# NEW GENE SOURCES FOR DEVELOPMENT OF AGRONOMIC PLANTS WITH TOLERANCES TO DROUGHT AND OTHER ABIOTIC STRESSES

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ABSTRACT

This project was an investigation in plant biochemistry/molecular biology/molecular genetics directed toward saving water through the development of water-conserving plants. The central, specific objective was to determine whether the thermophilic eubacterium, Thermus thermophilus HB8, produced one specific enzyme protein responsible for the biosynthesis of a class of compounds newly discovered in higher plants called uncommon polyamines. Uncommon polyamines may be protectants against abiotic stresses such as drought and heat. The rationale of the project was that if the central objective could be achieved, then the gene in T. thermophilus which produced the unique enzyme protein would be a valuable objective for cloning and transfer from the bacterium to plants using recombinant DNA methods. This transfer might confer improved tolerances in plants towards drought and heat stresses. The major conclusions of the project were the following: (i) Numerous criteria based on purification trials of the protein from T. thermophilus indicated that one unique protein is responsible for all uncommon polyamine biosyntheses. (ii) The enzyme protein demonstrated extraordinary catalytic efficiency indicating that it would be an excellent candidate for isolation of its gene and its transfer to plants. (iii) Unexpectedly, plants themselves were discovered to produce this enzyme protein and the uncommon polyamines in preselected drought-tolerant alfalfa and heat-tolerant cotton strains. (iv) Metabolic inhibitors were identified which could be exploited to develop cell selection protocols which may yield crop plants with improved drought tolerances.

Keywords: drought, plant-water relationships, salinity, water use efficiency, genetic engineering, plant physiology

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# INTRODUCTION AND LITERATURE REVIEW

## Justification of Project

This project was an investigation in plant biochemistry/molecular biology/molecular genetics directed toward saving water through the development of water-conserving crops. The major emphasis of the project was the long-term development of plants of agronomic value with enhanced heat and drought tolerances.

The lack of sufficient high quality water is the most limiting factor in crop productivity for many areas of the western United States and other arid or semiarid regions of the world. Over 80% of water usage in New Mexico and adjoining states is dedicated to agricultural irrigation. Drought, inconsistent surface water supplies, high energy costs for pumping ground water, depletion of aquifers, competition of agriculture with industry, increased water usage by cities, recreational demands for finite water sources, water pollution, and water-limiting legislation have all focused attention on the need to develop agronomic plants with the capacity to produce an economic-return with less water usage than those plants currently being used.

Additional problems in water usage worldwide call for a similar research focus. First, of the world's 3.2 billion hectares of potentially arable land, 1.3 billion hectares are considered to be irrigable. It can therefore be anticipated that irrigation, in a variety of forms, will increase on a global scale accompanied by increasing salinity of soils. Second, economic factors mainly related to mechanization, energy consumption, increased human population, or the need to hold agricultural crop markets to prevent loss of income, have created pressures to cultivate marginal arid or semiarid lands. Plant species with heat, drought, and saline resistances will be needed to cope with the environmental challenges of crop production of arid lands. Optimizing water-use efficiency and combining this with an appropriate degree of environmental stress resistances in specific crops will require far more advanced knowledge about biochemical mechanisms of plant resistances to environmental stresses than is currently available.

Research directed toward development of crop plants with enhanced environmental resistances to drought, heat or saline damages has been slow and success has been limited. The reasons are evident. Historically, plant breeding has been the major strategy for developing stress-resistant plant strains. This approach will continue to be the mainstay for plant improvement programs for many years.

However, classical plant breeding has limitations. First, plant breeding for any form of drought, heat, or saline resistance has been hampered principally by lack of identification of specific parameters that represent the significant traits which collectively confer survival and productivity for each stage of the plant life cycle. A great number of subtle changes are brought about in plants by these stresses that cumulatively are seen as more obvious gross changes (for example, senescence of leaves, reduced growth, and early maturation). It is highly improbable that any single parameter can adequately reflect the general response of the plant to drought, heat, or saline stress, whether for a short stage of development or for the entire ontogeny of the plant. Second, the source of beneficial genes a plant breeder can exploit is limited to the species undergoing selection. The advent of modern plant biotechnology has expanded the potential source of beneficial genes from within the species to virtually all living organisms. New strategies made available by recombinant DNA technology opens the possibility of improving plants with beneficial genes transferred from any other plant or animal species, microorganisms or viruses. This strategy has already led to the development of laboratory plant models with enhanced insect resistance, herbicide resistance, and selective secondary-metabolite productions of economic value. Many more specialized features of agronomic plants promise to be forthcoming in the very near future. An additional research approach is needed to supplement plant breeding in order to develop agronomic plants with improved tolerances to abiotic stresses. The project addressed this fundamental problem through exploitation of biochemical, and eventually recombinant DNA methods.

Literature Review

The Polyamines. The term "polyamines" is used throughout this report as a collective term for the naturally occurring diamines (1,3-diaminopropane, putrescine, cadaverine), triamines (spermidine, norspermidine, homospermidine), tetramines (spermine, norspermine, canavalmine, thermospermine), pentamines (caldopentamine, homocaldopentamine), and hexamines (caldohexamine, homocaldohexamine) shown in Table 1. The aliphatic diamine, putrescine, and polyamines, spermidine and spermine, occur in various complements in all living organisms. These are referred to as "common" polyamines in this report. Many aspects of the biosyntheses, occurrences, and potential functional roles of these compounds in animals, microorganisms, and plants have been reviewed extensively (Heby 1981; Bachrach, Kaye, and Chayen 1983, Slocum, Kaur-Sawhney, and Galston 1984; Tabor and Tabor 1984; Tabor and Tabor 1985; Flores, Young, and Galston 1985; Smith 1985a; Marton and Morris 1987).

Prior to the investigation summarized in this report, studies of common polyamine profiles in microorganisms led to the discovery of "uncommon" polyamines. "Uncommon" polyamines is a term used in this report for norspermidine, norspermine, homospermidine, aminopropylcadavarine, thermospermine, caldopentamine, homocaldopentamine, caldohexamine, and homocaldohexamine. One or more of these "uncommon" polyamines has been found in thermophilic bacteria (Oshima 1975; 1978; 1979a; 1982; 1986; Oshima and Baba 1981; Oshima and Kawahata 1983; 1986; Oshima and Kawahata 1983), methanogenic bacteria (Schrer and Kneifel 1983), Vibro (Yamamoto et al 1983), Cyanobacteria (Hamana, Miyagawa, and Matsuzaki 1983), Acetobacteria (Paulin et al 1983b), Rhizobium (Smith 1977a), Lactobacilli (Poso et al 1976), Rhodopseudomonas viridis (Tait 1979), and select algae (Villanueva, Adlakha, and Calvayrac 1980). Homospermidine was found in some plants (Kuttan et al 1971; Hamana and Matsuzaki 1984). However, none of the polyamines comprised entirely of repeating aminopropyl moieties (norspermidine, norspermine, caldopentamine, caldohexamine) were reported to occur in higher plants until recent studies on drought-tolerant strains of alfalfa and heat-tolerant strains of cotton (Rodriguez-Garay, Phillips, and Kuehn 1989; Phillips 1987). The discovery of these compounds in drought-tolerant, higher plants is a direct consequence of this project originally made possible by funding from the New Mexico State Water Resources Research Institute.

General Background. Abiotic stresses are critical limitations of crop productivity throughout the world. Losses in crop production due to water shortages, which

TABLE 1

Structures of Naturally Occurring Aliphatic Diamines and Polyamines

| Name | Structure |
| --- | --- |
| | Diamines |
| 1,3-Diaminopropane | $H_2N(CH_2)_3NH_2$ |
| Putrescine | $H_2N(CH_2)_4NH_2$ |
| Cadaverine | $H_2N(CH_2)_5NH_2$ |
| | Triamines |
| Norspermidine (also called caldine) | $H_2N(CH_2)_3NH(CH_2)_3NH_2$ |
| Spermidine | $H_2N(CH_2)_3NH(CH_2)_4NH_2$ |
| Aminopropylcadaverine | $H_2N(CH_2)_3NH(CH_2)_5NH_2$ |
| Homospermidine | $H_2N(CH_2)_4NH(CH_2)_4NH_2$ |
| | Tetramines |
| Norspermine (also called thermine) | $H_2N(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH_2$ |
| Spermine | $H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$ |
| Thermospermine | $H_2N(CH_2)_3NH(CH_2)_3NH(CH_2)_4NH_2$ |
| Canavalmine | $H_2N(CH_2)_4NH(CH_2)_3NH(CH_2)_4NH_2$ |
| | Pentamines |
| Caldopentamine | $H_2N(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH_2$ |
| Homocaldopentamine | $H_2N(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH(CH_2)_4NH_2$ |
| | Hexamines |
| Caldohexamine | $H_2N(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH_2$ |
| Homocaldohexamine | $H_2N(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH(CH_2)_4NH_2$ |

frequently occur with high ambient temperatures, may be greater than those caused by all other environmental conditions combined (Kramer 1980). Yet, understanding of environmental stress-tolerance mechanisms in plants at the biochemical, cellular, or molecular levels, which allow some plants to tolerate and survive specific types of stresses, is very limited. Moreover, the process of breeding improved crop varieties that tolerate water deficiency and heat stress has been slow. In addition, recombinant DNA technology cannot contribute to this problem until specific genes are identified which contribute to abiotic-stress tolerances. The central hypothesis of this project was that the aliphatic polyamines may serve as drought and heat stress protectants in plants, working in concert with osmoregulation and other unidentified tolerance mechanisms. The project addressed this hypothesis with a strategy that has potential in the long-term for developing drought, heat, and/or saline tolerant plants using biochemical and recombinant DNA methods or possibly also, in vitro plant cell selection methodologies.

Polyamines and Abiotic Stress Responses in Plants. Over the past 15 years, research has implicated the naturally occurring common polyamines (see Table 1) in processes controlling cellular growth in prokaryotes and higher animals. They have also been implicated in various aspects of plant growth, developmental processes, and the response of plants to specific stress stimuli (Smith 1985a; Slocum, Kaur-Sawhney, and Galston 1984; Smith 1984; Galston 1983; Bachrach, Kaye, and Chayen 1983; Tabor and Tabor 1983; Tabor and Tabor 1984). Putrescine accumulates in plants subjected to many types of stress conditions, notably in potassium-ion and magnesium-ion deficiencies (Basso and Smith 1974), with ammonium ion feeding (LeRudulier and Goas 1971), during acid stress (Smith and Sinclair 1967), in high salinity (Priebe, Klein, and Jager 1978; Shevyakova, Arutyunova, and Strogonov 1981; Friedman, Levin, and Altman 1986; Cohen, Altman, and Levin 1986; Young and Galston 1983), and during osmotic shock or desiccation by withholding water (Flores and Galston 1982; Flores and Galston 1984a; 1984b). Under the influence of these stress conditions, putrescine and common polyamines are derived entirely from the amino acid arginine, via the pathway (Flores and Galston 1984b):

arginine ---> agmatine ---> N-carbamoyl putrescine ---> spermidine ----> spermine ---> uncommon polyamines (our work, this proposal)

This contrasts with the situation in mammalian cells and fungi where the rate-limiting step in polyamine biosynthesis is the decarboxylation of ornithine to form putrescine

(Tabor and Tabor 1984). Although arginine decarboxylase is widespread in plants, it is virtually absent in animals.

There is increasing evidence that uncommon and higher molecular weight ($M_r$) common polyamines may serve as abiotic stress protectants for plants and other cells. Topical application of uncommon polyamines to crop plants protects them from adverse effects of extreme moisture and temperature conditions (Okii et al 1980). Evidence suggests a protective role for higher $M_r$ polyamines at the membrane level in plants (Altman 1977; Naik, Sharma and Srivastava 1980; Papovic et al., 1979; Altman and Bachrach 1981; Apelbaum et al., 1981; Speranza, Calzoni, and Bagni 1984). In microbial systems, polyamines modulate membrane fusion, exhibit differential preferences for alignment of membrane phospholipids, and may mediate attachment or expulsion of membrane proteins (Meers et al 1986). Uncommon and higher $M_r$ penta- and hexa-polyamines were first found in thermophiles and halophiles (Oshima 1982; Hamana et al 1985). These high $M_r$ polyamines confer thermoprotection during in vitro protein synthesis (Oshima 1983), and may serve specific roles in conferring tolerance to high temperature (Oshima and Senshu 1985; Paulin, 1983b) through their capacities to stabilize the double helix of DNA (Wilson and Bloomfield 1979) and to promote DNA condensation at high temperature (Basu and Marton 1987). In mammalian cells, spermidine and spermine confer heat protection to cells previously depleted of their cellular polyamines through the use of specific metabolic inhibitors of polyamine biosynthesis (Mivechi et al 1986). Thus, the syntheses of spermine and higher $M_r$ polyamines by plants could be a key protective factor for the environmentally stressed cell.

Virtually no published works describing the effects of high temperature on polyamine titers in plants exist. However, some research indicates there are heat protective actions of exogenously applied polyamines on plant tissues. Exogenous spermidine and spermine exert a protective action on seed germination at high temperatures (Nezovora and Borisova 1967), reduce protein efflux from apple pollen grains (Speranza, Calzoni, and Bagni 1984), inhibit leakage of betacyanin from beet root discs damaged by high temperature treatment (Altman 1982; Naik, Sharma, and Srivastava 1980) and exhibit temperature-dependent inhibitory effects on ethylene biosynthesis in apple tissue. The latter observation correlates with the prevention of membrane microviscosity associated with senescence (Apelbaum et al 1981).

The topical application of synthetic diamines and natural polyamines to a number of agronomically and horticulturally important crops (wheat, rice, peanut, lettuce, barley, oat, rye corn, millet, soybeans, buckwheat, rapeseed, cotton, potato, a

variety of fruit trees and flower plants), all under field conditions, has been found to protect these plants against frost damage, heat loss of chlorophyll, photochemical oxidation, and wilting. Several patents have been awarded in Germany, Japan, and the United States to a Japanese group for the practical use of these amines in field applications (Okii et al 1980). An important conclusion from this work is the recognition that the higher $M_r$ uncommon polyamines provide the greatest protection to plants at exceedingly low concentrations, some as low as 0.1 mole per hectare.

Uncommon Polyamines in Thermophilic Bacteria, a Potential Source of Thermal-Tolerance Genes. The most consistent association between cellular tolerance for high-temperature environments and the polyamines has been found in thermophilic bacteria. The tetramine spermine and/or one or more uncommon polyamines, such as caldine (also called norspermidine), homospermidine, thermine (also called norspermine), thermospermine, caldopentamine, homocaldopentamine, caldohexamine and homocaldohexamine (see Table 1), have been identified in certain bacterial strains grown between 70 - 90°C (Oshima and Senshu 1985). Some, but not all, of these compounds have been found in select halophilic eubacteria (Hamana et al 1985). The association of uncommon polyamines with increased capacity of extreme thermophiles to survive elevated culture temperatures has led to the suggestion that high $M_r$ uncommon polyamines serve a specific role in conferring tolerance to high temperature growth environments (Oshima 1975; Paulin et al 1983b). Indeed, the discovery by Oshima (1983) that the high $M_r$ uncommon polyamines confer thermoprotection during in vitro protein synthesis supports this claim. Caldine, thermine and those uncommon amines larger than tetramines have not previously been reported in higher plants (Hamana and Matsuzaki 1985). Our research in the present project established for the first time their occurrence under drought and heat stress conditions in drought-tolerant alfalfa and heat-tolerant cotton strains (see below). Thus, significant correlative evidence has been gained for the hypothesis that these uncommon polyamines have adaptive significance in plant tolerances to abiotic stresses. Strategies to enhance the syntheses of these protective agents in stress-susceptible plants may produce strains with improved thermal and drought stress tolerances. This project addressed such a strategy.

The enzymology of biosynthesis of these uncommon polyamines had not been studied before this project began. However, the molecular structures of the various uncommon polyamines identified thus far, suggested a likely pathway for their respective syntheses. Figure 1, below, shows the structure of caldohexamine.

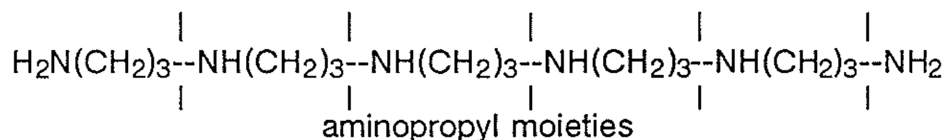
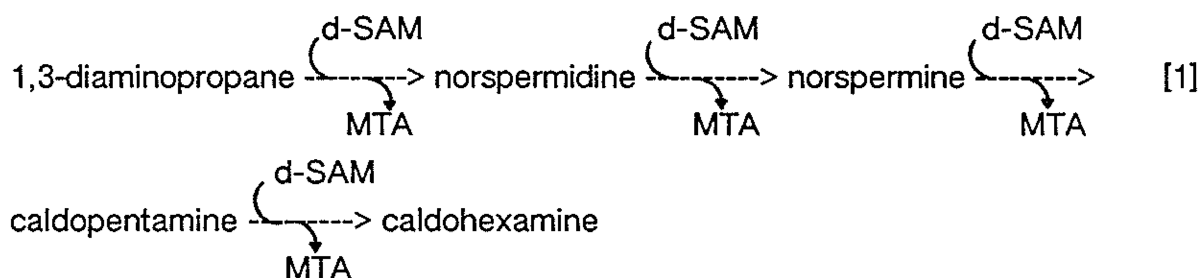


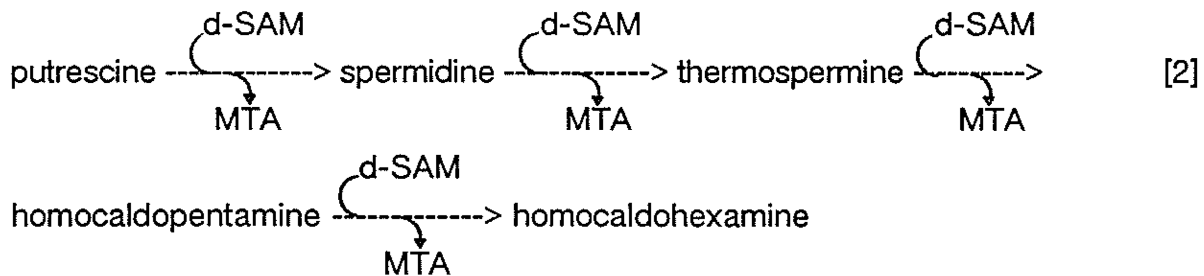

Figure 1. Diagrammatic structure of caldohexamine emphasizing its probable origin from sequential additions of aminopropyl moieties derived from decarboxylated S-adenosylmethionine.

