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Transgenic manipulation of polyamine metabolism in poplar

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TRANSGENIC MANIPULATION OF POLYAMINE METABOLISM IN POPLAR

By

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DISSERTATION

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DEDICATION

This dissertation is dedicated to my parents.

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ABBREVIATIONS

ACC= 1-aminocylcopropane-1-carboxylic acid; Ag= aminoguanidine; Arg= arginine; ADC= arginine decarboxylase; AO= amine oxidases; APCHA= (N-{3-aminopropyl}cyclohexylamine) ; CaMV= cauliflower mosaic virus; CHA= cycloheximide; 2,4-D= 2, 4- dichlorophenoxyacetic acid; DAO= diamine oxidase; DAP= diaminopropane; DEPC= diethyl pyrocarbonate; DFMA=α-DL- difluoromethylarginine; DFMO= α-DL- difluoromethylornithine; DTT= dithiothreitol; dcSAM= decarboxylated SAM; DW= dry weight; FW= fresh weight; GABA= γ- aminobutyric acid; GAD= glutamate decarboxylase; Gln= glutamine; Glu= glutamate; GOGAT= glutamate synthase; GS=glutamine synthetase; HPLC= high performance liquid chromatography; JA= jasmonic acid; MeJA= methyl jasmonate; MCHA= (trans-4-methylcyclohexylamine); MGBG= Methylglyoxal (bis-guanylhydrazone); MSX= methionine sulfoximine; N= nitrogen; NR= nitrate reductase; NiR= nitrite reductase; NPT II= neomycin phosphotransferase II gene; NT= non-transformed; NT-HP = non-transformed-high putrescine; OAT= ornithine amino transferase; Orn= ornithine; ODC= ornithine decarboxylase; PAO= polyamine oxidase; PCA= perchloric acid; PDH= pyrroline dehydrogenase; PLP= pyridoxal phosphate; Pro= proline; Put= putrescine; SA= salicylic acid; SAM= S-adenosyl methionine; SAMDC= S-adenosyl methionine decarboxylase; Spd= spermidine; Spm= spermine; SSAT= spermidine/spermine acetyl transferase; TLC= thin layer chromatography.

ABSTRACT

GENETIC MANIPULATION OF POLYAMINE METABOLISM IN POPLAR

by

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University of New Hampshire, May, 2002

Polyamines are low molecular weight polycations found in all living organisms. They are involved in plant stress response and development. The study was aimed at analyzing the effects of altered polyamine metabolism on the polyamine and related pathways in transformed poplar cells overexpressing a mouse *odc* cDNA under the control of 2X 35S CaMV promoter. The transgenic cells (line 2E) showed elevated levels of mouse ornithine decarboxylase enzyme activity, several fold higher amounts of putrescine, a small increase in spermidine, and a small reduction in spermine as compared with non-transgenic (NT) cells. The conversion of labeled ornithine into putrescine was significantly higher in the transgenic than the NT cells.

The results show that: (a) Transgenic expression of a heterologous *odc* gene can be used to modulate putrescine metabolism in poplar cells, (b) accumulation of putrescine in high amounts does not affect the native arginine decarboxylase activity, (c) ornithine biosynthesis occurs primarily from glutamine/glutamate and not from catabolic breakdown of arginine, (d) ornithine biosynthesis may become a limiting factor for putrescine production in the *odc*

transgenic cells, (e) assimilation of nitrogen into glutamine keeps pace with an increased demand for its use for biosynthesis, (g) increased putrescine degradation occurs without major changes in the activity of diamine oxidase, (h) rate of spermidine turnover is slower in the transformed cells than the NT cells, and (i) the expression of genes involved in the related pathways may be altered.

INTRODUCTION

Polyamines in Plants

Polyamines are low molecular weight polycations found in all living organisms. The commonly occurring polyamines in plants are spermidine (Spd), spermine (Spm) and their diamine precursor, putrescine (Put) (Fig.1). The uncommon polyamines norspermidine (caldine), norspermine (thermine), pentamine and hexamine were first discovered in halophilic and thermophilic bacteria (Kuehn et al., 1990). These uncommon long chained polyamines are required for the activation of the ribosomal ternary complex during protein synthesis and affect the rate of chain elongation in these organisms (Hamana et al., 1992). Norspermidine and norspermine have been detected under stress conditions in plants and are also known to be present in mosses. Homospermidine, another uncommon polyamine, is present in ferns and algae. Cadaverine is formed from lysine and is found in all members of the family Leguminosae. It serves as a precursor of quinolizidine alkaloids (Smith and Wilshire, 1975; Schoofs et al., 1983). A role for cadaverine in plant development has been proposed by many researchers (Torrigiani and Scoccianti, 1995; Shevyakova et al., 2000).

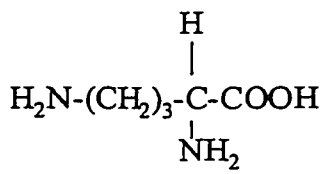
Polyamines exist in free, bound and conjugated forms. Free polyamines are soluble in perchloric acid (PCA) whereas bound forms are not. They are not disassociated with salt nor can be exchanged with free polyamines. Polyamines are generally conjugated with hydroxycinnamic acids, fatty acids or alkaloids (Flores and Filner, 1985; Ghosh, 2000; Bagni and Tassoni, 2001). The conjugated forms are not water- soluble whereas free polyamines are. Polyamines also serve as precursors for secondary metabolites such as nicotine, and can be conjugated with phenolic acids to produce plant defense-related compounds (Martin-Tanguy, 1997).

Putrescine $H_2N\text{-}(CH_2)_4\text{-}NH_2$

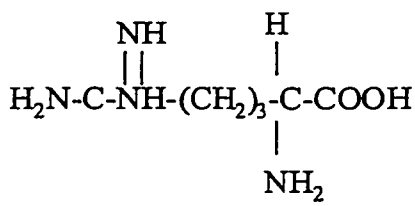
Spermidine $H_2N\text{-}(CH_2)_4\text{-}NH\text{-}(CH_2)_3\text{-}NH_2$

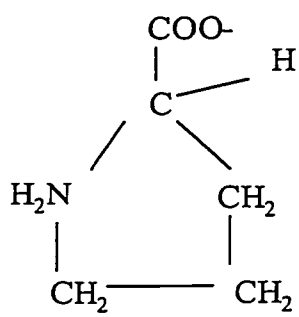
Spermine $H_2N\text{-}(CH_2)_3\text{-}NH\text{-}(CH_2)_4\text{-}NH\text{-}(CH_2)_3\text{-}NH_2$

Ornithine

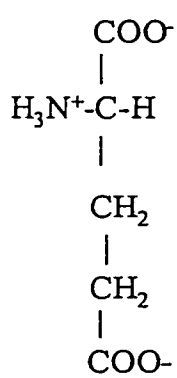


Arginine

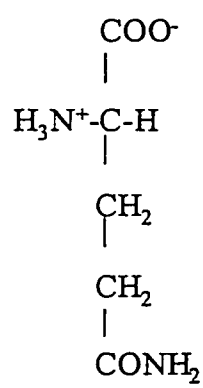




Proline



Glutamate



Glutamine

Fig. 1. Structure of the polyamines and amino acids of the glutamate family.

Rates of polyamine biosynthesis and degradation, their conjugation with phenolic acids, and intercellular transport all contribute to cellular levels of free polyamines in plants.

Polyamine Functions

In higher plants, polyamines are involved in many processes such as stress (Zhou et al., 1995; Minocha et al., 1992; 1997; 2000; Watson and Malmberg, 1996; Chattopadhyay et al., 1997; Bouchereau et al, 1999; Liu et al., 2000); embryogenesis (Robie and Minocha, 1989; Singh and Rajam, 1998; Minocha et al., 1993; 1999b; Martinez et al., 2000; Shoeb et al., 2001), pollen formation, floral initiation, fruit development (Slocum and Flores, 1991; Rodriguez et al., 1999), and senescence (Srivastava, 1987; Pandey et al., 2000). It is unclear at present whether the increase in Put in response to stress is beneficial to the plant or causes injury. Due to their richness in amine groups, and their presence in millimolar quantities in plant cells, polyamines could also play a role in the modulation of reduced nitrogen (N) and in the sequestration of free ammonia (NH_3) produced inside the cells (Lovatt, 1990; Slocum and Weinstein, 1990). Polyamines also serve as storage products of N and are suggested to have a role in reducing NH_4/NH_3 toxicity (Cohen, 1998). Most studies reported thus far have emphasized the correlative changes in cellular polyamines and a developmental and/or a physiological response of the plant (Evans and Malmberg, 1989; Walden et al., 1997; Cohen, 1998, and references therein).

Polyamines are able to interact with polyanions, such as the phosphate residues in nucleic acids, acidic phospholipids and membranes. Hence, this property can explain their role in enhancing transcription, translation and membrane stabilization in the cells. Spermine plays an important role in stabilizing DNA configuration (Egli et al., 1991). Spermidine and Spm also perform crucial functions in DNA replication, transcription and translation. They stabilize interactions between macromolecules; thus Spd is often included in microprojectile coating mixtures used for biolistic bombardment (Christou and Ford, 1995). Spermidine and Spm cause DNA bending as well as change the conformation of B-DNA to Z-DNA (Feuerstein et al., 1990).

Post-translational covalent linkage of polyamines to proteins is catalyzed by transglutaminases (TGases) that are located both extra- and intracellularly (Serafini-Fracassini et al., 1995). The polyamines are acyl-acceptors for TGases. This crosslinking of proteins to polyamines protects them from proteolysis. They can also affect several mitochondrial functions via electrostatic interactions (Votyakova et al., 1999). Attachment of polyamines to mitochondrial proteins also occurs via TGases. Spermidine and Spm have been known to stabilize molecular complexes of thylakoid membranes (Besford et al., 1993). Polyamine binding is known to modify activities of some enzymes. These two polyamines have also been shown to inactivate fructose-1,6 bis-phosphate phosphatase of chloroplasts *in vitro* (Carley et al., 1983).

Spermidine and Spm biosynthesis increases during the transition from G1 to S phase of the cell cycle. Adding polyamines allows the cells to progress to the S phase. Inhibition of Spd and Spm biosynthesis blocks the cells in the G1 stage of the cell cycle. Hence, the suggestion that polyamines are involved in the mitotic cell division cycle (Rupniak et al., 1978). In animal systems, a relationship between polyamines (Spd and Spm) and cytoskeletal structures has been seen and the inhibition of polyamine biosynthesis resulted in blocking cytokinesis (Pohjanpelto et al., 1985). Pohjanpelto and Hölttä (1996) also suggest a role for polyamines in joining the Okazaki fragments.

One of the roles of polyamines in macromolecular function is in the formation of hypusine, a post translationally modified lysyl residue in eukaryotic initiation factor 5A. The aminobutyl group that is donated by Spd to a specific lysine residue (Lys-50) is oxidized and leads to hypusine synthesis (Davis et al., 1992; Ober and Hartmann, 1999). In several plants, Spd is a substrate for alkaloid production (Graser and Hartmann, 2000). Spermidine provides its aminobutyl group to Put yielding homospd, the building block of the necine base moiety of pyrrolozidine alkaloids. *In vitro* studies have shown that polyamines bind to polygalacturonic acid. Spermidine has been implicated in the chilling tolerance of cucumber by preventing the activation of NADPH oxidases in the microsomes (Shen et al., 2000). Liu et al. (2000) showed

that application of polyamines inhibited the inward K (+) movement across the guard cell plasma membrane and that Spd inhibited the KAT1-like inward K (+) channel. Spermidine and Spm may displace Ca^{++} ions and block the signal transduction cascade in the cell. This is started by changes in the galacturonic acid conformation once Ca^{++} binding has taken place (Messiaen et al., 1997). The inhibition of polyamine synthesis has also been shown to affect the middle lamella decomposition and cell enlargement (Berta et al., 1997).

It has been known that polyamine transport across the plasma membrane is energy dependent and Ca^{++} is involved in the process (Antognoni et al., 1995). Treatments inhibiting calmodulin action or the kinase and phosphatase activities significantly reduced Ca^{++} activated Put uptake. It was shown in *Neurospora crassa* that the uptake of polyamines shows the presence of a biphasic system: one saturable and one non-saturable (Davies and Ristow, 1988). At the whole plant level, polyamines have been reported in the xylem and phloem exudates of various plant species (Friedman et al., 1986), and translocation via xylem vessels is dependent on the rate of transpiration (Bagni and Pistocchi, 1991). The long distance transport of polyamines is nonpolar. In carrot cell cultures, there were two saturable uptake components for low and high concentrations of Put, respectively (Bagni and Pistocchi, 1991). For Spd uptake, there was one saturable component followed by a linear one; Spm had the same pattern for uptake like Put. Studies were carried out using protoplasts since substantial amounts of the polyamines bind to the cell walls after uptake. Spermidine uptake in the vacuole showed a biphasic pattern, a saturable one followed by a linear one. pH dependence for polyamines was seen for both uptake by the protoplast as well as vacuole (Bagni and Pistocchi, 1991).

Polyamine Biosynthesis

The diamine Put is synthesized in almost all organisms by decarboxylation of ornithine (Orn) in a single step reaction brought about by ornithine decarboxylase (ODC; E.C. 4.1.1.17). In plants, bacteria and some fungi, it is also formed from arginine (Arg) via arginine decarboxylase

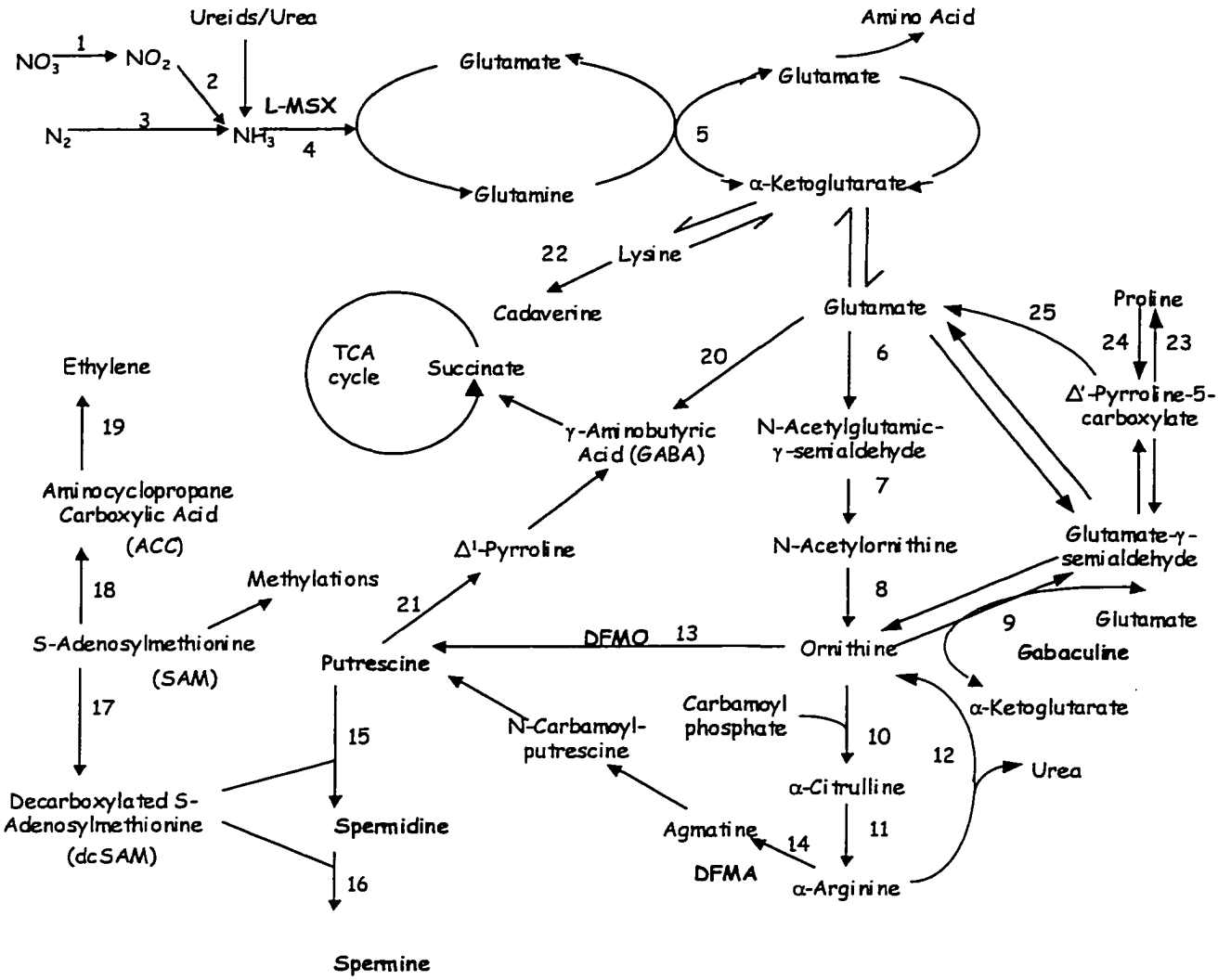


Fig. 2. Polyamine biosynthesis and related nitrogen metabolism. The enzymes are: 1. Nitrate reductase, 2. Nitrite reductase, 3. Nitrogenase, 4. Glutamine synthetase (GS), 5. Glutamate synthase (GOGAT), 6. Glutamate reductase, 7. Acetylglutamic-γ-semialdehyde transaminase, 8. Acetylornithinase, 9. Ornithine aminotransferase (OAT), 10. Ornithine transcarbamylase, 11. Arginine synthase, 12. Arginase, 13. Ornithine decarboxylase, (ODC) 14. Arginine decarboxylase (ADC), 15. Spermidine synthase, 16. Spermine synthase, 17. SAM decarboxylase (SAMDC), 18. ACC synthase, 19. ACC oxidase, 20. Glutamate decarboxylase (GAD), 21. Diamine oxidase (DAO), 22. Lysine decarboxylase (LDC), 23. P5C Reductase, 24. Proline oxidase, 25. P5C Dehydrogenase.

(ADC; E.C. 4.1.1.19) through the intermediates agmatine and N-carbamoylputrescine (Fig. 2). N-carbamoylputrescine formed from agmatine by agmatine iminohydrolase is converted to Put by N-carbamoylputrescine amidohydrolase. Bacterial metabolism is closer to plants than animals or fungi, in that, they utilize both pathways for Put production. *Arabidopsis* is the only plant and one of only two eukaryotic organisms (the other being the protozoan *Trypanosoma cruzi*) that have been demonstrated to lack ODC activity. As ODC is a key enzyme in polyamine biosynthesis, *Arabidopsis* is totally reliant on the ADC pathway (Hanfrey et al., 2001). However, ADC activity as well as the presence of agmatine has been recently reported in animals also (Li et al., 1994; Gilad et al., 1996). Agmatine in animals is presumably converted into Put by a single step reaction involving the enzyme agmatinase (Cohen, 1998). A recent report suggests that agmatine also serves the dual regulatory role of suppressing polyamine biosynthesis (suppresses ODC activity) and polyamine uptake (affecting polyamine transporters) through the induction of antizyme (Satriano et al., 2002). Also, this pathway is not considered a significant source of Put in animals. The presence of two alternative pathways (ODC and ADC) for Put production in many plant tissues complicates the situation regarding their metabolic regulation, particularly, since the substrates of the two pathways (Orn and Arg) are also interconvertible (Fig. 2).

The presence of more than one pool of a compound in a cell can be achieved by two mechanisms: (A) the metabolite has a single origin and is compartmentalized after its synthesis, or (B) the two pools have different origin, are specifically connected to distinct pathways, and are not in equilibrium. The two mechanisms however are not mutually exclusive and can operate simultaneously and intermediate situations are also possible (Lips and Beevers, 1966; Knight et al., 1996). Lips and Beevers (1966) found that two distinct pools of malate were produced from acetate-^{3}H and bicarbonate-^{14}C (mechanism B). There can be preferential utilization of one pool over another depending upon relevant enzymes and other catabolic machinery. Putrescine can be synthesized from Orn and Arg and a situation similar to malate might exist for it as well.

