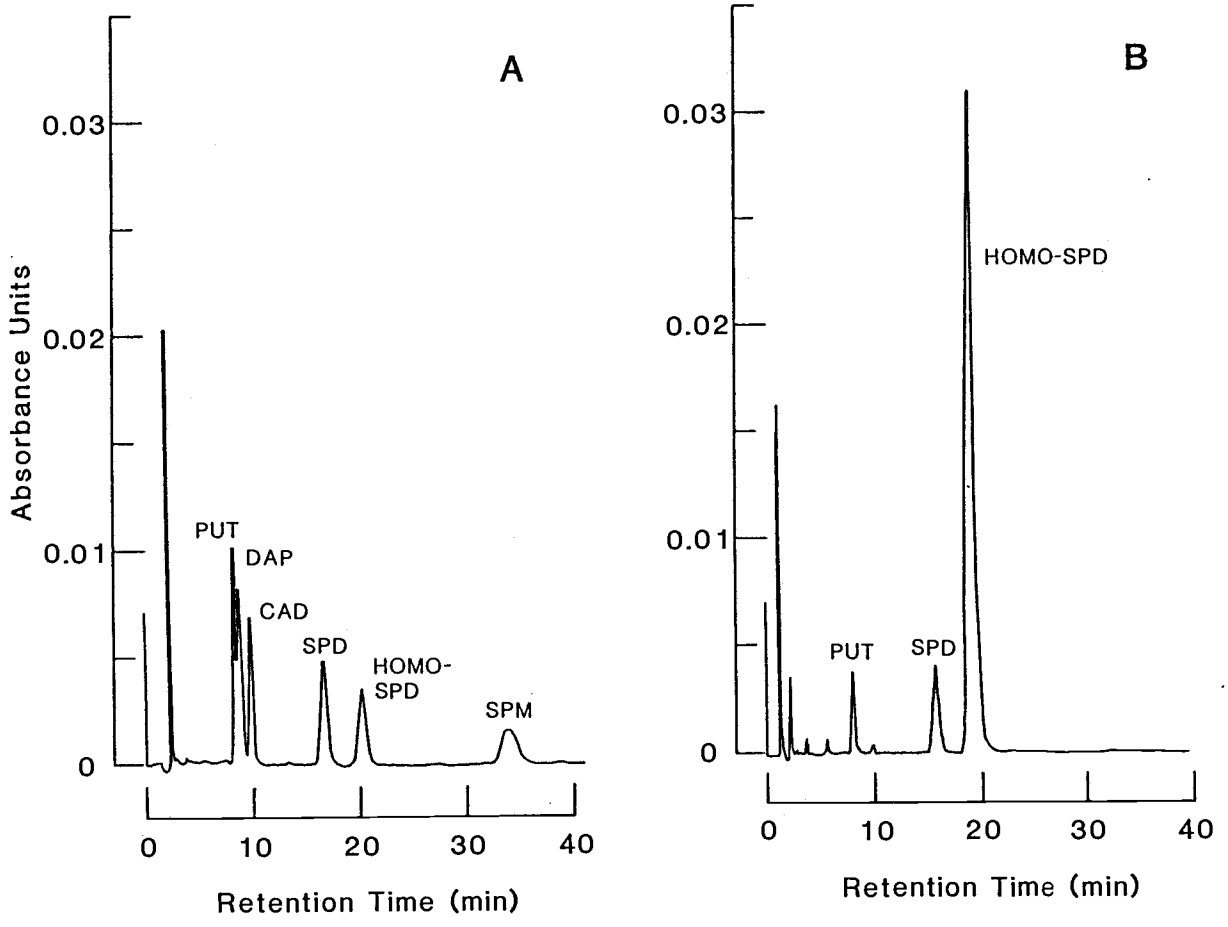
A
B
Absorbance Units
0.03
0.02
0.01
0
PUT
DAP
CAD
SPD
HOMO-
SPD
SPM
HOMO-SPD
0
10
20
30
40
Retention Time (min)

Figure II.1

Table II.1 Intracellular Polyamine Levels in *Rhizobium meliloti* 41[a]

Polyamine	Concentration[b] (pmol/10^8 cells)
Putrescine	16 ± 15
Spermidine	150 ± 40
Spermine	ND[c]
Homospermidine	620 ± 100

[a] *R. meliloti* was cultured on defined medium for 24 h and harvested. Perchloric acid extracts of these cells were derivatized with benzoyl chloride and the polyamine levels were quantitated by HPLC.

[b] Results are mean values (± S.E.) from ten experiments.

[c] Not detected.

arginine decarboxylase were detected in Rhizobium meliloti at activities of 0.44 nmol/min/mg protein and 0.098 nmol/min/mg protein, respectively.

Effects of Exogenous Polyamines on Polyamine Levels

When R. meliloti was cultured in the presence of 5 mM-putrescine or 1 mM-homospermidine, spermidine levels decreased greater than 70 percent (Table II.2). The addition of putrescine, spermidine, or homospermidine resulted in a 2- to 8-fold increase in the intracellular putrescine concentration. Exogenous spermidine led to a 3-fold increase in the intracellular spermidine level as well as an increase in the homospermidine concentration. These compounds had no effect on the growth rate of R. meliloti (data not shown).

Effect of DFMO on Ornithine Decarboxylase In Vitro

DL-α-Difluoromethylornithine (DFMO), a substrate analog which specifically and irreversibly inhibits ornithine decarboxylase (4), an enzyme involved in putrescine synthesis, was used to study polyamine biosynthesis in R. meliloti. We found DFMO to inhibit the in vitro activity of the rhizobial ornithine decarboxylase in a time-dependent manner (Figure II.2), with loss of linearity after several minutes. Inactivation of the enzyme by DFMO required pyridoxal phosphate, as does the decarboxylation of ornithine, and could not be reversed upon dialysis of the DFMO-treated preparation against

Table II.2. Effects of Exogenous Polyamines on Polyamine Levels in *Rhizobium meliloti* 41.

Addition	pmol Polyamines[a] (x 10^8 cells)		
	PUT	SPD	HOMO-SPD
None (Control)	6 (100)	120 (100)	630 (100)
5 mM-Putrescine (PUT)	47 (783)	25 (21)	500 (79)
5 mM-Spermidine (SPD)	47 (783)	360 (300)	760 (121)
1 mM-Homospermidine (HOMO-SPD)	12 (200)	34 (28)	790 (125)

[a]Numbers in parentheses represent percentage of control.

Results from a single representative experiment.

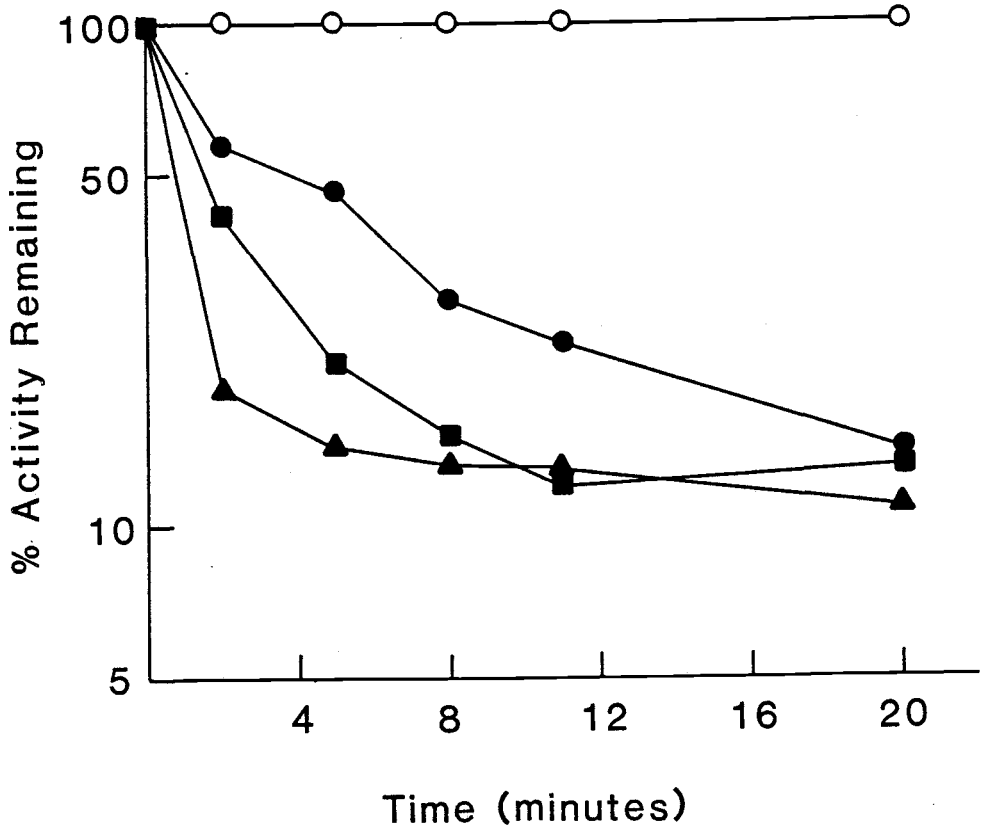

Figure II.2. Time-dependent inhibition of Rhizobium meliloti ornithine decarboxylase by difluoromethylornithine (DFMO). Ornithine decarboxylase was extracted from R. meliloti, incubated in the absence (○) or presence of 10 uM (●), 25 uM (■), or 50 uM (▲) DFMO, and assayed as described in Materials and Methods.

the extract buffer for 24 h (conditions which maintain activity of the native enzyme). A plot of the half-life of enzyme activity ($\underline{t}_{1/2}$) versus the reciprocal of DFMO concentration (Figure II.3) permitted calculation of the apparent dissociation constant ($\underline{K}_i$) and the $\underline{t}_{1/2}$ at infinite inhibitor concentration (29, 30). In this rhizobial system, DFMO had a $\underline{K}_i$ of 120 μM with a $\underline{t}_{1/2}$ of 0.3 min at infinite concentration. Fifty percent inhibition of ornithine decarboxylase occurred in 3.3 min with as little as 10 μM DFMO.

Effects of DFMO In Vivo

R. meliloti incubated in the presence of 5 mM-DFMO exhibited the same exponential growth rate, but grew to twice the final optical density, of control cultures (Figure II.4). The polyamines from these cultures were extracted, derivatized with benzoyl chloride, and analyzed by HPLC. As seen in Table II.3, the addition of 5 mM-DFMO resulted in a dramatic shift to spermidine as the major polyamine, as opposed to homospermidine for the control culture. The percentage of total polyamines which was homospermidine decreased two-fold, in contrast to spermidine which increased by a factor of about 5. The total polyamine pool decreased about 50 percent with the addition of DFMO.

In order to determine if the shift in polyamines seen with DFMO could be altered, exogenous putrescine or homospermidine was added in addition to DFMO. In contrast to the increase in the spermidine pool found in the presence of DFMO alone, the resulting polyamine

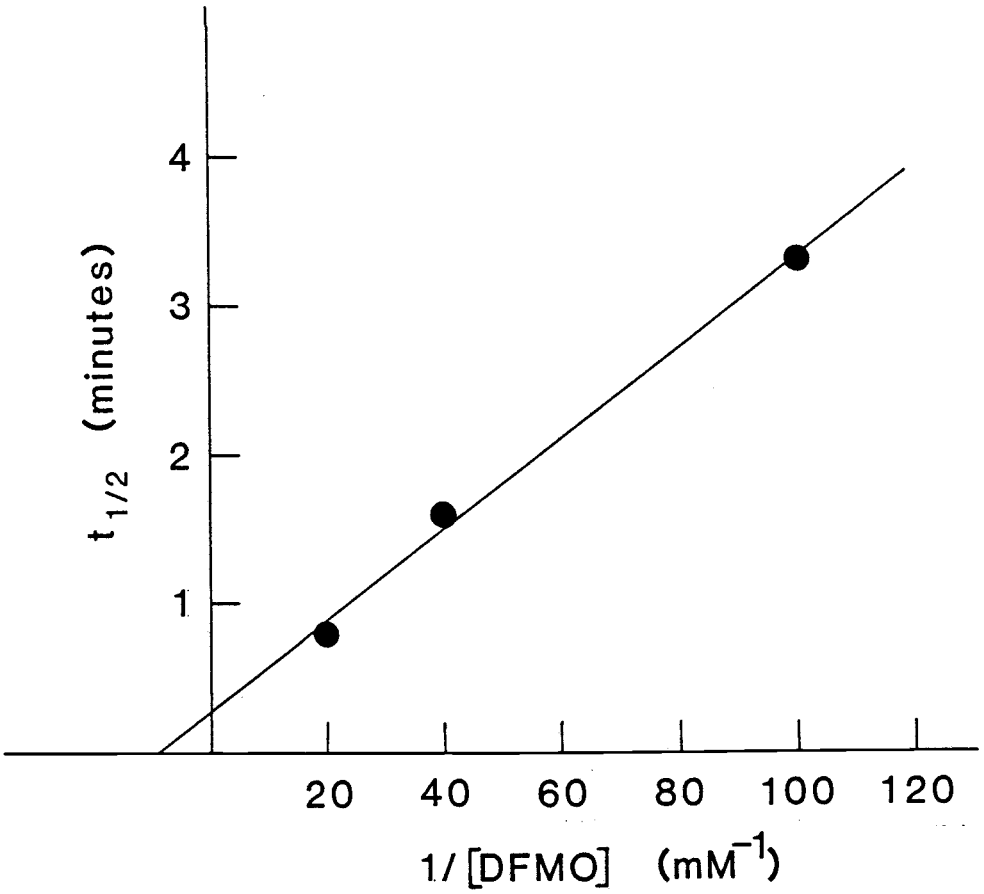

Figure II.3. Dependence of the half-life ($t_{1/2}$) of Rhizobium meliloti ornithine decarboxylase activity on the concentration of difluoromethylornithine (DFMO). At different concentrations of DFMO (0.01-0.05 mM), the half-life of enzyme activity was determined.