The molecule is comprised of repeating units of identical aminopropyl groups. This suggests that caldohexamine may be biosynthesized by four successive repetitious reactions that add an aminopropyl group to an initial precursor molecule of 1,3-diaminopropane, $H_2NCH_2CH_2CH_2NH_2$. Decarboxylated S-adenosylmethionine is an aminopropyl group donor in biochemical reactions. Thus, the biosyntheses of the triamine, norspermidine, the tetramine, norspermine, the pentamine, caldopentamine, and the hexamine, caldohexamine, could be accomplished by a single pathway through the successive transfer of an aminopropyl group from decarboxylated S-adenosylmethionine (d-SAM) to 1,3-diaminopropane, norspermidine, norspermine, and caldopentamine, respectively (DeRosa et al 1978). Methylthioadenosine (MTA) would be a product of each aminopropylation reaction. Thus:




Repetition of a single type of reaction is the common characteristic of all four reactions required to biosynthesize caldohexamine and the intermediates of this pathway from precursor, 1,3-diaminopropane. A single enzyme protein, an aminopropyltransferase enzyme, with high specificity for transfer of an aminopropyl group from decarboxylated S-adenosylmethionine to the aminopropyl end of each precursor in scheme [1] above, could accomplish this biosynthetic route. Isolation of the gene for such a putative enzyme is the eventual objective of this research. Similar aminopropyltransferases from plants have been characterized for the biosyntheses of spermidine from putrescine and of spermine from spermidine (Yamanoha and Cohen

1985; Greenberg and Cohen 1985). The aminopropyltransferases which catalyze these reactions in common polyamine biosynthesis are specifically called spermidine synthase and spermine synthase, respectively. However, these catalysts do not exhibit any capacities to catalyze the reactions shown in reaction scheme [1], above. A similar single type of aminopropyltransferase might also account for an analogous pathway to homocaldopentamine and homocaldohexamine from putrescine by the pathway:




Although the uncommon polyamines caldine (norspermidine) and thermine (norspermine) were first discovered nearly a decade and a half ago (Oshima 1975), investigations of their biosynthetic pathways and enzymes have only recently begun. The work of this author and that of an Italian group are the only known investigations of this type. During the course of this project, the latter group reported in an abstract the isolation of an aminopropyltransferase from an extreme acidothermophilic archaebacterium, Solfalolous sulfataricus.

Investigation of an aminopropyltransferase capable of producing thermo-protecting uncommon polyamines in a thermophilic bacterium has potential for developing a single-gene source which may confer drought, heat, or saline-stress tolerances when cloned, isolated, and transferred into higher plants. This is an especially attractive objective since the two essential precursors for the biosynthetic pathway required by the aminopropyltransferase enzyme, either 1,3-diaminopropane (see scheme [1]) or putrescine (see scheme [2]), are produced naturally in virtually all plants (Fuhrer et al 1982; Shih et al 1982). The aim of this project was to characterize the putative unique aminopropyltransferase enzyme from the eubacterial thermophile, Thermus thermophilus HB8, which catalyzes sequential additions of aminopropyl groups to these precursors, thus producing the heat-protectant higher-polyamines. From the amino acid sequence of a portion of this protein, the putative gene which carries the genetic coding sequences for the aminopropyltransferase enzyme could be

isolated and cloned. The gene would be a valuable resource for testing the transformation of plants for possible environmental stress tolerances.

Biosynthetic Relationship Between Common and Uncommon Polyamines. Several metabolic pathways have been delineated for the common polyamines in diverse organisms. The major pathways were first established in microorganisms. They were later found to be similar in animals and plants. These major pathways are summarized in Figure 2. Putrescine, the precursor for spermidine and spermine, is formed in nearly all cells by direct decarboxylation of L-ornithine. This reaction is catalyzed by ornithine decarboxylase (ODC; see reaction 1, figure 2) and it appears to be the rate-limiting step in polyamine biosynthesis. Plants and certain bacteria possess an additional pathway for synthesis of putrescine, involving the decarboxylation of L-arginine by arginine decarboxylase (ADC; see reaction 2, figure 2) to form agmatine, and the subsequent conversion of agmatine to putrescine and urea. ADC is virtually absent in animal cells. Spermidine and spermine are subsequently synthesized from putrescine by the combined actions of S-adenosylmethionine decarboxylase (SAMDC), putrescine aminopropyltransferase (PAPT; formerly called spermidine synthase), and spermidine aminopropyltransferase (SAPT; formerly called spermine synthase). In the reactions catalyzed by PAPT and SAPT, putrescine (see reaction 5, figure 2) and spermidine (see reaction 6, figure 2), respectively, serve as the acceptor of an aminopropyl group transferred from decarboxylated S-adenosyl-L-methionine (d-SAM). The latter is formed through decarboxylation of S-adenosylmethionine (SAM) by the action of SAMDC.

The absence of a diaminobutyl group in the structures of norspermidine (also called caldine), norspermine (also called thermine), caldopentamine, and caldohexamine, and the occurrence of a repeating structural motif of aminopropyl groups [$-NH(CH_2)_3-$], suggest these uncommon polyamines might be biosynthesized by successive, repetitious reactions that add an aminopropyl group to an initial precursor molecule of 1,3-diaminopropane, $H_2NCH_2CH_2CH_2NH_2$, as discussed earlier. DeRosa et al (1978) proposed such a biosynthetic pathway in the extreme thermoacidophilic bacterium, Caldariella acidophila, which exploited the suggestions of Oshima's earlier work. In DeRosa's proposed pathway (see figure 2 above), decarboxylated S-adenosylmethionine donated the aminopropyl group in successive reactions that converted 1,3-diaminopropane to norspermidine to norspermine to caldopentamine to caldohexamine. These reactions were analogous to the highly specific reactions catalyzed by PAPT and SAPT in spermidine and spermine biosynthesis, respectively.

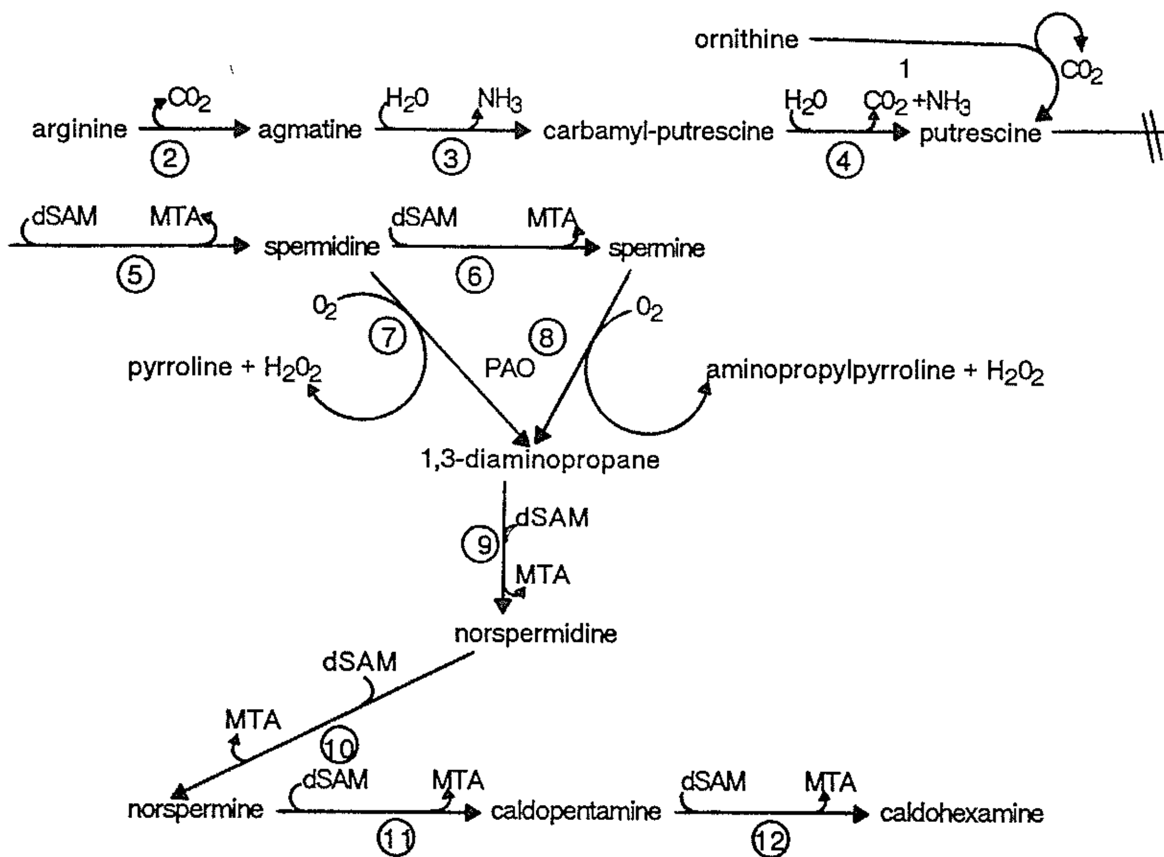



Figure 2. Known biosynthetic pathways for the common polyamines in plants and postulated biosynthetic pathways for the uncommon polyamines beginning with 1,3-diaminopropane. Structures for the polyamines are presented in Table 1.

It is not known if these reactions are catalyzed by four independent aminopropyltransferases, each specific for a unique reaction. Alternatively, a single aminopropyltransferase enzyme, with a broad specificity to transfer an aminopropyl group from decarboxylated S-adenosylmethionine to the aminopropyl end of each precursor in figure 2 above, could accomplish this biosynthetic route. The preliminary report of Cacciapuoti et al (1986) suggests, however, that a single aminopropyltransferase is the only enzyme required to convert the metabolite, 1,3-diaminopropane, to the four uncommon polyamines in S. solfataricus. This possibility extends to the biosynthesis of uncommon polyamines in other species where the uncommon polyamines might be found.

The role of 1,3-diaminopropane as a metabolic precursor for reactions 9 through 12 is hypothetical but seemingly essential. There are only two confirmed reactions in metabolism known to generate 1,3-diaminopropane as a product. Both reactions are catalyzed by polyamine oxidase (see reactions 7 and 8, figure 2). Polyamine oxidase is an enzyme specific for the oxidation of the polyamines, spermidine or spermine. It has virtually no effect on diamines such as putrescine or cadaverine (Smith 1985b). The enzyme has been widely reported to occur only among various species of the Gramineae plants (the grasses), from which it has been characterized in oats (Smith 1977b), barley (Smith 1976), corn (Suzuki and Yanagisawa 1980), and millet (Hibasawa et al 1986). However, polyamine oxidase has recently been purified from Eichhornia crassipes Solms (water hyacinth), which does not belong to the Gramineae but which is also a monocot (Yanagisawa et al 1987). Numerous polyamine oxidases catalyze oxygen-dependent cleavage of spermidine to pyrroline, and spermine to aminopropylpyrroline. In each case, the additional reaction products are 1,3-diaminopropane and hydrogen peroxide.

If the action of polyamine oxidase was found to be the major or sole source of 1,3-diaminopropane in biological cells capable of producing the uncommon polyamines (norspermidine, norspermine, caldopentamine, caldohexamine), then this enzyme would assume a pivotal position as a connecting catalyst between the recognized biosynthetic pathway for common polyamine biosyntheses, in the pathways shown in figure 2. Assignment of such a role to polyamine oxidase in higher plants was irrelevant in the absence of the discovery that higher plants were capable of producing the uncommon polyamines, norspermidine, norspermine, caldopentamine or caldohexamine. However, one of the results of this project has been the chemical structural elucidations of isolated uncommon polyamines from plants. Thus, a major finding of this project is that some higher plants do indeed

produce uncommon polyamines when grown under conditions of drought-stress (Rodriguez-Garay, Phillips, and Kuehn 1989), and heat-stress (Phillips 1987). Thus, the potential role of polyamine oxidase in abiotic stress responses through the pathway proposed (reactions 7 and 8) gains importance. The detailed enzymology that accounts for the biosynthess of the uncommon polyamines had not been studied in these or any other plants prior to this project. This became an unplanned central objective of this project during its second year.

Specific Aims of Project. The original research aims for this project were the following:

i. to establish the identity of the precursors and intermediates of the biosynthetic pathway to the uncommon polyamines (norspermidine, norspermine, caldopentamine, caldohexamine, homocaldohexamine) in the thermophilic bacterium, Thermus thermophilus HB8.

ii. to determine whether the enzymatically-catalyzed reactions for higher-polyamine syntheses of tetramines, pentamines and hexamines, shown in reaction schemes [1] and [2], in Thermus thermophilus HB8 are due to the action of a single enzyme protein, an aminopropyltransferase.

iii. to purify to homogeneity the putative single enzyme protein responsible for biosynthesis of uncommon polyamines and to prepare antibody reagents against the protein.

The rationale for these objectives was that if a single aminopropyltransferase is responsible for uncommon polyamine biosynthesis from 1,3-diaminopropane in T. thermophilus HB8, then cloning, isolation, and transfer of the gene for this protein into plants, may confer the capacity to produce thermal and drought protective uncommon polyamines in transgenic plants.

## METHODOLOGY

### Microorganism

The obligate thermophilic eubacterium, Thermus thermophilus HB8 was used. This organism has an optimum growth temperature of about 70°C, but grows well throughout the range of 60-90°C. The organism was precultured in petri plates containing the following components per one liter of aqueous medium: 4 g of yeast extract, 6 g of tryptone or peptone, 1.8 g of NaCl, 0.3 g of KCl, 35 mM glycerol (5 ml of 7 M stock aqueous solution), 7 g of HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer adjusted to pH 7.4 at room temperature with NaOH, 0.55 g

of $CaCl_2 \cdot 2H_2O$, and 11 g of Gelrite (Scott Laboratories, Inc., Carson, CA 90746). This medium was autoclaved for 50-70 minutes. After autoclaving, 0.5 ml of "1000x" vitamins was added. The vitamin solution was sterilized by filtration through a Nalgene type S CN filter of 0.22 µm pore size before addition to the sterilized medium. The stock "1000x" vitamins solution contained the following components per one liter of aqueous medium: 0.01 mg of d-biotin, 1 mg of thiamine hydrochloride, 1 mg of pyridoxine hydrochloride, 0.7 mg of nicotinic acid, 2 mg of calcium pantothenate, 1 mg of p-aminobenzoic acid, and 10 mg of inositol. This strain of T. thermophilus HB8 is auxotrophic for biotin. Petri plates were placed in self-sealing plastic bags to retard dehydration when incubated in a laboratory oven at 65-70°C. Heavy growth was achieved within 24 - 48 hours. Liquid cultures of 2-liter size were inoculated with cells scraped from 10 to 20 petri plates and transferred in a suspension with 10 ml of the rich medium as described above, but lacking Gelrite and $CaCl_2 \cdot 2H_2O$. Liquid cultures lacking Gelrite and $CaCl_2 \cdot 2H_2O$ were grown at designated temperatures in a 2-liter jacketed growth vessel using a Virtis Omni-Culture benchtop fermentor equipped with a Neslab controlled water bath. Generally, 16 to 20 hours of growth to a Klett density of 250 yielded about 7 gm of wet-packed washed cells. Stocks of the organism were maintained on the petri plates described above and stored at 3°C for several months.

When this project began, the author's laboratory had no prior experience in culturing thermophilic bacteria. No facilities were available for their culture. This grant allowed for the purchase of a benchtop fermentor. Dr. James Fee from Los Alamos National Laboratory consulted with this author on how to culture T. thermophilus HB8.