Both ODC and ADC enzymes require pyridoxal phosphate (PLP) and a sulfhydryl reagent, such as, dithiothreitol for *in vitro* activity. Ornithine decarboxylase is the first and often the rate- limiting enzyme in polyamine biosynthesis in animals. It is a dimer and is conserved among eukaryotes (Coffino, 1989; Michael et al., 1996). Ornithine decarboxylase is chiefly regulated by feed back control (controlling both synthesis and degradation) of the enzyme in animals. Exposing cells to high concentrations of intracellular polyamines causes a rapid degradation of ODC. A regulatory protein called antizyme, induced by polyamines, targets the ODC for degradation (Coffino, 2001). It inactivates the enzyme by forming an ODC-antizyme complex (Kanamoto et al., 1986; Coffino, 2001) and facilitates its degradation by 26S proteasome and inhibits further polyamine uptake (Hayashi et al., 1996). Two plant antizymes and the existence of an inactive ODC-antizyme complex has been reported in barley (Koromilas and Kyriakidias, 1988); however, this report has not been confirmed. It appears that the activities of ODC and ADC are regulated in a developmental and tissue specific manner (Minocha et al., 1995, Walden et al., 1997), and biochemical mechanisms of their turnover is not known.

Like ODC, the regulation of ADC is also dependent upon the physiological condition, developmental stage and type of tissue. Nam et al. (1997) cloned the *adc* cDNA from soybean hypocotyls and studied the regulatory mechanisms of the enzyme activity. They found that the change in the content of *adc* mRNA played an important role in the regulation of enzyme activity during early development, in different tissues and under acid stress. It has been suggested that ADC synthesis is post translationally regulated by Spm during osmotic stress (Borrell et al., 1996). Spermine inhibits the processing of the ADC proenzyme keeping it in the inactive state and avoiding over activity of the enzyme. Arginine decarboxylase activity in the leaves of *Pharbitis nil* was induced by light either by inducing the synthesis of ADC protein *de novo* or by activating it, and this was reflected by an increase in the Put levels (Yoshida and Hirasawa, 1998).

The triamine, Spd is synthesized from Put by the enzyme Spd synthase (E.C. 2.5.1.16); the reaction involves the transfer of an aminopropyl moiety from decarboxylated S-adenosylmethionine (dcSAM) to Put, and Spm is synthesized using the enzyme Spm synthase (E.C. 2.2.1.22), the reaction involves the transfer of an additional aminopropyl moiety from decarboxylated S-adenosylmethionine (dcSAM) to Spd (Fig. 2). S-adenosylmethionine decarboxylase (SAMDC; E.C. 4.1.1.50) converts S-adenosylmethionine (SAM) into dcSAM. S-adenosylmethionine is formed from L-methionine and ATP by SAM synthetase. Spermidine and Spm are both predominant in mammals as end products of the polyamine pathway and the Put pool is often low (Kashiwagi et al., 1986). The acetylpolyamine catabolic pathway (discussed later) allows cells to dispose of excess Spd and Spm.

The activities of the Put biosynthetic enzymes, ADC and ODC as well as SAMDC were shown to increase three fold upon exposure to methyl jasmonate in tobacco control explants (Biondi et al., 2001). A dose dependent increase in the mRNA levels of the three enzymes was seen. An overall upregulation of the polyamine metabolism was observed. Activity of the catabolic enzyme, diamine oxidase (DAO; Fig. 2., step 21) was also elevated mainly in the cell wall fraction.

In animals and fungi, ODC and SAMDC have been postulated to regulate polyamine biosynthesis (Tiburcio et al., 1997). In contrast to ODC, which is feedback regulated, the synthesis of SAMDC in plants is regulated by the amount of *samdc* mRNA available. Spermidine and Spm but not Put exert a negative control on SAMDC (Tiburcio et al., 1997). In other words, when Spd and Spm are present, SAMDC is rapidly degraded. Conversely, SAMDC is induced when these two polyamines are reduced. Also, Put is an obligatory activator of SAMDC in mammals and fungi and it has a direct stimulatory effect on the enzyme activity (Pegg and Williams Ashman, 1968). However, in bacteria it is Mg^{++} rather than Put that is the activator of SAMDC. In plants, SAMDC is not stimulated by Put and the accumulation of cellular polyamines blocks synthesis of the enzyme (Hiatt et al., 1986).

All the three key enzymes (ADC, ODC and SAMDC) have a short half-life indicating that they could be important metabolic control points in the cell. In most animals, except some parasites, the presence of a PEST (Proline, Serine, Threonine and Glutamic acid) sequence at the carboxy terminal part is responsible for the short half- life of ODC (Ghoda et al., 1989; 1992). The cloning of Datura *odc* cDNA (Michael et al., 1996) and *Nicotiana glutinosa odc* cDNA (Lee and Cho, 2001) in plants has revealed that the plant gene does not possess the PEST extension, unlike the animal gene responsible for the rapid turnover, thus, imparting stability to this enzyme. Two functional Spd synthase genes, psSPDSYN1 and psSPDSYN2 have been isolated from pea and found to be regulated differentially. psSPDSYN1 is upregulated early after fruit set whereas psSPDSYN2 is expressed later (Alabadi and Carbonell, 1999).

Polyamines and Ethylene

The ethylene and the polyamine biosynthetic pathways are linked through SAM (Fig. 2.). For ethylene production, SAM is converted into 1-aminocylcopropane-1-carboxylic acid (ACC) by ACC synthase, which is then converted to ethylene by ACC oxidase (Miyazaki and Yang, 1987). S-adenosylmethionine donates methyl groups for various transmethylation reactions (Kushad and Dumbroff, 1991). In addition, the methyl moiety can be transferred to Put by Put-N-methyltransferase, to form N-methyl-Put that serves as a precursor of nicotine and tropane and pyridine-type alkaloids (Hibi et al., 1994; Moyano et al., 2002).

There are several reports that illustrate the competing relationship of the polyamine and ethylene pathways. Polyamines are associated with growth promoting processes whereas ethylene is associated with senescence. Locke et al. (2000) found that exogenous polyamines promoted barley seedling growth in a fashion similar to ethylene biosynthetic inhibitors. Also, blocking endogenous ethylene production with aminoethoxyvinylglycine (AVG) enhanced the free Put and Spd content of the germinating barley grains. It has been reported that ethylene can also inhibit polyamine biosynthesis (Galston and Kaur- Sawhney, 1987) and vice versa. Lee et al. (1997)

found that Spd could inhibit ethylene biosynthesis and other degradative processes such as membrane leakiness, rise in RNAse and protease activities and chlorophyll loss.

In contrast to the above studies, reports are also available in the literature that suggest that the biosynthetic routes of ethylene and polyamines may not compete with each other. Cut carnation flowers that were treated with 50 mM aminotriazole showed no effect on the levels of Put and Spd but retarded the ethylene climacteric peak as well as senescence (Serrano et al., 1999). Kushad et al. (1988) reported that in water stressed apple leaves and avocado fruit, ACC and ethylene levels rose but SAM and polyamine pools remained unchanged. Cohen and Kende (1986) found that in growing rice internodes, ethylene stimulated the SAMDC and ADC enzyme activities preventing a decline in polyamine pool.

Polyamines and Inhibitors

The role of specific inhibitors is important in understanding the function of polyamines. DL-α-Difluoromethylornithine (DFMO) and DL-α-Difluoromethylarginine (DFMA) are suicide inhibitors of ODC and ADC, respectively. Both the inhibitors combine irreversibly with the active site of the respective enzyme and inhibit enzyme activity. Methylglyoxal (bis-guanylhydrazone) (MGBG) is a powerful, competitive inhibitor of SAMDC; cyclohexylamine (CHA) and trans-4-methylcyclohexylamine (MCHA) inhibits Spd synthase and APCHA (N-{3-aminopropyl}cyclohexylamine) inhibits Spm synthase. Cereal leaves pretreated with DFMA had reduced Put but significantly higher Spd and Spm (Tiburcio et al., 1986) indicating that ADC pathway is the primary source of Put in this case. Khan and Minocha (1989) investigated the role of ADC in phytopathogenic fungi using these inhibitors. It is generally believed that the ADC pathway is operational under stress conditions while ODC under developmental conditions (Minocha and Minocha, 1995). In the case of carrot, DFMA blocked somatic embryogenesis whule DFMO did not affect development, Put negated the inhibitory effect of DFMA (Feirer et al., 1984). To specifically demonstrate the function of the two enzymes by using DFMA or

DFMO is also confusing in intact plants because of the conversion of DFMA to DFMO by arginase. Plants rich in arginase, such as tobacco, are able to hydrolyze DFMA or DFMO (Slocum et al., 1988). In salinized tomato leaf explants, the decrease in Put and Spd was found to be correlated with the increase in Pro accumulation. It has also been found that DFMA that blocks Put biosynthesis under stress and normal conditions also behaved, as an inhibitor of Pro accumulation while DFMO, which reduced the levels of Spd, Spm and diaminopropane (DAP) had no effect on Pro content under salt stress. Putrescine supplied at low concentrations stimulated the accumulation of Pro. When these salinized tomato plants were treated with aminoguanidine (Ag), a specific inhibitor of the catabolic DAO (Fig. 2., enzyme 21), Pro accumulation was strongly inhibited. These results suggest that in stressed tissues, Put and Pro are connected by a precursor-product relationship involving DAO and γ- aminobutyric acid (GABA) metabolism (Aziz et al., 1998). α-Difluoromethylornithine also inhibited root growth and arbuscular mycorrhizal infection of *Pisum sativum*. When exogenous Put was supplied with DFMO, the effect was reversed indicating a role of ODC in the growth and fungal infection (Ghachtouli et al., 1996).

Methylglyoxal (bis-guanylhydrazone), a known inhibitor of SAMDC, is also known to stabilize SAMDC enzyme *in vivo* against proteolytic degradation (Hiatt et al., 1986). So, the reversible binding of this inhibitor, thus conferring stability, could explain unexpected rise in SAMDC activity in some cases when MGBG was present. High concentrations of MGBG are known to inhibit embryogenesis and also block growth in *Picea abies* but low concentrations are ineffective in doing so probably because of the presence of high Spd in the embryogenic callus (Santanen and Simola, 1992). In tobacco cells, MGBG inhibited the formation of Spd from methionine via SAM but enhanced the formation of Spd from aspartic acid (Lee and Park, 1991). Spermidine increased significantly in the chilling tolerant cucumber cultivars. MGBG prevented this rise in Spd and thus enhanced NADPH oxidase activity resulting in chilling injury (Shen et al., 2000). This also confirmed that the regulation of Spd synthesis is at the level of SAMDC. The

insect polyamine biosynthesis has been targeted using inhibitors to explore the possibility of insect pest control. Rajam (1991) used DFMO, MGBG and CHA to study the insecticidal activities of these inhibitors. All inhibitors had insecticidal activities and found that MGBG was the most effective.

Polyamine Catabolism

In plants, polyamine catabolism is not simply a degradative process but also a link between amino acid and carbon metabolism. The amine oxidases (AO) are a group of enzymes that catalyze the oxidation of amines, in particular diamines and polyamines, to the corresponding aldehydes. Two main types of AO are known: the copper-containing copper-amine oxidases (Cu-AOs, E.C.1.4.3.6) and the flavin-containing FAD-amine oxidases (FAD-AOs, E.C. 1.5.3.3) (Medda et al., 1995). The AOs can also be grouped into those that act on the primary amino groups of di- and polyamines (DAO; E.C.1.4.3.6) and those that act on secondary amino groups of polyamines (polyamine oxidases, PAO; E.C. 1.5.3.3). Diamine oxidases are dimers formed from identical subunits. Diamine oxidase has been purified and characterized from rice (Chaudhri and Ghosh, 1984), barley (Cogoni et al., 1989), maize (Suzuki and Hagiwara, 1993) and wheat (Suzuki, 1996). Four isoenzymes of DAO have been isolated from pea (McGovan and Muir, 1971). They are subject to inhibition by a variety of chelating agents, carbonyl reagents, and hydrazine (Bieganski et al., 1982; Smith, 1985). Metal chelators all inhibit Cu-AOs non-competitively. Genes encoding DAO have been cloned from *Lens culinaris* (Rossi et al., 1992), *Pisum sativum* (Tipping and McPherson, 1995) and *Brassica juncea* (Jiao and Pua, 2001). Generally, DAOs are known to be localized in the cell wall (Slocum and Furey, 1991), although Federico et al. (1985) suggest a cytosolic location for DAO in lentils. The lentil AO activity is greatest in growing parts of the plant but decreases with maturity and senescence (Medda et al., 1995). The best substrates for DAO are Put and cadaverine and their pH optimum has been found to be around 7.0 (Smith, 1985). These two substrates have been found to compete with each other

for DAO (Pec and Frebort, 1991). Lentil DAO also oxidized L-Orn and L-lysine, and to some extent, β-alanine and GABA at high concentrations of the enzyme (Medda et al., 1996), although both L-Orn and L-Arg were found to be poor substrates for lentil Cu-AO. L-ornithine was oxidized to Glu-5-semialdehyde and NH_3 in a manner similar to oxidation of usual substrates and Glu-5-semialdehyde spontaneously cyclized to Δ^1-pyrroline-5-carboxylic acid (P5C). However, L-Arg is oxidized by an unusual mechanism yielding Glu-5-semialdehyde, NH_3 and urea as reaction products (Medda et al., 2000). Diamine oxidase is an unspecific enzyme in that it has a broad pH curve and its activity with Spd is about 40% as compared to Put at pH 7.5 (Cogoni et al., 1990). Polyamine oxidases are symplastic in barley primary leaves unlike oat and maize where they are localized in the cell wall (Li and McClure, 1989). The enzyme is an acidic protein, and is inhibited by acridine dyes and hydrazines (Radova et al., 2001).

Polyamine catabolism in plants not only regulates the cellular levels of polyamines but it is also associated with lignification, cell wall stiffening and cellular defense. The activity of DAO is regulated by plant hormones and environmental stress factors (Federico et al., 1985; Maccarone et al., 1991). It has been suggested that DAO and PAO may be involved in auxin oxidation (Park and Park, 1987) and the regulation of transport of amines between the cells. Rabiti et al. (1989) have proposed a possible role for DAO in the regulation of polyamine transport in addition to its catabolism. The catabolic products of polyamine oxidation also help protect plants from pathogen infections. The degradation activity of polyamines generally parallels biosynthetic activity. Catabolic activity was absent in resting *Lathyrus* seeds but increased when germination began and this coincided with activated polyamine biosynthesis (Suresh and Adiga 1977; Torrigiani et al., 1988). In synchronized cultures of *Helianthus tuberosus*, Put was high in the G1 and S stages of the cell cycle and so was the DAO activity (Torrigiani et al., 1989). Diamine oxidase and PAO activities were monitored in callus (both embryogenic and non embryogenic) cultures of *Picea abies* by Santanen (2000); the oxidation activities correlated well with changes in Put levels in the

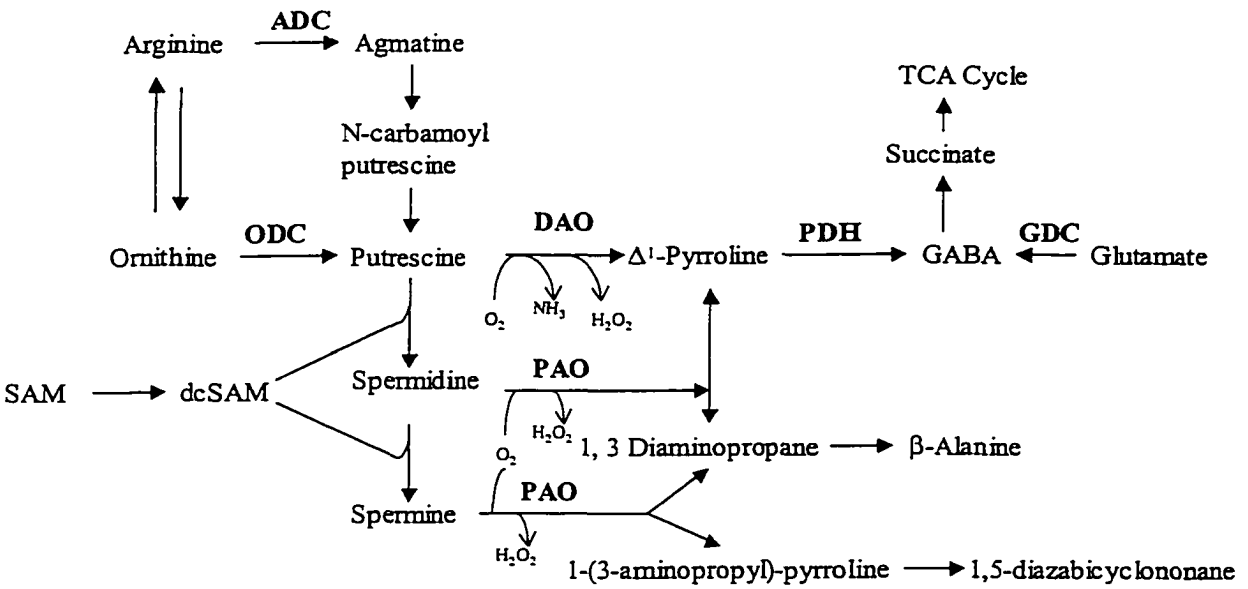


Fig. 3. Pathway of polyamine biosynthesis and catabolism in plants. ADC, Arg decarboxylase; DAO, diamine oxidase; GAD, Glu decarboxylase; ODC, Orn decarboxylase; PAO, polyamine oxidase; PDH, Δ^1-pyrroline dehydrogenase; SAMDC, S-adenosylmethionine decarboxylase.

callus cultures. A positive correlation between DAO activity and the content of cadaverine in hypocotyls of soybean seedling was seen by Scoccianti et al. (1990); however, there was no correlation in the root where cadaverine dropped sharply and the DAO activity remained unaltered. As a result of DAO action, Put is converted into Δ^1-pyrroline along with the release of hydrogen peroxide and NH_3 (Fig. 3). Pyrroline dehydrogenase (PDH) then converts Δ^1-pyrroline to γ-aminobutyric acid (GABA); NAD is required for the activity of PDH. GABA is transaminated and oxidized to succinic acid entering Krebs cycle. This catabolic pathway in the cells recycles carbon and N from Put. Synthesis of GABA is seen under a variety of stress conditions (Shelp et al., 1999). Putrescine catabolism is not only a means of eliminating cellular Put but also generating products important in other physiological processes (Hausman et al., 1997; Martin-Tanguy, 1997).

In microorganisms, two major catabolic pathways for Spd and Spm have been described, one via Δ^1-pyrroline and the other via N-acetyl derivatives. The two routes converge at γ-aminobutyrate (Large, 1992). In yeasts, a catabolic route for Spd and Put has been described via N-acetyl derivatives. In rat liver peroxisomes and fungal cells, it was found that Spd is converted by DAO to Put, 3-aminopropanal and H_2O_2 (Bagni and Pistocchi, 1992). In barley leaves, labeled Spd produced Put, DAP and aminopropylpyrroline (Smith, 1970). Diaminopropane is also a metabolic precursor for uncommon polyamines, such as, caldine, thermine and caldopentamine (Kuehn et al., 1990). It is also found to be important in retarding senescence by inhibiting the rise of protease activity and the loss of chlorophyll (Tiburcio et al., 1993; Bouchereau et al., 1999).