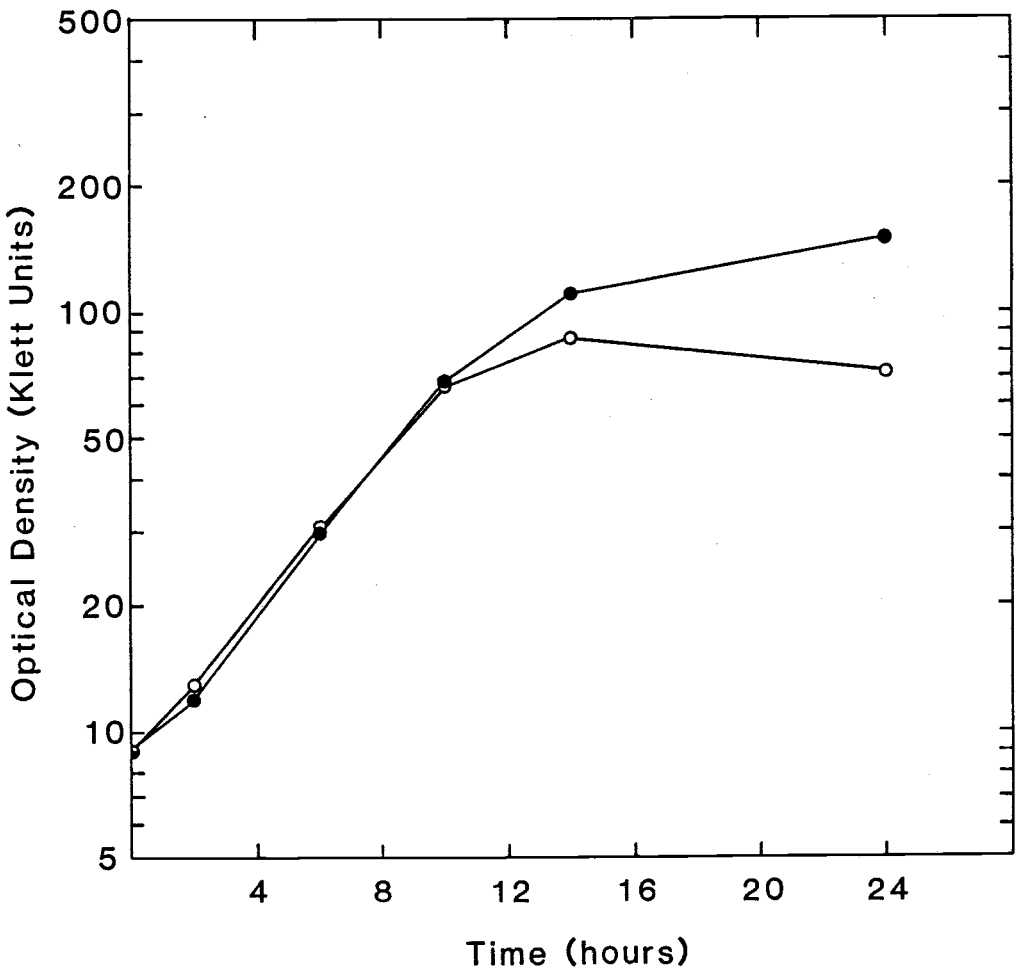

Figure II.4. Effect of difluoromethylornithine (DFMO) on the growth of Rhizobium meliloti 41. Rhizobium meliloti was cultured in defined medium in the absence (○) or presence (●) of 5 mM-DFMO. Data are from a single representative experiment.

Table II.3. Effect of Difluoromethylornithine (DFMO) on Polyamine Levels in Rhizobium meliloti 41.

Addition	pmol Polyamines[a] (x 10^8 cells)		
	PUT	SPD	HOMO-SPD
None (Control)	31 (100)	73 (100)	560 (100)
5 mM-DFMO	11 (35)	180 (247)	120 (21)

[a]Numbers in parentheses represent percentage of control.

Results from a single representative experiment.

profiles exhibited a dramatic decrease in spermidine similar to that found in Table II.2 (data not shown).

The activity of ornithine decarboxylase was inhibited *in vivo* over 90 percent after incubation of *R. meliloti* 41 in the presence of 5 mM-DFMO for one hour (data not shown). If, however, *R. meliloti* 41 was grown in the presence of 5 mM-DFMO for 10 hours, the specific activity of ornithine decarboxylase was restored to control levels (0.44 nmol/min/mg protein versus 0.42 nmol/min/mg protein for the control and DFMO exposures, respectively).

Interconversion of Polyamines

To study incorporation and interconversion in the polyamine pool, suspensions of *R. meliloti* were incubated in the presence of [1,4-^{14}C]-putrescine or [^{14}C]-spermidine. Aliquots were removed at various times, and the polyamines in each sample were analyzed by HPLC. The amount of radioactivity incorporated into each amine as a percentage of the total radiolabeled polyamine pool was determined and is shown in Tables II.4 and II.5 for ^{14}C-putrescine and ^{14}C-spermidine, respectively. With ^{14}C-putrescine, the majority of the putrescine accumulated was incorporated into homospermidine, with a small percentage appearing in spermidine. The radiolabel in both triamines relative to the total radiolabeled polyamine pool increased with time, with a concomitant decrease in putrescine itself. Over time, the addition of ^{14}C-spermidine resulted in a decrease in radioactive spermidine with respect to the total labeled

Table II.4. Incorporation of [1,4-^{14}C]-Putrescine into the Polyamine Pool of *Rhizobium meliloti* 41.

	% Total Radiolabeled Polyamines		
Time	PUT	SPD	HOMO-SPD
10 minutes	19.6%	0.92%	79.4%
20 minutes	14.5%	1.37%	84.1%
30 minutes	11.4%	1.73%	86.9%
60 minutes	6.9%	2.22%	90.9%

Table II.5. Incorporation of [^{14}C]-Spermidine into the Polyamine Pool of Rhizobium meliloti 41.

Time	% Total Radiolabeled Polyamines		
	PUT	SPD	HOMO-SPD
10 minutes	0.61%	95.9%	3.53%
20 minutes	2.53%	90.1%	7.41%
30 minutes	2.79%	86.2%	11.0%
60 minutes	3.02%	79.9%	17.1%

polyamine pool, but increases in both the diamine and homospermidine. The size of the individual polyamine pools relative to each other did not change over the course of the experiment (data not shown).

Accumulation of Polyamines

In order to determine if the various polyamines were accumulated by similar mechanisms, ^{14}C-polyamines were incubated in the presence of competing amines. As shown in Figure II.5, the presence of excess homospermidine restricted the accumulation of [1,4-^{14}C]-putrescine to 15 percent of the amount taken up in the absence of the triamine. Likewise, the amount of [^{14}C]-spermidine transported into Rhizobium in the presence of its precursor, putrescine, was limited to 16 percent of the control incubated in the absence of putrescine (Figure II.6). In the absence of competing polyamines, both putrescine and spermidine were accumulated at equal rates.

Polyamine Analysis of Alfalfa

Putrescine, spermidine, and spermine were detected in both roots and leaves when Medicago sativa L. (cv Anchor) was analyzed for polyamine content. In addition to these three polyamines, homospermidine was found in nodules harvested six weeks after inoculation with R. meliloti 41 (Table II.6). However, homospermidine was not found in the leaves or roots from uninoculated plants (data not shown).

The addition of 1 mM exogenous putrescine or spermidine to the

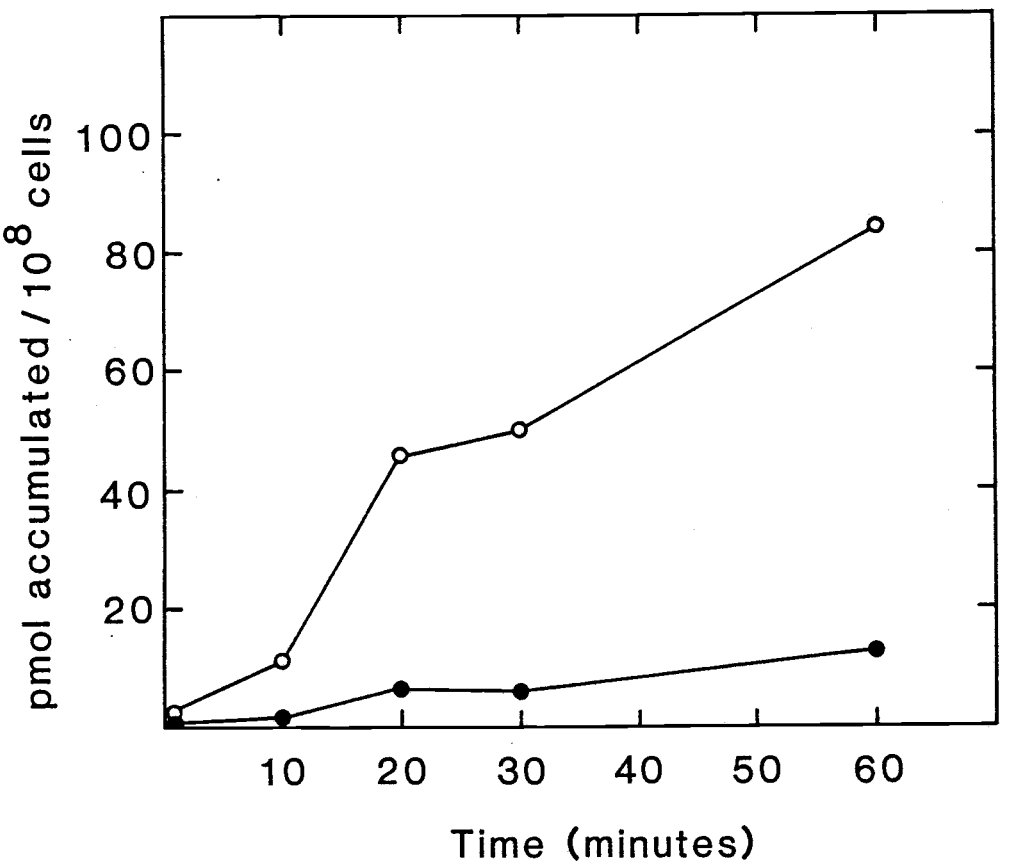

Figure II.5. Accumulation of [1,4-^{14}C]-putrescine in the absence and presence of homospermidine. R. meliloti suspensions were incubated with radiolabeled putrescine in the absence (○) and presence (●) of 100-fold excess homospermidine as a measure of polyamine transport. Aliquots were removed and filtered at various times as described in Materials and Methods.