Analytical Quantitation of Polyamines by High Performance Liquid Chromatography

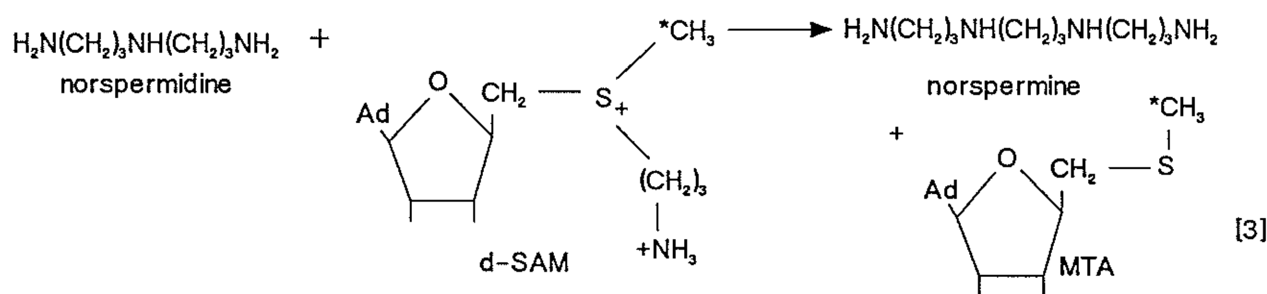
To accomplish research aim (i) listed above, methods were needed for separation and identification of the uncommon and higher polyamines: norspermidine (caldine), norspermine (thermine), and caldopentamine. Postdoctoral research assistant, Dr. Benjamin Rodriguez, and the author worked jointly in developing a high performance liquid chromatographic (hplc) method which detects and quantitates these polyamines. We exploited the Pharmacia Prep RPC HR5/5 silicate $C_2/C_{18}$ reversed-phase chromatographic medium in our hplc methods because of its superior capacity to resolve the dansylated derivatives of the higher uncommon polyamines from the common polyamines. The dansylated derivatives of caldine and thermine cochromatograph with those of the common polyamines, spermidine and spermine, respectively, in most other rapid hplc separation protocols (Walter and Geuns 1987).

However, the Pharmacia $C_2/C_{18}$ medium successfully separates these derivatized polyamines under isocratic elution with 80:20 (v:v), methanol:water solutions.

Briefly, our procedures include the following. Cells of T. thermophilus HB8 are extracted with 10% trichloroacetic acid. After centrifugation to remove insoluble cell debris, the supernatant fraction is loaded onto a 1 x 5 cm column of Dowex 50WX8 cation-exchange resin. The column is washed successively with 20 ml of 0.1 M sodium phosphate, pH 8.0, containing 0.7 M NaCl, then 20 ml of 0.5 M HCl. The collective polyamines are eluted with 15 ml of 6 M HCl (Fujita et al 1980). The solution of polyamines is evaporated to dryness under a stream of air on a warm plate and the polyamines are converted to their dansylated derivatives (Kremmer, Holczinger, and Boldizar 1984). The dansylated polyamines are separated and quantitated at 50°C using a Pharmacia automated FPLC system equipped with the reversed-phase $C_2/C_{18}$ column and an interfaced integrator (Hewlett-Packard 3390A). A full analysis is complete in 20 minutes. This hplc system detects and quantitates diamines and the tri-, tetra-, penta, and hexa-polyamines shown in Table 1. Polyamines in amounts a low as 20 pmole can be readily quantitated. Five weeks of project time were required to develop these methods for detection and quantitation of the uncommon polyamines.

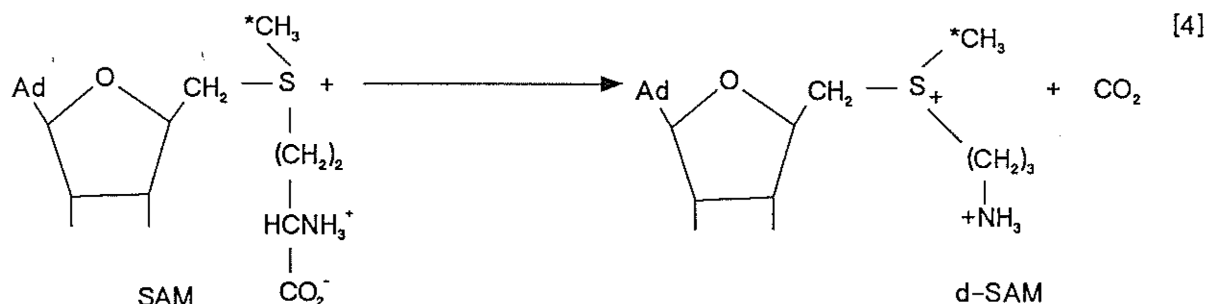
Preparation of $^{14}$C-Labelled Substrates for Aminopropyl-transferases

One of the potential reactions catalyzed by aminopropyltransferase is reaction [3] given below.




To assay the catalytic activity of aminopropyltransferase, the substrate, decarboxylated S-adenosylmethione (d-SAM) is required. d-SAM is not commercially available either in nonradioactive or radioactive forms. It must be synthesized in the local laboratory. We synthesized d-SAM both in a nonradioactive form and as a [methyl- $^{14}$C] d-SAM radioactive form. The latter reagent is depicted in reaction [3] where the asterisk

denotes the position of the radioactively-labeled carbon atom. The reagent, S-adenosylmethionine (SAM), is commercially available in nonradioactive and radioactive forms. Thus, to prepare d-SAM from starting material SAM, the enzyme S-adenosylmethionine decarboxylase is required to catalyze the conversion in reaction [4]:




SAM decarboxylase was purified from wild type Escherichia coli K12 using affinity chromatography on methylglyoxal(bisguanylhydrazone) -Sephrose 6B (Markham, Tabor, and Tabor 1982; 1983). This enzyme preparation was subsequently used to convert SAM to d-SAM as shown in reaction [4] using modified procedures of Pegg (1983). The radioactive form of d-SAM, [methyl- $^{14}$C]d-SAM, was prepared from commercially available [methyl- $^{14}$C]SAM (Research Products International Corp., cat no. cmm-270), depicted in equation [4] with the $^{14}$C-radioactive label at the asterisk. Approximately one month of laboratory effort was required to develop these procedures to prepare a stock of d-SAM for our subsequent studies. Once prepared, the stocks could be stored indefinitely with no deterioration (Matos and Wong 1987).

Recently we have received a sample of E. coli K12 strain HT527 from Dr. Herbert Tabor at the National Institutes of Health. This strain produces 15-fold more S-adenosylmethionine decarboxylase than wild type E. coli K12 used earlier in our project (Markham, Tabor, and Tabor 1982). This organism will greatly improve our capacity to synthesize [methyl-$^{14}$C] d-SAM in the future.

Enzymatic Assay Procedures for Aminopropyltransferase

The enzymatic assay procedure developed for aminopropyltransferase is a modified method of that reported by Raina et al (1983). In the original method, the radioactively labeled [methyl-$^{14}$C]decarboxylated S-adenosylmethionine (d-SAM), as shown in reaction [3] above by asterisks, in the aminopropyltransferase reaction was separated from the starting substrate by chromatography on a column of Dowex 50W-

X4 ($H^+$ form). However, we found that the recovery of [methyl-$^{14}C$] MTA from the Dowex column was incomplete and variable. We substituted a weak cation exchange column media, namely a phosphocellulose cation exchanger (Cellex-P, Bio-Rad Laboratories) for this separation, which gave excellent results.

The procedures we developed to determine caldine- and thermine-aminopropyltransferase enzyme activities are the following. Approximately 0.5 ml of phosphocellulose cation exchanger is packed in a Pasteur pipette using glass wool as a bed support. The column is generated to the $H^+$-form with 0.5 M HCl and washed with water. The void volume of the column is approximately 0.8 ml.

The reaction mixture to carry out reaction [3] shown above is 0.1 ml total volume and contains 100 mM potassium phosphate, pH 7.4, 5 mM dithiothreitol, 0.5 mM caldine (for caldine aminopropyltransferase), and 0.02 mM [methyl-$^{14}C$]decarboxylated S-adenosylmethionine (d-SAM). This mixture is preincubated for 10 min at the designated reaction temperature before the addition of the enzyme preparation. The reaction is allowed to continue for 10 min, then is stopped by adding 0.5 ml of 25 mM HCl. The pH of the mixture falls below 3. Centrifugation of the mixture is needed only if a precipitate appears. A sample of 0.5 ml of the mixture is applied to the phosphocellulose column, previously equilibrated with 25 mM HCl. The first 0.5 ml of effluent from the column is discarded. Radioactive methylthioadenosine (MTA) and its degradation products are then eluted directly into a scintillation vial with 1.8 ml of 25 mM HCl. Ten milliliters of scintillation solution (3a70B, Research Products Inc.) is added to the vial, mixed vigorously, and counted for radioactivity in a liquid scintillation spectrometer.

The phosphocellulose columns are regenerated with 4 ml of 0.5 M HCl and washed with 2 ml of water. To avoid accumulation of precipitated protein, the columns are occasionally treated with 4 ml of 0.1 M NaOH, washed with water, and regenerated as described above.

The method described here in detail is sensitive and rapid for the assay of any type of aminopropyltransferase catalyst. Only the type of polyamine substrate (1,3-diaminopropane, caldine, thermine, caldopentamine, etc.) needs to be varied to assay for a particular unique aminopropyltransferase activity. The labeled substrates are expensive and laborious to prepare. The method, however, has marked advantages. First, only small amounts of d-SAM (about 1-2 nmol per assay mixture) are required for standard assays. Second, the sensitivity for detecting low catalytic activity is very high. Third, the method is especially suitable for a rapid test to determine substrate specificity as regards different polyamine acceptors. This latter advantage was of

prime importance to this project and it was the reason why we chose to develop this method of assay.

Enzymatic Assay Procedures for Polyamine Oxidase

Two enzymatic assay procedures were developed for polyamine oxidase. The colorimetric method was that described by Yanagisawa et al (1987). The radioisotope tracer method was developed in our laboratory.

In the colorimetric method, enzyme activity is assayed by measuring colorimetrically ı-pyrroline or ı-aminopropylpyrroline products of the oxidation of spermidine, (see reaction scheme [5] and [6]) or spermine, respectively, with o-aminobenzaldehyde (Suzuki and Hirasawa 1973).

$$H_2N(CH_2)_3NH(CH_2)_4NH_2 + O_2 \longrightarrow H_2N(CH_2)_3NH_2 + \text{ı-pyrroline} + H_2O_2 \quad [5]$$

spermidine

$$H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2 + O_2 \longrightarrow H_2N(CH_2)_3NH_2 + \text{ı-aminopropylpyrroline } (H_2N(CH_2)_3N\text{-ring}) + H_2O_2 \quad [6]$$

spermine

The reaction mixture consisting of 50 mmoles each of a three-component buffer (TES, MES, and BICINE), pH 7 with KOH, 5 mmoles of spermidine · 3HCl or spermine · 4HCl, 0.066 ml of 0.2% o-aminobenzaldehyde (Sigma, cat. no. A9628) in absolute ethanol, 80 mg of beef liver crystalline catalase (Sigma, cat. no. C-30), 1 mmole of diethyldithiocarbamic acid, and deionized distilled water to 0.8 ml, was incubated at 30°C. The reaction was initiated by adding 0.2 ml of enzyme preparation. Total reaction time was 30 minutes. The reaction was terminated by adding 0.066 ml of 50% trichloroacetic acid. A precipitate formed on acidification was removed by centrifugation at 11,000 x g for 3 minutes. The absorbance of the supernatant solution was determined spectrophotometrically at 435 nm. The extinction coefficient for the yellow quinazolium complex product is $1.86 \times 10^3\ M^{-1}\ cm^{-1}$ (Holmstedt, Larrson, and Tham 1961). Protein concentrations were determined according to Bradford (1976).

In the radioisotope assay, enzyme activity is assayed by measuring radioactive [$^{14}$C]spermine (see reaction scheme [6], above). Components for the assay reaction mixture and for termination of the reaction are the same as those described previously for the colorimetric method. A 10-ml sample of the terminated reaction mixture and a 10-ml sample of standard 30 mM 1,3-diaminopropane are co-streaked across the end of a 4 x 15 cm strip of Whatman 3MM paper that is previously soaked and lightly blotted with 44.4 mM sodium citrate buffer, pH 4.3. The standard diamine serves as a carrier. The paper strip is placed in an electrophoresis chamber with the sodium citrate buffer in both anode and cathode chambers. The reaction sample on the strip is situated in the chamber such that the [$^{14}$C]1,3-diaminopropane reaction product migrates throughout the length of the strip toward the cathode chamber (negative pole). Paper strips streaked with 10 ml of 30 mM 1,3-diaminopropane and spermine standards are electrophoresed simultaneously with the strip containing the reaction sample. Electrophoresis is conducted at 200 volts, 12 mamps, for one hour. The strips containing the standards are air-dried, sprayed with a 0.5% solution of ninhydrin in water-saturated butanol, air-dried again, then developed at 100°C in an oven for 20 to 30 min. The location of stained standards on the paper strips are used to identify the [$^{14}$C]1,3-diaminopropane and [$^{14}$C]spermine zones on the reaction sample strip. These zones are cut from the paper strip and counted in a liquid scintillation spectrometer. The scintillation cocktail contains 4 g of Preblend 2a70 (Research Products International Corp., Elk Grove Village, IL) per liter of toluene.

Plant Materials

Both whole plant tissues and corresponding cell suspension cultures of two select Medicago sativa L. (alfalfa) strains were used in this project. One strain was the drought-susceptible strain of 'Mesilla' labeled MES-O. The second strain was the third cycle population, designated MES-3, derived from 'Mesilla' by phenotyic recurrent selection for productivity under less than optimum moisture. MES-3 is drought tolerant and water-use efficient (Currier, Melton, and Wilson 1987). Whole plants were field grown. For initiating cell suspension cultures, approximately 100 seedlings of each population were germinated. The epicotyls were excised and utilized as explants following the general procedures of McCoy and Walker (1984).

Culture media used the composition of Phillips (1983). Each population of cell culture was replicated and subcultured every two weeks to maintain growth in the exponential and linear phases. Cultures were incubated on an oscillating shaker (120 rpm) at 25°C under diffuse light.

Treatment of Plant Cell Cultures with Inhibitors

2-Hydroxyethylhydrazine (HEH) was added to the culture media at 0, 0.5, 5, and 50 mM prior to autoclaving. Polyethylene glycol 8000 MW (PEG) at 20% or mannitol at 15% was used in culture media to simulate water-deficit stress. Cultures were subjected to stress and inhibitor treatments for seven days at which time replicate samples of cells were removed, washed once, and stored as frozen pellets for later polyamine oxidase analyses.

Plant Extracts

Extracts of whole plant tissues or cells from suspension cultures were prepared from two-g samples. Samples were frozen in liquid nitrogen and pulverized with mortar and pestle in five volumes of 50 mM TES buffer, pH 7.5. After thawing, the suspension was centrifuged at 12,000 x g for 30 minutes. The supernatant fraction was used as the crude extracted enzyme preparation.

Thermus thermophilus HB8 Extracts

Extracts of T. thermophilus HB8 were prepared by passing a 50% suspension of cells in 100 mM potassium phosphate buffer through a French pressure cell once at 15,000 psi. The broken cell suspension was centrifuged at 100,000 x g for one hour and the supernatant fraction was used as the enzyme preparation.

## RESULTS AND DISCUSSION

Demonstration of Polyamine Oxidase in Thermus thermophilus HB8

To establish a biochemical pathway for uncommon polyamine biosynthesis in T. thermophilus HB8, detection of polyamine oxidase enzyme activity was attempted. The supernatant fraction derived from 100,000 x g centrifugation of ruptured cells routinely yielded a maximum specific activity of $4.11 \pm 0.20$ µmole of pyrroline/min/mg protein. This catalytic rate was nearly the same for cells grown at 60, 70, 80, or 90°C. A molar extinction coefficient of $1.86 \times 10^3$ $M^{-1}cm^{-1}$ was used to convert the $A_{435}$ data from the colorimetric method to the quantity of pyrroline produced (Holmstedt et al., 1961). Approximately, 10-13% of the total polyamine oxidase activity was detectable in the lysed cell debris after centrifugation at 100,000 x g. Thus, unlike the polyamine oxidases of higher plants which are cell-wall associated, the enzyme in T. thermophilus HB8 appears to be readily soluble and of comparable activity to that in maize (Torrigiani, Scoccianti, and Bagni 1988).

Using the radioactive method for polyamine oxidase, which measures [$^{14}$C]1,3-diaminopropane produced from [terminal methylenic -$^{14}$C] spermidine, a specific enzyme activity of 5.23 $\pm$ 0.17 μmole of [$^{14}$C]1,3-diaminopropane/min/mg protein was detected. Since the stoichiometry of the polyamine oxidase reaction predicts equimolar yields of pyrroline and 1,3-diaminopropane from spermidine, these data are in excellent agreement with those obtained by the colormetric assay method.

Demonstration of Aminopropyltransferase in Thermus thermophilus HB8

The occurrence of polyamine oxidase in Thermus thermophilus HB8 predicted that 1,3-diaminopropane should be detectable in extracts of this thermophile (see below) and that aminopropyltransferase enzymatic activity might be demonstrable, which utilizes the diamine as a propylamine acceptor from decarboxylated S-adenosylmethionine. Indeed, attempts to demonstrate various polyamine-dependent aminopropyltransferase enzymatic activities in crude extracts of T. thermophilus HB8 were successful. 1,3-Diaminopropane, norspermidine (caldine), norspermine (thermine), putrescine, spermidine, and spermine-dependent aminopropyltranferases were all detectable. For example, norspermidine (caldine) aminopropyltransferase was assayed with a $V_{max}$ of 2.34 μmole/min/mg protein and a $K_m$ for norspermidine (caldine) of 46.5 μM at 60 °C. The specific enzymatic activity of norspermidine aminopropyltransferase is exceptionally high in extracts of T. thermophilus HB8 compared to that of spermidine aminopropyltransferase reported in mammalian tissues (Raina, Pajula, and Eloranta 1976; Raina et al 1983). For example, the thermophilic enzyme is 1,850 times more active than the best mammalian source, which is the pancreas. Rat pancreas extracts demonstrate a specific activity of 1.26 nmoles/min/mg protein (Raina et al 1983). Spermidine aminopropyl transferase purified to apparent homogeneity from rat prostate gland only exhibits a specific catalytic activity of 206 nmoles/min/mg protein. This value is only 9% of the specific enzymatic activity demonstrated by norspermidine aminopropyltransferase in crude, unfractionated extracts of T. thermophilus HB8 at 60°C. Thus, T. thermophilus HB8 is one of the best sources for highly enzymatically active aminopropyltransferase demonstrated heretofore in a biological source.