Flavin containing PAOs occur throughout the Gramineae family (Šebela, 2001). The enzyme was first isolated from *Hordeum vulgare*. All the enzymes are monomeric and some of them are known to be glycoproteins. Antibodies that recognize PAO in maize have been generated and these could help in understanding their regulation (Angelini et al., 1995). The PAOs oxidize Spd and Spm at their secondary amino groups (Federico and Angelini, 1991).

Polyamine oxidases from cereals are inhibited by acridine compounds, such as quinacrine, basically because of the presence of the flavin cofactor (Hirasawa et al., 1986). Degradation of Spd by PAO yields Δ^1-pyrroline and that of Spm produces 1,3 aminopropylpyrroline and 1,5 diazabicyclononane, along with DAP and hydrogen peroxide (Morgan, 1985; Bouchereau et al., 1999) (Fig. 3). Diaminopropane is eventually converted into β-alanine. Polyamine oxidases have a more narrow substrate specificity than DAOs that will oxidize a large number of substrates. Barley PAO has a pH optimum of 7.0-8.0 for Spd while that for Spm is 4.8 (Santanen, 2000). Maize and oat PAO have a pH optimum of 5.5-6.5 for both the substrates (Smith, 1985). Maize PAO is partially oxidized under fully aerobic conditions and the oxygen concentration could be a rate -limiting factor *in vivo* (Bellelli et al., 1997). Diaminopropane has been known to increase under stress conditions (Smith, 1985) reflecting salt stress induced polyamine oxidation. Diamine oxidase and PAO activities are stimulated in these conditions. The somatic embryos of *Picea abies* had higher Spd content than nonembryogenic callus but no rise in SAMDC activity was seen in the former. This suggested the possible role of a catabolic enzyme in regulating Spd levels (Santanen and Simola, 1992). Also, most of the Spd was bound to the cell wall. This correlated with the high activity of PAO.

Polyamine oxidases may be correlated with peroxidase and may have a role in the production of reactive oxygen intermediates in the apoplast (Angelini et al., 1995). They have also been known to play an important role in polyamine homeostasis and cell death (Federico and Angelini, 1991).

The pathway that converts glutamate to succinate via GABA is called the GABA shunt (Shelp et al., 1999). The three enzymes involved are: (1) glutamate decarboxylase (GAD), a cytosolic enzyme, that decarboxylates glutamate, (2) GABA transaminase (GABA-T) that catalyses the reversible conversion of GABA to succinic semialdehyde, and (3) succinic semialdehyde dehydrogenase (SSADH), that irreversibly oxidizes succinic semialdehyde to succinate. The latter two are mitochondrial enzymes. Recent studies on GABA metabolism have

focused largely on the GABA production via GAD (Shelp et al., 1999) with little attention paid to the synthesis of GABA from Put. Scott-Taggart et al., (1999) evaluated the hypothesis that GABA synthesis *in situ* is regulated by glutamate availability. Synthesis of GABA increases rapidly in response to hypoxia, acidosis, mechanical stress, cold stress or under long term conditions such as those that limit Gln synthesis, reduce protein synthesis or enhance protein degradation. It has been suggested that one conformation of the GABA molecule can mimic ACC and therefore, might share common binding sites on interacting proteins involved in ethylene biosynthesis (Kathiresan et al., 1997). These authors also investigated the possibility that an increase in GABA might increase ethylene production and it would do so either by acting as a direct precursor or indirectly promoting ethylene production. However, it was observed that GABA did not act as a direct precursor since [^{14}C] GABA did not result in [^{14}C] ethylene. Also, it was found that endogenous ACC levels were several fold higher in GABA treated tissues. This suggested higher ACC synthase activity in GABA treated tissues.

The interconversion of Spm into Spd and Spd into Put is commonly observed in animal cells. It does not take place by a reversal of the aminopropyltransferase reactions involved in their biosynthesis but by acetylation and oxidation steps (Fig. 4) (deAgazio et al., 1996). The polyamines are acetylated by the activity of Spd/Spm 1-N-acetyltransferase (SSATs) and the acetylated polyamines are either excreted out of the cell or rapidly degraded by PAO, yielding Spd or Put and acetylaminopropanal. These enzymes are strictly regulated *in vivo* at the level of gene expression and post transcriptionally by polyamines (Veress et al., 2000). In general, the SSAT activity is much lower than PAO activity and the cellular concentration of acetylated derivatives is often very low. Acetylated polyamines have not generally been found in plant tissues but a polyamine acetylating activity has been reported in the chloroplasts of *Helianthus tuberosus* (Del Duca et al., 1995). Also, Spd to Put interconversion has been suggested in tobacco thin layer explants (Torrigiani et al., 1993), in chloroplast of *Helianthus tuberosus* (Del Duca et

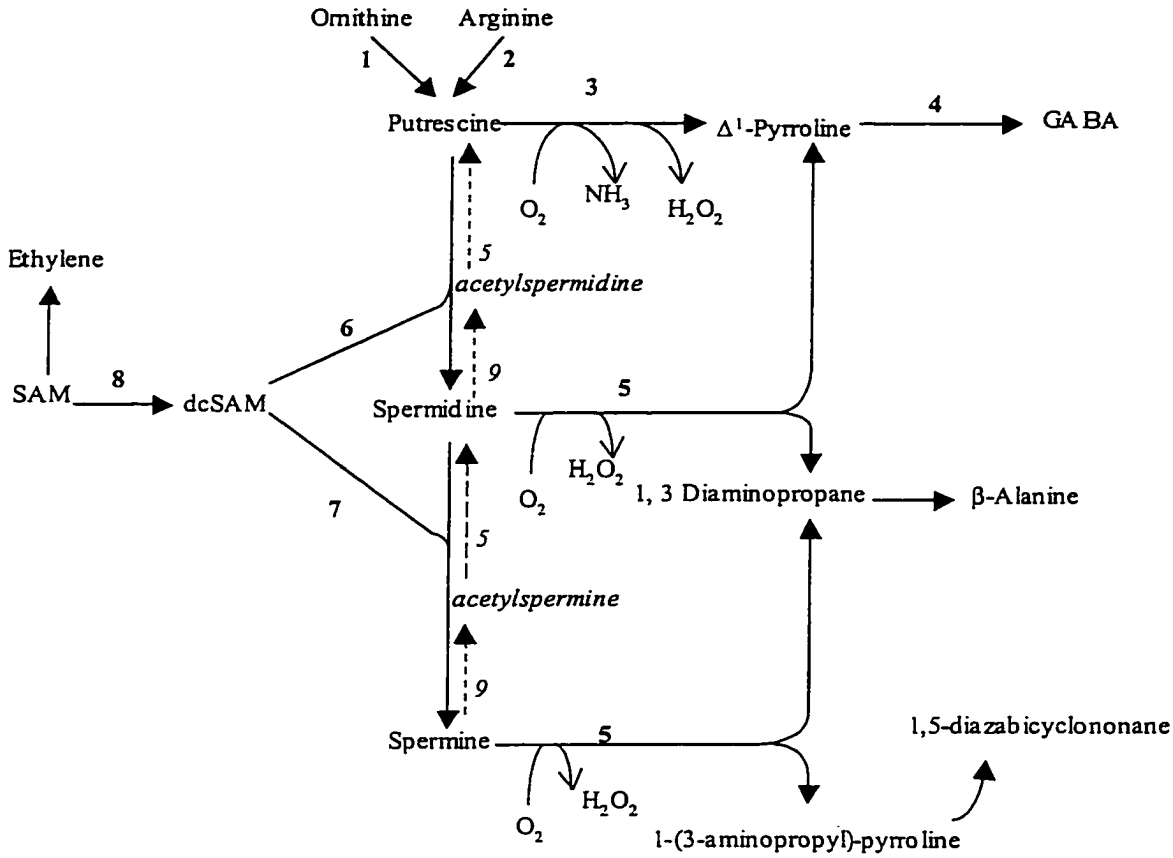


Fig. 4. Pathway of Polyamine biosynthesis and catabolism in plants and animals. (1) ODC, Orn decarboxylase; (2) ADC, Arg decarboxylase; (3) DAO, diamine oxidase; (4) PDH, Δ^1-pyrroline dehydrogenase; (5) PAO, polyamine oxidase; (6) Spd synthase; (7) Spm synthase; (8) SAMDC, S-adenosylmethionine decarboxylase; (9) Spd/Spm N^1- acetyltransferases. Dotted arrows represent catabolic pathways in animals (modified from deAgazio et al., 1996).

al., 1995), soybean (Caffaro et al., 1993), maize seedlings (de Agazio et al., 1996) and in *Arabidopsis thaliana* (Tassoni et al., 2000). Three genes have been isolated from maize and genomic Southern analysis indicates the existence of a gene family for PAO in other monocots. The production of the enzyme is most probably controlled at the transcriptional level (Cervelli et al., 2000). Scaramagli et al. (2000) found that cultured potato cells that had been gradually acclimated to low water potential (by increasing PEG concentrations) had higher DAO activity as well as ADC and ODC activities as compared to the control and water stressed shocked potato cells. The absence of DAO or PAO and PDH that catalyze Put oxidation had been reported in the PUT cells of the XD line of cultured tobacco cells (Balint et al., 1987). These cells have the ability to utilize Put as the sole source of N. Putrescine catabolism in these cells was chiefly accomplished by using hydroxycinnamoylputrescines, caffeoyl GABA and GABA as obligatory intermediates (Balint et al., 1987). The formation of caffeoyl GABA in PUT cells was absent in XD line.

Nitrogen Metabolism in Plants

Nitrogen is taken up from the soil largely as nitrate (NO_3^-) and is reduced to nitrite via nitrate reductase (NR) (E.C. 1.6.6.1) and then to ammonium (NH_4^+) by nitrite reductase (NiR) (Fig. 2; step 1-3). All of the enzymes that regulate the entry of N into the metabolic pathway have been well characterized and their genes have been cloned from a variety of organisms (Forde and Clarkson, 1999). The activity of NR regulates the amount of NO_3^- available for amino acid biosynthesis. Nitrate reductase is a cytosolic enzyme and is induced by exogenous NO_3^- and polyamines inhibit this response (Vance, 1997). However, polyamines do not inhibit the activity of the enzyme or the uptake of NO_3^-. The enzyme is also induced by light and upon illumination, partial dephosphorylation of the enzyme occurs. This is the active state of the enzyme. Sucrose can replace light in the NR mRNA induction in *Arabidposis*. Glutamate, Gln and other amino acids have a negative effect on NR expression and activity (Lillo, 1994). Treatments that inhibit

amino acid biosynthesis cause an increase in NR mRNA. Plants treated with inhibitors of Gln synthetase (GS) and those expressing a NiR antisense cDNA have higher amounts of NR mRNA than control plants. Nitrite reductase is a plastid localized enzyme and is similar to NR in its regulation. It requires NO_3^- and light for induction. The transit peptides of NiR are among the shortest known in plants. Reduced N also exerts a negative feedback control on the synthesis of NiR mRNA and protein (Vance, 1997).

Assimilation of NH_4^+ involves linking of N metabolism with carbon metabolism through both cytosolic and organellar enzymes. The initial step in NH_4^+ assimilation is catalyzed by GS (E.C. 6.3.1.2). The activity of GS increases significantly during legume root nodule development, in etiolated leaves exposed to light, and in leaves and roots of plants grown in NH_4^+ or NO_3^-. There exist two forms of GS, a cytosolic (GS1) and chloroplastic (GS2) that assimilate NH_3 produced by different physiological processes in different plant organs. The two forms are immunologically distinct. Antibodies to cytosolic GS will recognize cytosolic GSs from different species but not the plastid forms. Glutamine synthetase is controlled at both the transcriptional and post-transcriptional levels (Cren and Hirel, 1999; Forlani, 2000). Freeman et al. (1990) demonstrated the importance of GS2 in photorespiration by molecular analysis of the photorespiratory mutants. These barley mutants lacked GS activity and analysis of GS mRNA and protein expression showed three classes of mutants. Class I showed no GS mRNA and protein, class II mutants had normal mRNA but no protein, and class III had normal protein but no GS activity. Class I mutants could arise from transcriptional control while the latter two could be the result of translational and post translational regulation. Light and NO_3^- play an important regulatory role in controlling the production of GS2 in leaves (Oliveira et al., 2001; Avila et al., 2001). Neither dicot nor monocot GS1 genes respond to light. In *Arabidopsis* leaves, GS2 expression depends upon light in a phytochrome-and sugar-dependent manner. The characterization of GS2 mutants has shown that this isoenzyme is essential in the reassimilation of the large quantities of NH_3 released from the photorespiratory N cycle. The expression of GS1

is induced during senescence and is probably involved in the remobilization of N compounds. Also, the inability of GS1 to replace GS2 function indicates that it has a distinct role to play in green leaf tissues (Lancien et al., 2000). The role of NH_4^+ and NO_3^- in GS expression is variable. In soybean and rice, the transcription and translation of some GS1 genes is induced by these N sources (Marsolier et al., 1993) but in other species such as *Phaseolus* and *Medicago*, initial gene expression requires neither N source. The mechanism of GS turnover in plants is the same as in bacteria (Ortega et al., 1999; Suganuma et al., 1999).

Glutamate synthase (GOGAT, E.C. 1.4.7.1) catalyzes the reductive transfer of amido group of Gln to α-ketoglutarate resulting in the formation of two molecules of Glu (Vance, 1997). Glutamate now can either be used to replenish the pools of Gln via GS or donate its N to form amino acids, alkaloids, ureides and polyamines. Glutamine pool increases strongly when GOGAT is inhibited by azaserine (AZA) (Alhama et al., 1998). In higher plants, GOGAT exists as Fd-GOGAT and NADH-GOGAT. The former is localized in the chloroplast and is involved in NH_4^+ assimilation derived from photorespiration as well as light dependent NO_3^- reduction. The latter is present in non-green tissues. The expression of GS and Fd-GOGAT genes in conifers appear to be controlled by a developmental program in the initial stages of tree seedling growth. In the later stages, when the reserves have been mobilized, the effect of environmental factors is more pronounced. The observed expression pattern of genes involved in N metabolism is closely associated with the light-independent chloroplast development observed in conifers (Cánovas et al., 1998).

Methionine sulfoximine (MSX), an inhibitor of GS, enhanced the NR activity and metabolic NO_3^- concentration in barley seedlings (Aslam et al., 2001). Kumar et al. (1990) studied the effect of MSX on GS activity and NH_3 accumulation in barley, sorghum and *Moricandia arvensis*. They also studied the changes in pool sizes of amino acids involved in photorespiratory N metabolism treated with MSX. Glutamine synthetase and GOGAT function to maintain a cyclic flow of N from NH_4^+ into Gln and Glu. The GS-GOGAT cycle also plays an

important role in anaerobic amino acid accumulation (Reggiani et al., 2000). Alanine and GABA were the main amino acids accumulated in rice roots under anaerobic conditions and their synthesis was strongly inhibited by MSX and AZA, inhibitors of GS and GOGAT, respectively. Inhibitors have been used to study high affinity transport of NH_4^+ and NO_3^-. Vidmar et al. (2000) treated barley roots with NO_3^- in the presence and absence of amino acids to investigate the regulation of HvNRT2, a gene encoding high affinity NO_3^- transporter. All amino acids caused a decrease in the mRNA levels as well as NO_3^- influx but since the amino acids are interconvertible, specific inhibitors were used to clarify the role of specific amino acids. Methionine sulfoximine, AZA and tungstate (inhibitor of NR) were used. Both MSX and AZA decreased the transcript levels of HvNRT2 as well as NO_3^- influx. Azaserine caused an increase in Gln levels suggesting that Gln is responsible for the downregulation of HvNRT2. Rawat et al. (1999) studied NH_4^+ uptake and expression of AtAMT1 gene (encoding a putative high-affinity NH_4^+ transporter) in *Arabidopsis*. Using MSX they found that end products of NH_4^+ assimilation were responsible for AtAMT1 gene expression. Gene expression and NH_4^+ influx decreased when Gln was supplied alone or NH_4NO_3 was used with MSX.

Most plant species are able to absorb and assimilate NO_3^-, NH_4^+, urea and amino acids but the response to a particular form of N varies from species to species, e.g. optimal growth of tomato roots is when NO_3^-: NH_4^+ ratio is 3:1, whereas white spruce prefers NH_4^+; some arctic sedges prefer amino acids (Crawford and Glass, 1998). Organic acids can facilitate the growth of plant cell cultures on NH_3 as a sole source of N. Behrend and Mateles (1976) studied the role of succinate in tobacco cell cultures. They suggest that the role of organic acids in facilitating growth is neither connected to NH_4^+ transport nor to relieving the cells of NH_3 toxicity but may be due to the need for additional carbon skeletons for amino acid synthesis. Organic N favors the growth of coniferous somatic embryos in cultures (Naylor, 1984; Santanen, 2000). Ammonium uptake in the cells follows a biphasic pattern, like the polyamines. In the low range, influx occurs by a saturable, high affinity transport system (HATS) that is dependent upon metabolic energy

and in the high range via a non-saturable low affinity transport system (LATS). Influx of NH_4^+ by the HATS was subject to negative feedback regulation. This may be due to the unmetabolized NH_4^+ in rice roots or reduced N (Wang et al., 1993). In the case of NO_3^- influx, vacuolar accumulation of NO_3^- may participate in flux regulation.

Polyamine content can be manipulated by modifying N sources (Altman and Lewin, 1993). Exogenous NH_4^+ increases the synthesis of free polyamines, particularly Put. This statement can be substantiated by the accumulation of free and total Put as well as Spd in the roots of peas supplied with NH_4^+ and plants with root nodules (Tonin et al., 1991). When Put was given to soybean cultures in the presence of NH_4^+ and NO_3^-, it was rapidly converted into GABA, succinate and malate and very little was converted into Spd. Polyamines play an important role in the sequestration of N in whole plants and data on the accumulation of polyamines in forest trees in response to nitrogen supplementation in soil supports this (Minocha et al., 2000).

Most higher plants develop severe toxicity symptoms when they are grown on NH_4^+ as the sole N source (Britto et al., 2001). Excessive NH_3 concentrations in the cells are toxic and this N may be channeled into amides such as asparagine. Rabe and Lovatt (1986) have suggested that all mineral nutrient deficiencies with the exception of molybdenum, required for NR, produce a significant accumulation of NH_3 and its removal occurs via Arg biosynthesis. Their observation was based on phosphate deficient citrus or squash plants that showed a ten fold increase in Arg biosynthesis as a result of NH_3 accumulation. The effect of NH_4^+, NO_3^-, and Put on ADC and arginase specific activity, free polyamine levels, endogenous Arg and Orn and ADC mRNA were studied in grapevine suspension cultures (Primikirios and Roubelakis-Angelakis, 1999). There was no correlation between ADC activity and ADC transcript levels. When NH_4^+ was fed to NH_4^+ free cultures, there was a four-fold increase in ADC activity leading to a concomitant increase in Put. Also, there was a decrease in Arg and arginase while Orn increased. On the other hand, NO_3^- addition did not lead to any marked changes in either of the parameters considered in the study. The addition of Put caused a four-fold decrease in ADC activity and increased Arg, Orn and

arginase activity (Primikirios and Roubelakis-Angelakis, 1999). Nitrogen metabolism was monitored in *Nicotiana plumbaginifolia* cell suspension cultures (Mesnard et al., 2000) by following the incorporation of ^{15}N label from $(NH_4)_2SO_4$ and KNO_3 into amino acids and polyamines using nuclear magnetic resonance. N-acetylated compounds composed of the intermediates in Arg biosynthesis, N-acetylGlu and N-acetylOrn and, possibly, the intermediate of Put degradation into GABA, N-acetylPut could be resolved. The occurrence of ^{15}N-label in agmatine and the low detection of labeled Put indicated that crucial intermediates of the pathway from Glu to polyamines and/or the tobacco alkaloids could be monitored.