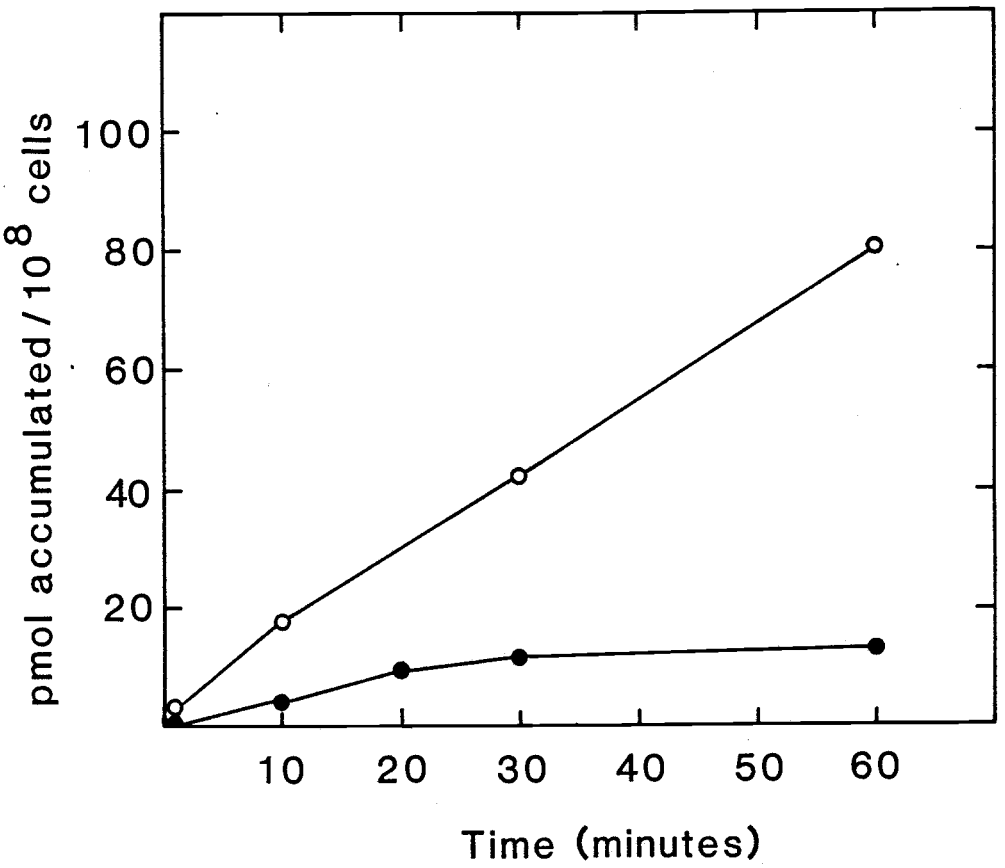

Figure II.6. Accumulation of [^{14}C]-spermidine in the absence and presence of putrescine. R. meliloti suspensions were incubated with radiolabeled spermidine in the absence (O) and presence (●) of 100-fold excess putrescine as a measure of polyamine transport. Aliquots were removed and filtered at various times as described in Materials and Methods.

seedling agar resulted in at least a 4-fold increase in the corresponding polyamine pool extracted from the nodules (Table II.6).

In order to determine if DFMO had the same effect on polyamine content on Rhizobium within the nodule, 5 mM-DFMO was added to the seedling agar before pouring. Polyamine analyses of the resulting nodules are shown in Table II.7, where the dominant triamine in the nodules of both cultivars shifts from homospermidine to spermidine in the presence of DFMO.

Table II.6. Effects of Exogenous Putrescine and Spermidine on the Polyamine Content of Medicago sativa L. (cv Vernal) Nodules

Addition	Polyamine Concentration[a] (nmol/g fresh wt.)			
	PUT	SPD	HOMO-SPD	SPM[b]
None (Control)	33	62	39	1800
1 mM-Putrescine	130	72	39	78
1 mM-Spermidine	72	300	34	320

[a]Based on nodules excised from four plants.

[b]Spermine

Table II.7. Effect of Difluoromethylornithine (DFMO) on the Polyamine Content of Medicago sativa L. Nodules

Addition	Polyamine Concentration (nmol/g fresh wt.)			
	PUT	SPD	HOMO-SPD	SPM[a]
cv Vernal[b]:				
None (Control)	68	29	50	2000
5 mM-DFMO	<1	150	74	28
cv Anchor[c]:				
None (Control)	2	390	430	ND[d]
5 mM-DFMO	290	840	300	ND

[a]Spermine

[b]Based on nodules excised from 10 plants.

[c]Based on nodules excised from 3 plants.

[d]Not determined.

DISCUSSION

Homospermidine is the dominant (79% of the total polyamine pool), but not the only, polyamine in extracts of Rhizobium meliloti. While the presence of homospermidine has been reported previously for two other rhizobial species - R. leguminosarum and R. phaseoli (8), our analyses of R. meliloti also detect spermidine and putrescine which were not reported to be present in these other species, probably as a result of the lower sensitivity of the method used. In contrast, putrescine is the dominant amine in Escherichia coli where the ratio of putrescine to spermidine is reported in the range of 3.4-7 to 1 and spermidine is present at 220-670 pmol/10^8 cells (31, 33, 34).

In addition to the differences in the levels and the polyamines present, our studies with the ornithine decarboxylase from R. meliloti suggest that it more closely resembles the enzyme from Pseudomonas aeruginosa than the ornithine decarboxylase from E. coli or Klebsiella pneumoniae (5). While DFMO has no apparent effect in vitro on the enzymes from the latter two species, ornithine decarboxylase from R. meliloti is more sensitive to the inhibitor, with a 12-fold lower dissociation constant (K_i = 120 vs 1400 μM) than the enzyme from the pseudomonad. The sensitivity of the rhizobial ornithine decarboxylase to DFMO is on the same order of magnitude in terms of both K_i and $t_{1/2}$ as other enzyme-activated inhibitors of putrescine biosynthesis in E. coli, such as α-monofluoromethylputre-

scine, ∝-monofluoromethylarginine, and (E)-∝-monofluoromethyldehydroarginine (32, 34).

Since homospermidine is synthesized from two molecules of putrescine (10), the inhibition of putrescine synthesis via ornithine decarboxylase by DFMO should and does directly result in diminished homospermidine levels, particularly in an organism like R. meliloti in which putrescine is normally present at low concentrations. In contrast to homospermidine, we found that spermidine concentrations increase in the presence of DFMO. Increases in the intracellular spermidine concentration in the presence of putrescine biosynthetic inhibitors have been reported previously for E. coli and Ps. aeruginosa (32-34), but not to the degree found for Rhizobium with added DFMO. In R. meliloti, this increase in spermidine may result from the conversion of homospermidine to spermidine via putrescine and a polyamine oxidase, a process which has not been shown to exist in any homospermidine-containing organisms. The action of a bacterial polyamine oxidase or dehydrogenase would likely yield putrescine as one product due to the symmetrical structure of homospermidine. On the other hand, since the activity of ornithine decarboxylase is restored to normal levels by 10 hours, DFMO may be acting at a secondary site, such as an effector or regulatory molecule, enhancing the synthesis of spermidine (or inhibiting homospermidine synthesis).

The ability of exogenous spermidine to augment both putrescine and homospermidine also may be due to the interconversion of the two

polyamines, probably via putrescine, as evidenced by the incorporation of radioactivity derived from spermidine, labeled in the tetramethylene moiety, into putrescine and homospermidine.

The addition of putrescine or homospermidine, in the presence or absence of DFMO, results in substantially reduced spermidine levels. Bitonti et al. (35) observed a similar reduction in spermidine concentrations in the presence of polyamine biosynthetic inhibitors and exogenous putrescine, but described it as paradoxical since spermidine synthesis was not completely inhibited. Since this effect is seen in Rhizobium both without inhibitor, as well as with DFMO, which, when used alone, stimulates spermidine synthesis, the phenomenon appears to be solely due to the presence of the exogenous amine.

Since the increase in spermidine seen when this amine is exogenously supplied is not accompanied by any change in the final optical density of the culture, the stimulation of final optical density by DFMO is probably due to the decrease in homospermidine or a combination of both responses. The ability to selectively diminish the homospermidine pool will have to await specific inhibitors of its synthesis and/or mutants deficient in the homospermidine synthase.

The rapid incorporation of [1,4-^{14}C]-putrescine into homospermidine, rather than spermidine, and its eventual appearance in spermidine, suggests that: (1) putrescine is a precursor for both triamines; (2) putrescine is preferentially synthesized into

homospermidine-whether based on substrate affinity, reaction rate, amount of enzyme present, and/or effector regulation; and (3) homospermidine may be degraded to putrescine.

Like E. coli (31), putrescine inhibits spermidine transport in non-growing R. meliloti. However, 10-fold excess spermidine did not inhibit putrescine uptake in the enteric (31), whereas homospermidine is effective at blocking the accumulation of putrescine in Rhizobium. Whether the amine in excess is acting as an analog of the labeled compound or actually competing for accumulation by the same transport mechanism is unknown, but the equivalent rates of accumulation of putrescine and spermidine suggest that these three polyamines utilize the same mechanism (which may be important for biosynthetic regulation).

The presence of homospermidine is not restricted to free-living rhizobia as evidenced by its detection in the nodules, but not the roots, of alfalfa. Previously, homospermidine has been reported in the root nodules of various legume species (8, 28). The polyamine content of alfalfa nodules responds much like the free-living rhizobia in the presence of putrescine, spermidine, or DFMO. Since the concentration of polyamines is highest in the nodules of leguminous plants (28), the changes seen with these various compounds are most likely due to the procaryotic member of the symbiosis, even though the plant itself is also affected.

A close relationship between homospermidine and nitrogen fixation has been suggested based on data for nitrogen-fixing cyano-

bacteria and procaryotes (13). Cyanobacteria capable of nitrogen fixation, like rhizobia, contain homospermidine as the dominant polyamine. However, spermidine predominates in the cyanobacterial species which lack the ability to fix nitrogen, and homospermidine is absent or present only at low levels. The significance of homospermidine in various nitrogen-fixing organisms (8, 13, 16) must await further investigation, but may be related to the particular environmental requirements of the nitrogen fixation process, much as the length of the polyamine chain correlates with the growth temperature of thermophilic bacteria (36).

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CHAPTER III

Spermidine is derived from aspartic acid in *Rhizobium meliloti*

Authors: Paula Allene Tower and Adolph J. Ferro

SUMMARY

The biosynthesis of polyamines, and spermidine in particular, in Rhizobium meliloti was examined. Incubation of R. meliloti with DL-[5-^{14}C]-ornithine, L-[U-^{14}C]-arginine, or L-[U-^{14}C]-aspartic acid resulted in the isolation of labeled putrescine, spermidine, and homospermidine. However, labeled polyamines were not found when L-[3,4-^{14}C]-methionine was used. Use of DL-α-difluoromethyl-ornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase, demonstrated that L-[U-^{14}C]-aspartic acid was incorporated into spermidine directly, rather than after first being metabolized to putrescine. In addition, spermidine was radiolabeled even upon incubation of L-[U-^{14}C]-aspartic acid with a methionine auxotrophic strain of R. meliloti and in the presence of DFMO. Although S-adenosylmethionine synthetase activity was present in cell-free extracts of R. meliloti, S-adenosylmethionine decarboxylase activity was never detected under a variety of growth or in vitro assay conditions. Therefore, spermidine biosynthesis in R. meliloti appears to be independent of the classical pathway involving S-adenosylmethionine decarboxylation, utilizing instead an alternate pathway related to aspartic acid.

INTRODUCTION

Spermidine is the polyamine most commonly found in procaryotic systems. The classical pathway of spermidine biosynthesis involves the decarboxylation of S-adenosylmethionine by S-adenosylmethionine decarboxylase. Subsequent transfer of the aminopropyl moiety from the resulting S-5'-deoxyadenosyl-(5')-3-methylthiopropylamine to putrescine yields spermidine and 5'-deoxy-5'-methylthioadenosine. However, an alternate pathway involving a Schiff base reaction between L-aspartic β-semialdehyde (the aminopropyl donor) and putrescine has been found in the bacteria, Rhodopseudomonas spheroides and Paracoccus denitrificans (1), as well as in the plant, Lathyrus sativus (2). This condensation and subsequent NADPH-dependent reduction results in the formation of "carboxyspermidine." A pyridoxal phosphate-dependent enzyme then decarboxylates carboxyspermidine to produce spermidine.

In this report, we present evidence for the existence of the alternate pathway of spermidine synthesis in Rhizobium meliloti, one which is derived from aspartic acid and is unrelated to methionine and S-adenosylmethionine.

MATERIALS AND METHODS

Chemicals

L-[1-^{14}C]-Ornithine (59 mCi/mmol), L-[U-^{14}C]-aspartic acid (224 mCi/mmol), and S-adenosyl-L-[carboxyl-^{14}C]-methionine (60 mCi/mmol) were purchased from Amersham Corporation. DL-[5-^{14}C]-Ornithine (49 mCi/mmol), L-[3,4-^{14}C]-methionine (57 mCi/mmol), and L-[U-^{14}C]-arginine (300 mCi/mmol) were obtained from Research Products International Corporation. DL-α-Difluoromethylornithine (MDL 71,782A) was a gift from Dr. Peter P. McCann (Merrell Dow Research Institute, Cincinnati, OH).

Bacterial Growth

Rhizobium meliloti strains 41, C15, and C15 M1 were gifts from Dr. Lyle R. Brown (Oregon State University, Corvallis, OR) and were cultured as described (3).

Escherichia coli B/2 was cultured in LB broth (4) at 37°C.

Enzyme Assays

Enzyme extracts were prepared as described (3).

S-Adenosylmethionine decarboxylase (EC 4.1.1.50) activity was measured as a function of $^{14}CO_2$ released from S-adenosyl-L-[carboxyl-^{14}C]-methionine by a modification of the procedure of Markham et al. (5). The reaction mixture contained (final concentrations): 50 mM-potassium phosphate (pH 7.0); 1.5 mM-dithiothreitol; 0.04 mM-S-adenosyl-L-[carboxyl-^{14}C]-methionine (0.05 μCi);

10 mM-$MgCl_2$ or 2.4 mM-putrescine·2HCl; and 1-2 mg protein in a final volume of 0.5 ml. The assay was carried out as previously described for ornithine decarboxylase (3).