Relevant kinetic parameters for other aminopropyl-moiety acceptors are presented in Table 2. All aminopropyltransferase enzymatic activities capable of producing norspermidine, norspermine, caldopentamine, spermidine, spermine, thermospermine, and homocaldopentamine, are readily demonstrable in crude extracts from T. thermophilus HB8. The $K_m$ values for the various propylamine-moiety

TABLE 2

Michaelis Constants at Different Temperatures For Various Aminopropyl-Acceptors of Aminopropyltransferase Catalytic Activities in Extracts of *Thermus thermophilus* HB8

| Substrates | Km, μM | | |
|---|---|---|---|
| | 60°C | 70°C | 80°C |
| 1,3-Diaminopropane | 40.7 | 51.1 | 68.9 |
| Norspermidine | 46.5 | 58.1 | 71.2 |
| Norspermine | 47.2 | 60.3 | 72.4 |
| Putrescine | 55.3 | 63.7 | 74.8 |
| Spermidine | 59.7 | 66.3 | 78.6 |
| Spermine | 59.1 | 67.8 | 80.1 |

acceptors are of similar value over the temperature range of 60-80°C. Similar values varying from 0.6 to 50 μM have previously been reported for mammalian aminopropyltransferases from various sources (Raina et al 1983). Such high affinity for these simple amines suggests that affinity chromatography on Sepharose media, to which has been linked select polyamines, should be an efficient purification method for the aminopropyltransferase from T. thermophilus HB8.

Temperature-Dependence of Aminopropyltransferase Enzymatic Activities

The temperature dependencies for three aminopropyltransferase activities are shown in figure 3. The general shape of the activity versus temperature curves for 1,3-diaminopropane, norspermidine, and norspermine aminopropyltransferases were similar. However, there was a general increase in catalytic rate at all temperatures that correlated with the length of the structural chain of the uncommon polyamine substrate. Thermine aminopropyltransferase activity was higher at all temperatures, followed by norspermidine aminopropyltransferase, then 1,3-diaminopropane aminopropyltransferase.

One interpretation of the results of figure 2 could be that uncommon polyamine substrates of greater chain length and amine groups provide greater stability to an aminopropyltransferase enzyme at higher temperatures. Consistent with this interpretation is the observation that enzyme assay reaction mixtures containing norspermine exhibited no cloudiness even at 90°C reaction temperatures. Cloudiness is indicative of denaturation of macromolecules. Assay mixtures containing norspermidine exhibited very slight cloudiness after reactions were completed in the 70-90°C range. Mixtures containing 1,3-diaminopropane were consistently and appreciably cloudy in this temperature range. Although these observations are qualitative, the protection against heat denaturation of components in crude soluble extracts of T. thermophilus HB8 provided by low concentrations of norspermine is visually dramatic. This property of this uncommon polyamine has not previously been reported in the general literature. The property supports the original hypothesis of the author that the uncommon polyamines may be heat and drought protectants at the cellular level in plants.

A second interpretation of the order of catalytic rates shown in figure 2 might be that increased temperatures result in a change in enzyme conformational structure which yields enhanced affinity of aminopropyltransferase for its uncommon polyamine substrates. For example, at higher temperatures aminopropyltransferase may exhibit an order of affinity for its substrates: norspermine > norspermidine > 1,3-

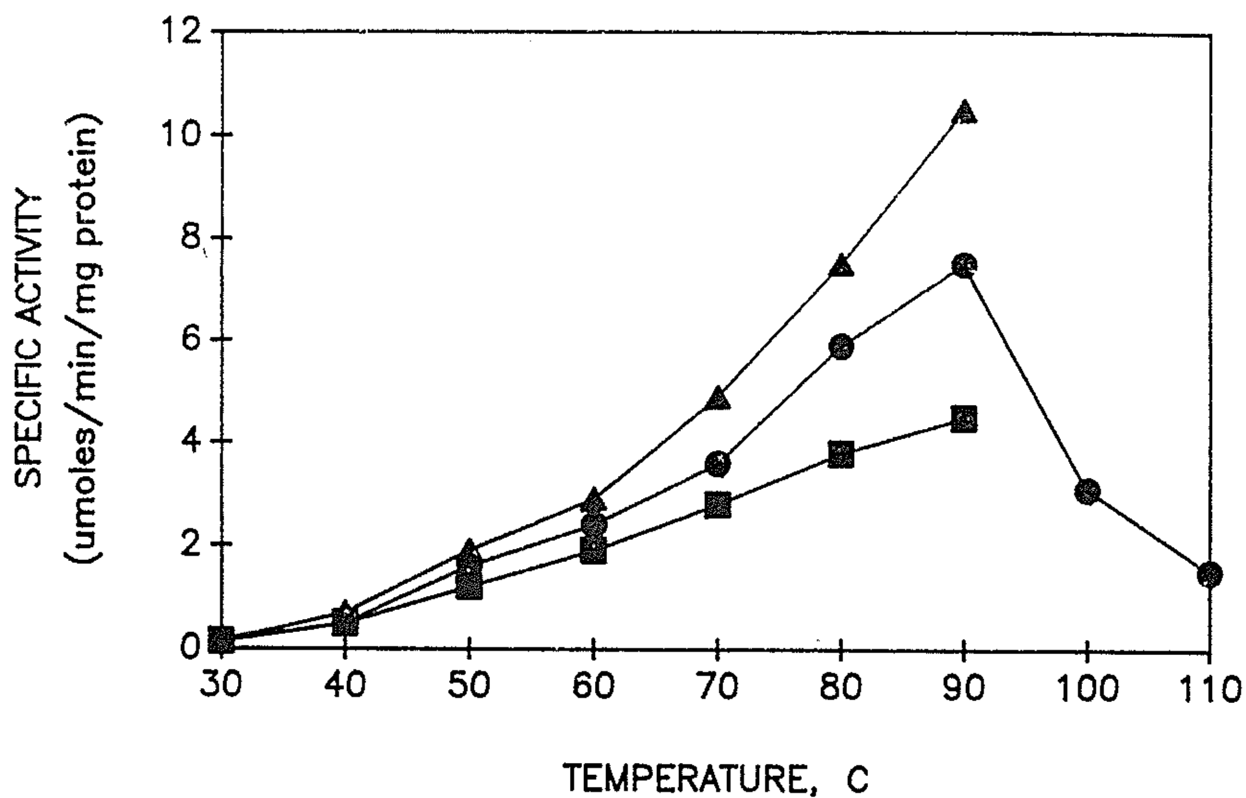



Fig. 3. Temperature dependence of norspermine, norspermidine, and 1,3-diaminopropane aminopropyltransferase enzymatic activities in crude extracts prepared from Thermus thermophilus HB8. Components of the reaction mixtures were described in the text. Each reaction mixture contained 500 µg of protein. The reaction time was 4 minutes. The specific radioactivity of [$^{14}$C]decarboxylated S-adenosylmethionine was 17.85 µCi/mmole.

diaminopropane. This interpretation is not supported by the data in Table 2. In fact, aminopropyltransferase demonstrates a slight incremental increase in $K_m$ at all temperatures in the order: norspermine > norspermidine > 1,3-diaminopropane. Since the $K_m$ is inversely proportional to the affinity of an enzyme for its substrate, there is clearly no preferential affinity of aminopropyltransferase for norspermine that could explain the results of figure 2. Thus, improved protection of the enzyme by norspermine is an attractive hypothesis.

Since T. thermophilus HB8 is a thermophilic bacterium that grows optimally between 70-85°C, an aminopropyltransferase enzyme from Thermus is expected to demonstrate a temperature optimum for catalytic activity above 50°C. A major concern of this author, which was presented in the grant application of two years ago, was that the enzyme may not be enzymatically active when cloned and produced in a plant which grows at significantly lower temperatures. Enzymes from thermophilic bacteria preferring growth temperatures in the range of 70-85°C generally exhibit optimum catalytic capacities at temperatures near or above 65°C (Lauwers, Heinen, and Mulders 1981). However, most of these enzymes retain 25-60% of their catalytic capacity in the range of 30-45°C and most are more thermally stable at the low temperature range.

From figure 3, it is evident that the enzyme is catalytically functional in the temperature range of 40-100°C. Even at a comparatively low temperature of 40°C, aminopropyltransferase from T. thermophilus HB8 demonstrates a high catalytic rate of 0.68 μmoles of product formed/min/mg. The exceptionally high catalytic effficiency of this enzyme provides for a high catalytic rate, even at temperatures which do not support growth of the host T. thermophilus HB8. Agronomic plants in temperate regions commonly experience companion stresses of drought and heat in the temperature range of 35-42°C. A functional aminopropyltransferase catalyst should thus be produced in transgenic plants bearing the aminopropyltransferase gene from T. thermophilus HB8. As simple as this result may appear, the author considers it to be one of the more important results obtained from this project. If the enzyme had failed to exhibit catalytic capacity below 45°C, there would have been no purpose to continue this project.

Demonstration of Uncommon Polyamines in Thermus thermophilus HB8

Using the analytical method of quantitating dansylated polyamines by hplc, the quality and quantity of polyamine titers in T. thermophilus HB8 were investigated at different growth temperatures. The rationale for this study derived from our early

assumption that growth conditions of the thermophile which produced the largest amounts of specific uncommon polyamines might provide cells enriched in aminopropyltransferase(s) specific for norspermine, norspermidine, or 1,3-diaminopropane. Results are given in Table 3. A representative hplc separation profile showing the types of polyamines present in a typical extract of T. thermophilus HB8 is shown in figure 4. Unexpectedly, cultures of T. thermophilus HB8 grown throughout the temperature range of 55-90°C yielded comparable levels of aminopropyltransferase catalytic activity regardless of polyamine substrate that was tested as the aminopropyl group acceptor. The enzyme activities shown in Table 3 were determined at 60°C. Significantly, periodic checks of extract preparations for catalytic capacity at temperatures higher than the growth temperature yielded similar catalytic rates as shown in the table. The data therefore are consistent with the interpretation that T. thermophilus HB8 produces a single type of aminopropyltransferase, which has broad substrate specificity and which is expressed constitutively at all growth temperatures.

Interestingly, data in Table 3 show that the higher molecular weight uncommon polyamines, norspermine and caldopentamine, are not detectable in cultures grown at 55°C and 60°C. Only when cultures are grown at temperatures above about 80°C are these uncommon polyamines consistently observed. Therefore, if T. thermophilus HB8 constitutively produces one type of aminopropyltransferase with capacity to catalyze biosyntheses of all of the uncommon polyamines, the question arises as to how this enzyme is regulated to provide only select populations of the uncommon polyamines at specific growth temperatures.

Effect of Inhibitors on Aminopropyltransferase Activities in Extracts of Thermus thermophilus HB8

A number of compounds structurally related to the substrates and products of aminopropyltransferase or previously known to inhibit aminopropyltransferases for common polyamine biosynthesis were tested for their capacity to inhibit the enzyme from T. thermophilus HB8. The rationale for this study was to gain evidence to support the hypothesis that a single aminopropyltransferase is responsible for uncommon polyamine biosynthesis in the thermophilic bacterium. Compounds tested for inhibition of the thermophilic enzyme are shown in Tables 4 and 5.

5-Methylthioadenosine (MTA) is a product of the aminopropyltransferase reaction. Its effect on the thermophile enzyme is shown in Table 4. The adenosylthioether, which inhibits mammalian putrescine aminopropyltransferase and

TABLE 3

Titers of Uncommon Polyamines and Levels of Aminopropyltransferase Catalytic Activities in Thermus thermophilus HB8 After Growth at Various Temperatures

| Growth temperature | Uncommon polyamine levels[a] nmoles/gm wet-packed cells | | | | Specific aminopropyltransferase µmoles/min/mg protein[c] | | |
|---|---|---|---|---|---|---|---|
| | DAP | Norspd | Norspm | Capm | DAP | Norspd | Norspm |
| 55 | 1,281 | 420 | -0-[b] | -0- | 2.35 | 2.55 | 2.66 |
| 60 | 1,134 | 631 | -0- | -0- | 2.74 | 2.57 | 2.86 |
| 70 | 987 | 604 | 127 | -0- | 2.81 | 2.43 | 2.77 |
| 80 | 855 | 576 | 514 | 233 | 1.91 | 1.78 | 1.86 |
| 90 | 624 | 519 | 1,041 | 381 | 2.12 | 2.88 | 2.27 |

[a] Abbreviations are: DAP, 1,3-diaminopropane; Norspd, norspermidine; Norspm, norspermine; Capm, caldopentamine.

[b] "0" means no polyamine was detected by the analytical method used.

[c] Enzyme assays for data shown were conducted at 60°C.

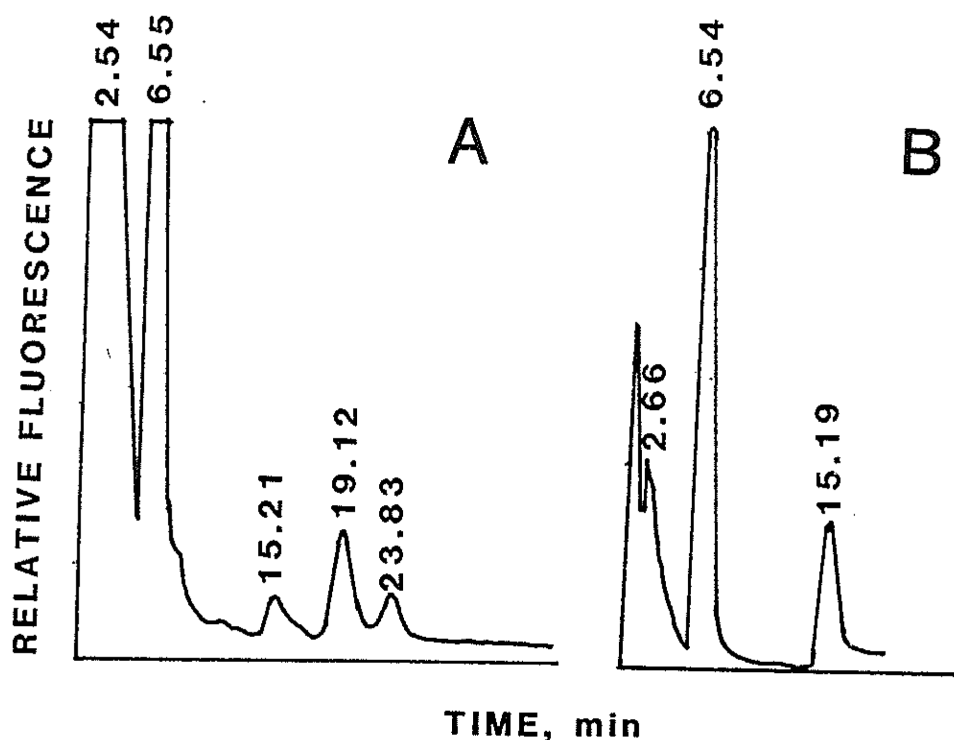



Fig. 4 HPLC elution profile for the uncommon polyamine in cells of Thermus thermophilus HB8. Cells were grown for 19 hours at 80°C, harvested, washed once with ice-temperature water, then extracted, with 10% trichloroacetic acetic acid (TCA). The soluble polyamines were derivatized and quantitated as their dansylated conjugates by reverse-phase hplc. Panel A: Shows a profile of cellular polyamines and retention times in parenthesis for 1,3-diaminopropane (2.54), norspermidine (6.55), norspermine (15.21), presumptive caldopentamine (19.12), and presumptive caldohexamine (23.83). Panel B: Shows a profile of authentic uncommon polyamines, 1,3-diaminopropane (2.66), norspermidine (6.54), and norspermine (15.19). Standards for caldopentamine and caldohexamine were not available.

TABLE 4

Inhibitors of Aminopropyltransferase from Thermus thermophilus HB8 with Respect to Decarboxy-SAM[a]

| Inhibitor tested | Concen., mM | Inhibition, % | Inhibition $K_i$ (μM) with polyamine substrate[b] | | |
|---|---|---|---|---|---|
| | | | DAP | Norspd | Norspm |
| 5'-Methylthioadenosine | 0.1 | 100 | 5.63 | 5.91 | 5.27 |
| 5'-Methylthioinosine | 0.5 | -0- | – | – | – |
| S-Adenosylhomocysteine | 1.0 | -0- | -- | -- | – |
| Cyclohexylamine sulfate | 0.1 | 100 | 0.84 | 1.11 | 0.98 |

[a] Enzyme assays were conducted at 60°C and decarboxylated S-adenosylmethionine was present at 0.02 mM.

[b] Abbreviations are the same as in Table 3.