Ornithine and Arginine Biosynthesis

Amino acid biosynthesis is an important link between N and carbon metabolism in photosynthetic organisms, since the primary products of both pathways are necessary for producing amino acids. Ornithine and Arg belong to the Glu family of amino acids. Other members of Glu family of amino acids are Pro and Gln. Glutamine synthesized from NH_4^+ is rapidly converted into Orn via a series of acetyl derivatives. The acetylation ensures the production of Orn and not Pro from Glu. This is then phosphorylated to N-acetyl-γ-glutamylphosphate and N-acetylOrn in several steps. The kinase leading to a series of reactions to produce Orn is inhibited by Arg. Orn transcarbamylase carries out the conversion of Orn and carbamyl phosphate into citrulline, which is converted into Arg via arginosuccinate synthetase and arginosuccinate lyase or arginine synthase (Fig. 2, step 11). Both the enzymes are cytoplasmic; and it was reported in soybean cell suspension cultures that high intracellular concentrations of Arg inhibit the former while enhance the latter (Cohen, 1998).

The conversion of Glu to Pro involves the activation and reduction of the γ-carboxyl group to form glutamic-γ-semialdehyde which spontaneously cyclizes to yield Δ^1-pyrroline-5 –

carboxylate (P5C). The action of P5C reductase (P5CR) yields Pro. The catabolic enzyme, Pro oxidase converts Pro back to Glu by the same route.

Ornithine can also be formed from Arg and Pro in addition to being formed from Glu. The induction of arginase by Arg in Arg degradation has been indicated in higher plants and is known in lower organisms such as fungi and lichens. Analogues of Arg could not replace it as a substrate for the enzyme and both agmatine and 5'-deoxymethylthioadenosine served as inhibitors (Hwang et al., 2001). Specific activity of the enzyme in ginseng roots was increased by GA, IAA, kinetin and Put and increases in arginase activity by these plant hormones could affect polyamine metabolism intracellularly (Hwang et al., 2001). In higher plants, products of Arg degradation, urea and Orn, are used in assimilation of N into amino acids and polyamines, respectively. Arg can feedback inhibit Orn biosynthesis. Also, Arg degradation by arginase occurs largely in mitochondria whereas Arg synthesis occurs in cytoplasm (Shargool et al., 1988). In animals also, Orn is formed by the action of arginase with the release of urea (Fig. 2).

Ornithine decarboxylase and ADC are cytoplasmic enzymes but can be distributed in the organelles such as, nuclei, chloroplasts and mitochondria (Torrigiani et al., 1986). Thus Orn as a substrate for Arg production and Orn as a catabolic product of Arg degradation would be separated by the mitochondrial membrane. Yu and Cho (1998) supplied exogenous Arg to germinating soybean seeds, which, resulted in an increase in arginase activity followed by a significant increase in the free amino acids. But, the stimulated arginase induction by exogenous Arg did not enhance polyamine accumulation. Arginase mRNA levels and activity were measured in soybean embryos. The activity was barely detectable in the embryonic stage but increased during germination stages as Arg supplied as a sole source of N did produce urea (Goldraij and Polacco, 1999). Further studies by Goldraij and Polacco (2000) demonstrated that the uptake of Arg into the mitochondria is similar in the embryonic and germination stages, there was no degradation in the former and Arg was used as a N reserve in this stage. The other route for Arg degradation found in many microorganisms is the Arg dihydrolase pathway. The first

enzyme Arg deiminase converts Arg back to citrulline and NH_3. Citrulline is cleaved by the Orn transcarbamylase to yield Orn and carbamyl phosphate.

Proline can also be formed from Orn by the action of Orn aminotransferase (OAT) forming Glu-γ-semialdehyde /P5C. The enzyme P5C reductase (P5CR) forms Pro from P5C while P5C dehydrogenase forms Glu from Pro (Ireland, 1997) (Fig. 2). Roosens et al. (1998) evaluated the importance of Orn in Pro formation. Increases in free Pro have been seen to play a key role for osmotic adjustment in a large number of plant species (Rhodes et al., 1986; Delauney and Verma, 1993). They observed that under salt stress, free Pro, activity of P5C synthetase (P5CS), OAT activity and OAT mRNA all increased in younger plantlets of *Arabidopsis*. This suggests that the Orn pathway in addition to the Glu pathway plays an important role in Pro production under stress conditions in younger plants. Delauney et al. (1993) monitored the levels of mRNA in *Vigna aconitifolia* for Δ^1-P5CS and δ-OAT, the two key enzymes for Pro synthesis under different physiological conditions. Salt stress and N starvation induced P5CS mRNA levels and depressed OAT mRNA levels. Conversely, OAT mRNA level was elevated in plants supplied with excess N while the P5CS mRNA level was reduced. These data suggest that the Glu pathway is the primary route for Pro synthesis in plants during conditions of osmotic stress and N limitation, whereas the Orn pathway assumes prominence under high N input. The enzyme OAT is readily inhibited by gabaculine.

Transgenic Manipulation of Polyamine Metabolism in Plants

Until recently, the most common approach to modulate cellular polyamines in plants has been to use chemical inhibitors. Some limitations of this approach include: the issues related to differential rates of uptake of the inhibitors, their metabolic conversions, the lack of their specificity, and their deleterious effects on membrane properties (Hiatt et al., 1986; Kumar and Minocha, 1998). Additionally, the inhibitors often do not allow an up-regulation of the cellular

polyamines. The transgenic gene expression, on the other hand, provides a means of both up- and down-regulating specific metabolic steps in a pathway (Kinney, 1998; Lindsey, 1998; Nuccio et al., 1999). The latter approach can reveal mechanisms of metabolic regulation that may not be seen simply by mutant analysis or inhibitor studies.

Only a few studies have been reported on the use of mutants (Balint et al., 1987; Hibi et al., 1994; Watson et al., 1998) to understand polyamine metabolism. The first report of a genetically mapped mutation in the polyamine biosynthetic pathway in plants was by Soyka and Heyer (1999) in *Arabidopsis*. The insertion of a transposable element in the ADC2 locus caused a knockout of the ADC2 gene, and it was determined that the induction of the ADC2 gene activates the polyamine pathway under osmotic stress. This was demonstrated by the fact that no induction of the ADC activity was observed by sorbitol in the homozygous mutant. The unstressed mutant had about 44% reduced ADC activity than the wild type but had no obviously different phenotype. Hanzawa et al. (2000) showed that inactivation of the ACAULIS5 (*ACL5*) gene (recessive mutations in the gene) in *Arabidopsis* causes defective stem internode elongation through reduction in cell expansion, and the gene encoding this protein shares sequence similarity with Spd synthase and Spm synthase. Also, expression of the recombinant protein in *E.coli* showed that *ACL5* possesses Spm synthase activity. The mutant phenotype of *acl5* was restored by somatic reversion of a transposon-induced allele, as well as the expression of the *ACL5* cDNA under the control of a heat shock gene promoter in *acl5* mutant restored the phenotype in a heat shock dependent manner.

In recent years, polyamine metabolism has become the target of genetic manipulation both in animals and in plants (reviewed in Kumar and Minocha, 1998). Most of the genes encoding polyamine biosynthetic enzymes have recently been cloned and some have been used for transgenic expression. A summary of current status of these efforts is given in Table I.

Transgenic Expression of Ornithine Decarboxylase (ODC)

Hamill et al. (1990) used a yeast *odc* cDNA in tobacco plants in order to modulate the metabolism of Put and nicotine, an alkaloid derived from Put. The transformed root cultures had an enhanced capacity to accumulate both Put and nicotine. The increase seen in Put was only two fold suggesting that other regulatory mechanisms might limit the potential increase in metabolic flux. The cellular contents of Spd and Spm were not affected. Following that, studies from our lab on the overexpression of a mouse *odc* gene were reported. Successful transformations with both the full length and 3'-truncated mouse *odc* cDNA under the control of 35S CaMV promoter were made in *Nicotiana tabacum* cv. Xanthi by *Agrobacterium* mediated transformation and the transformants were analyzed (DeScenzo and Minocha, 1993). Several transgenic plants had increased amounts of Put with only small increases in Spd. Bastola and Minocha (1995) transformed carrot cells using the same gene under the control of a 35S CaMV promoter. The transformed carrot cells showed not only higher levels of Put but also showed improved somatic embryogenesis in auxin-free medium as well as the production of somatic embryos in the presence of inhibitory concentrations of 2,4-D. The transformed cells were also tolerant to DFMA (a potent inhibitor of ADC) and inhibitor of growth and embryogenesis of nontransformed cells (Robie and Minocha, 1989), thus, indicating that in the transformed cells, mouse ODC could potentially replace the function of carrot ADC for Put biosynthesis. The regenerated plants appeared phenotypically normal. Andersen et al. (1998) reported preliminary metabolic study of the transformed lines of carrot and found that higher rates of Put production in the transformed cells were accompanied by higher rates of conversion into Spd and Spm as well as the catabolism of Put.

In my study, poplar (*Populus nigra X maximowiczii*) suspension cultures have been transformed with a PCR amplified truncated mouse *odc* cDNA under the regulation of a 2X 35S CaMV promoter and an in depth study of the polyamine and N metabolism and ethylene

Table I. List of transgenic experiments in plants with genes encoding polyamine biosynthetic enzymes (updated from Kumar and Minocha, 1998).

Gene	Promoter-orientation	Plant	Reference
Yeast ODC	35S CaMV-sense	Tobacco	Hamill et al., 1990
Mouse ODC	35S CaMV-sense	Tobacco	DeScenzo and Minocha, 1993
Mouse ODC	35S CaMV-sense	Carrot	Bastola and Minocha, 1995
Mouse ODC	2X 35S CaMV-sense	Poplar	Bhatnagar et al., 2001
Human SAMDC	35S CaMV-sense	Tobacco	Noh and Minocha, 1994
Potato SAMDC	35S CaMV-sense	Potato	Kumar et al., 1996
Potato SAMDC	Tet-inducible-anti/ sense	Potato	Kumar et al., 1996
Potato SAMDC	Patatin-anti/ sense	Potato	Pedros et al., 1999
Oat ADC	Tet-inducible-sense	Tobacco	Masgrau et al., 1997
Oat ADC	ABA -inducible -sense	Tobacco	Leach et al., 1997
Oat ADC	maize ubiquitin-sense	Rice	Noury et al., 2000
Oat ADC	CaMV antisense	Rice	Capell et al., 1998
Oat ADC	ABA inducible-sense	Rice	Roy and Wu, 2001
Oat ADC	35S CaMV-sense	Tobacco	Burtin and Michael, 1997
ADC	35S CaMV-sense	*Cajanus cajan*	Sivamani et al., 2001
Bacterial LDC	35S CaMV-sense	Tobacco	Fecker et al., 1993
Pea DAO	35S CaMV-anti/ sense	Pea	Wisniewski and Brown, 2000
Pea DAO	pENOD 12-inducible-sense	Rice	Bassie et al., 2000
Pea DAO	35S CaMV-antisense	Rice	Bassie et al., 2000

biosynthesis were done in these cell lines. Parts of these results have been published (Bhatnagar et al., 2001, 2002; Quan et al., 2002).

Transgenic Expression of Arginine Decarboxylase (ADC)

Masgrau et al. (1997) found that overexpression of an oat *adc* gene (cloned by Bell and Malmberg, 1990, Accession number X56802) under the control of a tetracycline-inducible promoter in tobacco led to high levels of Put. The high level of endogenous Put resulted in abnormal vegetative growth characterized by short internodes, thin stems and leaves, leaf-chlorosis and necrosis as well as reduced root growth. Flowering of these plants was not affected. The results were somewhat similar to those shown by DeScenzo and Minocha (1993). Masgrau's group did a follow up study on the T2 generation of a selected tobacco transgenic line (line 52) under the control of tetracycline-inducible promoter (Panicot et al., 2002). This cell line was characterized by moderate increase in ADC activity and polyamine levels but no phenotypic alterations. The authors discuss the possible causes of toxicity and suggest that either Put or its oxidation products (through the action of DAO) are responsible for the deleterious effects observed in these transgenic plants.

Burtin and Michael (1997) also manipulated the expression of the *adc* gene in tobacco plants with the same oat *adc* cDNA as used by Masgrau's group. By overexpressing the gene under the control of 35S CaMV promoter, they found that second generation transgenic plants contained high levels of oat *adc* transcript, high ADC enzyme activity, as well as ten to twenty fold higher agmatine. No increases in polyamines (Put, Spd and Spm) were found, and the ODC and SAMDC activities were unaffected. There was no diversion of the polyamine metabolism into the hydroxycinnamic acid-polyamine conjugate pool or the tobacco nicotine pool. The activity of the chief catabolic enzyme, DAO, was similar in the wild type and transgenic leaves. The authors suggested that the higher ADC activity and agmatine production were subjected to a metabolic block preventing increased polyamine accumulation. No change was observed in the

morphology of the transformed plants as compared to the wild type. The oat *adc* cDNA has also been overexpressed under the control of an abscissic acid-inducible promoter (rab 16A) from rice in transgenic tobacco plants using *Agrobacterium*-mediated transformation (Leach et al., 1997). The transgenic plants grew faster, had larger leaves and increased internode distance, flowered earlier, and produced more flowers and capsules than the controls.

Capell et al. (1998) produced rice cell lines with oat *adc* in an antisense orientation under the control of 35S CaMV promoter, where they observed marked decreases in both ADC and ODC activities as well as significant decrease in Put and Spd content. The oat *adc* cDNA was also introduced into rice under the control of a constitutive maize ubiquitin 1 promoter, and significant increases were observed in the mRNA levels, ADC enzyme activity and polyamines (Noury et al., 2000). Significant increases (up to ten fold) in Put were observed in the seeds of one cell lineage as compared to controls and *hpt* selectable marker transformed controls. Bassie et al. (2000) studied the polyamine levels during morphogenesis of these transgenic rice cell lines at three developmental stages. In the dedifferentiated stage, the amounts of Put and Spd were significantly lower than the wild type and *hpt* transformed controls while Spm increased two fold, whereas the trend was reversed in the regenerating tissues. In the differentiated shoots, there was a general increase in all the polyamines. The results contrasted with previous work reported by the same group using the same gene in rice plants but under the control of a 'weaker' 35S CaMVpromoter, in that, the ubiquitin promoter showed higher mRNA measurements and ADC activity (Bassie et al., 2000). They proposed a threshold model that high ADC expression leading to production of Put above a basal level is necessary to generate a metabolic pool to cause polyamine flux through the pathway, leading to an increase in concentration of Spd and Spm.

An *adc* cDNA from oat under the control of an ABA-inducible promoter, introduced into rice by *Agrobacterium*-mediated transformation has been reported by Roy and Wu (2001). Salinity stress-induced an upregulation of ADC activity and polyamine accumulation, and second

generation transformed plants showed an increase in biomass when compared to non-transformed controls under salinity-stress conditions.

Transgenic Expression of S-adenosylmethione Decarboxylase (SAMDC)

Attempts have also been made to genetically manipulate Spd and Spm levels by transgenic expression of SAMDC. In most cases, the changes in the cellular content of these two polyamines are much smaller than those seen in similar studies with *adc* or *odc* overexpression. Noh and Minocha (1994) demonstrated that tobacco plants transformed with a human *samdc* cDNA driven by a constitutive 35S CaMV promoter showed a two-to-four fold higher SAMDC enzyme activity, significantly reduced Put levels, and higher Spd levels. All the transgenic plants appeared phenotypically normal and produced fertile flowers, and fruits. The explants of *samdc* transgenic plants showed increased shoot regeneration ability on callusing medium.

The first report on transgenic manipulation by SAMDC in a homologous system was by Kumar et al. (1996) in potato. Both sense and antisense constructs were made under the control of 35S CaMV promoter. The transformation by potato *samdc* cDNA affected not only the biosynthesis of polyamines but also ethylene. The production of transgenic plants with the 35S CaMV-*samdc* sense construct was unsuccessful, indicating that the constitutive expression of *samdc* may be lethal. The transgenic plants expressing the antisense *samdc* transgene had decreased level of SAMDC transcript, and showed stunted phenotype with highly branched stems, short internodes, small and chlorotic leaves, and inhibited root growth. However, successful transgenic potato plants were produced when transformation was done under the control of tetracycline inducible promoter using the same gene in both orientations (Kumar et al., 1996). They suggested that highly variable expression of the *samdc* transgene in *Tet-* antisense and sense plants may be due to the difficulties associated with the uptake of tetracycline under hydroponic and/ or soil conditions or with its degradation in the cells.

Pedros et al. (1999) reported significantly smaller sized but larger number of tubers in potato plants transformed with the sense construct for *samdc* gene under the control of tuber-specific patatin promoter. There were increased *samdc* transcript levels, SAMDC enzyme activity and Spd levels in these plants as compared to vector-transformed controls. The antisense *samdc* constructs had no obvious phenotypic effect. A transgenic cell line, PS-18 transformed with a *Datura samdc* gene under the control of 35S CaMV promoter was produced by J.S. Lee et al. in our lab (unpublished data). The cell line had lower Put levels at 3 days and 6 days; however, the levels of Spd and Spm were the same as NT cells. No differences in ethylene production and ACC levels were observed in this cell line (Quan et al., 2002).

There are few reports available on the cDNA clones for Spd synthase and Spm synthase (Hashimoto et al., 1998; Franceschetti and Michael, 1999; Hanzawa et al., 2000).

Transgenic Expression of Diamine Oxidase (DAO)

Pea lines transformed with the sense and antisense *dao* constructs under the control of a tissue-specific promoter showed strong cosuppression of DAO activity in nodule and epicotyl extracts with sense constructs, whereas antisense transgenics were not affected (Wisniewski and Brown, 2000). No difference in nodule number was observed between the transformed and the control cell lines, suggesting that DAO may not have a role in nodule initiation. However, cosuppressed lines were less sensitive to the inhibitory effects of exogenous Put and less active in the crosslinking of glycoproteins, suggesting that Put degradation products could retard nodule development. The authors concluded that the most critical role of DAO in pea nodule development is in the regulation of diamines in the host cells.

Bassie et al. (2000) produced transgenic rice cell lines with the pea *dao* cDNA in the antisense orientation under the control of 35S CaMV (plasmid p35S*daoa)* and pea ENOD12 (plasmid pE*daoa*) nodulin promoter. The molecular analysis revealed not only the stable integration of the transgene but also the active transcription with both the constructs. There was a

two-fold decrease in DAO activity in transformed callus lines expressing p35S*daoa* as compared to wild type and *hpt* controls. In cell lines transformed with pE*daoa*, a 2.5 fold reduction in enzyme activity was observed. Biochemical analysis of cellular polyamines from p35S*daoa* transformants indicated a six-fold increase in Put, and an 8 and 3.5 fold increase in Spd and Spm, respectively. They reported a maximum of 2.5 fold increase in Put and Spd with no effect on Spm. The authors are the first to report the down regulation of an enzyme involved in polyamine metabolism that causes an increase in the concentration of Put and Spd.

Transgenic Expression of Lysine Decarboxylase (LDC)

Like the common polyamines, expression of a bacterial lysine decarboxylase gene (*ldc*), producing cadaverine, in tobacco was done by Herminghaus et al. (1991) with an attempt to alter alkaloid biosynthesis. Two strategies were employed: one focused on the expression of the gene in the cytosol and the other on the targeting of the protein to the choloplast. In plants that expressed the processed bacterial enzyme, cadaverine levels increased from zero to 0.3-1% of dry mass. In another study, Fecker et al. (1993) transformed hairy root cultures of *Nicotiana tabacum* with an ldc gene controlled by a 35S CaMV promoter. The cadaverine levels rose from 50 micrograms to 700 micrograms per gram dry mass. Formation of the alkaloid anabasine increased three fold, and feeding of lysine to root cultures further enhanced cadaverine and anabasine levels in the transformed cell lines.