S-Adenosylmethionine synthetase (ATP:L-methionine adenosyltransferase; EC 2.5.1.6) activity was determined by measuring the conversion of L-[3,4-^{14}C]-methionine to ^{14}C-S-adenosylmethionine, using the affinity of a boronate derivative for cis-diols to separate the product. The reaction mixture, based on (6), contained (final concentrations): 130 mM-Tris·HCl (pH 8.0); 20 mM-ATP; 200 mM-KCl; 200 mM-$MgCl_2$; 1 mM-L-[3,4-^{14}C]-methionine (0.25 μCi); and 2 mg protein in a final volume of 0.5 ml. After incubation at 37°C for 60 min, the reaction was terminated with the addition of perchloric acid, deproteinized, and neutralized. One half of the mixture was applied onto an Affi-Gel 601 (Bio-Rad) column (0.7 cm I.D. x 4 cm; 2 ml bed volume) equilibrated with 10 mM-Tris·HCl (pH 7.2). Unreacted methionine was removed with 6 ml 10 mM-Tris·HCl (pH 7.2). S-Adenosylmethionine was eluted with two 3 ml washes of 0.1 N formic acid and collected directly into scintillation vials.

Accumulation/Incorporation of ^{14}C-Amino Acids

A culture of R. meliloti 41 was grown in defined medium (3), harvested, washed twice, and resuspended to a density of 8.8 x 10^8 cells/ml in defined salts (per liter: 0.1 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.5 g NH_4Cl, 0.23 g KH_2PO_4, 0.23 g Na_2PO_4, pH 7.0) with or without 5 mM-DFMO. After preincubating 10 ml suspensions at 30°C for 15 min,

50 nmol L-amino acid (49-57 μCi/umol) were added to each flask. To monitor accumulation, aliquots of 0.1 ml were removed at various times and applied onto 0.2 μm cellulose triacetate filters under vacuum filtration. After washing with 10 ml ice cold buffer (10 mM-potassium phosphate, 1 M-KCl, pH 6.8), the filters were dried and the radioactivity quantified in a liquid scintillation counter. At one hour, the cells were harvested by centrifugation (6,400 x g, 4°C, 15 min), washed twice, and the cell pellets were frozen at -20°C. Polyamines were analyzed by high performance liquid chromatography (HPLC) as described (3) using methanol/water (51:49; v/v) solvent; one ml fractions were collected and the radioactivity determined by liquid scintillation spectrometry. R. meliloti C15 and C15 M1 were grown in defined medium supplemented with 50 μg/ml L-methionine, resuspended to a density of 1.3×10^9 cells/ml, and otherwise treated as above.

RESULTS

Although S-adenosylmethionine synthetase was detected in Rhizobium meliloti 41 extracts at a specific activity comparable to that found in Escherichia coli B/2, S-adenosylmethionine decarboxylase activity was never found under a variety of growth or in vitro assay conditions (Table III.1). Similar assay conditions yielded positive results for E. coli extracts.

Therefore, the ability of R. meliloti 41 to use various amino acids as polyamine precursors was examined in order to investigate the mechanism of spermidine biosynthesis. As seen in Figure III.1, radioisotopes of arginine and aspartic acid were taken up and accumulated at comparable rates, as were those of ornithine and methionine, although at a reduced level. When di- and polyamines were examined by HPLC, putrescine, spermidine, and homospermidine were radiolabeled upon prior incubation of R. meliloti 41 with DL-[5-^{14}C]-ornithine, L-[U-^{14}C]-arginine, or L-[U-^{14}C]-aspartic acid (Table III.2). However, labeled polyamines were not found when L-[3,4-^{14}C]-methionine was used. In the case of ^{14}C-ornithine and ^{14}C-arginine, greater than 95 percent of the total polyamines labeled occurred as putrescine and homospermidine, with the vast majority as homospermidine. With ^{14}C-aspartic acid, 97% of the radioactivity in the polyamine fraction was equally divided between spermidine and homospermidine.

Table III.1. S-Adenosylmethionine (SAM)-Related Enzyme Activities in *Rhizobium meliloti* 41 versus *Escherichia coli* B/2

Enzyme Activity	Specific Activity (nmol/min/mg protein)	
	R. meliloti 41	*E. coli* B/2
SAM synthetase	0.30	0.24
SAM decarboxylase	ND[a]	0.046

[a]Not detected.

Figure III.1. Accumulation of Amino Acids by Rhizobium meliloti 41. R. meliloti suspensions were incubated with amino acid radioisotopes (49-57 uCi/umol) in the presence and absence of 5 mM-difluoromethylornithine (DFMO). As a measure of amino acid accumulation, aliquots were removed and filtered at various times. (○) DL-[5-^{14}C]-ornithine, (●) DL-[5-^{14}C]-ornithine + DFMO, (△) L-[U-^{14}C]-arginine, (▲) L-[3,4-^{14}C]-methionine, (□) L-[U-^{14}C]-aspartic acid, (■) L-[U-^{14}C]-aspartic acid + DFMO.

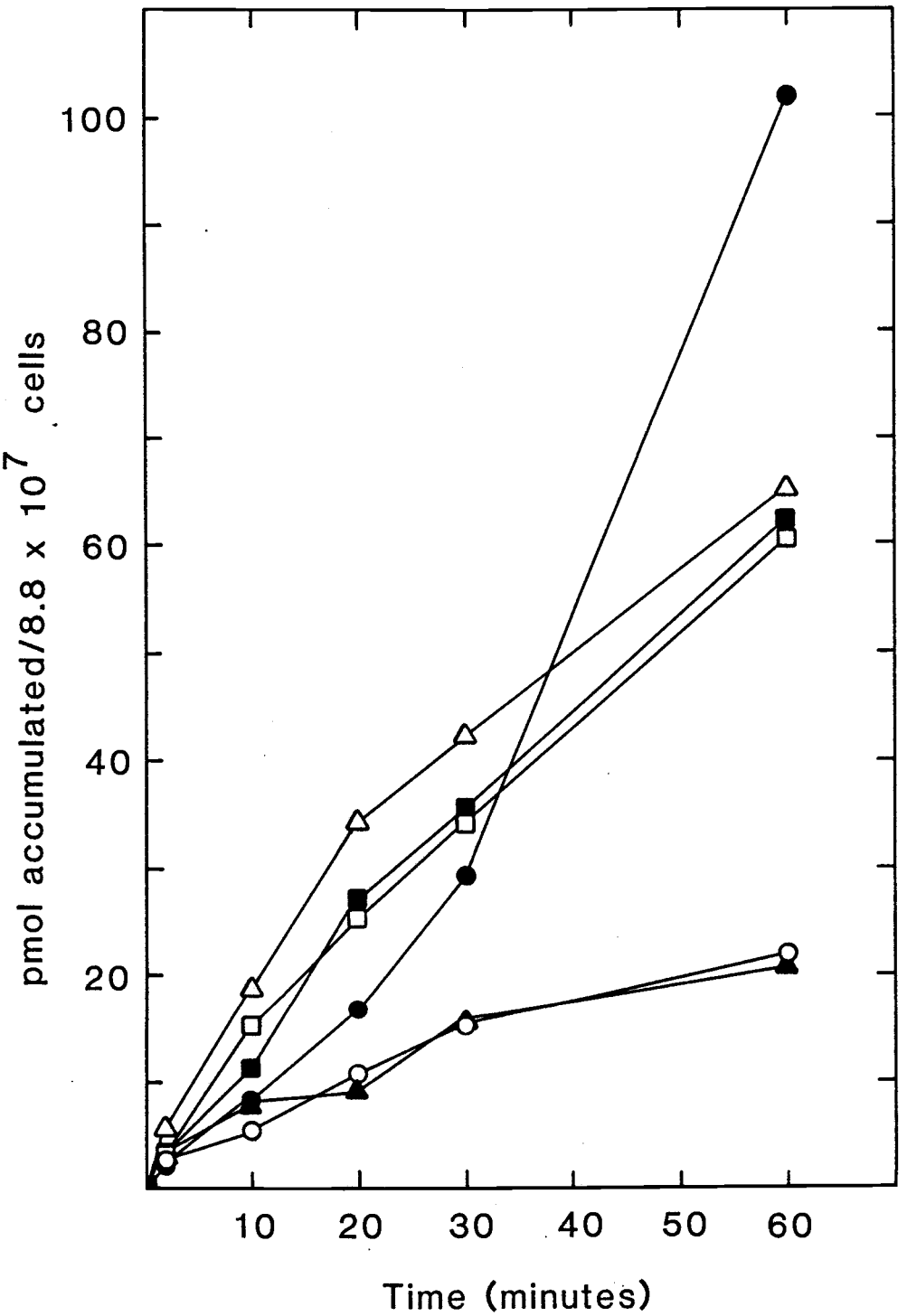
100
80
60
40
20
pmol accumulated/8.8 x 10^7 cells
10
20
30
40
50
60
Time (minutes)

Figure III.1

Table III.2. Incorporation of ^{14}C-Amino Acids into Putrescine (PUT), Spermidine (SPD), and Homospermidine (HOMO-SPD) by Rhizobium meliloti 41.

Addition	pmol Polyamines (x 10^{10} cells)			% Total Label as Polyamines		
	PUT	SPD	HOMO-SPD	PUT	SPD	HOMO-SPD
DL-[5-^{14}C]-ornithine	14	8.2	170	0.55	0.32	6.5
L-[U-^{14}C]-arginine	14	3.1	100	0.19	0.04	1.3
L-[3,4-^{14}C]-methionine	0.13	0.70	0	<0.01	0.03	0
L-[U-^{14}C]-aspartic acid	1.8	29	29	0.03	0.41	0.41

To determine if L-[U-^{14}C]-aspartic acid was incorporated into spermidine directly or after first being metabolized to putrescine, R. meliloti 41 suspensions were incubated in the absence or presence of DL-α-difluoromethylornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase, and either DL-[5-^{14}C]-ornithine or L-[U-^{14}C]-aspartic acid. At the 5 mM-DFMO concentration used, the activity of ornithine decarboxylase from R. meliloti 41 is inhibited in vivo 93 percent after one hour (3). As seen in Figure III.1, the presence of DFMO stimulated the accumulation of ^{14}C-ornithine 4.6-fold over suspensions incubated in the absence of this analog. In contrast, DFMO had no effect on ^{14}C-aspartic acid accumulation. Despite the higher level of the amino acid taken up, the amount of ^{14}C-ornithine incorporated into the three amines declined with the addition of DFMO, with the greater decreases reflected in spermidine and homospermidine (Table III.3). However, the incorporation of ^{14}C-aspartic acid into spermidine remained relatively constant in the presence of DFMO, with reduced levels of both putrescine and homospermidine. Total polyamine pools remained constant under these conditions (data not shown).

Since aspartic acid also could be converted to methionine, we examined the incorporation of L-[U-^{14}C]-aspartic acid into polyamines by R. meliloti C15 M1, a methionine auxotroph defective in cystathionine synthase (7), and C15, the corresponding wild-type strain. After confirming the presence of putrescine, spermidine, and homospermidine in the two strains by HPLC analysis, suspensions

Table III.3. Incorporation of ^{14}C-Amino Acids into Putrescine (PUT), Spermidine (SPD), and Homospermidine (HOMO-SPD) by Rhizobium meliloti 41 in the Presence of 5 mM-Difluoromethylornithine (DFMO).

Addition	pmol Polyamines[a] (x 10^{10} cells)			% Total Label as Polyamines		
	PUT	SPD	HOMO-SPD	PUT	SPD	HOMO-SPD
DL-[5-^{14}C]-ornithine + DFMO	8.6 (61%)	1.7 (21%)	36 (21%)	0.07	0.01	0.31
L-[U-^{14}C]-aspartic acid + DFMO	0.13 (7%)	26 (90%)	3.0 (10%)	<0.01	0.36	0.04

[a]Numbers in parentheses represent percentage of control incubated in the absence of DFMO (see Table III.2 for actual values).

of C15 (wild-type) and C15 M1 (*met*$^-$) were incubated in the absence or presence of 5 mM-DFMO and L-[U-^{14}C]-aspartic acid. As with strain 41, the rate of accumulation of ^{14}C-aspartic acid was found to be the same regardless of the strain (wild-type vs. *met*$^-$) or the addition of DFMO (data not shown). Both the C15 wild-type strain and the methionine auxotroph incorporated ^{14}C-aspartic acid into putrescine, spermidine, and homospermidine (Table III.4). As with *R. meliloti* 41, the levels of radiolabeled putrescine and homospermidine were dramatically reduced with the addition of DFMO. In the case of strain of C15 M1, spermidine was radiolabeled despite defective methionine biosynthesis and the presence of DFMO.

Table III.4. Incorporation of ^{14}C-Amino Acids into Putrescine (PUT), Spermidine (SPD), and Homospermidine (HOMO-SPD) by Rhizobium meliloti C15 Wild-Type and Methionine Auxotrophic Strains in the Absence and Presence of 5 mM-Difluoromethylornithine (DFMO).