TABLE 5

Inhibitors of Aminopropyltransferase from Thermus thermophilus HB8 with Respect to Polyamine Substrates[a]

| Inhibitor tested | Concen., mM | Inhibition, % | Inhibition $K_i$, (μM) with respect to[b] | | |
|---|---|---|---|---|---|
| | | | DAP | Norspd | Norspm |
| 1,10-diaminodecane | 1.0 | -0- | – | – | – |
| 1,9-diaminononone | 1.0 | -0- | – | – | – |
| 1,8-diaminooctane | 1.0 | -0- | – | – | – |
| 1,6-diaminohexane | 1.0 | -0- | – | – | – |
| 1,5-diaminopentane (cadaverine) | 1.0 | -0- | – | – | – |
| 1,5-diaminobutane (putrescine) | 1.0 | 91 | 61 | 57 | 64 |

[a] Enzyme assays were conducted at 60°C and uncommon polyamine substrates were present at 0.2 mM.

[b] Abbreviations are the same as in Table 3.

spermidine aminopropyltransferase (Raina et al 1983; Pajula and Raina 1979), acts as a powerful competitive inhibitor with respect to decarboxylated-SAM, with an apparent $K_i$ that was virtually identical (average 5.60 μM) when determined with different polyamine substrates, 1,3-diaminopropane, norspermidine, or norspermine. The high similarity of $K_i$ values suggests that there may be only one aminopropyltransferase in T. thermophilus HB8. The substitution of the 6-amino group of adenosine by a hydroxyl group results in a complete loss of inhibitor activity. This demonstrates the relevance of the purine amino group in the binding of decarboxylated-SAM with the enzyme. The structural analog of MTA, S-adenosylhomocysteine, which is known to inhibit the rat prostate spermidine aminopropyltransferase (Hibasawa et al 1980), did not demonstrate any inhibition of the thermophile enzyme. Cyclohexylamine sulfate, a potent inhibitor of putrescine aminopropyltranferase in higher plants (Sindhu and Cohen 1983) is similarly an inhibitor of the thermophile enzyme. Again as in the case of the inhibitor 5-methylthioadenosine, the $K_i$ for cyclohexylamine is essentially the same for 1,3-diaminopropane, norspermidine, and norspermine aminopropyltransferase activities in extracts from T. thermophilus HB8. This again suggests that there is one type of transferase in Thermus.

Table 5 lists diamine analogs tested for their inhibitory activity. Long aliphatic-chained diamines do not affect the three aminopropyltransferase activities in T. thermophilus HB8. Only the diamine, putrescine, with four methylenic carbons, can function as an inhibitor. It also can serve as a substrate for the aminopropyltransferase of Thermus (see Table 2). These results indicate that the length of the aliphatic spacer between the primary amino groups is critical for recognition by the aminopropyltransferase.

Attempts to Fractionate Putative Multiple Forms of Aminopropyltransferase from Thermus thermophilus HB8

All four of the enzymes involved in the biosynthesis of the common polyamines, putrescine, spermidine, and spermine in mammalian tissues are readily separable from one another. Notably, size-exclusion chromatography on Sephacryl S-200 Superfine (Pharmacia) can resolve ornithine decarboxylase ($M_r$ 53,000), S-adenosylmethionine decarboxylase ($M_r$ 78,000), putrescine aminopropyltransferase ($M_r$ 70,000, a dimer) and spermidine aminopropyltransferase ($M_r$ 90,000 a dimer) (Raina et al 1983). Thus, Sephacryl S-200 chromatography was employed to fractionate crude extracts of T. thermophilus HB8. The objective was to gain evidence for putative multiple aminopropyltransferase enzymes.

Using a column 1.6 x 110 cm, a single fraction was resolved which demonstrated aminopropyltransferase enzyme activity with all three substrates, 1,3-diaminopropane, norspermidine, and norspermine substrates at 60°C and at 80°C. The elution profile is shown in figure 5. Clearly only one aminopropyltransferase fraction was evident from the elution profile that normally would have separated putrescine and spermidine aminopropyltransferases from mammalian sources. When this fraction was rechromatographed by anion exchange column chromatography on Whatman DE52 DEAE cellulose (figure 6) and eluted with a salt gradient, a single fraction containing aminopropyltransferase activity was again obtained. This fraction also demonstrated catalytic activity with 1,3-diaminopropane, norspermidine, and norspermine at 60°C and 80°C. The active fractions from DE52 column chromatography were desalted, concentrated, and loaded into a glass isoelectric focusing column (0.5 cm i.d. x 8 cm) containing a 0-48% sucrose gradient plus 1% ampholine 5-7 (Pharmacia Fine Chemicals). After isoelectric focusing as detailed in Figure 6, fractions were collected from the column and assayed. As shown in figure 7, only one fraction of aminopropyltransferase activity was isolated which focused at a single isoelectric point of pI= 6.58. This enzyme was also catalytically active with polyamine substrates, 1,3-diaminopropane, norspermidine, and norspermine at 60°C and 80°C. As this report is being written, the purity of this preparation is being assessed.

These fractionation properties strongly support the hypothesis that T. thermophilus HB8 produces a single aminopropyltransferase with the capacity to synthesize all the uncommon polyamines, and perhaps also the common polyamines, which bear aminopropyl groups from decarboxylated S-adenosylmethionine. If T. thermophilus HB8 produces multiple forms of this enzyme, each specific for a different polyamine substrate, then one must conclude that these enzymes have identical molecular weights, ionic surface charges, and isoelectric points. While the latter situation is possible, it is seemingly unlikely. A definitive resolution of this question will derive from final confirmation of purification of the enzyme with all three catalytic properties. Work is continuing on this objective.

Discovery of Uncommon Polyamines and a New Biosynthetic Pathway in Higher Plants

With the development of hplc methods to quantitate polyamines and procedures to assay for polyamine oxidase and aminopropyltransferase, it was decided to use these methods on higher plant samples. The rationale for this investigation was that

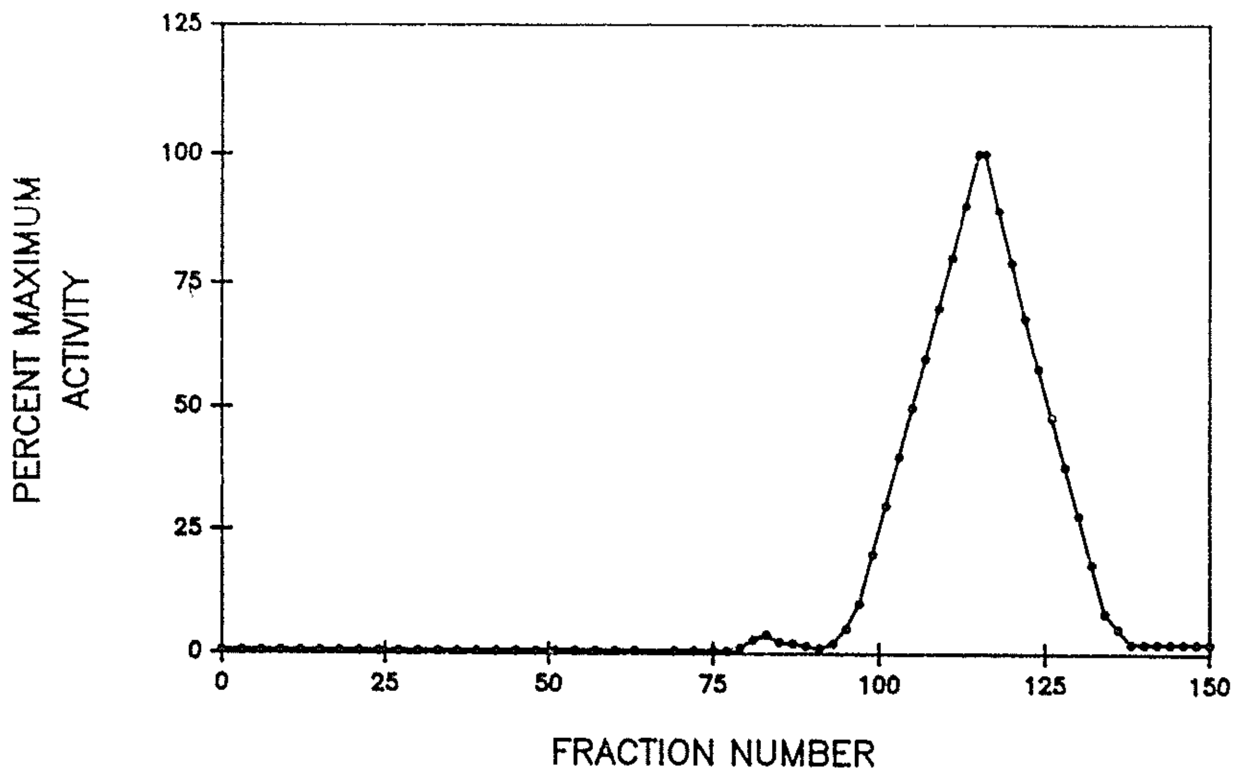



Fig. 5 Elution profile of aminopropyltransferase enzyme activity in a crude extract of Thermus thermophilus HB8 from a Sephacryl S-200 Superfine (Pharmacia Fine Chemicals) Column (1.6 cm i.d. x 91 cm). The column was developed with 0.1 M potassium phosphate buffer, pH 7.4, and 1 mM 2-mercaptoethanol. The flow rate was 6 mL/hr. Enzyme activities in each fraction (1.4 mL) are expressed as percent of the peak value.

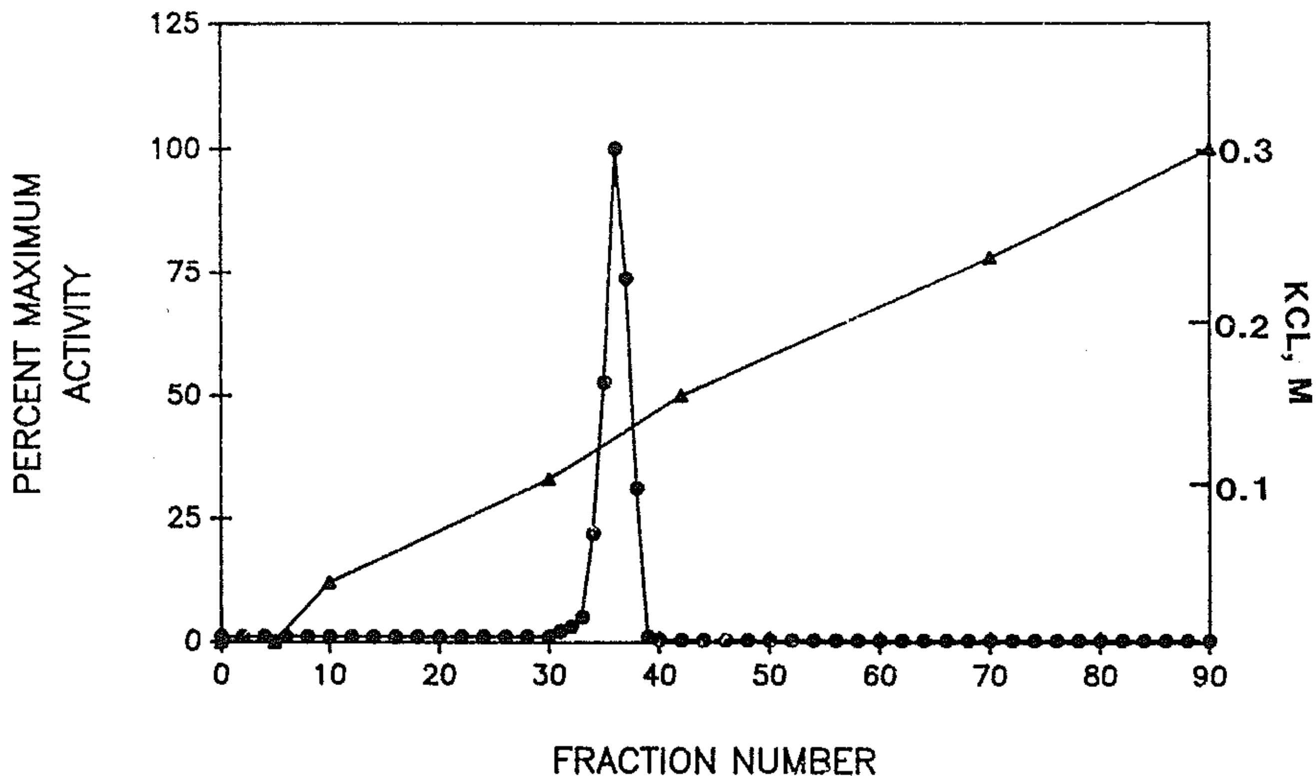



Fig. 6 Elution profile of aminopropyltransferase enzyme activity partially purified as in fig. 5, from a 2.6 cm i.d. x 30 cm column of Whatman DE52 DEAE-cellulose. The column was developed with 600 ml of 10 mM potassium phosphate buffer, pH 7.4, and a linear gradient of 0 - 0.3 M KCl (▲). The flow rate was 42 mL/hr. Enzyme activities (●) in each fraction (6.6 mL) are expressed as percent of the peak value.

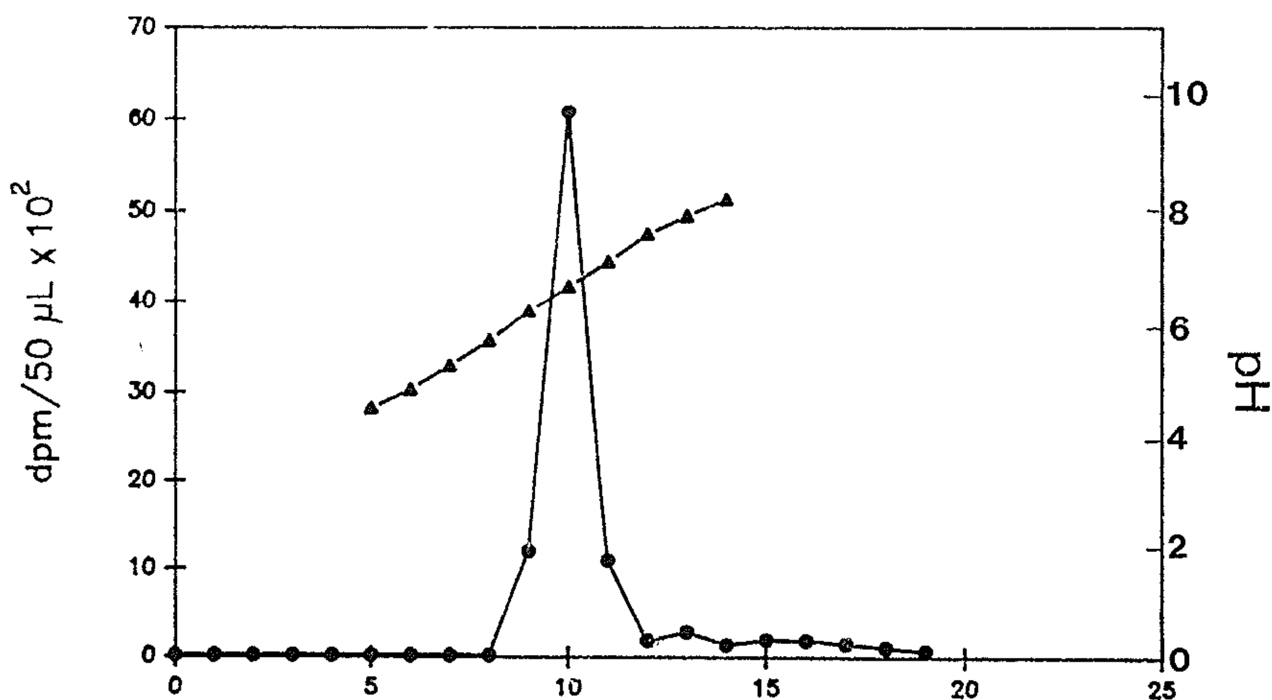



Fig. 7 Aminopropyltransferase profile after isoelectric focusing from pH 5 to 7. Sucrose gradient columns (0.5 cm i.d. x 8 cm) contained 0 to 48% sucrose in 1% ampholine 5 - 7. Samples (90 µL) of the fraction from DEAE cellulose column chromatography, that contained aminopropyltransferase activity, were diluted with 60 µL of a solution that contained 48% sucrose and 1% ampholine. The 150-µL sample was layered 5 cm from the bottom of the sucrose gradient column. The sample was focused at 500 volts for 5 hr followed by an increase to 750 volts for 1.5 hr. Fractions (0.25 µL) were collected and assayed for aminopropyltransferase enzymatic activity. Data are represented as the dpm of [$^{14}$C]5′-methylthioadenosine produced per assay of 50 µL of each fraction versus the fraction number (●). The pH of each fraction was determined and is plotted versus the fraction number (▲). The maximum activity was isolated in fraction #11, which had a pH of 6.58.

uncommon polyamines have not previously been reported to exist in higher plants (Hamana and Matsuzaki 1985). Also, polyamine oxidase has not been previously reported to exist outside the Gramineae, nor has it been found in dicotyledenous plants (Smith 1985b). Finally, only putrescine and spermidine aminopropyltransferases have been investigated in higher plants (Sindhu and Cohen 1983). It was decided to apply the techniques we had developed for investigating uncommon polyamines in T. thermophilus HB8 to higher plants, namely alfalfa, to determine whether specific strains preselected for tolerance to drought might produce polyamines other than putrescine, spermidine, and spermine. This investigation was undertaken with the combined funding of WRRI and the USDA through the Southwest Consortium on Plant Genetics and Water Resources.