Manipulation of Polyamine Metabolism in Animals

The regulation of polyamine metabolism in animals using the transgenic approach also provides insight into our understanding of polyamine metabolism in plants. A synopsis of such studies in animal systems is presented here. In addition to *odc*, research has been active in the animal field for the manipulation of Spd and Spm synthases and Spd and Spm acetyltransferases (SSAT).

The overexpression of a human *odc* cDNA in transgenic mice led to higher Put accumulation (Halmekytö et al., 1991) in several organs, particularly the brain and testis. This did not however, result in its metabolism into Spd and Spm (Halmekytö et al., 1993; Suppola et al., 2001). Polyamine pattern changed strikingly in brain and testis. Activities of SAMDC and Spd and Spm synthases were increased in the testis of transgenic mice. Activity of SSAT and PAO were similar. The results of Halmekytö et al. (1993) are more compatible with the view that it is not the inhibition of SAMDC but Put sequestration through some unknown mechanism that is the cause of this diamine not being converted to higher polyamines. In the tissues of transgenic mice carrying the human *Spd synthase* gene, as well as those overexpressing both *odc* and *Spd synthase*, the levels of Spd and Spm both remained within the normal limits (Heljasvaara et al., 1997). The same group also produced transgenic mice overexpressing both *samdc* and *odc* as well as *samdc* alone. The latter did not have higher Spd and Spm levels. This means that the dcSAM is not the only limiting factor in these tissues to cause an elevation of Spd and Spm levels. The authors determined the metabolic flux of polyamines using pulse labeling in transgenic primary fibroblasts isolated from these transgenic mice to study how homeostasis in polyamine pools is maintained. They found that the polyamine metabolic flow is faster in the transgenic fibroblasts than the non transgenic controls. The intracellular homeostasis is maintained partly by acetylation of the polyamines followed by degradation, and also their secretion into the medium.

A transgenic mouse line having a human *Spd synthase* gene was generated by Kauppinen et al. (1993). The tissue Spd synthase activity in the transgenic mice was two to six times higher than in their syngenic littermates. However, no changes were observed in tissue Put, Spd and Spm levels. This suggests that perhaps SAMDC plays a more important role in the accumulation of Spd and Spm as compared to Spd synthase. This view is supported by the observation that tissue Spd and Spm content remained normal in hybrid transgenic mice overexpressing both *odc* and *Spd synthase* genes.

Pietilä et al. (1997) generated transgenic mouse lines overexpressing *ssat* resulting in activated catabolism and secretion of polyamines. Tissues of transgenic mice showed distorted polyamine pools, presence of N^1-acetylSpd, not normally found in mouse tissues, Put accumulation, and decreases in Spd and Spm pools. Permanent hair loss occurred in three to four weeks accompanied by follicular cysts in the dermis. Female infertility was also seen in the transgenic mice. McCloskey et al. (1999) also stably overexpressed *ssat* in chinese hamster ovary (CHO) cells and observed similar perturbations in polyamine pools. There was striking similarity between the phenotypes of *ssat* transgenic mice and those overexpressing *odc* under the control of keratin (skin specific) promoter (Peralta-Soler et al., 1996), such as, timing of hair loss and wrinkling of the skin. The results were surprising in the sense that although *odc* overexpression leads to an increase in tissue polyamine pools and *ssat* overexpression causes a decrease, yet similar phenotypes were observed. However, both transformations resulted in Put overproduction. This suggests a role for Put imbalance in the phenotype observed. Pietilä et al. (2001) further confirmed the role of Put in disturbed hair follicle development by showing that doubly transgenic mice (*odc* and *ssat*) with extremely high Put levels had more severe skin damages. O' Brien et al. (1997) studied whether *odc* overexpressed constitutively is sufficient condition for skin tumor promotion. Transgenic mice with *odc* targeted to hair follicle keratinocytes did not need treatment with tumor promoters for tumors to develop as compared to littermate controls. Overexpression of epidermal *odc* conferred a growth advantage on skin tumors *in vivo* (Halmekytö et al., 1992). Putrescine was also found to play a role in synaptic neurotransmission and this was studied using *odc* overexpressing mice (Pussinen et al., 1998).

Effects of SSAT on cell growth have been difficult to analyze because of the transient (i.e. fast degradation) nature of this enzyme, and insights into the cellular consequences of *ssat* overexpression have been provided by using polyamine analogs, such as, N^1-N^{12} diethyl Spm. These analogs induce SSAT and deplete intracellular pools of higher polyamines. Short term induced expression may be a better way to study the regulatory responses of SSAT

overproduction rather than long term studies (Pietilä et al., 1997). Analog induction has generally been correlated with inhibition of cell growth. Alhonen et al. (1998) isolated primary fibroblasts from these transgenic mice lines overexpressing *ssat* to study consequences of polyamine catabolism. Treatment of *ssat* overexpressing fibroblasts with the analog N^1-N^{11}-diethylnorSpd (DENSPM) increased enzyme protein and SSAT activity. However, no major changes were seen in mRNA. The pools of Spd pools were depleted more rapidly in the non-transgenic fibroblasts, and the cells were more sensitive to DENSPM induced growth inhibition. Further, Alhonen et al. (1999) indicate a role for SSAT in whole animal toxicity by these analogs. Treatment for four days with DENSPM caused profound changes in the transgenic animals expressing *ssat*. Putrescine pools increased while Spd and Spm pools nearly disappeared, also resulting in a compensatory rise in ODC activity. The treatment resulted in 50% mortality in the transgenic animals; non-transgenics exhibited no toxicity and SSAT activity increased only slightly upon induction. Transgenic mice died three days earlier (i.e. day 7) than their non-transgenic (i.e. day ten) littermates.

More recently, gene expression in uterus and ovary of *ssat* transgenic mice and non transgenic mice have helped reveal the role of SSAT and polyamines in controlling the molecular pathways underlying reproductive tract tissue growth and function (Min et al., 2002).

Vujcic et al. (2000) transfected human breast carcinoma cells with tetracycline regulated *ssat* human cDNA or murine gene. They conditionally overexpressed *ssat* using doxycycline and concluded that spontaneous and specific induction of SSATs can negatively affect cell growth. The authors reported for the first time the accumulation of N^1-N^{12}-diacetylSpm in addition to N^1-acetylSpd and N^1-acetylSpm. Their results demonstrate the ability of SSAT to regulate polyamine pools against a huge extracellular gradient; diacetylate Spm, inhibit cell growth and enhance cell growth inhibition by analogs.

The tissue polyamine pools in the *ssat* overexpressing mice under its own promoter were affected and the mice were hairless from an early age. Mice transformed with *ssat* gene under the

control of a metallothionein (MT) promoter also suffered permanent hair loss but at a later age (Suppola et al., 1999). MT-*ssat* transgenics showed Put accumulation, significant reduction in Spd and Spm pools, treatment with DENSPM resulted in immense induction (more than 40, 000 fold increase of SSAT enzyme activity and depletion of Spd and Spm pools from the liver within 1–2 days of the analog treatment). In addition, there was marked mortality (60%) with ultrastructural changes in the liver and mitochondrial swelling. Suppola et al. (2001) found that there was enhanced polyamine catabolism in mice overexpressing both *odc* and *ssat* as compared to mice over expressing *ssat* alone. In comparison with *ssat* overexpressing mice, the doubly transgenic mice were reported to have Put levels that were increased five times and the Spd and Spm pools declined by 60%. There was a much greater efflux of Spd from the transgenic lines than the syngenic controls.

Gene Expression Studies in the polyamine pathway

Changes in polyamine metabolism have been observed in a variety of events, such as plant stress and development; however, their precise role is not yet understood. Gene expression analysis has helped understand how the level of expression and the enzyme activity may change under abiotic/ biotic stress conditions and different stages of development.

Tomato ADC is encoded by a single gene. It was reported that during fruit ripening the ADC transcript peaked at breaker stage. No significant differences in steady state ADC mRNA levels were seen when a normal and long-keeping Alcobaca variety were compared although the latter had higher ADC activity and Put content (Rastogi et al., 1993). This suggests that changes in ADC mRNA alone cannot account for the differences in ADC activity and that other regulatory mechanisms must be operating. Expression of *adc* in pea is high in young developing tissues but lower in fully expanded leaflets and roots, while it is developmentally regulated in the ovary and fruit (Perez-Amador et al., 1995). Chattopadhyay et al. (1997) studied the effect of salinity stress in salt tolerant (Pokkali) and salt sensitive (M-1-48) rice (time course and

increasing concentrations of NaCl) on ADC activity and mRNA levels. Results suggest that in Pokkali the transcript accumulates as well as ADC activity increases, however, in M-1-48 this mechanism is absent where salinity stress downregulates both the transcript level and enzyme activity. Nam et al. (1997) suggest that changes in the content of *adc* mRNA have an important role in the regulation of ADC enzyme activity during early development, tissue-specific activity and acid stress.

Methyl jasmonate (MJ) induced mRNAs for ODC, SAM-synthase (SAM-S) and Put-N-methyltransferase, and auxin significantly reduced the MJ- inducible accumulation of mRNAs for ODC, SAM-S and Put-N-methyltransferase. Levels of mRNAs for ADC and SAMDC were not affected in response to MJ (Imanishi et al., 1998). Their results suggest that MJ induces expression of a series of genes involved in nicotine biosynthesis by multiple regulatory mechanisms. Ornithine decarboxylase gene expression was found to be rapidly inducible by MJ in tobacco BY-2 (bright yellow-2) cells in response to wounding (Imanishi et al., 2000). This enzyme was also shown to be upregulated by 2, 4-D and gibberellic acid in tomato early fruit development (Alabadi and Carbonell, 1998). Sucrose induction of the ODC gene was seen in roots, hypocotyls and flowers of tomato (Kwak and Lee, 2001). Wang et al. (2000) found differentially expressed gene products encoding ODC, ADC and SAM-S by subtractive hybridization among a set of cDNAs generated from root mRNAs isolated from tobacco before and three days after topping. Steady state levels of ODC and ADC mRNAs increased after topping whereas little changes were observed in SAM-S transcripts.

Mad-Arif et al. (1994) isolated the SAMDC gene and characterized its expression pattern in potato and found that the gene expression was high in the young and actively dividing tissues, and low in mature and non dividing tissues of both vegetative and reproductive organs. *Tritordeum* cDNA encoding SAMDC was isolated and northern analysis showed that its transcript accumulated in response to wounding, and the levels changed in response to a circadian rhythm. There was a peak in the middle of the light period and this periodicity continued in light

conditions but changed under darkness (Dresselhaus et al., 1996). Northern analysis showed a differential expression of the pea *samdc* gene in the vegetative and reproductive tissues. The highest *samdc* mRNA levels were found in callus and undifferentiated tissues with high rates of cell division and at the onset of fruit set. Gene expression was also induced in senescing ovaries and it was found that the levels of transcripts in leaves and shoots were differentially affected by ozone treatment (Marco and Carrasco, 2001). The differences in *samdc* gene expression were compared between salinity stressed (var. Lansheng) and control rice seedlings (var. 77-170) (Li and Chen, 2000). The authors found that elevation of the *samdc*1 transcript occurred earlier in Lansheng than 77-170 under salt stress, and higher levels of expression were detected in Lansheng relative to the controls under low salt stress. This suggests that the expression of the SAMDC gene is correlated to the salt tolerance in rice seedlings. A time course analysis in the rice shoots revealed that the transcript levels changed during drought, salinity and ABA stress conditions. In *Arabidopsis*, it was found that *samdc*1 is ubiquitous whereas *samdc*2 is expressed only in leaves and inflorescences (Franceschetti et al., 2001).

Rea et al. (1998) found that the transcript level and activity of Cu-AO were modulated during seedling development in parallel to wall maturation. Mechanical wounding induced an increase of the Cu-AO mRNA accumulation and enzyme activity, which remained high during the wound healing process. The AO inhibitor, Ag, decreased the deposition of lignin-suberin barrier along the lesion. They suggest that Cu-AO may be a limiting factor in H_2O_2 production and its expression is associated with the remodeling of cell wall during ontogenesis and wound healing (Rea et al., 1998).

Objectives of this study

As we move towards modulating specific aspects of cellular metabolism in plants through genetic engineering, it would be informative to analyze the impact of manipulating single reactions in a pathway on the regulation of the entire pathway, and also on other related pathways

that utilize the same precursors and intermediates. Most of the studies reported so far have been aimed at the transgenic manipulation of polyamine metabolism and analysis of the changes in the polyamine content, and, in some cases on its developmental effect on plants. The primary objective of my research was to analyze how Put overproduction in poplar cells affects the related pathways, such as, substrate (Orn and Arg) biosynthesis and N metabolism, as well as to study polyamine catabolism in response to transgenic manipulation of cellular Put. Some of the specific questions pertaining to the above objective that were addressed are:

1. *What are the effects of mammalian ornithine decarboxylase expression on cellular levels of putrescine, spermidine and spermine and the rate of their biosynthesis?*
2. *Is there a feedback regulation of native arginine decarboxylase pathway as a result of increased putrescine accumulation by the transgenic odc?*
3. *Does ornithine become limiting in the transgenic cells due to its excessive utilization by ODC?*
4. *What is the primary source of ornithine in the cells? Is it glutamate or arginine?*
5. *What is the fate of excess putrescine and its rate of turnover in the transgenic cells?*
6. *How is the turnover of spermidine and spermine affected in the transgenic cells?*
7. *Does the overproduction of putrescine have an effect on adc, Spd synthase, glu decarboxylase and mouse odc gene expression?*

The poplar cells were transformed using biolistic bombardment with the plasmid pCW122-*odc*, containing the truncated mouse *odc* cDNA regulated by a 2X 35S CaMV promoter resulting in Put overproduction. Transformed cells so produced were characterized for the presence and expression of the gene as well as the polyamine content. Biochemical studies were done using radioactive substrates and analyzing the contents of total and radioactive polyamines in the treated nontransformed and transformed cells. The effect on the gene expression of

enzymes of the polyamine and N pathway in these poplar cell lines were studied using northern blot analysis.

MATERIALS AND METHODS

Plasmid Construction and Transformation

The plasmid pCW122-*odc* used in my study was prepared by Drs. S.C. Minocha and R. Minocha as follows. The plasmid *puc*ODC-1 (DeScenzo and Minocha, 1993) was used to amplify a PCR product containing the coding sequence of the mouse *odc* gene (Accession number S64539). The PCR product was gel purified and ligated into the pCW122 expression vector (Walter et al., 1998) from which the *gus* gene had been removed by restriction with *Hind*III and *Bam*H1. Blunt-end ligation was performed following a filling-in reaction (Klenow polymerase) and dephosphorylation of the vector. *Escherichia coli* (DH10b) containing the reconstituted plasmid were electroporated and selected on ampicillin, and tested for correct orientation of the mouse cDNA by restriction analysis and by sequencing of the junction between the promoter and the coding sequence. The reconstituted plasmid, called pCW122-*odc*, contains the truncated mouse *odc* cDNA regulated by a 2X 35S CaMV promoter and a CaMV 3'-termination sequence. The plasmid also contains an *nptII* gene under the control of a single 35S CaMV promoter for selection of transgenic plant cells on kanamycin (Fig. 5). The transformed cell lines used in this study were generated by Bernadette Glasheen and Suneet Bains in our laboratory.

The biolistic bombardment technique was modified from Walter et al. (1998) for transformation of suspension cultures. Gold particles (1 μm, Bio Rad Labs, Hercules, CA) were coated with either the plasmid pCW122-*odc* (Fig. 5) (*odc* + *nptII* gene) or pCW122 (Fig. 6) (*gus* + *nptII* gene) DNA (2 μg DNA/ μg gold particles) in the presence of 1 M $CaCl_2$ and 16.7 mM spermidine (Spd). Rupture discs of 1350 psi were used for bombardment. For preparation of tissue, 1 mL of three day (d) old cell suspension (containing about 100 mg FW of cells) of hybrid poplar *(Populus nigra x maximowiczii)* was vacuum-filtered onto a sterilized 60 mm (diameter)

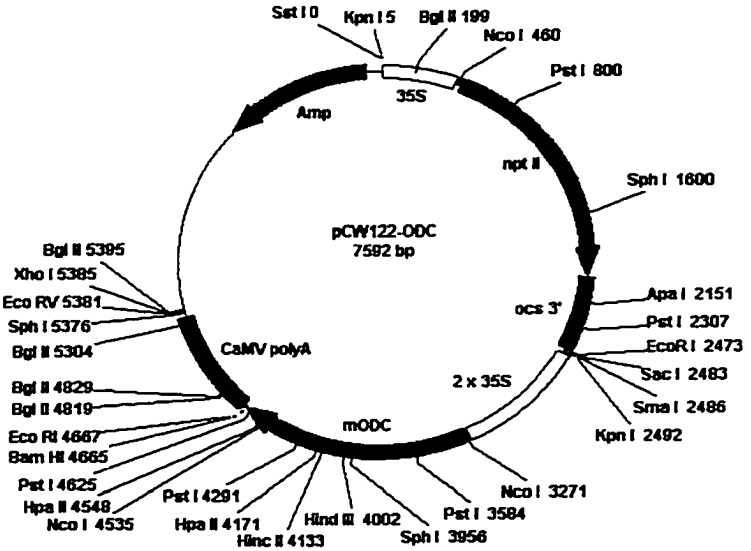


Fig.5. Plasmid pCW122-*odc*

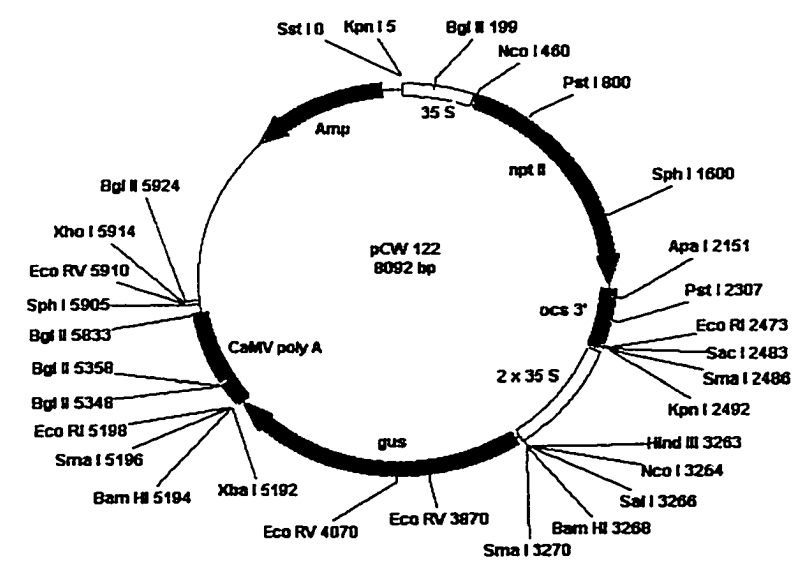


Fig.6. Plasmid pCW122

Whatman #1 filter paper. The filter paper was placed in the center of a petri dish containing MS (Murashige and Skoog, 1962) medium with 0.2 M sorbitol for 16-20 h prior to bombardment. Following bombardment, the cells were kept for 3 d on the same medium and then the filter papers were transferred to the selection medium containing 100 mg L^{-1} kanamycin (Eastman Kodak Company, Rochester, NY, Cat # IBO2120). When the cells had grown to 5 mm clumps on the filter paper, they were transferred directly onto solid medium containing kanamycin. Following several subcultures, suspension cultures were initiated by transferring cell masses from solid medium to liquid medium and placing them on the shaker.