Addition	pmol Polyamines (x 10^{10} cells)			% Total Label as Polyamines		
	PUT	SPD	HOMO-SPD	PUT	SPD	HOMO-SPD
C15 (wild-type):						
L-[U-^{14}C]-aspartic acid	2.0	15	25	0.02	0.17	0.29
L-[U-^{14}C]-aspartic acid + DFMO	0.27	19	0.22	<0.01	0.26	<0.01
C15 M1 (met$^-$):						
L-[U-^{14}C]-aspartic acid	2.1	18	21	0.03	0.22	0.26
L-[U-^{14}C]-aspartic acid + DFMO	0.58	19	0.30	0.01	0.27	<0.01

DISCUSSION

The inability of methionine to be incorporated into spermidine *in vivo* and the lack of detectable S-adenosylmethionine decarboxylase activity *in vitro*, despite the capacity to synthesize S-adenosylmethionine, could not account for the obvious presence of spermidine. Initial reports of an additional pathway of spermidine biosynthesis utilizing L-aspartic β-semialdehyde as the "aminopropyl donor" (1, 2) led us to examine this possibility. While L-aspartic acid is readily converted into spermidine in free-living *Rhizobium meliloti*, a significant percentage of the radioactivity in the amine fraction appears as homospermidine and, to a lesser extent, putrescine. Srivenugopal and Adiga (2) similarly found radiolabeled putrescine with L-[U-^{14}C]-aspartic acid infiltration into *Lathyrus sativus* seedlings and suggested metabolic conversions related to diamine synthesis as a possible explanation.

We used DFMO, an irreversible inhibitor of ornithine decarboxylase, to investigate the involvement of putrescine biosynthesis in the incorporation of L-aspartic acid into polyamines. DFMO not only inhibits the rhizobial ornithine decarboxylase activity *in vivo* (3) and the incorporation of ornithine into spermidine, but also effectively blocks the conversion of aspartic acid into putrescine and, thereby, into homospermidine. The metabolism of aspartic acid through the tricarboxylic acid cycle via oxaloacetate and α-ketoglutarate, glutamic acid, and ornithine (or arginine) is the

likeliest possibility for this conversion to putrescine. If de novo synthesis of putrescine by ornithine decarboxylase is reduced, the existing pool of putrescine, although small, could serve as the precursor of spermidine, as could the potential degradation of homospermidine into putrescine or the synthesis of putrescine from arginine via arginine decarboxylase. Why DFMO should stimulate ornithine accumulation in non-growing cells is both unexpected and unknown, but the additional amino acid appears to be directed away from the polyamine pool.

Although aspartic acid can be converted into methionine, the ability of R. meliloti C15 M1, a methionine auxotroph, to incorporate L-[U-^{14}C]-aspartic acid into spermidine is additional evidence suggesting that spermidine biosynthesis in this procaryote bypasses the classical pathway involving methionine, S-adenosylmethionine, and decarboxylated S-adenosylmethionine.

It may be of ecological advantage for Rhizobium meliloti to derive its spermidine from aspartic acid since aspartic acid, but not methionine, is relatively abundant in its symbiotic partner as well as in the bacteroid itself (8).

It is interesting to note that according to the 16S ribosomal RNA procaryotic phylogeny (9), Rhizobium is closely related to Rhodopseudomonas, Paracoccus, and Rhodospirillum which are reported to synthesize spermidine via L-aspartic β-semialdehyde and putrescine and/or contain homospermidine (1, 10, 11). The enzymes for this alternate pathway also have been detected in Agrobacterium

tumefaciens (13), another close relative of Rhizobium, as has the presence of homospermidine (12).

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CHAPTER IV

Enzymatic Synthesis of Homospermidine in *Rhizobium meliloti*

Authors: Paula Allene Tower and Adolph J. Ferro

SUMMARY

Homospermidine synthase from Rhizobium meliloti 41 was partially purified and characterized. This enzyme, which synthesizes homospermidine from putrescine, was stimulated by NAD^+ and exhibited a pH optimum of 7.6 in Tris·HCl buffer. Maximal activity was attained at 34°C. Kinetic analysis revealed a K_m of 1.4 mM with respect to putrescine.

Of the substrate and product analogs tested in vitro, spermidine was the most effective inhibitor of homospermidine synthase, exhibiting competitive inhibition kinetics and a K_i of 0.08 mM. Other inhibitory compounds included diaminooctane and diaminopropane, as well as homospermidine itself, which inhibited the activity greater than 50 percent at equimolar concentration to the substrate.

INTRODUCTION

Polyamines less common than spermidine have been detected in a variety of organisms. One such polyamine, homospermidine [H_2N-$(CH_2)_4$-NH-$(CH_2)_4$-NH_2], was first reported as a naturally occurring compound in sandal leaves by Kuttan et al. (1). This polyamine has since been found in a number of plant, animal, procaryotic, and archaebacterial systems (2-11). In plants, it has been identified as a precursor in the synthesis of pyrrolizidine alkaloids (12).

Tait (5) has shown that, in Rhodopseudomonas, homospermidine is synthesized from two molecules of putrescine via a NAD-requiring enzyme, in what is thought to involve a Schiff base reaction with 4-aminobutyraldehyde as the postulated intermediate (5, 13) (Figure IV.1). Our studies on polyamine metabolism in Rhizobium meliloti revealed ornithine, arginine, and putrescine as precursors to homospermidine (14, 15) in agreement with other studies (5, 13, 16).

Interestingly, several nitrogen-fixing organisms are included among the organisms in which homospermidine has been detected (3, 8, 11). Within the cyanobacteria, only those species capable of nitrogen fixation contain homospermidine as the major polyamine (8). However, spermidine predominates in the cyanobacterial species which lack the ability to fix nitrogen, and homospermidine is absent or present only at low levels.

The reasons for attempting to purify the homospermidine synthase from R. meliloti are three-fold. First, the ability of

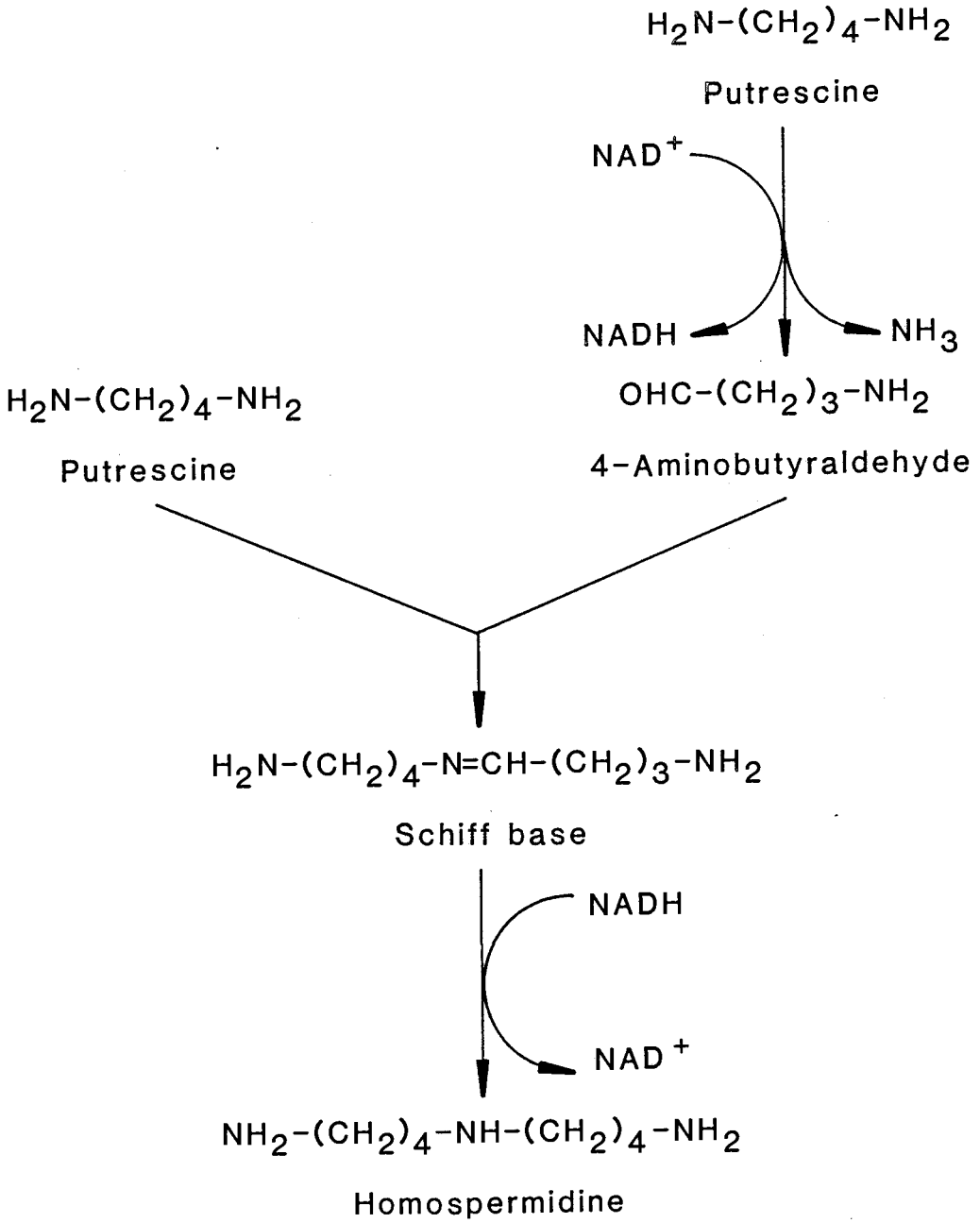

Figure IV.1. Postulated Synthesis of Homospermidine

DL-α-difluoromethylornithine (DFMO), an inhibitor of putrescine biosynthesis, to reduce the intracellular concentration of homospermidine, while augmenting spermidine levels, suggests that DFMO inhibits homospermidine synthesis in addition to ornithine decarboxylase (14). Secondly, if DFMO does not inhibit the enzyme in vitro, is the decrease in homospermidine concentration due to the degradation of homospermidine? The isolation of enzymatically synthesized [^{14}C]-homospermidine would help to determine whether homospermidine can be degraded to putrescine and act as a putrescine reservoir in R. meliloti. Finally, a mutant deficient in the synthesis of homospermidine would be highly desirable in elucidating any role(s) homospermidine plays in the nitrogen fixation-nodulation processes. Since polyamine mutants do not exhibit selectable phenotypes, purification of the homospermidine synthase is the first step towards site-directed mutagenesis of this enzyme.

In this report, we have partially purified and characterized the homospermidine synthase from R. meliloti.

MATERIALS AND METHODS

Chemicals

Authentic homospermidine trihydrochloride used as a standard was a gift from Dr. Seymour S. Cohen (SUNY, Stony Brook, NY); DL-α-difluoromethylornithine (MDL 71,782A) was a gift from Dr. Peter P. McCann (Merrell Dow Research Institute, Cincinnati, OH). Homospermidine hydrochloride was synthesized from the free base of putrescine (Sigma) and 4-bromobutylphthalimide (Aldrich) (2:1; mmol/mmol) according to the procedure of Okada et al. (17). [1,4-^{14}C]-Putrescine (118 mCi/mmol) was purchased from Amersham Corp. All other compounds were purchased from commercial sources.

Polyamine Analysis

Enzyme assay mixtures and di- and polyamine standards were derivatized with benzoyl chloride as previously described (18). The benzoyl amines were analyzed by high performance liquid chromatography (HPLC) using a Waters Model M-6000A system equipped with a Model U6K injector, a reverse phase μBondapak C18 column (3.9 mm I.D. x 30 cm; 10-μm particle size), Model 440 UV absorbance detector (254 nm), Model 740 data module, plus a Co:Pell ODS guard column (2.1 mm I.D. x 7 cm; Whatman). The mobile phase consisted of methanol/water (55:45; v/v) at a flow rate of 1.0 ml/min. Fractions were collected every two minutes and the radioactivity quantitated.

Assay mixtures and standards were also derivatized with dansyl

chloride (5-dimethylamino-1-naphthalene sulphonyl chloride) according to the method of Seiler and Weichmann (19) as modified by Flores and Galston (20). Thin layer chromatography (TLC) of the dansylated derivatives was performed with sheets of aluminum oxide F_{254}, neutral, Type T, on aluminum, in a solvent system consisting of chloroform/n-butanol (49:1, v/v). Dansyl derivatives were visualized with a mineral lamp.

Separation of underivatized polyamines was achieved using descending paper chromatography (Whatman No. 1) with a solvent system comprised of ethylene glycol monomethyl ether/propionic acid/water (70:15:15) saturated with NaCl (21). The compounds were detected by spraying with ninhydrin, followed by brief heating at 100°C.

R_f (R_t) values for the three methods are shown in Table IV.1.