Discovery of Norspermidine (Caldine) and Norspermine (Thermine) in Alfalfa

Analyses were performed by hplc of dansylated derivatives of the polyamine fraction obtained after cation exchange chromatography of TCA extracts from alfalfa shoot meristem tissues. Tissues were sampled from separate drought-tolerant and drought-susceptible strains. These fractions revealed unidentified polyamine-like components in addition to the common polyamines, spermidine and spermine (Fig. 8). Dansylated, authentic spermidine and spermine exhibited retention times of 11.38 and 35.63 min, respectively (Fig. 8D), at 20°C. Unidentified dansylated components were detected in alfalfa meristem tissues with retention times of 10.59, 13.72, 19.12, 21.64 and 33.25 min (Fig. 8A). Retention times for dansylated standards of monoacetylated putrescine, monoacetylated spermidine, monoacetylated spermine, tyramine and tryptamine did not coincide with the retention times of the unidentified components (profiles not shown). However, dansylated derivatives of authentic norspermidine and norspermine exhibited retention times of 10.58 and 33.18 min, respectively (Fig. 8C), which were nearly identical to retention times for two unidentified components observed from different alfalfa plant samples (Fig. 8, A and B).

Structural evidence for the occurrence of norspermidine and norspermine in alfalfa was obtained by mass spectrometry. Quantities of each respective polyamine were purified by paper electrophoresis from eluants collected after cation exchange chromatography. Trifluoroacetic acid (TFA) derivatives were prepared for the purified alfalfa polyamines and authentic standards of norspermidine and norspermine. The compound which demonstrated a retention time of 10.59 min by hplc as a dansylated derivative (Fig. 8A), gave a mass spectrum (Fig. 9A) which was identical to standard norspermidine (Fig. 9B). Similarly, the compound whose dansylated derivative

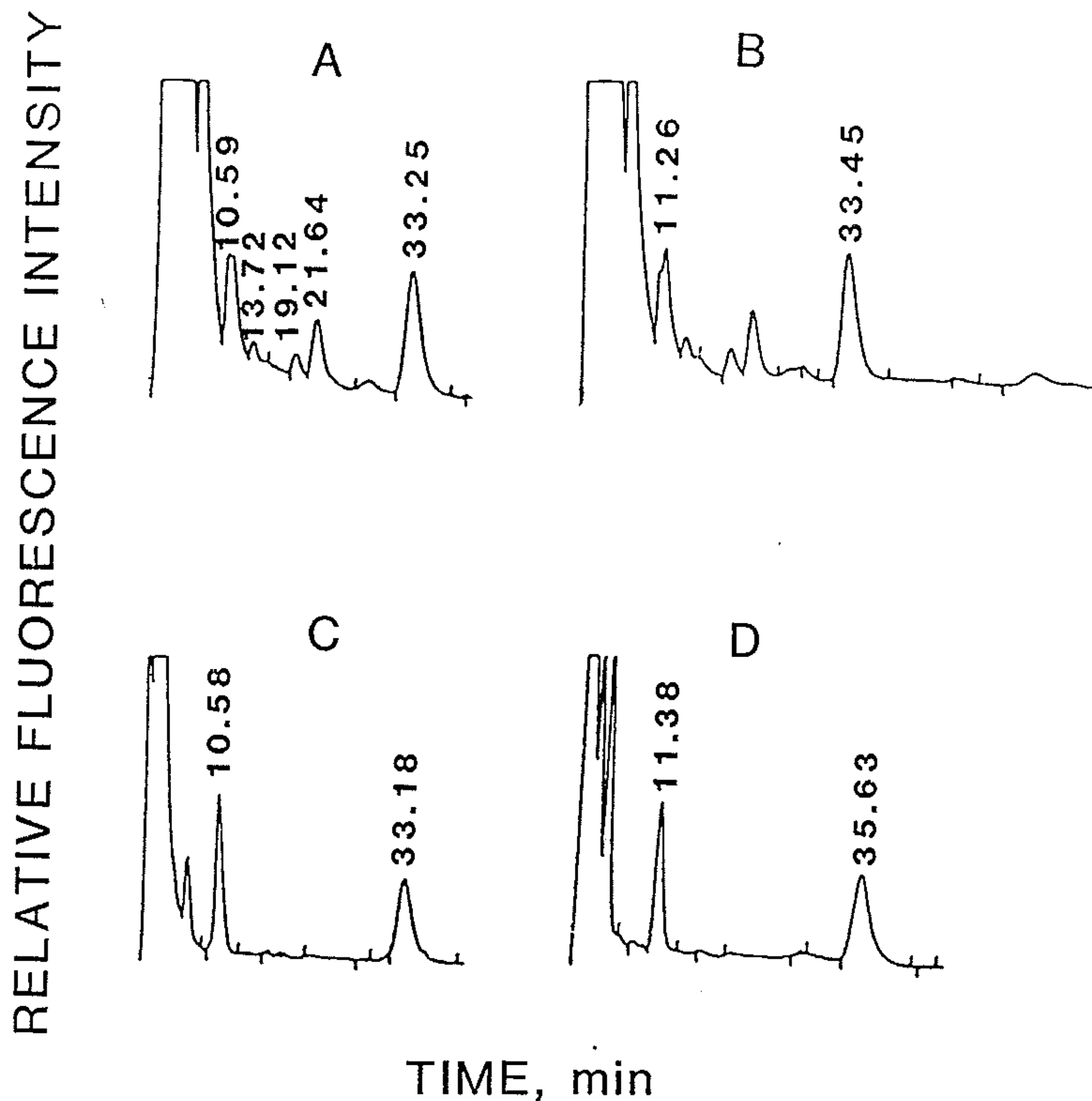



Fig. 8 HPLC separations of dansylated-polyamines from shoot meristem tissues of alfalfa (tracings A, B) and comparison of retention times with authentic standards (tracings C, D). The elution peaks and corresponding retention times in min for separated, identified fractions are: (A) norspermidine, 10.59; norspermine, 33.25; (B) spermidine, 11.26; norspermine, 33.45; (C) norspermidine, 10.58; norspermine 33.18; (D) spermidine, 11.38; spermine, 35.63. Other dansylated, polyamine-like compounds separated from plant tissues shown in tracings A and B (from two different alfalfa samples) have not been identified.

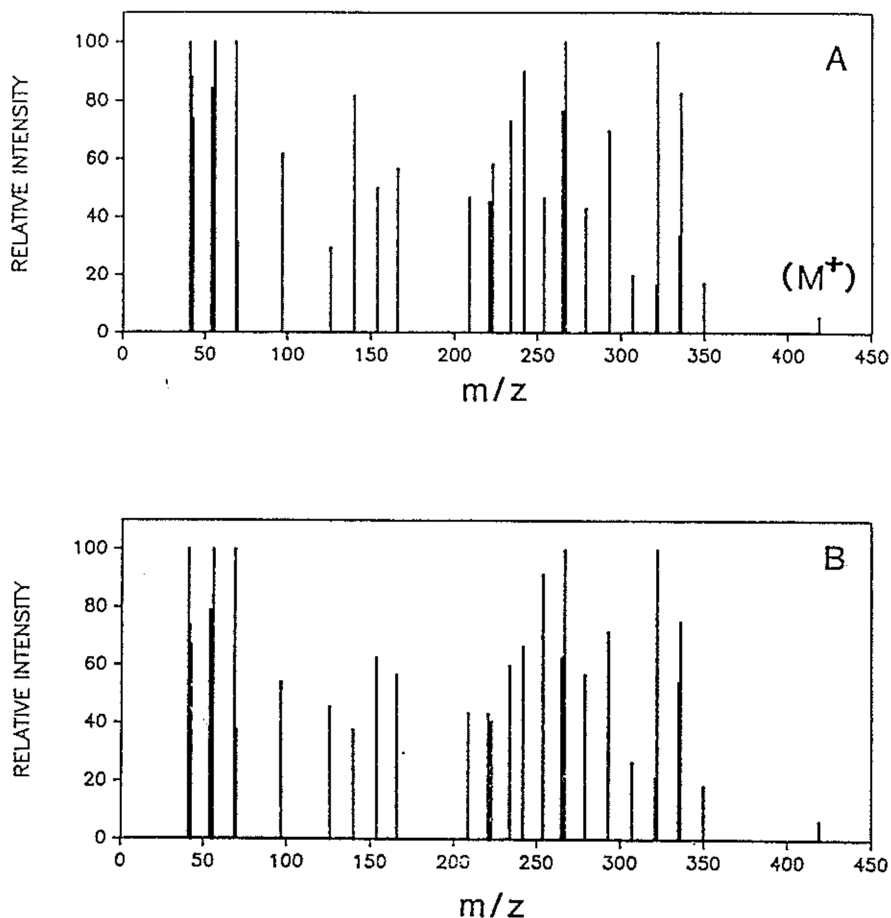



Fig. 9 Normalized mass spectra of TFA derivatives of norspermidine isolated from an alfalfa plant (A) and authentic standard norspermidine (B). Analyses were performed on a Hitachi model RMU-6E mass spectrometer using an ionizing voltage of 70 eV. Intense fragment ions obtained at m/z 69, 126, 140, 154, 279, 293, 307, 322, and 350 are particularly indicative of norspermidine. A weak molecular ion ($M^+$) peak at m/z 419 identifies norspermidine. The identity of norspermidine from alfalfa is established by comparing the mass spectrum of the plant sample (A) with authentic derivatized norspermidine (B). Relative fluorescence intensity values for m/z between 0 and 200 were taken from a relative scale of 1x sensitivity, between 200 and 269 from a scale of 5.3x sensitivity, and between 270 and 419 from a scale of 16.3x sensitivity.

showed a retention time of 33.25 min by hplc (Fig. 7A), yielded a mass spectrum (Fig. 10A) identical to standard norspermine (Fig. 10B). The mass spectra of TFA-derivatized spermine and spermidine standards were also compared with the alfalfa components of interest, and they were clearly different (spectra not shown).

Table 6 presents data representative of the amounts of the uncommon polyamines, norspermidine and norspermine, that can be quantitated in shoot meristem tissues from different alfalfa populations. Amounts of putrescine, spermidine and spermine present in the same samples were comparable to the amount of norspermine detected (data not shown). The simultaneous occurrence of the uncommon polyamines, norspermidine and norspermine, is variable. The sample profile shown in Figure 8B, for example, contained spermidine and norspermine, but not putrescine or spermine. In contrast, the sample profile shown in Figure 8A contained norspermidine and norspermine, but not the other common polyamines.

The detection of the uncommon polyamines norspermidine and norspermine in a higher plant such as alfalfa, as presented in this section, is unprecedented (Hamana and Matsuzaki 1985). Earlier identification of these polyamines has been restricted to bacteria that thrive in extreme environments, certain fungi, eukaryotic algae, lichens and mosses. Numerous studies have reported the wide distribution of the common polyamines, putrescine, spermidine and spermine, among higher plants. Also, the uncommon triamine, *sym*-homospermidine, has been found in higher plants including sandalwood (Kuttan et al 1971), water hyacinth (Yamamoto et al 1983), *Heliotropium* (Birecka et al 1984) and grass pea (Srivenugopal and Adiga 1980). Thus, the current finding of norspermidine and norspermine in alfalfa extends knowledge concerning the variety of triamines and tetramines that can be synthesized by higher plants.

This finding has important implications for future investigation of polyamines and their biosynthetic enzymes in higher plants. First, the occurrence of norspermidine and norspermine in alfalfa implicates a previously unrecognized biosynthetic pathway in higher plants. These reactions could proceed through successive additions of an aminopropyl moiety from decarboxylated S-adenosylmethionine by the action of an aminopropyltransferase enzyme, beginning with the precursor 1,3-diaminopropane, as proposed for thermophilic bacteria (DeRosa et al 1978). We speculate that a nonspecific aminopropyltransferase may exist in alfalfa that catalyzes elongation of 1,3-diaminopropane to norspermidine, and norspermidine to norspermine. Such an aminopropyltransferase with broad specificity has recently been isolated from an extreme acidothermophilic archaebacterium (Cacciapuoti et al 1986).

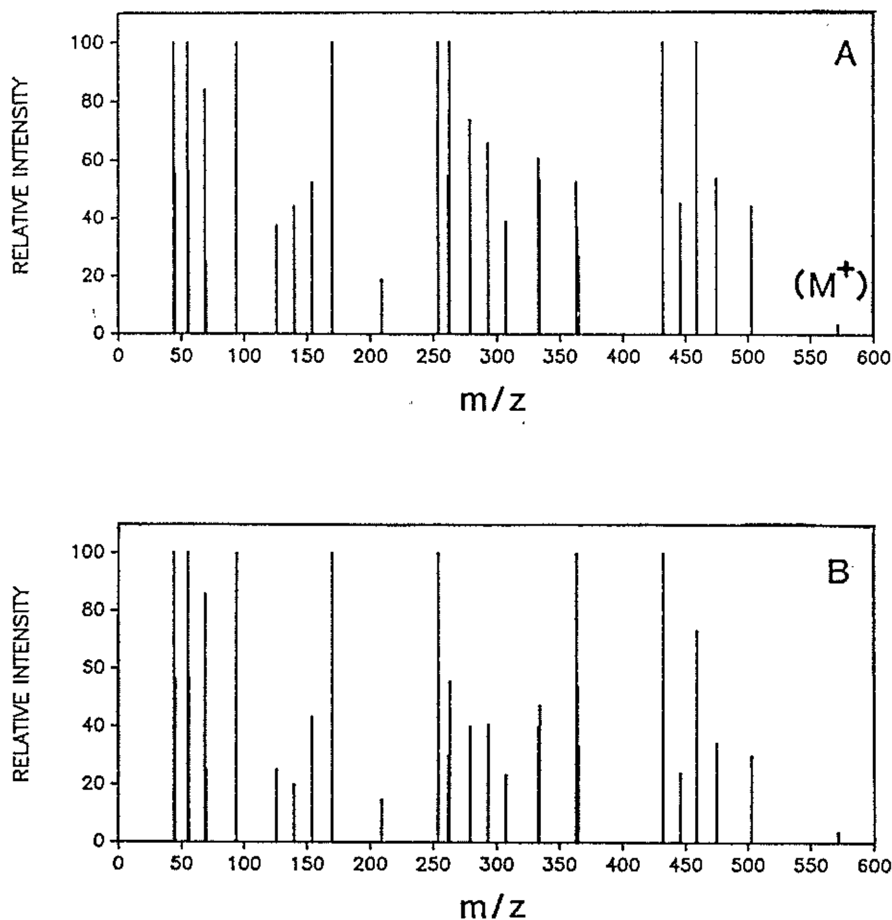



Fig. 10. Normalized mass spectra of TFA derivatives of norspermine isolated from an alfalfa plant (A) and authentic standard norspermine (B). Analyses were performed on a Hitachi model RMU-6E mass spectrometer using an ionizing voltage of 70 eV. Intense fragment ions obtained at m/z 69, 126, 140, 154, 279, 293, 307, 432, 446, 459, 475, and 503 are particularly indicative of norspermine. A weak, but visible, molecular ion ($M^+$) at m/z 572 identifies norspermine. The identity of norspermine from alfalfa is established by comparing the mass spectrum of the plant sample (A) with authentic derivatized norspermine (B). Relative fluorescence values for m/z between 0 and 210 were taken from a relative scale of 1x sensitivity, between 211 and 335 from a scale of 2.6x sensitivity, and between 336 and 572 from a scale of 8.5x sensitivity.

TABLE 6

Representative Amounts of Uncommon Polyamines Quantitated in Shoot Meristem Tissues of Different Alfalfa Populations[a]

| Population | Norspermidine | Norspermine |
|---|---|---|
| | nmol x $g^{-1}$ fresh wt of tissue | |
| Mesilla-0 | Not detected | 50.2 |
| Mesilla-1 | 12.5 | 98.7 |
| Mesilla-2 | 12.3 | 47.3 |
| Mesilla-3 | 16.5 | 65.1 |

[a] Quantities of purified, dansylated polyamines from plant samples were determined from areas of peaks derived from hplc tracings, which were normalized by peak areas generated from authentic standards of dansylated norspermidine or norspermine, respectively. The alfalfa populations were previously selected by phenotypic recurrent selection for increasing productivity under conditions of limiting moisture, and represent the original, unselected cv Mesilla (Mesilla-0) and the three subsequent cycles of selection (Mesilla-1, -2, and -3, respectively). The plants were grown in drought boxes with limited irrigation water as described by others (Salter et al 1984). Each data point represents at least 15 plants.

Second, the occurrence of norspermidine and norspermine in alfalfa implicated the existence of a polyamine oxidase in this dicotyledonous plant. The sole known origin of 1,3-diaminopropane in plant metabolism is from oxidative cleavage of spermidine or spermine through the action of polyamine oxidase (Slocum, Kaur-Sawhney, and Galston 1984; Smith 1985a). 1,3-Diaminopropane is the essential precursor for biosynthesis of norspermidine and norspermine (Hamana and Matsuzaki 1985). Polyamine oxidases demonstrate specificity for spermidine and spermine, and they occur throughout the Gramineae in cereals such as barley, oats and maize (Slocum, Kaur-Sawhney 1984; Smith 1985b), and in other monocots such as water hyacinth (Yanagisawa et al 1987). Its existence has also been implicated in *Helianthus tuberosus* (Bagni et al 1981) through the detection of 1,3-diaminopropane. A possible role for this enzyme in higher plants may be to generate the precursor, 1,3-diaminopropane, for the biosynthesis of the uncommon polyamines, norspermidine and norspermine.

The uncommon polyamines have been implicated in the adaptation of thermophilic and halophilic bacteria to extreme environments (DeRosa et al 1976; Oshima 1983; Yamamoto, Shinoda, and Makita 1979). In the present study, norspermidine and norspermine were identified in alfalfa populations after growth in drought boxes under limited moisture conditions. Additional study is needed to ascertain the functional significance of their occurrence.