Cell Culture

Liquid and solid cultures were routinely maintained on MS medium (Murashige and Skoog, 1962; Gibco BRL, Cat # 11117-074 or Sigma, St.Louis, MO., Cat # M-5524) containing vitamins of B-5 medium (Sigma, G-1019) (Gamborg et al. 1968), 2% sucrose (w/v), and 0.5 mg L^{-1} 2, 4-dichlorophenoxyacetic acid (2, 4-D) (Sigma, D-7299). The pH of the medium was adjusted to 5.7 before autoclaving. Suspension cultures were maintained by transferring 7 mL of the 7 d old cell suspensions to 50 mL of fresh medium in a 125 mL Erlenmeyer flask, and kept on a gyratory shaker at 160 rpm. Stock cultures were maintained on solid medium (0.8% agar type A, Sigma, A-4550) and subcultured at 3-4 week intervals. All cultures were kept at 25±1^{0}C under 12 h photoperiod (80±10 $\mu E.m^2 sec^{-1}$). The medium for maintenance of transgenic cells contained 100 mg L^{-1} kanamycin, however, the antibiotic was not present during the experimental treatments. My project was initiated at this stage. Transformed cell lines were provided to me, which I characterized by molecular and biochemical analysis before conducting further experiments.

Isolation of Plant Genomic DNA

The Cetyltremethylammonium bromide (CTAB) method was used for isolation of genomic DNA (Webb and Knapp, 1990). Mortar, pestle and spatulas were pre-chilled at 4°C. Two grams of cells were frozen and ground to fine powder in a three-inch mortar and pestle using liquid nitrogen. The powder was poured into a 50 mL Teflon centrifuge tube containing 10 mL of CTAB extraction buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA and 100 mM Tris-HCl, pH 8.0 and 20 μL β- mercaptoethanol added just prior to use). The powder was mixed by inversion and then incubated at 60°C in a water bath for 30 min with inversion at regular intervals. The tubes were incubated on ice and then extracted with an equal volume of chloroform (molecular biology grade) (Sigma, C-2432): isoamyl alcohol (Fisher, BP 1150-500) (24:1 v/v). The tubes were vortexed gently but thoroughly and centrifuged at 3000 X g for 5 min at 4°C using JS-25.50 or JS-13.1 swinging bucket rotor. The upper aqueous layer was transferred to a new tube and an equal volume of phenol (Sigma, P-1037): chloroform: isoamylalcohol (25: 24:1) (v/v/v) was added. The phenol used was equilibrated with 50 mM Tris, 10 mM EDTA at pH 8.0. The contents were vortexed gently but thoroughly and centrifuged at 10000 X g for 5 min at 4°C. The upper aqueous layer was transferred to a clean Teflon tube and an equal volume of ice-cold isopropanol was added and mixed well by inversion. The tubes were placed at –20°C for at least 1 h (in some cases overnight), to precipitate DNA. The contents were centrifuged at 10000 X g for 10 min at 4°C and the supernatant was carefully poured without disturbing the pellet. The DNA pellet was washed with 76% ethanol containing 10 mM ammonium acetate by swirling. The samples were centrifuged again at 10000 X g for 10 min at 4°C. The supernatant was poured off and the pellet was allowed to dry in the SpeedVac centrifuge (Savant Instruments Inc., Farmingdale, NY). The pellet was dissolved in 100 μL distilled water containing 2 μL RNase A (10 μg mL^{-1}) (5 Prime -> 3 Prime Inc., West Chester, PA).

DNA was quantified using a fluorometer (Dyna Quant 200, Hoefer Scientific Instruments, San Francisco, CA). Two μL of calf thymus DNA was used as a standard in 2 mL of DNA Standard assay solution. Then, the DNA in the samples was quantified by taking 2 μL of the sample in 2 mL of assay solution, and measuring fluorescence.

DNA Standard Assay solution used was (High range: 100 to 5000 ng mL^{-1} final DNA concentration) containing Hoechst dye (H33258) stock solution made as follows: 100 μL; 10X TNE Buffer: 10 mL and distilled water: 90 mL. The dye is made as 1μg mL^{-1} H33258 in 1X TNE). 10X TNE is made as follows: 12.11 g of 100 mM Tris; 3.72 g of 10 mM EDTA and 11.62 g of 2 M NaCl in 1 L of distilled water, pH to 7.4 and filtered prior to use).

PCR Analysis

The PCR reaction was carried out using 'Ready-to-go' PCR beads (Amersham-Pharmacia, Piscataway, NJ) as per instructions of the supplier. The PCR beads are designed for a 25 μL reaction. Each bead contains 1.5 units of Taq polymerase, 10 mM Tris-HCl (pH 9.0 at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$ and 200 μM of each dNTP. One μL of the forward and reverse primer mix was added (working stock of 10 μM each) and 1 μL DNA (approximately 50 ng of genomic DNA or 50 pg of plasmid DNA) was added to distilled water to bring the reaction to 25 μL. The reaction was run in PTC 100 Programmable Thermal Controller (MJ Research Inc., Watertown, MA). PCR primers were 5'GAACCATGGGCAGCTTTAC3' and 5'CTACTACATGGCTCTGGATCTGTTTCA3' for *odc* gene. The cell lines were also tested for the presence of the *nptII* gene. The primers used were: 5' GAG GCT ATT CGG CTA TGA CT 3' and 5' TCG GGA GCG GCG ATA CCG TA 3'. The profile for the *nptII* gene was 93^{0}C for 1 min, 94^{0}C for 30 sec (denaturation), 65^{0}C for 30 sec (annealing), 72^{0}C for 30 sec (extension). The reaction was repeated for 35 cycles. The program for amplification of *odc* gene was: 94^{0}C for 2 min, 94^{0}C for 30 sec (denaturation), 62^{0}C for 30 sec (annealing), 72^{0}C for 30 sec (extension). The

reaction was repeated for 35 cycles. For both the reactions, this was followed by 72°C (extension) for 5 min and set to hold at 4°C.

Five μL of the PCR reaction products and 1 Kb DNA ladder (Promega, Cat # 5711) were electrophoresed on a 1% agarose gel in 1X TAE buffer (0.30 g in 30 mL) at 80V for 50 min. One μL of 6X loading dye was added to each sample before loading on the gel. The gel was stained in ethidium bromide (0.5 μg mL^{-1} final concentration) for 10 min and destained for 15 min in distilled water and viewed under UV light using Nucleovision 760 Gel Documentation System (Nucleotech, San Mateo, CA). A negative and a positive control reaction were set up for the PCR reaction of each gene.

Free Polyamine Analysis

Sample collection for polyamine analysis was done by vacuum filtering 5-10 mL of cell suspensions onto Miracloth (Calbiochem, La Jolla, CA, Cat # 475855) and transferring 200 mg fresh weight of cells to 800 μL of 5% perchloric acid (PCA) (1:4; w/v) in a 1.7 mL microfuge tube. Samples were kept on ice throughout the collection process and were then frozen (-20°C) and thawed three times (room temperature) before dansylation (Minocha et al., 1994).

Samples were vortexed for 1 min and after centrifugation (13000 X g, 8 min), 100 μL of the PCA extract was dansylated. One hundred μL of the mix of three polyamines (concentration of 0.02 mM) was dansylated as standard parallel to the samples. The three standards used were Put-di HCl (Sigma, P-7505), Spd-tri HCl (Sigma, S-2501) and Spm-tetra HCl (Sigma, S-2876). Twenty μL of 0.1 mM heptanediamine was added to both standards and samples as an internal standard. To this, 100 μL of saturated sodium carbonate was added followed by 100 μL of dansyl chloride (Sigma, D-2625) made as 20 mg mL^{-1} in acetone. The samples were incubated for 1 h at 60°C. The reaction was terminated by adding 50 μL L-alanine (Sigma, A-5824) or L-proline (Sigma, P-8849) (made as 100 mg mL^{-1} distilled water). Samples were briefly vortexed. Another

30 min incubation was done at 60°C to remove excess dansyl chloride. The sample tubes were placed in the SpeedVac with the caps wide open for 5 min to evaporate acetone under vacuum. The dansyl-polyamines were extracted with 400 μL toluene (J.T. Baker, Cat # 9456-03). After addition of toluene, the samples were vortexed and then allowed to sit for 5 min to separate the aqueous and toluene phases, and centrifuged at 13000 X g for one min. Of this, 200 μL toluene was transferred to a new microfuge tube and dried in SpeedVac. The dried dansyl-polyamines were dissolved in 1 mL methanol (Fisher Scientific, Lot# 970153). The samples were vortexed and centrifuged for 2 min each and 0.5 mL methanol extract was transferred to autosampler vials for polyamine analysis.

Samples were analyzed by HPLC using a gradient of acetonitrile (40-100%) and 10 mM heptanesulfonic acid (contains 10% acetonitrile to inhibit fungal and bacterial growth), pH 3.4 on a reversed-phase Pecosphere C18 column (4.6 x 33 mm, 3 μm) using a Perkin-Elmer HPLC system (Minocha et al., 1990). The Perkin-Elmer HPLC system consisted of a PE series 200 autosampler fitted with a 200 μL loop (the sample volume injected was 10 μL) and a PE series 200 gradient pump. The flow rate was set at 2.5 mL min^{-1}. Polyamines were quantified by a fluorescence detector (Perkin Elmer LC 240) set at excitation and emission wavelengths of 340 nm and 515 nm, respectively. Data were integrated using the PE Turbochrom (Tc4) (version 4.1) software (PE Corporation, Norwalk, CT). The output was obtained as polyamines (nmol g^{-1} FW) and the multiplication factor was incorporated in the software for analysis.

One of the mouse *odc* transformed cell lines, called 2E, was selected for further comparative and molecular analysis with the non-transformed (NT) cell line after PCR and polyamine analysis. Another NT cell line, named, non-transformed-high Put (NT-HP) produced significantly higher Put amounts as compared to NT and tested negative for the PCR for mouse *odc* gene as well as other molecular analysis described later. This cell line was included in some experiments.

Isolation of Plant RNA

RNA was isolated by the single step method of RNA isolation (modified from Chomczynski and Sacchi, 1987). Two grams fresh weight of cells were frozen in liquid nitrogen and ground to fine powder. The powder was added to 10 mL extraction buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sodium lauryl sarkosyl and 100 mM β-mercaptoethanol (70 μL) (added just prior to use) in screw cap Teflon tubes and vortexed well. One mL of 2 M sodium acetate, pH 4.0 (5.75 mL of glacial acetic acid and 40 mL of water, add NaOH to bring pH to 4.0) was added and vortexed. *Note: this solution is 2M acetate and not 2M sodium.* Ten mL of water saturated phenol (non buffered, pH<8.0) was added and vortexed. To the above slurry, 2 mL of chloroform was added and vortexed. The contents were centrifuged for 10 min at 10000 X g and the upper aqueous phase was transferred to a 50 mL new Teflon tube. These tubes were filled with 2% SDS overnight and rinsed well with 0.1% diethylpyrocarbonate (DEPC) (Sigma, D-5758) treated water. To the total volume of aqueous phase, one-fourth of chloroform: isoamylalcohol (24:1) was added. The contents were vortexed and centrifuged at 10000 X g for 6 min. The upper aqueous phase was transferred to a new RNAse free 50 mL Teflon tube.

All the solutions used from this point forward were treated with 0.1% DEPC, left overnight with caps loose in the fume hood and then autoclaved. An equal volume of ice-cold isopropanol was added to the tubes containing the aqueous phase from previous centrifugation, and allowed to chill at -20^{0}C for at least 1 h. Four mL of 4 M lithium chloride was added to the samples and the pellet was vortexed. It was ensured that the pellet dissolved and the contents were centrifuged at 5000 X g for 10 min. The supernatant was discarded and the pellet was resuspended in 2 mL CE (10 mM sodium citrate, 1mM EDTA). To the dissolved pellet, 2 mL chloroform was added and vortexed. The contents were transferred to an RNase free tube (Fisher Scientific Company, Pittsburgh, PA). These tubes are narrower and allow picking the aqueous phase easily without drawing out the interphase. The samples were centrifuged for 10 min at 5000

X g. The aqueous phase was transferred to a new RNase free tube and $1/10^{th}$ the volume (180 μL for 1.8 mL) of 2 M sodium acetate, pH 5.0 (2 M sodium acetate, add acetic acid to bring to pH 5.0) and 2.5 volume of ethanol were added. *Note: This solution is 2 M sodium and not 2 M acetate.* The tubes were allowed to chill at -20°C for at least 1 h to overnight. The samples were centrifuged for 15 min at 10000 X g to pellet the RNA by removing the cap. The pellet was washed with 1 mL of 70% ethanol and briefly centrifuged at 10000 X g. The tubes were placed in the SpeedVac by removing the cap and covering with parafilm and puncturing holes in it to evaporate ethanol. The pellet was reconstituted in 200 μL CE and the RNA was quantified spectrophotometrically as follows:

$$\text{Yield} = (\lambda_{260})(\text{dilution})(40\ \mu g\ mL^{-1}) = x\ \mu g\ mL^{-1}$$

Absorbance at 260 and 280 nm was measured using the spectrophotometer (Hitachi, U-2000). One μL of the sample in 1mL of water for quantification gives a dilution of 1000. An OD of 1 at A_{260} corresponds to approximately 40 $\mu g\ mL^{-1}$ of single stranded RNA. Typical 260/280 nm ratios were from 1.7 to 2.0.

Non-radioactive Probe Preparation

Non-radioactive probe was prepared by using digoxigenin (DIG) DNA Labeling and Detection Kit (Roche Biochemical Co., Indianapolis, IN, Cat # 1093657). The PCR product (1.3Kb) of *odc* cDNA from pCW122-*odc* was labeled by random primed incorporation of DIG-labeled deoxyuridine-triphosphate DIG-11-dUTP. Briefly, the protocol is as follows: PCR reaction was set up from the positive plasmid control (pCW122-*odc*) using the program for mouse *odc* gene as described earlier. A 1% agarose gel was run after pooling the PCR product of three tubes. The desired 1.3 Kb band was cut out and the DNA was gel purified either by using QIAquick Gel Extraction protocol (Qiagen Inc., Valencia, CA) or GFX™ PCR and Gel Band Purification Kit (Pharmacia Biotech, Piscataway, NJ). The procedure is described in the

manufacturer's protocol. The DNA was eluted in water and quantified. Probe making requires 0.5 μg-3.0 μg DNA.

In cases where PCR purified product was used, DNA was purified using the Qiagen Wizard PCR Product Purification Kit after amplification (centrifugation method). The procedure is described in the manufacturer's protocol. PCR amplified DNA was quantified, purified and then used for probe making. The DNA was denatured in a boiling water bath or Thermocycler for 10 min and chilled on ice for 5 min. To a 0.5 mL microfuge tube, the following reagent components were added on ice: DNA template (15 μL); hexanucleotide mix (2 μL), dUTP labeling mixture (2 μL) and Klenow Enzyme (1 μL) or DNA template (16 μL) and DIG High Prime labeling Mix (4 μL).

The contents were collected by brief centrifugation and the tube was incubated at 37°C for 20 h. The reaction was stopped by adding 2 μL of 0.2 M EDTA or heating at 65°C for 10 min.

Probe Quantification

After labeling, quantification of the probe was done by adding 1 μL of the DIG labeled probe to 9 μL of water to make a 10^{-1} (1: 10) dilution. One μL of this dilution was transferred to 9 μL of water to make a 10^{-2} dilution. Dilutions of 10^{-3} and 10^{-4} were made in the same way. One μL of the DNA dilutions as well as control dilutions named, A B, C and D where A is 1 ng μL^{-1} and B is 10^{-1} dilution, i.e. 100 pg μL^{-1}, C and D are 10 pg μL^{-1} and 1 pg μL^{-1} dilutions, respectively, was spotted on the 0.22 μM nytran membrane (Schleicher & Schuell, Keene, NH). The membrane was baked between two clean sheets of Whatman filter paper at 80°C in a hybridization oven (Unitherm, Integrated Separation Systems) for 30 min. The membrane was then wetted in washing buffer, agitated in antibody blocking solution for 10 min and transferred to antibody solution (anti-DIG- alkaline phosphatase conjugate, 1: 10000) for 10 min. The membrane was washed twice with washing buffer for 5 min each and incubated in 20 mL

detection buffer to equilibrate the membrane. All these steps were performed in clean glass petri dishes on an orbital shaker (Hoefer Scientific Instruments, San Francisco, CA). The membrane was finally placed in detection buffer containing the color substrate (NBT/BCIP) (40 μL substrate in 2 mL detection buffer) in the dark without shaking till color developed. The probe concentration was estimated by comparing the spot intensities with those of the control DNA provided in the kit. The Dot/ Blot Image Software on the Gel Documentation was used to graph the intensities of the resulting spots. The probe of appropriate dilution was added to the hybridization solution for Southern and northern hybridizations and reused three to four times. All stock and solutions used are given in Table II.

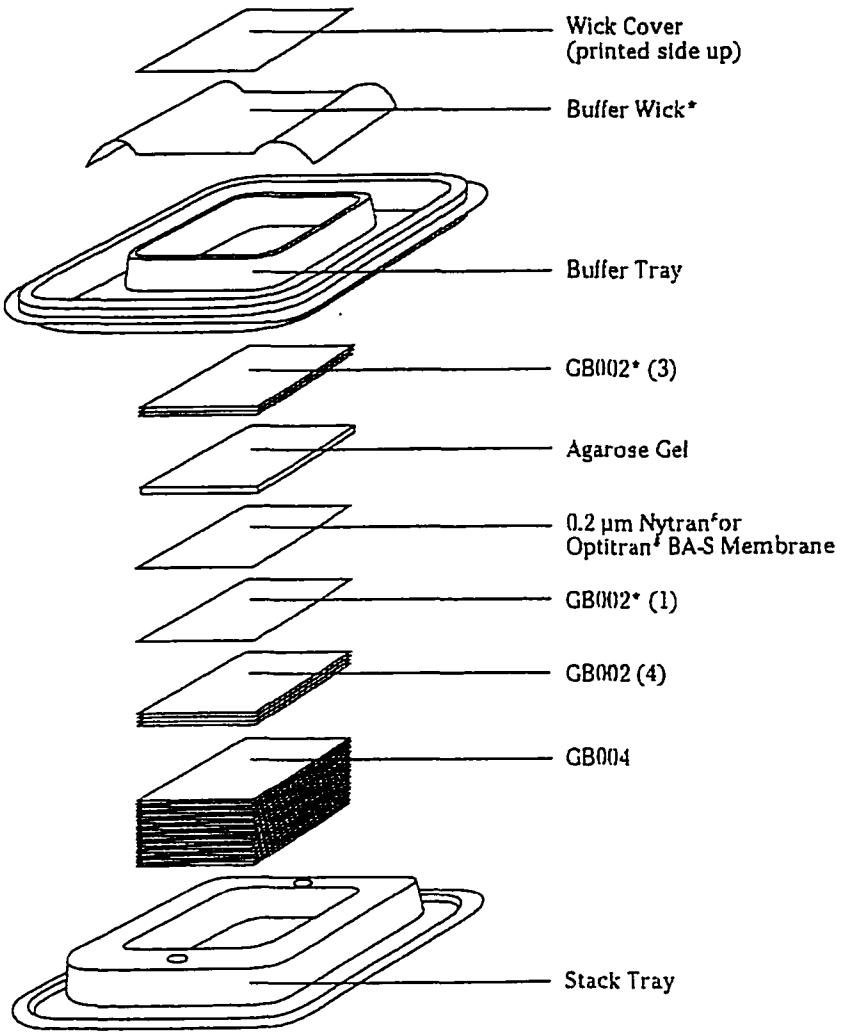
PCR and Genomic Southern Hybridization

Southern hybridization was carried out according to the Genius System User's Guide for membrane hybridization version 3.0. For PCR product hybridization, 5 μL of the PCR reaction was run on a 1% agarose gel at 80V for 45 min. For genomic Southern hybridization, 15 μg of genomic DNA was digested with *Eco*RI or *Hind*III (5 units of enzyme per μg of DNA; New England Biolabs, Beverly, MA) overnight at 37^{0}C. *Eco*RI flanks the mouse *odc* cDNA in the plasmid while *Hind*III cuts only once in the plasmid. The latter will be useful in determining the number of copies integrated in the genome. Additional enzyme was added after 4 h to ensure that enough active enzyme was available to digest genomic DNA. A 1% agarose gel was run at 40V for 3 to 4 h. The gel was photographed after staining for 15 min and destaining for 20 min. A uniform smear in all samples was expected to ensure complete digestion and then hybridization was carried out.