Enzyme Extracts

Cells were harvested by centrifugation (10,600 x g), washed twice, and resuspended in extract buffer (50 mM-potassium phosphate, 10% glycerol, and 1 mM-dithiothreitol, pH 6.8). After passing the suspension through a French pressure cell (18,000 psi) three times, cellular debris was removed by centrifugation (20,000 x g) for 30 min at 4°C, and the supernatant was stored frozen at -20°C after dialysis against the extract buffer. Protein concentration was determined as described by Bradford (22), using bovine serum albumin as the standard.

Table IV.1. R_f (R_t) Values for Analysis of Putrescine and Homospermidine.

	R_f (R_t) Values	
Method	Putrescine	Homospermidine
Paper Chromatography	0.36	0.23
TLC	0.50	0.78
HPLC	8.5 min	19.5 min

Homospermidine Synthase Assay

Initial determinations of homospermidine synthase activity were based on the reaction conditions of Tait (5) and the separation procedure of Kuttan and Radhakrishnan (13). The reaction mixture contained (final concentrations): 40 mM-Tris·HCl (pH 8.8); 40 mM-KCl; 0.2 mM-NAD^+; 4 mM-dithiothreitol; 1 mM-[1,4-^{14}C]-putrescine (0.1 μCi); and up to 0.3 mg protein. After incubation for 30 min at 37°C, the reaction was terminated with the addition of 0.02 ml 1.8 M-trichloroacetic acid and deproteinized. The supernatant was applied onto Dowex 50W-X8 (H^+) column (0.7 cm I.D. x 10 cm; 0.5 ml bed volume) and washed with 10 ml water. Unreacted putrescine and homospermidine were eluted with 15 ml 2.5 N-HCl and 8 ml 3 N-HCl, respectively. One ml aliquots of the acid washes were quantified by liquid scintillation spectrometry.

Partial Purification of Homospermidine Synthase

A frozen cell paste (9 g wet wt.) of R. meliloti 41, grown in yeast extract-mannitol broth (14), was resuspended in two volumes cold Buffer A (50 mM-Tris·HCl, 100 mM-KCl, 1 mM-dithiothreitol, 5% (v/v) glycerol, pH 8.0) and disrupted with a French pressure cell as described above. After removing debris, the supernatant was ultracentrifuged at 100,000 x g for one hour. The resulting supernatant was transferred to dialysis tubing and placed in contact with cold Sephadex G100 (Pharmacia) for 12 h in order to concentrate the preparation. The concentrated extract was dialyzed against two

changes of Buffer A.

An aliquot of the concentrated crude extract (equivalent to 1.2% column volume) was applied onto a Sephacryl S-200 (Pharmacia) gel permeation column (2.1 cm I.D. x 62 cm) equilibrated with Buffer A and eluted at a flow rate of 7.4 cm/h. Protein was monitored by absorbance at 280 nm. Fractions exhibiting peak homospermidine synthase activity were pooled and used for further study.

Determination of pH/Temperature Optima and K_m

Optimum pH was determined with 0.14 mg of the partially purified extract after incubation for one hour at 37°C using 40 mM-Hepes, -Tris·HCl, or -CAPS buffers in the pH range of 6.6-10.

The effect of incubation temperature on the activity of the homospermidine synthase was determined over a range of 1-50°C at pH 7.6 (Tris·HCl). The buffers were corrected for pH variations due to temperature.

The K_m for putrescine was determined over a range of substrate concentrations [0.1-0.25 mM-putrescine (2750 cpm/nmol); 0.5-4.0 mM-putrescine (550 cpm/nmol)] at 34°C (pH 7.6).

Inhibition of Homospermidine Synthase

The effect of various compounds on the activity of the homospermidine synthase was determined by incubating the partially purified extract for 1 h at 34°C, pH 7.6, in the presence of 10 mM-[1,4-^{14}C]-putrescine (550 cpm/nmol) and an equimolar concentration

of the test chemical. The 2.5 N-HCl wash of the Dowex 50W columns was increased to 18 ml to lower background radioactivity.

The $\underline{K}_i$ for spermidine was determined over a range of putrescine concentrations (0.25-2.0 mM; 2200 cpm/nmol) at 0.10 and 0.20 mM-spermidine.

RESULTS

Upon incubation of cell-free extracts of Rhizobium meliloti 41 with [1,4-^{14}C]-putrescine and NAD^+, the synthesis of a non-spermidine polyamine was observed. By comparing R_f values of dansylated standards by TLC, or underivatized polyamines by paper chromatography, with the radioactivity associated with the visualized spots, the product of the reaction was tentatively identified as homospermidine. The radioactive compound was also found to co-chromatograph with authentic benzoylated homospermidine standard by HPLC (Figure IV.2), further establishing its identity.

In R. meliloti extracts, the enzymatic synthesis of homospermidine by homospermidine synthase was observed at specific activities up to 14 nmol/min/mg protein. However, this activity was not detected in extracts from Escherichia coli B/2. The omission of NAD^+ from the reaction mixtures reduced the activity of dialyzed extracts over two-fold (Table IV.2).

The enzyme was partially purified (2.3-fold) from a cell-free extract of Rhizobium meliloti 41 by gel permeation chromatography on Sephacryl S200, after first concentrating with dry Sephadex G100. Fractions exhibiting homospermidine synthase activity were pooled, and the assay conditions optimized. As seen in Figure IV.3, a pH of 7.6 (Tris·HCl) yielded optimal activity and was used in all subsequent experiments. The temperature optimum for the homospermidine synthase was 34°C, with less than fifty percent of the maximal

Figure IV.2. Identification of [^{14}C]-homospermidine upon incubation of Rhizobium meliloti 41 cell-free extract with [1,4-^{14}C]-putrescine. R. meliloti 41 cell-free extract was incubated with 0.5 mM [1,4-^{14}C]-putrescine (0.3 mCi/mmol) for 1 h at 37°C and analyzed by HPLC. (A) HPLC chromatogram of benzoylated-putrescine (PUT) and -homospermidine (HOMO-SPD) standards. Radioactivity associated with each 2 ml fraction eluted by HPLC: (B) Control incubated with heat-denatured extract; (C) R. meliloti 41 cell-free extract (0.6 mg protein).

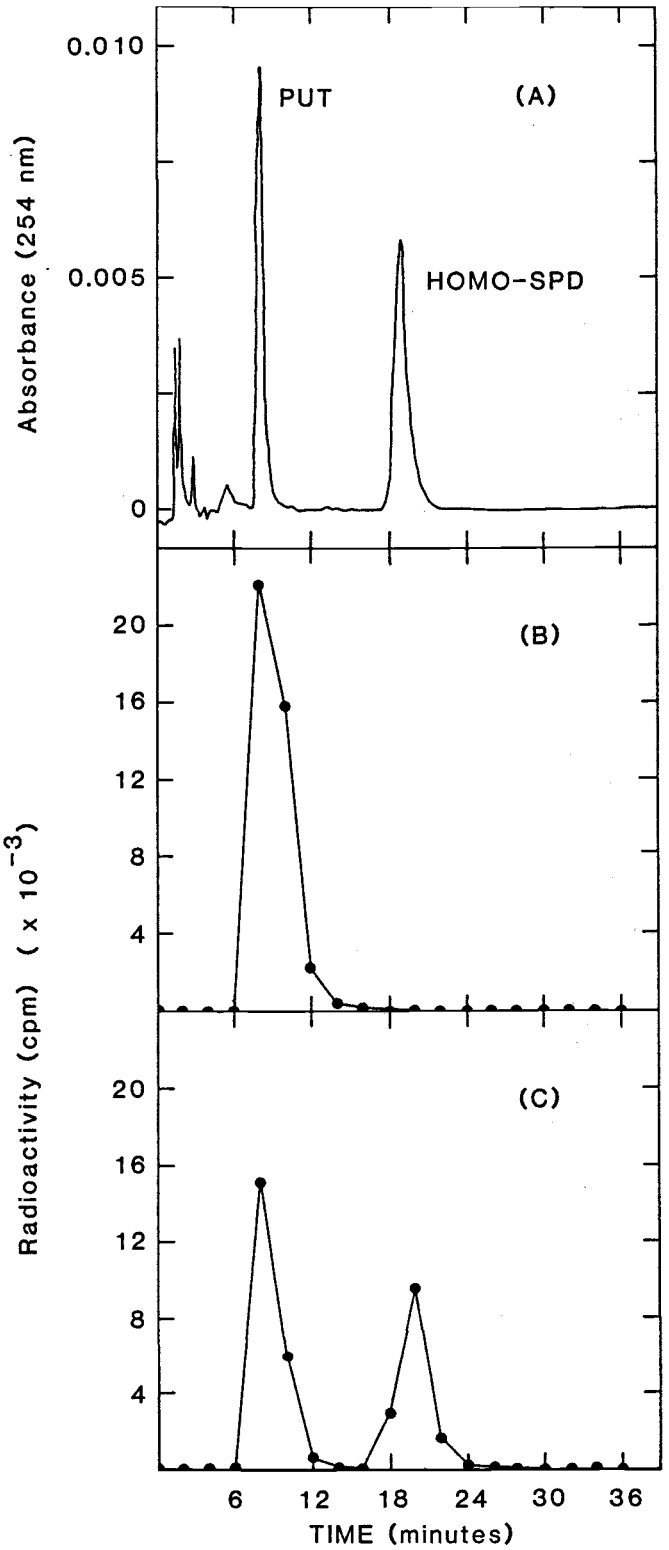
0.010
0.005
0
Absorbance (254 nm)
PUT
(A)
HOMO-SPD
20
16
12
8
4
(B)
20
16
12
8
4
(C)
Radioactivity (cpm) (x 10^{-3})
6
12
18
24
30
36
TIME (minutes)

Figure IV.2

Table IV.2. Effect of NAD^+ on the *in vitro* Synthesis of Homospermidine in Cell-free Extracts of *Rhizobium meliloti* 41

Component Omitted	Radioactivity[a] (cpm less background)
None	5024
NAD^+	1952

[a]Assay was carried out at 10 mM-putrescine (0.25 mCi/mmol) for one hour.

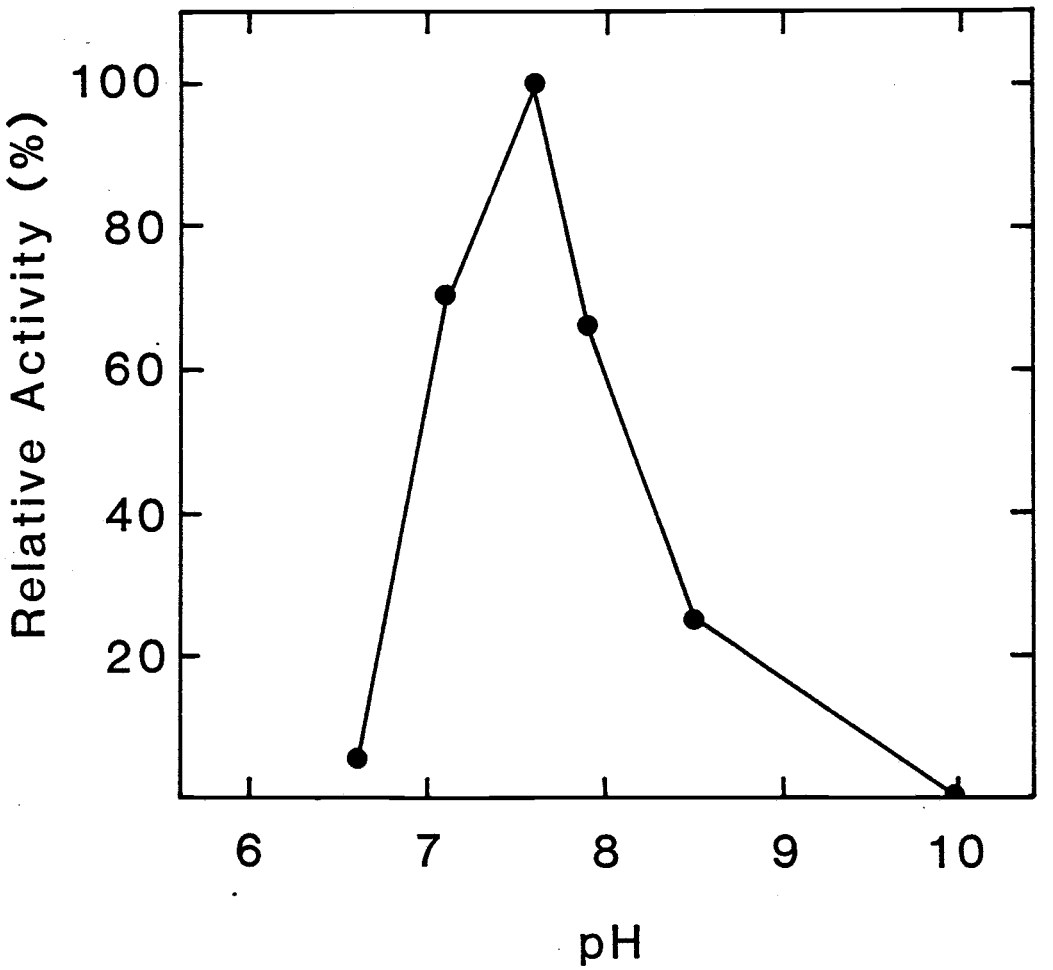

Figure IV.3. Effect of incubation pH on homospermidine synthase from Rhizobium meliloti 41. A partially purified extract of R. meliloti was incubated with 1 mM-[1,4-^{14}C]-putrescine for 1 h at 37^{o}C at various pH values.

activity remaining at 37°C (Figure IV.4). An incubation temperature of 34°C was subsequently used.