Discovery of Polyamine Oxidase in Extracts from Alfalfa

The discovery of norspermidine and norspermine in alfalfa meristem tissues of drought-tolerant alfalfa strains establishes for the first time that uncommon polyamines occcur in some higher plants and their occurrence correlates with tolerances to an abiotic stress. The finding immediately suggests the existence of an unrecognized biochemical pathway in higher plants involving polyamine oxidase, which converts spermidine and/or spermine to 1,3-diaminopropane, and aminopropyltransferase(s), which converts 1,3-diaminopropane to norspermidine to norspermine to caldopentamine to caldohexamine. The occurrences of these two enzyme catalysts were investigated in meristem tissue of preselected, drought-resistant strains of alfalfa and in corresponding cell suspension cultures.

The presence of polyamine oxidase enzymatic activity in alfalfa was confirmed by five criteria. Extracts from meristem tissue of MES-3 strain of alfalfa convert radioactive spermine to radioactive 1,3-diaminopropane as shown in Table 7. The diamine product was isolated and identified from the reaction mixture by co-

TABLE 7
Demonstration of Polyamine Oxidase in Extracts of Alfalfa Meristem Tissue By Conversion of [$^{14}C$] Spermine to [$^{14}C$] 1,3-Diaminopropane (DAP) and By Conversion of Spermine to 1-(3-Aminopropyl)-pyrroline (APP)[a]

| Experiment[a] | dpm recovered as DAP | µmole DAP formed[b] | Experiment[a] | $A_{435}$ for product | µmole APP formed[c] |
|---|---|---|---|---|---|
| 1 | 2281 | 0.253 | 3 | 0.350 | 0.188 |
| 2 | 1728 | 0.192 | 4 | 0.325 | 0.180 |

[a] Reaction mixtures contained: 50 µmoles each of TES/MES/BICINE buffers, pH 7 with KOH; 5 µmoles of spermine tetrahydrochloride, 1 µmole of diethyldithiocarbamic acid, 1 µmole 0-aminobenyaldehyde, 80 µg of beef liver catalase, and 500 µg of protein in 1 ml. The reaction time was 30 min at 30°C. Columns 2 and 3 show data from duplicate reactions for conversion of [terminal methylenes-$^{14}C$] spermine to [$^{14}C$]1,3-diaminopropane (DAP). The specific radioactivity of [$^{14}C$]spermine was 1.80 x $10^4$ dpm/µmole. Columns 5 and 6 show data for duplicate reactions for conversion of spermine to 1-(3-aminopropyl)-pyrroline (APP). APP was determined as a yellow quinazolinium complex with a molar extinction coefficient of 1.86 x $10^3$ $M^{-1}cm^{-1}$ (Holmstedt et al 1961).

electrophoretic mobility with authentic 1,3-diaminopropane and was quantitated by liquid scintillation spectrometry. Second, also shown in Table 7 are results from two spectrophotometric experiments which quantitated the product, 1-(3-aminopropyl)-pyrroline, from a polyamine oxidase-mediated reaction. Since the polyamine oxidase reaction yields equimolar quantities of the products, 1,3-diaminopropane and 1-(3-aminopropyl)-pyrroline (see reaction [6] earlier in report), the data in Table 7 are in agreement with this stoichiometry. Figure 11 shows the dependency of the plant polyamine oxidase on spermine concentration. A $K_m$ of 0.117 µM spermine can be calculated from this curve.

Third, the putative polyamine oxidase activity measured in alfalfa tissues is not due to contaminating diamine oxidase catalytic activity. Diamine oxidases require a copper (II) ion cofactor (Smith 1985b). Strong chelators of $Cu^{2+}$ inhibit diamine oxidases. Figure 12 shows that an alfalfa extract treated with the $Cu^{2+}$ chelator, diethyldithiocarbamic acid (DTC), exhibits marked inhibition of diamine oxidase activity as measured with putrescine as a substrate. The $K_i$ for DTC is 77.8 mM. However, the same extract preparation exhibits only slight inhibition of polyamine oxidase activity by the copper chelator as measured with spermine as a substrate. Because of this result, diethyldithiocarbamic acid was included in all subsequent enzymatic measurements of polyamine oxidase.

Fourth, polyamine oxidases studied among the Gramineae are inhibited by guanidine-type compounds. Figure 13 shows the putative polyamine oxidase in alfalfa is inhibited by the guanidine analog, aminoguanidine. The $K_i$ = 40.5 µM.

Fifth, by far the most effective guanidine yet found to inhibit polyamine oxidases is guazatine, which inhibits the enzyme from barley with a $K_i = 10^{-8}$ M (Smith 1985b). Potent inhibition by guazatine of the putative polyamine oxidase from alfalfa is shown in figure 14. Inhibition is strong as evidenced by the calculated $K_i$ = 10.2 µM.

These findings partially characterize an enzyme that oxidizes the polyamines spermine and spermidine at the secondary amino group to give respectively, 1-(3-aminopropyl) pyrroline and 1-pyrroline. 1,3-Diaminopropane is formed in the case of both substrates. The discovery of such a polyamine oxidase (E.C.1.5.3.3) in a higher plant outside the Gramineae is unprecedented. Heretofore, polyamine oxidase has been studied extensively only in the Gramineae (Smith 1985b). Recently, such an enzyme has been purified from a water hyacinth (Yanagisawa et al 1987) which is a monocot, but which does not belong to the Gramineae. An amine oxidase (E.C.1.4.3.6) in the Leguminosae, such as the pea seedling, is the only other plant enzyme known heretofore to be capable of oxidizing polyamines (Werle et al 1961).

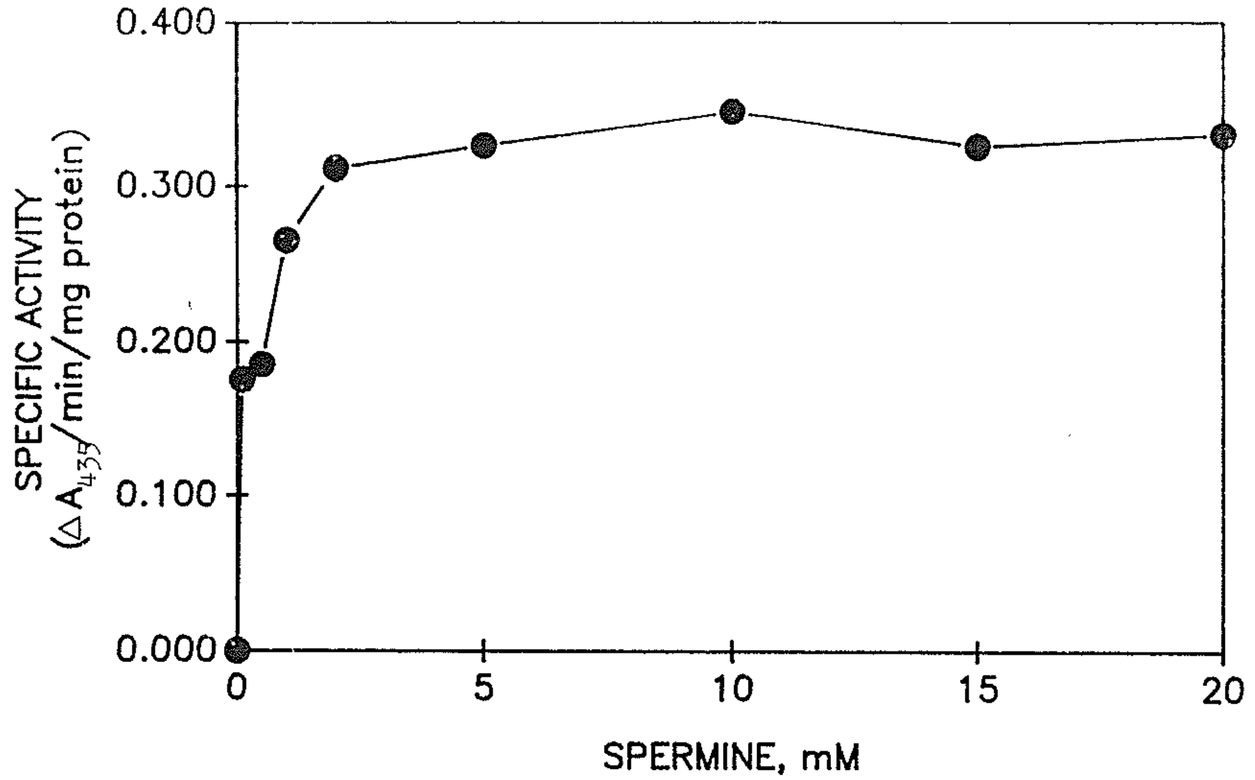



Fig. 11. Dependency of polyamine oxidase from alfalfa meristem tissue on spermine concentration. A $K_m$ for spermine of 0.117 mM can be calculated from a double-reciprocal plot of the graph.

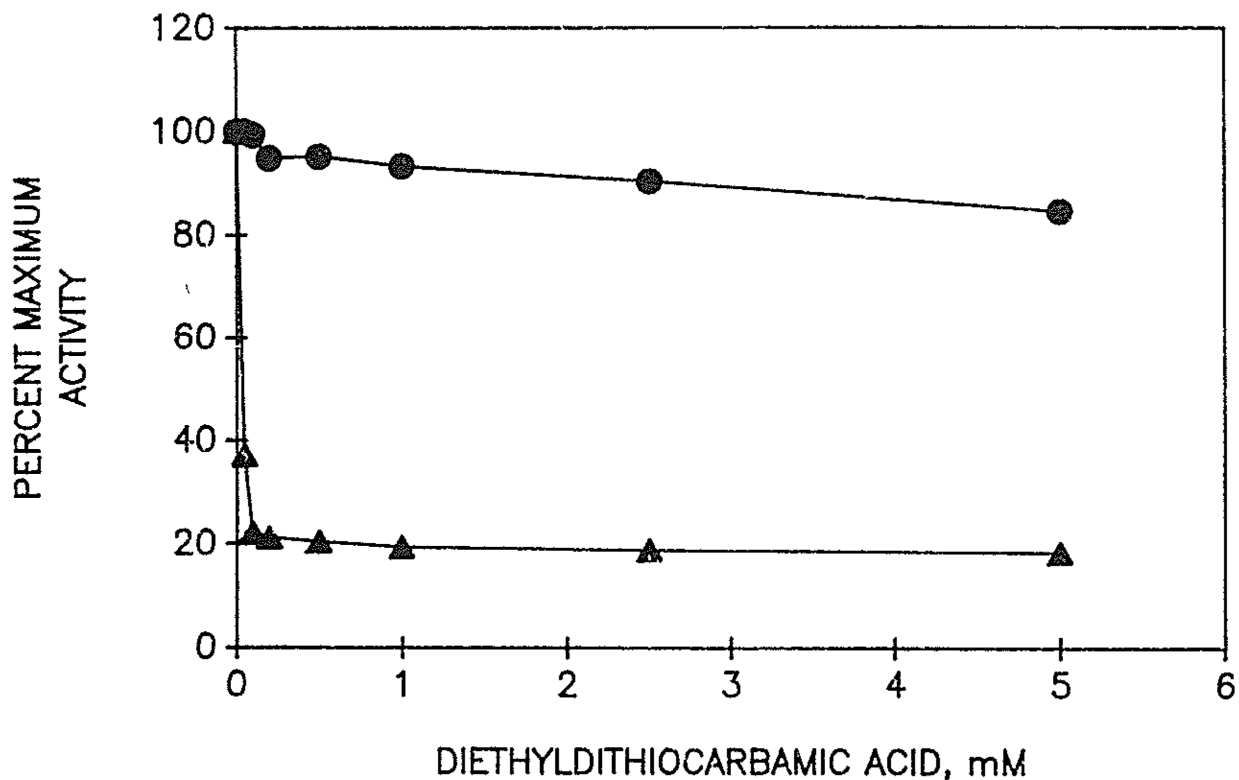



Fig. 12. Insensitivity of polyamine oxidase (●) and inhibition of diamine oxidase (▲) to the copper (II) ion chelator, diethyldithiocarbamic acid (DTC). Enzyme preparations were from alfalfa meristem tissue. Spermine (●) was the substrate used to measure the rate of polyamine oxidase catalysis. Putrescine (▲) was the substrate used to measure the rate of diamine oxidase catalysis. A $K_i$ for DTC can be calculated from diamine oxidase inhibition (▲) to be 77.8 mM.

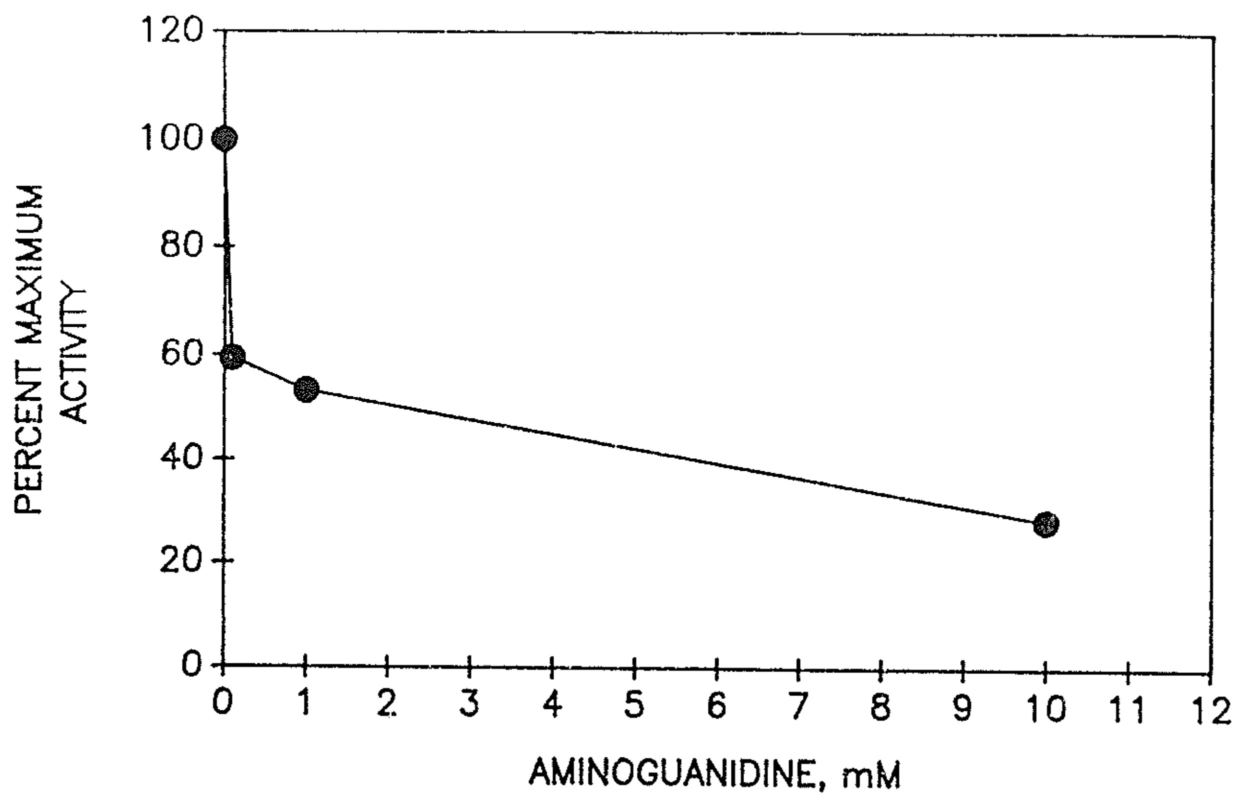



Fig. 13 Inhibition of polyamine oxidase from alfalfa meristem tissue by aminoguanidine. A $K_i$ for aminoguanidine can be calculated from polyamine oxidase inhibition to be 40.5 μM.