The gel was denatured for 30 min in 0.5 M NaOH, 1.5 M NaCl. This was followed by neutralizing the gel in 0.5 M Tris-HCl, pH 7.0; 3 M NaCl for 30 min. The DNA on the gel was transferred overnight to a 0.22 μm nytran membrane using the Turbo blotter kit (Rapid downward

transfer system) (Schleicher and Schuell) (Fig. 7). A corner of the membrane was cut for orientation purposes before setting up the transfer system. The transfer buffer used was 10X SSC. The system was set up using the manufacturer's protocol. The gel was discarded the next day after viewing under UV light to check if complete transfer had taken place. The position of wells on the membrane was marked with a soft pencil while disassembling the transfer apparatus. The membrane was placed in 2X SSC for 1-2 min to wash off any agarose particles and then baked at 80°C in the hybridization oven for 1 h by placing between two clean sheets of Whatman # 1 filter paper. Although the baked membrane can be stored at room temperature up to one year, the membrane was usually treated immediately.

For prehybridization and hybridization, the membrane was placed in a 50 mL hybridization bottle (with the side where the DNA was transferred facing inwards). Prehybridization was performed in 20 mL of prehybridization solution at 65°C for 2-3 h. The prehybridization solution consisted of: 5X SSC, 0.1% sarkosyl, 0.2% SDS, 1X blocking solution and distilled water. This was followed by hybridization overnight at 65°C. Both prehybridization and hybridization were performed under constant gentle rotation. A total of 10 mL of hybridization buffer was used with the probe concentration of 1:1000. The hybridization buffer contained the same components as prehybridization buffer. Following hybridization, the membrane was washed twice at room temperature for 5 min each in 2X SSC, 0.1% SDS and then twice at 68°C in 0.1X SSC, 0.1% SDS. After the post hybridization washes, the membrane was taken out of the hybridization bottles, transferred to a Tupperware container, placed for 1-5 min in washing buffer (99.7 mL 1X maleic acid buffer; 300 μL Tween 20) and then transferred to 40 mL of antibody blocking solution (1X blocking solution in 1X maleic acid buffer). The membrane was transferred to antibody solution (anti-DIG-alkaline phosphatase conjugate was added to fresh 1X blocking solution in a ratio of 1: 10000), i.e. 4 μL in 40 mL blocking solution for 30 min. The membrane was washed twice for 15 min each in 50 mL washing buffer



*Prewet in transfer buffer.

Fig. 7. Rapid downward Transfer system using Turbo blotter Kit.

(99.7 ml 1X maleic acid buffer; 300 μL Tween 20). Next, it was equilibrated for 2-5 min in 20 mL detection buffer (10 mL of 1 M Tris-HCl pH 9.5; 2 mL of 5 M NaCl; 5 mL of 1 M $MgCl_2$ and 83 mL deionized water). $MgCl_2$ enhances color detection. The membrane was constantly agitated on a shaker. Finally, the membrane was incubated in 10 mL of color substrate solution (200 μL of NBT (nitroblue tetrazolium)/BCIP per 10 mL detection buffer). The membrane was left undisturbed in the dark to allow color to develop. The reaction was allowed to proceed until desired bands appeared for PCR Southern and up to a maximum of 16 h for genomic Southern hybridization analysis. The recipes for all the stocks and solutions used are given in Table II.

Denaturing Gel Electrophoresis and Northern Blot Hybridization

All solutions and apparatus used were treated with 0.1% DEPC. The gel box was washed with detergent, rinsed with 95% ethanol, dried, treated with RNAse Zap (Ambion, Cat # 9780. 9782) and then washed with DEPC treated water. All glassware, forceps and spatulas were wrapped in aluminum foil and baked at 250°C for at least one day. Benchtop and Tupperware container were wiped clean with RNAse Zap. The gel was run in the fume hood. A 39.88 mL 1.2% agarose formaldehyde denaturing gel was prepared as follows: 0.48 g agarose, 1X MOPS Buffer (5 Prime→ 3 Prime, Cat # 5302-594115) (4 mL of stock 10X) and 28.2 mL water was mixed in a 125 mL baked Erlenmeyer flask. The agarose was melted in the microwave and allowed to cool to 60°C. A 2.2 M formaldehyde (Sigma, F-8775) (7.2 mL of 12.3M stock) was added and the gel was poured immediately. The gel running buffer consisted of 1X MOPS Buffer (27 mL of 10X MOPS buffer), 2.2 M formaldehyde (48 mL of 12.3 M stock) and 195 mL DEPC treated water. The RNA samples were prepared for electrophoresis by adding the components: 4.5 μL of total RNA (up to 30 μg); 10 μL formamide (total concentration of 50%); 2 μL of 10X MOPS buffer

Table II. Solutions used in Southern and northern hybridizations using DIG-labeled probes.

Final concentration	Compound	Stock	Volume (mL)
Prehybridization Buffer			**Total 20 mL**
5X	SSC	20X	5
0.10%	Sarkosyl	20%	0.1
0.20%	SDS	20%	0.2
1X	Blocking solution	10X	2
	Deionized water		12.7
			Total 40 mL
Hybridization Washes			
2X	SSC	20X	4
0.10%	SDS	20%	0.2
	Deionized water		35.8
0.5X	SSC	20X	1
0.10%	SDS	20%	0.2
	Deionized water		38.8
Antibody Blocking Solution			**Total 100 mL**
1X	Blocking solution	10X	10
1X	Maleic Acid buffer	2X	45 mL buffer + 45 mL water
Antibody washing Solution			**Total 100 mL**
0.30%	Tween Twenty		0.3
1X	Maleic Acid buffer	2X	50 mL buffer + 50 mL water
Maleic Acid Buffer			**Total 1 L**
0.1 M	Maleic acid		11.6g
0.15 M	Sodium chloride		8.77g
	Deionized water		950
Add sodium hydroxide pellets to adjust pH to 7.5 and bring to final volume of 1L			
Detection Buffer			**Total 100 mL**
100 mM	Tris-HCl pH 9.5	1 M	10
100 mM	NaCl	5 M	2
50 mM	$MgCl_2$	1 M	5
	Deionized water		83

and 3.5 μL of 12.3 M formaldehyde. The samples were denatured for 15 min at 65°C and then cooled on ice. Two μL of 10X loading dye (RNase free) was added to each sample (total volume of each sample loaded was 22 μL) before loading. The formaldehyde denaturing gel was run at 40V for 3 h. A portion of the gel was cut and stained to check for RNA integrity. Four μL (2-3 μg) of the RNA ladder was also run parallel to the samples in some cases to determine the size. The ladder was treated the same way as the samples prior to loading. The gel was stained for 30 min in ethidium bromide and destained overnight in water. The next morning, a picture was taken with a fluorescent ruler next to the ladder starting from the origin to mark the size of the ribosomal bands and to determine the transcript size of the samples. The other half of the gel was blotted using the Turboblotter kit and treated for northern analysis. The gel was washed thrice for 15 min per wash in 150 mL each of DEPC treated water under constant gentle agitation. The gel was then equilibrated for 45 min in 250 mL of 10X SSC with gentle but constant agitation. The RNA was transferred overnight using the Turbo blotter kit. The blotter was made RNAse free by washing well with detergent, treated with 95% ethanol, air dried, treated with RNAse Zap and rinsed with DEPC treated water. The membrane was baked at 80°C for 1 h. The gel was stained with ethidium bromide in the morning before discarding to ensure that no bands were seen and efficient transfer had taken place. Prehybridization and hybridization were done the same way as described earlier for Southern hybridization except that all the solutions used for northern hybridization till the step of hybridization were RNAse free, such as, SSC, blocking solution and water.

The membrane was also stripped and reprobed in some cases. In this case, all the solutions used upto detection were DEPC treated. The membrane was stripped only in cases where chemiluminiscent detection (described later) was done. It is imperative to prevent the membrane from drying so it was stripped the same day detection was done. After detection, the membrane was place in stripping solution (50% formamide, 50 mM Tris-HCl, pH 8.0 and 5%

SDS). The stripping solution was also RNAse free. *Note: this stripping solution is specific for chemiluminiscent detection.* The membrane was placed in a baked RNAse free hybridization bottle and 20 mL of stripping solution was added. The membrane was incubated at 80°C in the hybridization oven for 1 h. After 1 h, the stripping solution was changed with fresh 20 mL of stripping solution and the membrane was incubated again at 80°C for 1 h. After this, the membrane was taken out of the hybridization oven, placed in an RNAse free Tupperware containing 2XSSC for 5 min and then prehybridized and hybridized with another probe the same day. The membrane can also be stored in 2X SSC but was usually treated the same day. The membrane was only stripped once and reprobed.

Dot Blot Hybridization

Dot blot hybridization was performed using S & S Minifold I (Schleicher & Schuell) following manufacturer's instructions. Total RNA (up to 20 μg) was mixed with three volumes of denaturing solution containing 500 μL of 100% formamide, 162 μL of 37% formaldehyde and 100 μL of MOPS Buffer. The samples were incubated at 65°C for 15 min and then chilled on ice. Two volumes of ice-cold 20X SSC was added to each of the denatured samples. The samples were now ready to be loaded in their respective positions on the dot blot apparatus. The apparatus was assembled according to the Manufacturer's instructions and the slots that were not used were covered with parafilm and sealed tight. The transfer buffer used was 10X SSC. A vacuum of 40 mm Hg was applied. Suction was applied and 1 mL of 10X SSC was allowed to filter through. The samples were loaded followed by two rounds of 1 mL each of 10X SSC to the wells. The suction was left on throughout the process. The apparatus was disassembled and the RNA was immobilized on the membrane by baking at 80°C for 1 h. From this point on, the membrane was treated the same way as described earlier for northern blot analysis.

Preparation of Single Stranded cDNA

Two tube RT-PCR was performed according to the instructions of Qiagen Reverse Transcriptase handbook (Qiagen Inc., Valencia, CA). This was used as another approach to look at differences in expression of the mouse *odc* gene in different transformed cell lines. The single stranded cDNA was also used as s ource of DNA template to run PCR reactions using degenerate primers to design probes for genes of interest (described later). Total RNA from cell lines 2E, 2F and 8B, 10X buffer RT, dNTP mix, oligo dT primer, RNasin (RNase inhibitor) and RNase free water were all thawed on ice. A total of 20 μL reaction with 2 μg of RNA was needed per sample. All the reagents were mixed separately in each tube and were kept RNAse free throughout the setup. The reagents and the volumes used are given in Table III.

After the template RNA was added, the mix was vortexed for 5 sec and centrifuged to collect residual liquid from the walls of the tube. The tubes were incubated at 37^{0}C for 1 h. The reaction mixture was heated at 93^{0}C for 5 min and rapidly cooled on ice. PCR was carried out by taking an aliquot one-fifth of the final PCR volume. Five μL was added to a final volume of 25 μL. The PCR was run as per the reaction parameters for the respective gene. For example, the PCR reaction for the mouse *odc* was: 94^{0}C for 2 min, 94^{0}C for 30 seconds (denaturing), 62^{0}C for 30 seconds (annealing), 72^{0}C for 30 seconds for 35 cycles, 72^{0}C for 5 min and then hold at 4^{0}C. A 1 % agarose gel was run at 80V for 45 min, stained, destained and viewed under UV light.

In case of a DNA contamination in the RNA sample, a DNase treatment was done (though there was only one case observed). The reaction was set up as follows: RNA in water- 1-8 μL; RNase free DNase 10X reaction buffer-1 μL; RNase free DNase- 1u μg^{-1} RNA, and nuclease free water to a final volume of 10 μL. Two μg of RNA was treated with DNase for cDNA preparation. The samples were incubated at 37^{0}C for 30 min. One μL of DNase stop solution was added to terminate the reaction and then incubated at 65^{0}C for 10 min to inactivate the DNase. One to two μL of the treated RNA was added to the RT-PCR reaction.

Table III. The components used in making single stranded cDNA using the Qiagen Reverse Transcriptase handbook.

Component	Volume/ reaction	Final Concentration
10X buffer RT	2 μL	1X
dNTP mix (5mM each dNTP)	2 μL	0.5 mM each dNTP
Oligo-dT primer (10 μM)	2 μL	1 μM
RNasin (10 units/ μl)	1 μL	10 units
Omniscript Reverse Transcriptase	1 μL	4 units
RNase free water	Variable	
Template RNA	Variable	up to 2 μg 20 μL^{-1} reaction

NPT Assay

The concentration of the NPT II protein in the NT and 2E cell lines were determined using the NPT II Elisa kit (5 Prime→ 3 Prime, Inc., West Chester, PA) following the manufacturer's suggested protocol. Cells in 125 mL Erlenmeyer flask were allowed to settle to the bottom and 1 mL of the cells was taken in a 1.7 mL microfuge tube. The cells were pelleted by centrifugation (13000 X g) and resuspended in 200 μL of phosphate buffered saline (PBS) (8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 in 800 mL distilled water, adjusted to pH 7.4 with HCl, and brought to a total volume of 1 L with distilled water). The cells were frozen (-70^{0}C) and thawed (room temperature) three times, spun and then the supernatant was collected for determination of protein concentration. Dilutions of the cell extract were made to make sure the protein concentration fell in the linear range of the assay (20-150 pg 200 μL^{-1}). The dilutions were made in the dilution buffer provided in the kit. A four point standard curve was made by diluting the NPT II standard solution provided in the kit. The absorbance was measured at 405 nm using Program KC Junior on an ELISA microplate reader (Bio-Tek Instruments EL 311).

Ornithine decarboxylase (ODC) and Arginine decarboxylase (ADC) Enzyme Assays

The decarboxylation rates of ODC and ADC were measured in intact cells using their respective radioactive substrates (Minocha et al., 1999a). The reaction was carried out in 16 X 100 mm Kimble test tubes. The reaction mix contained 100 mg fresh weight of intact cells in 250 μL of 0.1 M Tris-EDTA buffer (pH 6.8 for mouse ODC and 8.4 for plant ADC and ODC) containing 5 mM pyridoxal phosphate (Sigma, P-3657), 1 mM DTT (Sigma, D-9779), the labeled substrate in 50 μL (0.05 or 0.1 μCi of [1-^{14}C] Orn, specific activity 58 mCi $mmol^{-1}$ (Moravek Biochemicals, Brea, CA., Cat # 142-173-953) or 0.1 μCi DL-[1-^{14}C] Arg, specific activity 57 mCi $mmol^{-1}$) (Amersham Life Sciences, Elk Grove, IL 60007., Cat # CFA.434) and the unlabeled substrate (2 mM L-Orn, Sigma, O-2375 or L-Arg, Sigma, A-5006). The total reaction volume was 300 μL. The reaction tubes were incubated at 37 ^{0}C for 60 min at 85 rpm in a shaking water

bath. The tubes were fitted with a rubber stopper holding a polypropylene well (Kontes Scientific Instruments, Vineland, NJ). Each well contained a 2 cm^2 Whatman 3MM filter paper soaked with 50 µL Scintigest (Fisher Scientific, Lot # 872729; Fair Lawn, NJ). The $^{14}CO_2$ liberated during the reaction was adsorbed on the paper. The reaction was stopped by injecting 0.5 mL of 0.5 N sulfuric acid into each tube through the stopper and the tubes were incubated for an additional 30 min at 37°C at 85 rpm to adsorb all of the released $^{14}CO_2$. The filter paper was removed and counted for radioactivity in 10 mL of Scintilene (Fisher Scientific, Lot # 980805; Fair Lawn, NJ) in Beckman-7000 LSC (Beckman, Fullerton, CA; LS 6000SC).

For inhibitor effects, 50 µL of either 60 mM DFMO or 6 mM DFMA stocks were used to achieve the desired concentration. The enzyme was pre-incubated with the inhibitor for 15 min at 37°C prior to the addition of substrate. The decarboxylation rates were expressed as $^{14}CO_2$ (nmol h^{-1} g^{-1} FW). The reaction components were mixed while the tubes were kept on ice before incubation at 37°C.

Calculations for determining the rates of decarboxylation:

DPM of sample-DPM blank = DPM recovered

DPM added = 0.1 µCi approximately = 2.2 X 10^5 dpm (actually counted)

DPM recovered/ DPM added X final substrate concentration in reaction = nmol CO_2 h^{-1}

nmol CO_2 h^{-1}/ amount of tissue = nmol CO_2 h^{-1} g^{-1} FW

Incorporation of Labeled Precursors into Polyamines

Incorporation of labeled precursors into polyamines was studied in NT and 2E cell lines. Seven day old cultures from several flasks were pooled on the day of subculture. Three flasks were used for each of the two cell lines. Throughout the study, maintenance cultures were started on Fridays. The cultures for these experiments were started by subculturing twice the volume (i.e. 14 mL cell suspension in 50 mL medium) and the cells were grown in kanamycin-free medium. After 3 d of culture, the flasks were allowed to sit in the laminar flow hood to settle cells to the

bottom and top 10 mL of the medium was removed from each flask. A sterile 500 mL Florence flat bottom flask was placed on a magnetic stirrer at low speed in the hood and the contents of the flasks were pooled in it. The magnetic stirrer was at a low speed so as not to stress the cells. The cells were transferred in 10 mL aliquots into 25 mL Erlenmeyer flasks to obtain a cell density of about 1 g per flask. To each flask, 25 μL of either 0.2 or 0.5 μCi of [U-^{14}C] Orn (specific activity 261 mCi mmol^{-1}, Amersham Radiochemicals, Cat # CFB.180) was added and the flasks were fitted with a polypropylene well containing 2 cm^2 3MM filter paper soaked with 50 μL Scintigest (Robie and Minocha, 1989). The flasks were capped immediately after addition of radioactivity and incubated for various lengths of time at 25^{0}C at 100 rpm on a gyratory shaker.

At the end of incubation, the cap was removed and the filter paper was transferred to a scintillation vial for counting of radioactivity to determine the rate of decarboxylation of [U-^{14}C] Orn. To each flask containing cell suspension, cold Orn was added to a final concentration of 2 mM (a total volume of 1 mL). The cells were collected on Miracloth by vacuum filtration, washed with 5 mL of 2 mM ice-cold Orn and weighed. Half a gram of cells were stored in 1 mL of 7.5% PCA at -20 ^{0}C. In order to study the effect of DFMO, 250 μL of 200 mM DFMO stock was added to 10 mL of culture. The flasks were incubated with the inhibitor for 15 min prior to the addition of the labeled substrate. Inhibitor treated samples were also tested for decarboxylation and incorporation of the labeled Orn into polyamines.

Rate of $^{14}CO_2$ production from [U-^{14}C] Orn was determined in another way. The rate of decarboxylation was studied every hour up to 8 h. In this case, the cells were collected only at the end of the experiment (i.e. at 8 h) while the filter paper was replaced quickly in the same flask after adding 50 μL of Scintigest at 1 h intervals. In other words, the $^{14}CO_2$ adsorbed on the filter paper was being released from the same flask at different time periods. Cells were collected in the same way as described before. The effect of 2 mM DFMO was also studied. The cells were incubated for 20 min before addition of the labeled substrate.