Kinetic analysis of the rhizobial enzyme with a double-reciprocal plot revealed a K_m value of 1.4 mM with respect to putrescine and a V_{max} of 3.6 nmol/min for one mg protein (Figure IV.5).

Several structural substrate and product analogs were evaluated for their ability to inhibit the activity of homospermidine synthase. As seen in Table IV.3, diaminopropane, which is one methylene group shorter than the normal substrate, inhibited the synthase about 70 percent, while cadaverine (1,5-diaminopentane) had little effect on activity. The product of the reaction, homospermidine, reduced the activity by about one half. Two other analogs of homospermidine were also effective inhibitors: di-n-butylamine and diaminooctane. γ-Aminobutyric acid (GABA), which is a potential degradative product of homospermidine, was slightly stimulatory.

Spermidine, which is normally present in Rhizobium, inhibited the activity of homospermidine synthase 91 percent when present in equimolar concentrations, making it the most effective inhibitor of the analogs tested. The triamine exhibited competitive inhibition kinetics with a K_i of 0.08 mM (Figure IV.6).

Two inhibitors of polyamine biosynthesis were also tested against homospermidine synthase. Methylglyoxyl bis(guanylhydrazone) (MGBG), a reversible inhibitor of S-adenosylmethionine decarboxylase, was inhibitory, whereas difluoromethylornithine (DFMO) had

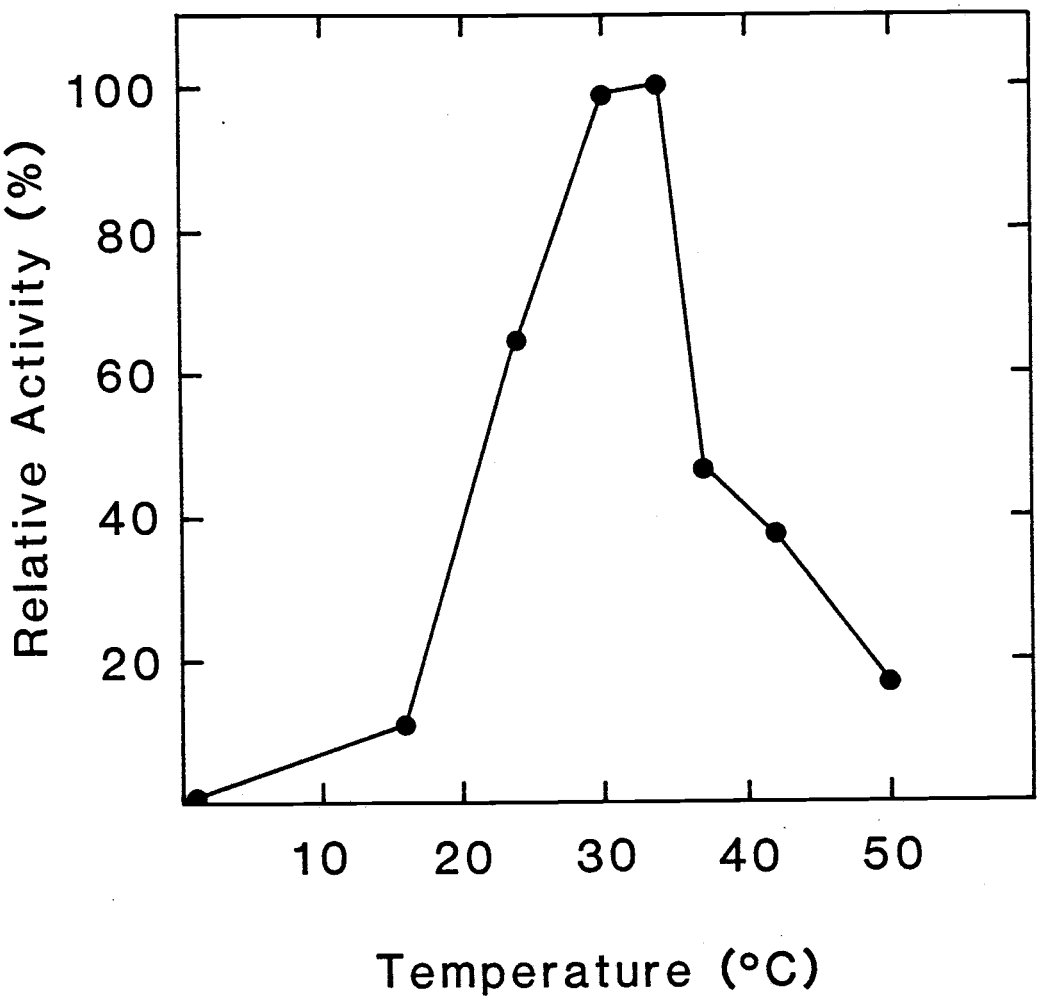

Figure IV.4. Effect of incubation temperature on homospermidine synthase from Rhizobium meliloti 41. A partially purified extract of R. meliloti was incubated at various temperatures with 1 mM-[1,4-^{14}C]-putrescine for 1 h at pH 7.6 (Tris·HCl). Controls were run at each temperature and each buffer corrected for pH variations due to temperature.

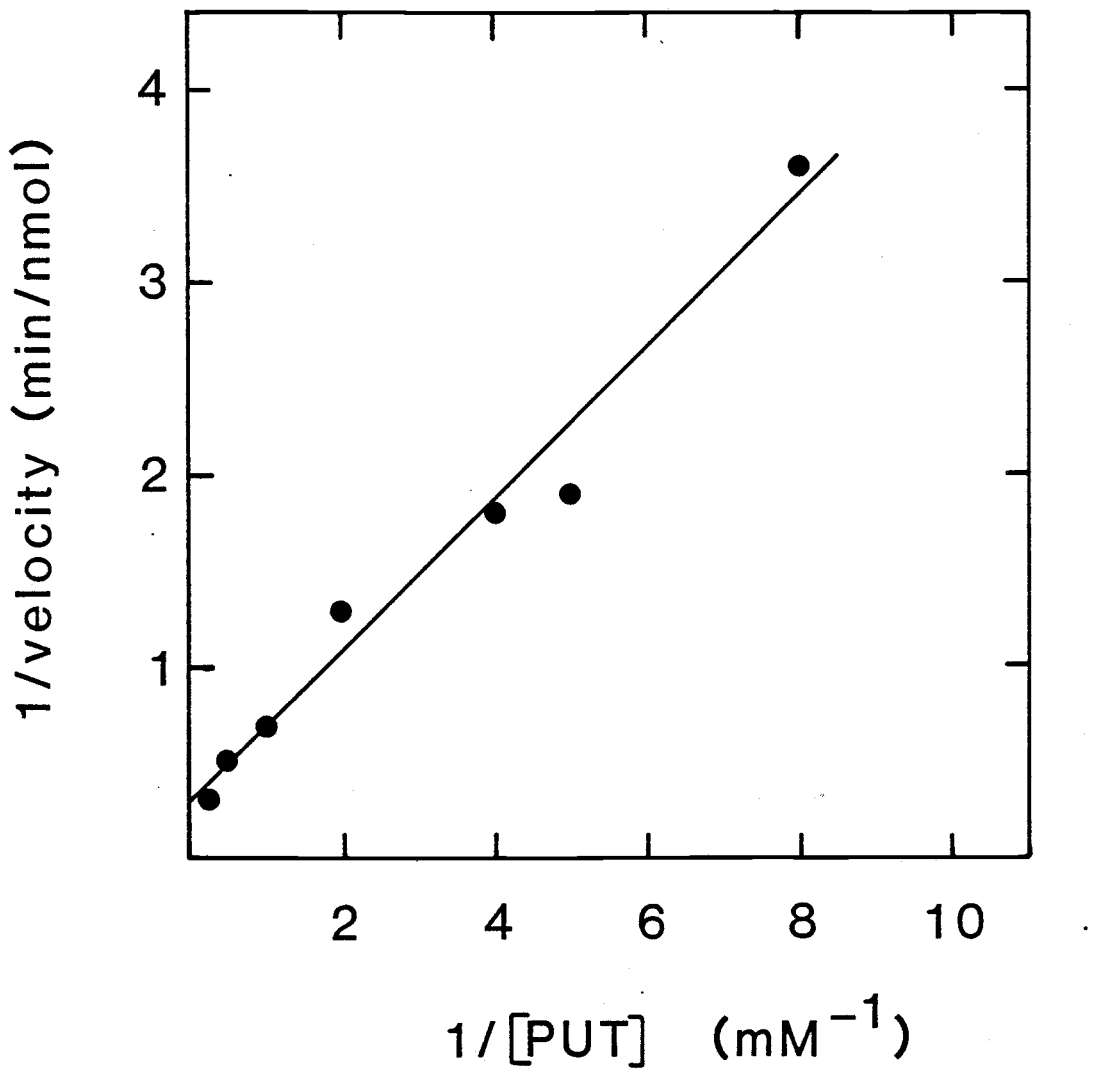

Figure IV.5. Lineweaver-Burk Plot for determination of putrescine K_m and V_{max} for homospermidine synthase from Rhizobium meliloti 41. Each point represents the average of two determinations. Velocities were nmol homospermidine formed per min per mg protein.

Table IV.3. Inhibition of Homospermidine Synthase from Rhizobium meliloti 41.

Addition	% Activity Remaining
None (Control)	100
Diaminopropane	29
Cadaverine	93
Diaminohexane	55
Diaminooctane	13
Spermidine	9
Homospermidine	42
Di-n-butylamine	67
DFMO	90
MGBG	49
GABA	126

A partially purified homospermidine synthase was incubated with 10 mM-[1,4-^{14}C]-putrescine and an equimolar concentration of each compound.

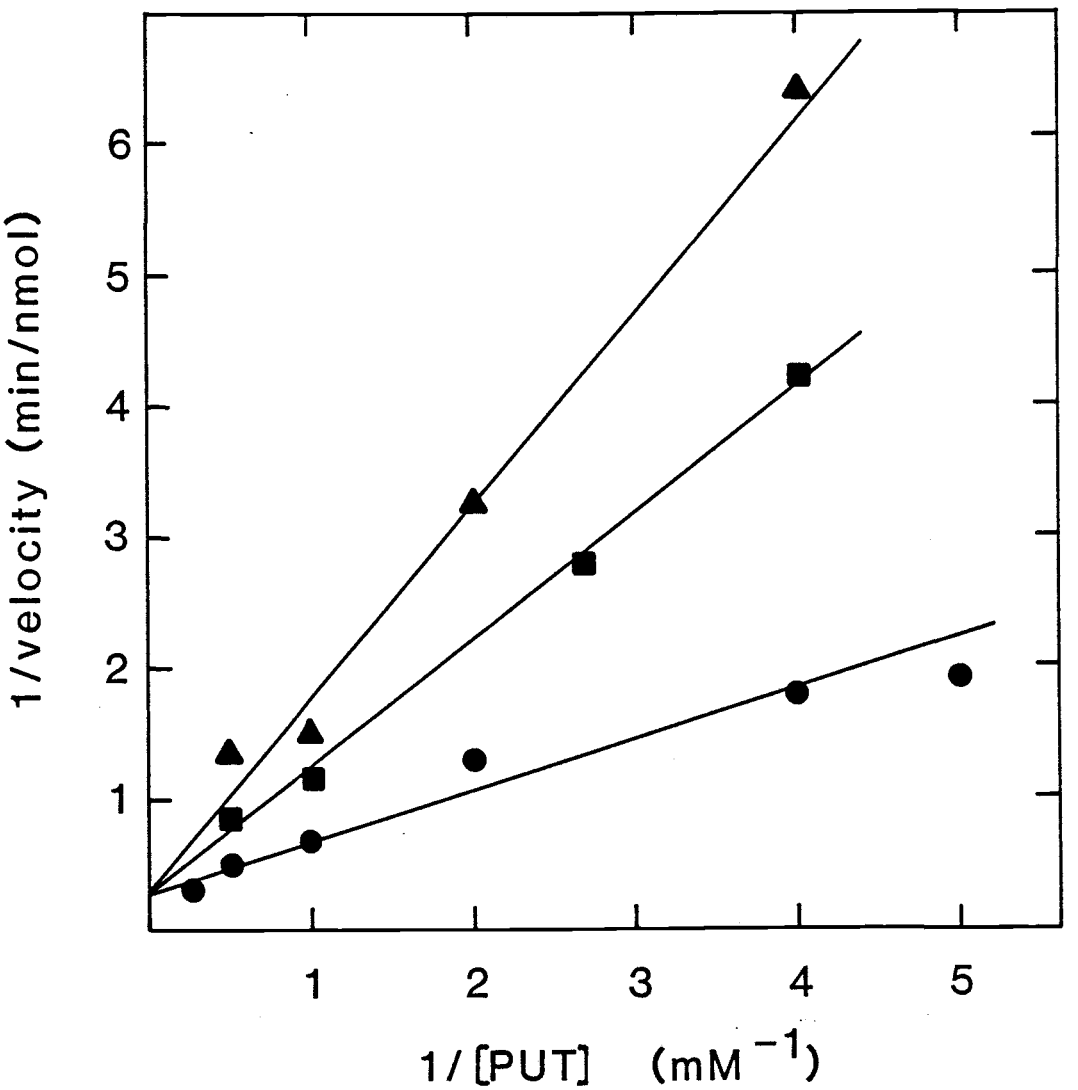

Figure IV.6. Inhibition of rhizobial homospermidine synthase by spermidine. Velocities were nmol homospermidine formed per min per mg protein. No inhibitor, (●); 0.10 mM-spermidine, (■); and 0.20 mM-spermidine, (▲).

little effect. Previous assays, using crude extracts and five-fold excess DFMO did not reveal any inhibition *in vitro* of homospermidine synthase by DFMO when analyzed by HPLC (data not shown).