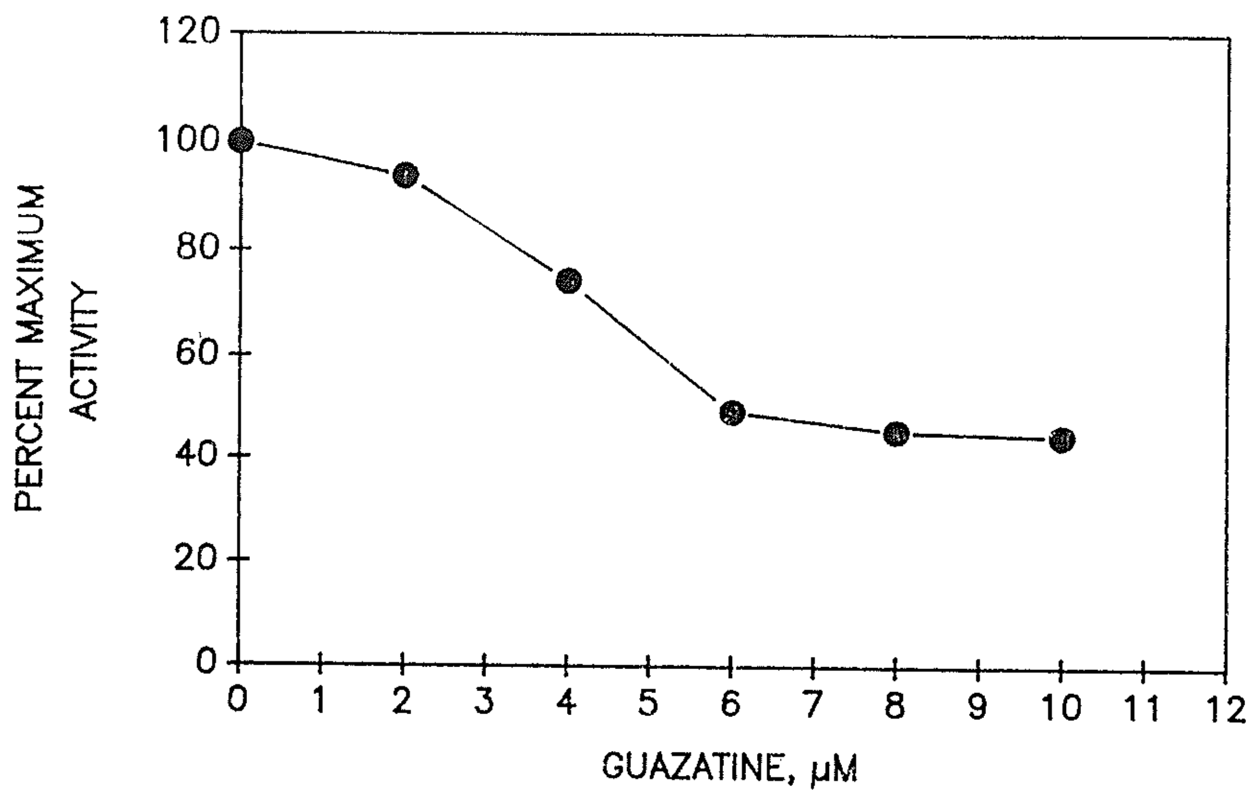



Fig. 14. Inhibition of polyamine oxidase from alfalfa meristem tissue by guazatine. A $K_i$ for guazatine can be calculated from polyamine oxidase inhibition to be 10.2 µM.

The latter enzyme, however, attacks primary amino groups only, with formation of an aldehyde, $NH_3$ and $H_2O_2$. It is particularly active with putrescine and cadaverine as substrate, but it will oxidize primary amino groups in spermidine and spermine and a variety of other mono- and di-amines (Werle et al 1961). Oxidation of putrescine by the pea seedling enzyme yields 1-pyrroline, and spermidine gives 1-(3-aminopropyl) pyrroline. Spermine is oxidized initially to a dialdehyde which breaks down spontaneously to putrescine, which is further oxidized to 1-pyrroline (Hasse and Schuhrer 1962; Alarcon 1970). The enzyme activity described in the present report exhibits substrate specificities and reaction products which clearly categorize it as a type of polyamine oxidase (E.C.1.5.3.3) which exists in the Gramineae. Thus, one likely function for the enzyme is to produce 1,3-diaminopropane, which serves as a connecting metabolic intermediate that extends the biosynthetic pathway from the common polyamines into a pathway that produces the uncommon polyamines (see figure 2 earlier). This hypothesis raised the question of whether alfalfa extracts contain 1,3-diaminopropane aminopropyltransferase activity or other uncommon polyamine aminopropyltransferase activities.

Discovery of Uncommon Polyamine Aminopropyltransferase Activities in Alfalfa

Two different aminopropyltransferase enzyme activities have been demonstrated by extracts prepared from the drought-tolerant Mesilla-3 strain of alfalfa. 1,3-Diaminopropane aminopropyltransferase, which catalyzes the formation of norspermidine from 1,3-diaminopropane has been found with a specific enzymatic activity of 0.47 µmole of 5'-methylthioadenosine produced/min/mg of protein. Norspermidine aminopropyltransferase has also been demonstrated with a similar catalytic efficiency of 0.53 µmole of 5'-methylthioadenosine produced/min/mg of protein.

Alfalfa appears to have the requisite enzymes for producing the uncommon polyamines from the common polyamines through two enzymatic activities, polyamine oxidase and aminopropyltransferase, just as has been found for the thermophile, Thermus thermophilus HB8. When this project was first proposed in 1986, these enzymes had never been reported in plants and their uncommon polyamine products were widely assumed to exist only in life forms adapted to extreme environments. The new discoveries of uncommon polyamines in a higher plant, and the likely biosynthetic enzymes that produce them, immediately suggest strategies in plant cell culture methods and genetic cloning methods which can exploit the uncommon polyamines for possible improvement of drought and/or heat tolerances in plant strains.

Differential Capacities of Expression of Polyamine Oxidase in Drought-Tolerant and Drought-Susceptible Strains of Alfalfa

A plant cell culture technique suggested by our findings, which may provide a method to select drought-tolerant cells, is to design cell selection methods based on metabolic inhibitors of uncommon polyamine biosynthesis. Such a technique is based on a two-fold hypothesis: First, the biosynthetic enzymes that produce the stress-protectant uncommon polyamines, may be most efficiently expressed by cells tolerant to water-deficit stress. Second, cells simultaneously subjected to water-deficit stress and a metabolic inhibitor of a specific polyamine biosynthetic enzyme may express more of the specific enzyme to overcome the metabolic inhibitor and thus produce more of the protective polyamines. Cells selected for their enhanced expression of uncommon polyamine biosynthetic enzymes may be regenerated to produce plants which are more drought tolerant.

As this report is being written, one correlative test of these proposals has been conducted, which supports the rationale for this approach. Drought-tolerant alfalfa strain (MES-3) and drought-susceptible strain (MES-0) were tested in cell suspension cultures for their respective capacities to express polyamine oxidase under conditions of water-deficit stress. Cell cultures of both strains were grown for one week in media containing mannitol to simulate water-deficit and varying amounts of the polyamine oxidase inhibitor, hydroxyethylhydrazine. Our hypotheses predict that MES-3 should have the capacity to express higher levels of polyamine oxidase than MES-0, owing to its preselection for drought-tolerance and water-use efficiency. Figure 15 confirms this prediction. MES-3 expresses an intrinsic level of polyamine oxidase that is six-fold greater than that expressed by MES-0, when the two strains are cultured under water-deficit without hydroxyethylhydrazine. However, after one week of growth in as little as 0.5 mM of the inhibitor, MES-3 cells induce their levels of polyamine oxidase 20-times that of MES-0. Control cultures of both strains grown under the influence of hydroxyethylhydrazine, but not mannitol, expressed uninduced levels of polyamine oxidase (data not shown in figure 15.)

The results described here present a rational bases for proceeding with further development of plant cell selection techniques for drought-tolerance which are based on uncommon polyamine biosyntheses. Experiments are in progress to determine whether inhibitors of aminopropyltransferase, such as cyclohexylamine, might also prove to be valuable for cell selection techniques. The ultimate test of this approach,

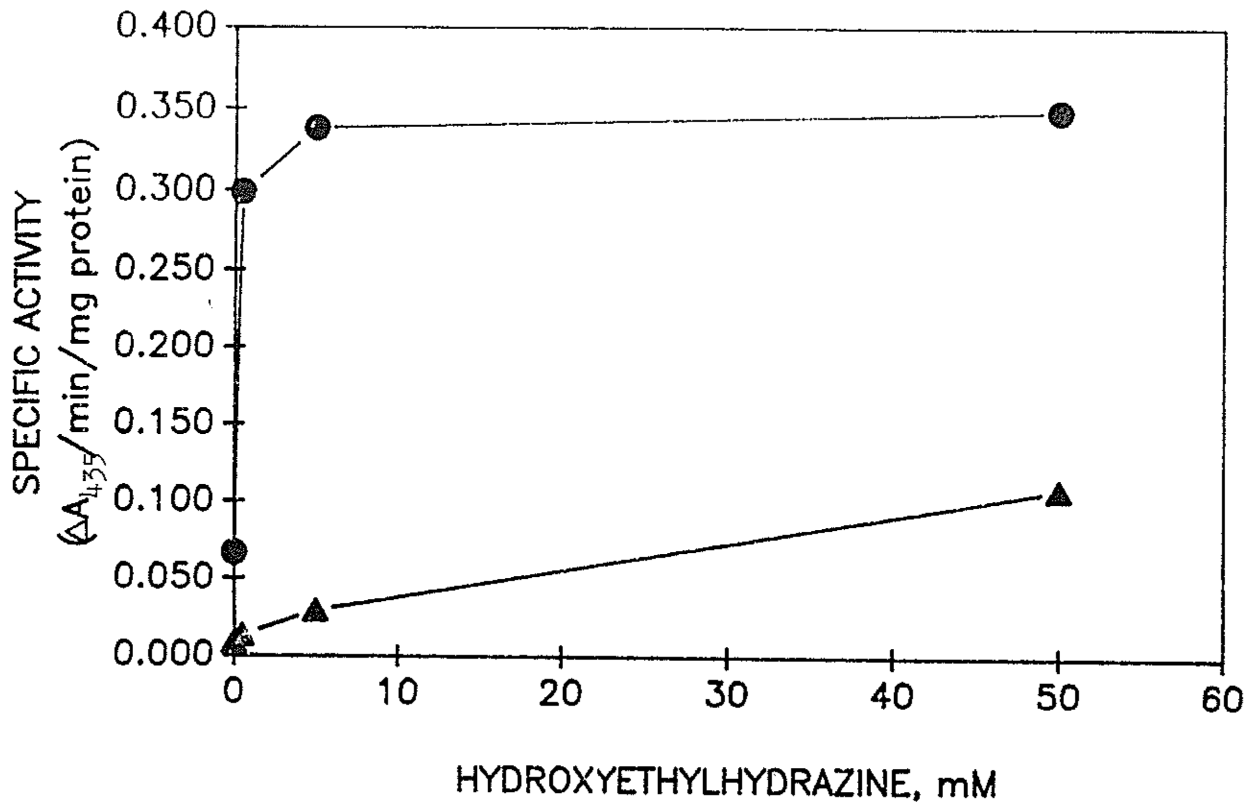



Fig. 15. Differential induction of polyamine oxidase enzymatic activity in cell suspension cultures of drought-sensitive (MES-O) and drought-tolerant (MES-3) alfalfa strains after growth for two weeks in the presence of hydroxyethylhydrazine (HEH). Cell suspension cultures for MES-O (▲) and MES-3 (●) were grown for two weeks in the presence of concentrations of HEH shown. HEH is a metabolic inhibitor of polyamine oxidase. After two weeks, the cells were harvested, washed with cold water, and then analyzed for polyamine oxidase activity.

of course, will be to treat unselected plant strains in cell culture with polyamine inhibitors and to test regenerated plants, with induced levels of uncommon polyamine biosynthetic enzymes, for tolerances to drought, and perhaps heat. This work is continuing in collaboration with Dr. Gregory C. Phillips, associate professor of Agronomy/Horticulture, at New Mexico State University.

## PRINCIPAL FINDINGS, CONCLUSIONS, RECOMMENDATIONS

### Findings, Basic Conclusions, and Summary

The principal findings of this project during the period July 1, 1987 through June 30, 1989, are the following:

A new gene product, an aminopropyltransferase enzyme, has been identified and characterized in the thermophilic eubacterium, Thermus thermophilus HB8. By numerous criteria, including chromatographic behavior, isoelectric focusing, and kinetic responses to select enzymatic inhibitors, T. thermophilus HB8 appears to produce a single aminopropyltransferase enzyme. This enzyme has the capacity to synthesize all of the uncommon polyamines norspermidine, norspermine, caldopentamine, and presumptively caldohexamine, from the precursor diamine, 1,3-diaminopropane. The enzyme demonstrates an exceptionally high specific activity, even at suboptimal temperatures around 40°C. Thus, if the gene for this enzyme is isolated, cloned, and transferred to plant species, a functional protein should be expressed at temperatures experienced by plants in high-temperature environments.

The metabolic pathway for biosynthesis of uncommon polyamines in T. thermophilus HB8 is likely to be that shown in figure 2. This conclusion is supported by the demonstration of polyamine oxidase and the aminopropyltransferase activities in extracts from T. thermophilus HB8. Thus, only two gene products are required to provide this organism with the capability to produce the uncommon polyamines. Although only one aminopropyltransferase appears to account for all uncommon polyamine biosyntheses, T. thermophilus HB8 differentially synthesizes these compounds in a manner dependent on growth temperature. How the regulation of this enzyme is achieved in the thermophile is unknown.

Using the assay methods developed to investigate polyamine titers, polyamine oxidase, and aminopropyltransferase in T. thermophilus HB8, an investigation of these amines and catalysts was initiated in whole plants and cell suspension cultures of preselected drought-tolerant alfalfa. Unexpectedly, the most important finding of this project was that drought-tolerant genetic lines subjected to stress conditions

synthesized uncommon polyamines in significant amounts. Norspermidine (caldine) and norspermine (thermine) were identified in alfalfa extracts. Their respective chemical structures were confirmed by mass spectrometry. This finding is unprecedented and our results (Rodriguez-Garay, Phillips, and Kuehn 1989) represent the first literature report of the occurrences of uncommon polyamines in a higher plant. The induction of biosynthesis of these compounds under osmotic and high temperature stress conditions is highly suggestive of a role in the tolerance mechanism(s) of these genetic materials. The drought-tolerant alfalfas, subjected to drought stress conditions, accumulate norspermine to dominant amounts over other polyamines in meristematic tissues.

Although not detailed in this report, preselected heat-tolerant cotton strains, subjected to heat stress conditions, were found to accumulate presumptively caldopentamine and/or homocaldopentamine in dominant amounts. One or both hexamines may also be produced in low amounts. This suggests the possibility that different individual polyamine species may play a specific role in protection against drought or osmotic stress, versus heat stress. These results are the bases for using meristematic tissues from whole plants in future work.

In drought-tolerant alfalfas subjected to stress conditions, a polyamine oxidase activity was found by the capacity to convert spermine or spermidine to $\Delta^1$-pyrroline derivatives and also radiolabeled spermine to radiolabeled 1,3-diaminopropane. This activity was sharply induced over 20-fold following drought or osmotic stress. This polyamine oxidase is distinguishable from diamine oxidase by virtue of its insensitivity to cupric ion chelators, and its potent inhibition by guazatine and 2-hydroxyethylhydrazine using in vitro assays (Smith 1985b). Thus, a polyamine oxidase is the likely source of 1,3-diaminopropane for the biosynthesis of the uncommon polyamines in higher plants (see figure 2). The finding of polyamine oxidase in a dicotyledenous plant such as alfalfa, is also unprecedented in the literature before this study. Most importantly for future work, preselected drought-stress tolerant alfalfa strains demonstrated a 20-fold greater capacity to induce polyamine oxidase in cell suspension cultures, under the combined influence of osmotic stress and the polyamine oxidase inhibitor, hydroxyethylhydrazine, than did preselected drought-susceptible strains. This result forms the bases for future investigations which will exploit cell selection techniques for drought tolerance in suspension cultures utilizing metabolic inhibitors of the uncommon polyamines.

Finally, in the drought-tolerant alfalfas subjected to stress conditions, an aminopropyltransferase activity was detected that is capable of converting

norspermidine to norspermine. The activity is also induced by exposure of cells to stress conditions, and it is inhibited by cyclohexylamine. This result also supports the putative pathways for uncommon polyamine synthesis as shown in figure 2. The same plant extracts contain aminopropyltransferase capable of converting spermidine to spermine, and 1,3-diaminopropane to norspermidine. It is not yet clear whether a single enzyme or multiple enzymes perform these functions.

Recommendations

Four recommendations derive from the results of this project.

First, it can be concluded with a significant degree of certainity that the uncommon polyamines in T. thermophilus HB8 are biosynthesized by the pathways shown in figure 2. Unexpectedly, this project has also shown that similar pathways exist in some higher plants and the capacity to express the pathways to uncommon polyamines is linked to tolerances against drought (alfalfa) and heat stresses (cotton). Much basic research can now be conducted which focuses on the putative roles of the uncommon polyamines as abiotic stress protectants. The determination of the extent of distribution of these pathways in the plant kingdom, precise definition of the conditions which elicit their biosynthesis, and the intracellular target structures or processes which gain protection as a result of their synthesis, are all intriguing areas for future basic research.

Second, T. thermophilus HB8 appears to produce a single type of aminopropyltransferase which can utilize a broad spectrum of polyamine substrates. The purification of this enzyme is nearly complete at this writing and may be completed by the end of the funding period for this project. From the purified protein, a partial amino acid sequence can be derived. From this sequence, a radiolabeled synthetic, single-stranded DNA probe can be synthesized and used to isolate the gene for aminopropyltransferase from T. thermophilus HB8. The availability of the gene will lead to numerous types of investigations. In the area of plant improvement, the gene can be transferred to plants to determine whether the gene product, aminopropyltransferase, is truly expressed as a functional enzyme. If it is, then the important question of whether enhanced capacity to produce the uncommon polyamines lends abiotic stress tolerances can be studied.

Third, the availability of the aminopropyltransferase gene from T. thermophilus HB8 will provide opportunities to study the consequences of uncommon polyamine synthesis in non-thermophilic microorganisms. Hence, a definitive test of the hypothesis that uncommon polyamines are abiotic stress protectants can be investigated in transgenic mesophiles into which has been transferred the

aminopropyltransferase gene from T. thermophilus HB8. A grant application to the National Science Foundation (Dec., 1988; June 1989) is pending to pursue this direction.

Fourth, the results of this project lead to the conclusion that in vitro cell selection methodology which exploits metabolic inhibitors of uncommon polyamine biosyntheses may be an effective and powerful approach to obtain plant lines that are tolerant to drought and/or heat stresses. This was elaborated earlier in this report. This author, in collaboration with Dr. G. C. Phillips at New Mexico State University, is actively pursuing this investigation.

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