DISCUSSION

Homospermidine was established as the polyamine produced upon incubation of cell-free extracts of Rhizobium meliloti with radiolabeled putrescine and NAD^+ on three distinct chromatography systems.

Unlike a previous study reporting a specific requirement for NAD^+ (16), the pyridine nucleotide only stimulated the activity of this homospermidine synthase. This difference may reflect an enzyme-bound cofactor implicated by the K_m (2.5 μM) for NAD^+ previously published (5).

The temperature and pH optima of 34°C and 7.6, respectively, for the rhizobial enzyme differ slightly from the values (37°C, pH 8.4) reported for Lathyrus (16).

Putrescine exhibits a lower affinity (higher Km) for the rhizobial homospermidine synthase, as compared to Rhodopseudomonas viridis (K_m 0.2 mM) (5), but a 2-fold lower K_m in contrast to Lathyrus sativus (K_m 3 mM) (16).

A number of inhibitors of the homospermidine synthase were identified. Diaminopropane, which is shorter in chain length than the normal substrate, inhibits the rhizobial enzyme about 70 percent as compared to 10 percent for Lathyrus (16). In Rhodopseudomonas, diaminopropane is reported to be a strong competitive inhibitor (K_i 2 μM) (5). Cadaverine, at one methylene group longer than putrescine, is a weak inhibitor in all three systems (5, 16).

Two analogs of homospermidine, spermidine and diaminooctane, were more effective at inhibition *in vitro* than the product itself, with spermidine being a competitive inhibitor. Spermidine also inhibited homospermidine synthase from *Lathyrus*, but only by 30 percent (16). Di-*n*-butylamine, another product analog, was also inhibitory, suggesting that product analogs as a class hold potential for the development of an inhibitor specific for homospermidine synthase.

Both *Rhizobium* and *Lathyrus* have been identified as using an aminopropyl moiety derived from aspartic acid in the synthesis of spermidine (15, 23), suggesting that spermidine might be an effector of homospermidine synthesis which also relies on a Schiff base reaction with putrescine. While spermidine inhibits homospermidine synthesis *in vitro*, this probably does not occur *in vivo* as exogenous spermidine does not reduce the homospermidine pool. In fact, since exogenous homospermidine results in greatly diminished spermidine concentrations (14), the opposite may be true in *Rhizobium*.

DFMO weakly inhibits the *in vitro* activity of homospermidine synthase, indicating that the decreased homospermidine levels observed *in vivo* in DFMO-grown cells are not due to direct action on the enzyme itself. It does not preclude, however, regulation by DFMO at the transcriptional level.

Ideally, an exogenous supply of [^{14}C]-homospermidine should be used to study homospermidine degradation *in vivo*. However, the

product inhibition exhibited by homospermidine and the difficulty in separating underivatized polyamines limit the feasibility of enzymatically synthesizing sufficient radiolabeled homospermidine. An alternative may be to prelabel the homospermidine pool with radioactive ornithine or arginine and subsequently trace the label.

Attempts to further purify the rhizobial homospermidine synthase using affinity chromatography could take advantage of the inhibition data since affinity matrices of CH-Sepharose-homospermidine and -putrescine were disappointingly inefficient at binding the enzyme (data not shown). However, ion-exchange chromatography on DEAE-cellulose (pH 8.0), has met with some success, reducing the number of proteins seen on SDS-PAGE gels to less than twenty bands. Further purification is necessary before initiating work on site-directed mutagenesis of homospermidine synthase. Studies using such mutants deficient in homospermidine synthase would improve our understanding of the involvement of homospermidine in the rhizobial-legume symbiosis.

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CHAPTER V

Summary

The focus of this research has been the biological synthesis and metabolism of the three polyamines found in free-living Rhizobium meliloti. A scheme of polyamine biosynthesis as proposed for this bacterial species is summarized in Figure V.1.

The main findings of this study are:

1. Homospermidine is the major polyamine and is synthesized from putrescine.
2. Spermidine is derived from aspartic acid by a pathway which does not involve methionine or S-adenosyl-methionine.
3. The presence of difluoromethylornithine (DFMO), an inhibitor of the rhizobial ornithine decarboxylase, shifts the major polyamine from homospermidine to spermidine and stimulates final cell density.

A number of questions have been left unanswered and are potential areas for further investigation. They include:

A. Is the shift in polyamines seen with the addition of DFMO (and DFMA-see Appendix) due to increased spermidine synthesis and/or decreased homospermidine production? Is homospermidine degraded to provide a source of putrescine and, thus, of spermidine under

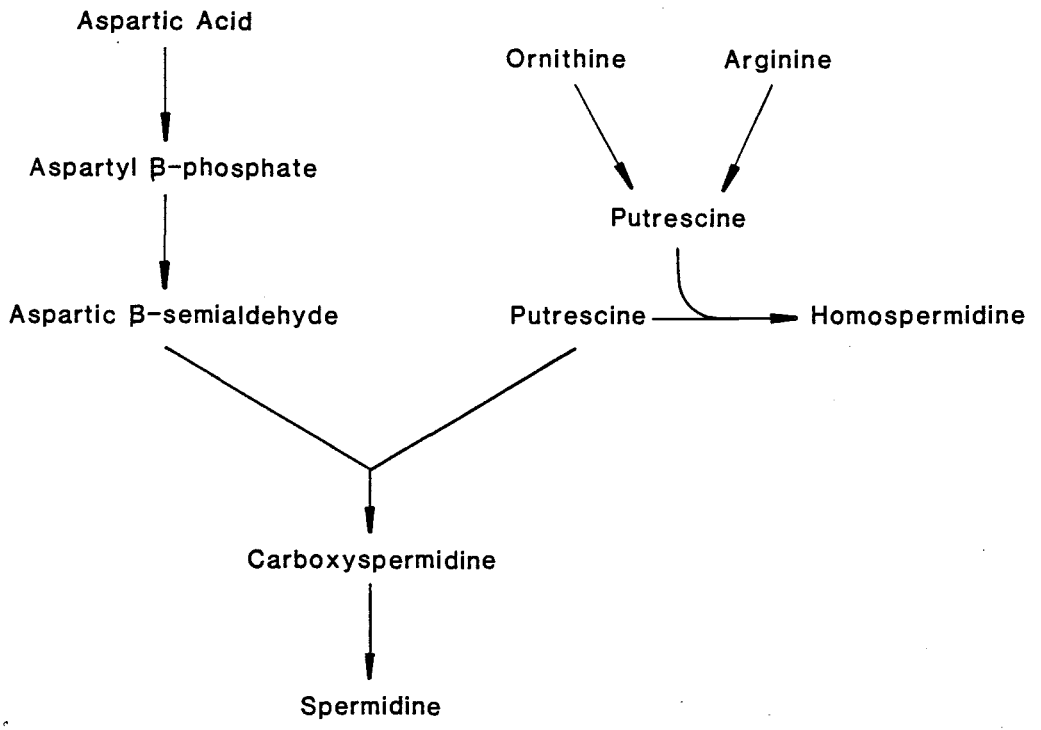

Figure V.1. Proposed Scheme of Polyamine Biosynthesis in Rhizobium meliloti

under these conditions?

B. Does extended exposure to DFMO further reduce the homospermidine level?

C. How does DFMO stimulate ornithine accumulation in non-growing cells?

D. If homospermidine is a reservoir of putrescine, of what significance is it?

E. Since putrescine marks the branch point between spermidine and homospermidine and the most dramatic changes occur with perturbations in the level of putrescine, is putrescine the key to regulating the biosynthesis of spermidine and homospermidine?

F. Is the presence of homospermidine significant for nitrogen fixation, i.e., is a mutant deficient in homospermidine synthase still infective and effective?

G. What is the function of S-adenosylmethionine (SAM) in Rhizobium since it is not involved in polyamine synthesis? SAM also plays a major role in transmethylation reactions. However Rhizobium appears to be hypomethylated as it is not resistant to cleavage by MboI restriction endonuclease (which requires unmethylated GATC sequences) and does not hybridize to the DNA adenine methylase (dam) gene from E. coli (Brooks et al., 1983). While many rhizobial strains require biotin, some do not. In any case, the supply of SAM

may exceed the demand for the vitamin.

H. Finally, if methylthioadenosine (MTA) is not a co-product of spermidine synthesis in this rhizobial system, why then is there a MTA-cleaving activity (see Appendix)? Perhaps ethylene is produced by Rhizobium in a SAM-dependent process similar to that found in plants. If ethylene is not produced, could SAM be involved in the formation of a novel nucleotide which could have significance for nitrogen fixation?

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APPENDIX

In order to determine if DL-∝-difluoromethylarginine (DFMA) had an effect on Rhizobium meliloti similar to DL-∝-difluoromethyl-ornithine (DFMO), R. meliloti 41 was cultured in defined medium in the absence or presence of 5 mM-DFMA, or in combination with 5 mM-DFMO, and the polyamines analyzed.

DFMA, like DFMO, stimulates the final optical density, increases spermidine, and decreases homospermidine and total polyamine concentrations (Table Appendix.1). These effects are also seen when DFMA is used in concert with DFMO. These data suggest that the synthesis of putrescine is a key step in the synthesis of polyamines in R. meliloti.

Table Appendix.1. Effect of Difluoromethylarginine (DFMA) on the Polyamine Content of Rhizobium meliloti 41.

Addition	Final Optical Density (Klett Units)	pmol Polyamines[a] (x 10^8 cells)		
		PUT	SPD	HOMO-SPD
None (Control)	72	31 (100)	73 (100)	560 (100)
5 mM-DFMA	169	16 (52)	120 (164)	320 (57)
5 mM each DFMA + DFMO	214	14 (45)	250 (342)	170 (30)

[a]Values in parentheses represent percentage of control.

Results from a single representative experiment.

Nodules were separated into bacteroid and nodule-soluble fractions as described previously (Fujihara et al., 1986), using a micro tissue grinder (Kontes; Vineland, NJ) and omitting the filtration step. Polyamine analysis of the two fractions revealed the presence of homospermidine in addition to putrescine, spermidine, and spermine (data not shown). The detection of homospermidine in the nodule-soluble fraction and spermine in the bacteroids suggests that polyamines are exchanged between the symbiotic partners, which might be important for either the status of the bacteroids within the nodule or for the nitrogen fixation process.

Although the presence of DL-α-difluoromethylornithine (DFMO) in the plant medium did not prevent nodulation, the leaves of both uninoculated and inoculated alfalfa (cv Vernal only) were chlorotic in comparison to inoculated or nitrogen-supplied controls. This chlorosis might indicate a possible cultivar distinction, nitrogen deficiency, and/or other metabolic deficiency. Another cultivar difference may be seen in the polyamine levels measured from the two sets of nodules (Table II.7).

In the course of characterizing polyamine biosynthesis in Rhizobium meliloti, cell-free extracts were assayed for activity of 5'-deoxy-5'-methylthioadenosine (MTA) nucleosidase (MTAase). Since the S-adenosylhomocysteine (SAH) hydrolase has MTA-degrading capability in some systems, SAH was tested as an inhibitor. MTAase activity was determined using [$^{14}CH_3$]-MTA as previously described (White et al., 1982) with slight modifications. The activity of MTAase was 0.12 nmol/min/mg protein in cell-free extracts of R. meliloti 41 and 0.19 nmol/min/mg protein in the same extract in the presence of an equimolar concentration of SAH. Therefore, a MTA-cleaving activity is present and it is not due to the action of an SAH hydrolase.