The rate of $^{14}CO_2$ production and incorporation of [U-^{14}C] Glu into polyamines was studied with 3 d old cells. The rate of $^{14}CO_2$ production was studied every hour up to 4 h and the incorporation was studied at the end of 4 h. Three cell lines were tested: NT, 2E and NT-HP. Three flasks each of the three cell lines (14 mL cells in 50 mL medium) were started on the day of subculture. When the cells were 3 d old, the cells were allowed to settle to the bottom of the flask in the hood. Top 10 mL of the medium was removed from each flask and the rest of the cell culture was swirled gently and pooled in a 500 mL Florence flat bottom flask. The Florence flat bottom flask was kept on a stirrer and 10 mL of cells were pipetted out in 25 mL flasks for the experiment. One μCi (20 μL) radioactivity was added to each experimental flask. Fifty μL Scintigest was added to the filter paper, which was placed in the well hanging from the cap and the flasks were capped immediately. The filter paper containing Scintigest was replaced every hour and the rate of $^{14}CO_2$ production was measured at 0-1 h, 1-2 h, 2-3 h and 3-4 h. The incorporation into polyamines was studied at the end of 4 h.

The samples were dansylated as per the protocol for radioactive samples described below. Dansyl-polyamines were separated on the TLC plate and the fractions of the respective polyamines scraped and counted for radioactivity.

Dansylation of Radioactive [^{14}C] Samples

For analysis of [^{14}C] precursor incorporation, 500 mg of cells were mixed with 1 mL of 7.5 % PCA (w/v) and frozen in 2 mL microfuge tubes. The samples were frozen and thawed three times as for non-radioactive samples. The samples were vortexed for 1.5 min and then centrifuged (14000 x g for 7 min). One mL of the PCA extract was dansylated in 2 mL microfuge tubes with modifications to the procedure described in Andersen et al. (1998). The PCA extract was distributed in 500 μL aliquots in two separate 2 mL microfuge tubes. A parallel set of 0.4 mM standards of polyamines (Sigma) was also prepared in the same way. To the PCA extract, 500 μL of the saturated sodium carbonate and 250 μL dansyl chloride (40 mg mL^{-1} acetone) were

added. The samples were vortexed for 50 sec and incubated for 2 h at 60°C. Samples were briefly centrifuged after allowing to cool for 5 min. After incubation, the reaction was terminated by adding 50 μL L-proline or L-alanine (100 mg mL^{-1} distilled water). Then the samples were incubated again for 30 min at 60°C to use up excess dansyl chloride. The samples were allowed to cool down and briefly centrifuged to spin down any liquid sticking to the sides of the microfuge tubes. The samples were placed in the SpeedVac with caps wide open for 7-8 min to evaporate acetone. The dansyl-polyamines were extracted in 500 μL of the toluene. Four hundred microliter of the toluene fraction from two replicate tubes representing the same cell sample were pooled together. Thus, each tube had 800 μL of toluene. The samples were vortexed and allowed to sit undisturbed for at least 5 min to separate the two phases and then centrifuged at 14000 X g for 1 min. A 20 μL aliquot of toluene and 20 μL of the aqueous phase were counted for radioactivity. The pH of the aqueous phase was also determined after removing the extra toluene phase. The aqueous fraction mostly contained the unused [^{14}C] Orn taken up by the cells or polyamine metabolites that are not partitioned into toluene, and provides data on the uptake of labeled substrates. Aqueous phase was counted in 10 mL Scintiverse (Fisher Scientific, S-X12-4). In some experiments, 20 μL aliquot of the PCA extract was also counted. Eight hundred μL of the toluene fraction was dried in a SpeedVac for 30 min and reconstituted in 50 μL of methanol. Of this, 2 μL was counted for radioactivity and 45 μL were spotted on 5 x 25 cm TLC plates (Whatman LK6D silica gel 60; Whatman Inc. Cat #4865-62). Each sample was loaded nine times (5 μL per loading) to get a concentrated spot and the loaded sample was allowed to dry using a dryer between the loadings.

A TLC chamber of 30 X 25 cm size was used. A Whatman filter paper of size 20.3 cm X 25.4 cm was placed in the chamber to saturate it with the solvent. The solvent mixture consisted of a 5:1 mix of chloroform (HPLC Grade, Sigma, C-2432) and triethylamine (Sigma, T-0886). When the solvent front had moved 15 cm from the origin, which took about 45 min, the plates

were removed from the chromatography chamber, air-dried and viewed under UV light in the Nucleovision 760 Gel Documentation System and photographed. The bands corresponding to the three polyamines were identified by the standards, marked with a lead pencil, scraped by using a pointed spatula and counted for radioactivity in Scintilene. The scrapings were collected on weighing paper (Fisher Scientific, 09-898-12A). To determine the identity, each of the polyamine standards (10 mM) was loaded separately and the R_f values were calculated for each polyamine. The polyamine standards were also mixed in different combinations and loaded.

Radioactivity tied up in Bands other than Polyamines

For some experiments, other fluorescent bands were also scraped to see if they accumulated significant amounts of radioactivity. In addition, in some cases, the TLC plate was scraped every 0.5 cm starting from the origin to the solvent front and the powder counted for radioactivity.

Effect of Cycloheximide on the Mouse ODC Enzyme Activity

The effect of cycloheximide (CHA), an inhibitor of protein synthesis, on the mouse ODC enzyme activity was determined with 3 and 4 d old cells. Only the cell line 2E was used. The routine amount of cells subcultured (i.e. 7 mL in 50 mL medium) in 125 mL flasks was used for the experiment. A total of four flasks were used (two with and two without the inhibitor). To two of the flasks, 75 μL (final concentration of 75 μg mL^{-1}) of 50 mg mL^{-1} CHA stock (made in 100% ethanol) was added. Aliquots were taken out from each of the flask (100 mg fresh weight cells) at different time periods and the reaction was set up as follows: Cells in 0.1 M Tris-EDTA buffer (pH 6.8 for mouse ODC) containing 5 mM pyridoxal phosphate (Sigma, P-3657), 1 mM DTT (Sigma, D-9779), the labeled substrate in 50 μL (0.05 or 0.1 μCi of [1-^{14}C] Orn, specific activity 58 mCi $mmol^{-1}$), containing unlabeled substrate (2 mM L-Orn; Sigma, O-2375) was added to a

final volume of 350 μL. The time periods tested were: 0 h, 30 min, 1, 2 and 4 h. The set up was the same as for ODC and ADC enzyme assays described earlier.

Effect of Inhibitors (DFMO and DFMA) on Polyamine Levels

The effect of 1 and 2 mM DFMA and 2, 5 and 10 mM DFMO was tested on 3 d old NT and 2E cells for polyamine levels. Both short term (4, 8 and 24 h) and long term duration (72 h) studies were performed. Two 500 mL Florence flat bottom flasks, one having 350 mL medium and the other having 200 mL medium were used. On the day of subculture, i.e. Friday, 49 mL of 7 d old culture were added to 350 mL medium and 28 mL were added to 200 mL medium. Ten mL of cells were pipetted under sterile conditions into 50 mL Erlenmeyer flasks, which were incubated on a shaker. On Monday, the respective inhibitor treatments were given to the cells. Inhibitors of desired concentrations were added in the range of 40 μL to 500 μL in a total of 10 mL medium. Control (no inhibitor) treatments were kept for each cell line in each experiment.

The experiment was also set up in another way where three flasks of 3 d old cells subcultured in the ratio of 50 mL medium to 14 mL of 7 d old cells for NT and 18 mL of 2E cells were used. On the third day, the three flasks of each cell line were pooled in a 500 mL Florence flat bottom flask and 10 mL aliquots were transferred to 25 mL Erlenmeyer flasks. The inhibitors were added as before. Following appropriate periods of incubation, cells were collected by vacuum filtration, and stored in 5% PCA. (1:4 ratio of cells: PCA, w/v). After freezing and thawing three times, the samples were dansylated and cellular polyamines were analyzed as described earlier.

Supplementation with Non-labeled Precursors and Inhibitors

The effects of several non-labeled precursors of the polyamine biosynthetic pathway, such as, L-Arg, L-Orn, L-Gln, L-Glu, urea, L-citrulline, L-lysine and agmatine on the cellular levels of polyamines were tested in the NT and 2E cells. The compounds and concentrations used

are given in Table IV. On the day of subculture, 49 mL of culture was added to 350 mL fresh medium and 28 mL was added to 200 mL fresh medium. Aliquots of 10 mL each were transferred to 50 mL Erlenmeyer flasks, cultured for three days and the respective treatments given using appropriate stocks of the test compound (see Table IV).

Following 24 h and 72 h incubation on a gyratory shaker, cells were collected by filtration and processed for polyamine analysis as described above. In addition, specific inhibitors in the nitrogen (N) assimilation pathway (such as, MSX for GS and gabaculine for OAT) were also used to determine their effect on cellular polyamine levels. The pH of the medium was monitored for the MSX experiments. Samples were collected at time zero to determine polyamine levels at start of the experiment.

Effect of Jasmonic Acid (JA) and Salicylic Acid (SA) on Polyamine Levels

The effect of 2, 10 and 50 μM JA (Sigma, J-2500) and 0.2, 1 and 5 mM SA (Sigma, S-6271) was tested on the cellular polyamine levels in NT and 2E cell lines. Three day old cells were treated with appropriate amounts of JA and SA in 10 mL culture in 50 mL Erlenmeyer flasks and the cells collected after 24 h and 72 h of treatment. The experimental set up was the same as described above for precursors and inhibitors. Salicylic acid powder was dissolved in 100% ethanol, as it was insoluble in water. Samples without SA (blank) were given respective μL of 100 % ethanol and were used as controls. Samples were collected at 0 h also to determine polyamine levels at the time of treatment.

Glutamine Synthetase Assay

Pea seeds were used as a positive control on the first trial of the experiment as they have significantly large amounts of this enzyme. Pea seeds were soaked in distilled water overnight and fully imbibed pea seeds (approximately 50 g fresh weight) were homogenized in a blender in 50 mL of cold tricine buffer (0.1M Tricine-KOH, pH 7.8; 20 mM $MgSO_4$, Na_2H_2 EDTA). The

Table IV. The compounds and concentrations of non-labeled precursors used.

Compound	Final Concentration in 10 mL culture
L-Arginine	10 μM, 50 μM, 250 μM, 500 μM, 2 mM, 5 mM, 10 mM
L-Ornithine	50 μM, 250 μM, 500 μM, 1 mM, 2 mM, 10 mM
L-Lysine	2 mM, 10 mM
Citrulline	2 mM, 10 mM
L-Glutamic acid	500 μM, 2 mM, 5 mM, 10 mM
L-Gutamine	250 μM, 500 μM, 1 mM, 2 mM, 5 mM, 10 mM
Urea	10 μM, 50 μM, 250 μM, 2 mM, 10 mM
GABA	1 mM
Agmatine	1 mM, 2 mM, 5 mM, 10 mM
L-MSX	20 μM, 100 μM, 1 mM, 5 mM
Gabaculine	10 μM, 50 μM, 100 μM, 1 mM

crude homogenate was filtered through Miracloth and the filtrate was centrifuged at 15000 rpm at 4^0C. The supernatant was used for the enzyme assay.

For poplar, cells from three flasks for each of the two lines were vacuum filtered, pooled and ground in cold Tricine buffer. The extract centrifuged at 15000 rpm at 4^0C was used for the assay. The assay was done at two time periods (0 and 20 min) and there were three replicates for each cell line. The following components were mixed: 0.6 mL Tricine buffer, 0.2 mL Glu solution (0.6 M), 0.1 mL hydroxylamine solution in water (96 mM), and 0.5 mL extract. The remaining extract was frozen immediately. The test tubes were incubated at 30^0C for 2 min in the a water bath. After incubation, 0.2 mL ATP solution (60 mM, neutralized to pH 6.9) was added to start the reaction. After 20 min, the reaction was stopped by adding 1.6 mL of stopping reagent (0.37 M $FeCl_3$, 0.67 N HCl). For blanks, the reaction was stopped by adding the stopping reagent immediately after adding ATP. The absorbance was read at 540 nm. Both standard and the zero time blanks were used in reading the absorbance. The standard blank contained all the mixtures of the assay except the extract. The standard curve was generated and used to quantify enzyme activity. The experiment was repeated three times.

Effect of Removal of Nitrogen Source in the Medium

Three day old cells of NT and 2E cell lines were used for this study. Four flasks of each cell line were used. Twice the normal volume of cells was subcultured (14 mL cells: 50 mL medium) for experimental flasks on Friday. On the day of the experiment (3 d old cells), the flasks were left undisturbed in the hood for 10 min and top 21 mL of the medium was removed. The contents were swirled and transferred to a 50 mL sterile centrifuge tube. The cells were spun at 1000 rpm in a swinging bucket rotor for 3 min to settle the cells and were then washed twice with modified MS Medium containing no N. This was achieved by having no ammonium nitrate (NH_4NO_3) in the medium and potassium nitrate (KNO_3) was replaced with potassium chloride of the same molarity.

Four Florence flat bottom flasks (500 mL) having 60 mL medium (two flasks with and two without N) were made for the two cell lines. Additionally, two 125 mL flasks having 30 mL medium with and without N each were made for each of the two cell lines. The cells in one of the centrifuge tubes were suspended in medium from one of these 125 mL flasks. Then, it was finally added to the round bottom flask having 60 mL medium already in it. The Florence flask was placed on the magnetic stirrer in the hood. The cells sticking to the centrifuge tube were washed with the remaining 30 mL medium in the 125 mL flask. This gave a final volume of 120 mL in the round bottom flask. This was done to achieve a desired concentration of about 1 g of cells per 10 mL of medium. Ten flasks of each cell line were made with 10 mL of medium in 25 mL flasks. Each flask provided two replicate samples and each treatment for each of the two cell lines was given in two separate flasks. Cells were collected at 0, 4, 12, 24, 48 and 96 h and processed for total fresh weight and polyamine analysis. The experiment was repeated twice.

Effect of Different Concentrations of NH_4NO_3 and NH_4Cl in the Medium

Six flasks of 3 d old cultures of NT and 2E each were taken and allowed to settle in the hood. Top 10 mL of medium was removed from each flask and the rest was transferred to sterile 50 mL centrifuge tubes. The cells were centrifuged at 1100 rpm for 2 min to settle the cells and not stress them. Once the cells had settled to the bottom, the medium was decanted. Now, the cells were washed twice with MS salts (premix powder without NH_4NO_3, Sigma, M-2909). The centrifuge tubes were gently inverted to allow uniform washing of the cells. The tubes were spun at the same speed again and the top wash medium was discarded.

To each centrifuge tube, approximately 42 mL of MS medium (without NH_4NO_3) was added and the contents were poured directly into a 500 mL sterile Florence flat bottom flask in the hood. Finally, 10 mL aliquots were distributed into 50 mL sterile flasks. Now, to these 50 mL flasks containing 10 mL of culture the following treatments were given:

(1) Control (untreated, unwashed cells).

(2) No ammonium nitrate.

(3) Normal concentration of ammonium nitrate (control for ammonium nitrate free MS medium washed cells) i.e. 20 mM

(4) One-fifth concentration of ammonium nitrate. i.e. 4 mM

(5) Ammonium chloride i.e. 20 mM

This experiment was done by Martha Munkaje.

The cells were collected as described before at 24 h and 72 h as well as at time zero (i.e. day of subculturing), mixed with PCA and frozen.

Putrescine Turnover Experiments

At the time of starting the experiments, twice the normal amount (i.e. 14 mL vs. 7 mL) of 7 d old NT and 2E cells were added to 50 mL of fresh culture medium in order to achieve a cell density of about 1 g per 10 mL of culture. After 3 d of culture, the flasks were placed in the hood for 10 min to let the cells settle at the bottom of the flask, and 24 mL of the medium was decanted from the top. To the remaining cells in the flask, either 50 μL of radioactive Put (1 μCi of [1,4-^{14}C] Put; specific activity 107 mCi mmol^{-1}, Amersham Life Science; Cat # CFA.301) or 20 μL of radioactive Orn (1μCi of [U-^{14}C] Orn; specific activity 261 mCi mmol^{-1}, Amersham Life Science; Cat # CFB.180) were added and the flasks placed back on the shaker. Following incubation with the radioactive substrates for 2 h, the suspensions were transferred to 50 mL sterile polypropylene centrifuge tubes and centrifuged at 1000 rpm in a swinging bucket rotor for 3 min to pellet the cells. The medium was decanted, the cells were washed with 30 mL fresh MS medium per tube by centrifugation, the pellet transferred to 50 mL fresh medium, and placed back on the shaker. Aliquots (5-8 mL) were removed from each flask at different times (0, 2, 4, 8, 24, 72 and 96 h for [U-^{14}C] Orn treated cells and 0, 2, 4, 8, 24, 72 and 84 h for [1,4 ^{14}C] Put treated cells). The cells were vacuum filtered on Miracloth, weighed and mixed with 7.5% PCA (w/v) (1 g cells: 2 mL of PCA), and frozen at -20° C for polyamine analysis by TLC and HPLC.

Tissue samples in PCA were frozen (-20^{0}C overnight) and thawed (3 to 4 h at room temperature) three times before dansylation (Minocha et al., 1994). After final thawing, the cells were vortexed and centrifuged at 14000 g for 10 min. For determination of the efficiency of extraction of polyamines and other radioactive derivatives, the pellet from the above centrifugation step was re-extracted twice with 2 mL of 5% PCA, and aliquots from each fraction counted for radioactivity. For analysis of radioactivity in different polyamines, 1 mL of the first supernatant fraction was dansylated as described earlier. The dansylated polyamines were reconstituted in 50 μL of 100% methanol. Radioactivity in the toluene and aqueous fractions was counted in order to follow the recovery of radioactive polyamines through various steps. Of the 50 μL methanol, 2 μL was counted to get an estimate of the number of counts in methanol fraction and 3.74 μL was added to 496.26 μL of 100% methanol in autosampler vials and used for determination of total cellular polyamines by HPLC. From the remaining extract, 40 μL of each sample was loaded on the TLC plate and the plates were developed as described earlier and the polyamine bands visualized under UV light. These bands were marked with a lead pencil, scraped and counted for radioactivity. Specific activity of each polyamine at each time period was calculated as dpm per nmol of the respective polyamines. Determination of initial half-life of Put (L_{50}) was done using the radioactivity data for the first 8 h only. Half-life of Put was determined using both substrates (Orn and Put). A linear regression was done ($y = mx + c$) on the first 8 h where, y was half the dpm value obtained in the Put fraction at time zero) and the value of 'x' (time) was calculated. The slope and the intercept values were obtained from the linear regression equation. The experiment was done three times and the data were pooled from three experiments for Orn feeding and two experiments for Put feeding.

Calculations for the determination of the amount of radioactive methanol to be taken for HPLC analysis

Original amount of sample in 1 mL PCA extract: 500 mg, thus total PCA extract is 1.5 mL.

Amount of PCA extract dansylated: 1mL thus, amount of sample dansylated is 333 mg equivalent.

Amount of sample in 1mL toluene is 333 mg and the amount in 800 μL is 266 mg. This was reconstituted in 50 μL methanol.

266 mg represents 50 μL methanol and 10 mg amount of cells is represented by 1.87 μL methanol. Ten mg of cells in 1 mL methanol represents a factor of 100. This is the factor incorporated for the analysis of non-radioactive samples. So, in these experiments taking 3.74 μL of radioactive sample in a total of 500 μL methanol represents a factor of 25, which, was used for analysis.

Calculations for Putrescine Loss and Conversion of Putrescine to Spermidine

Following the determination of radioactivity in different polyamine fractions by TLC and the amounts of total soluble polyamines in the cells by HPLC, the following formulae were used for various calculations presented here.

1. Loss of Put over 2 h = [(dpm in Put at 0 h - dpm in Put at 2 h) / dpm in Put at 0 h] X Mean[a] amounts of Put at 0-2 h.
2. Loss of Put over 8 h = [(dpm in Put at 0 h - dpm in Put at 8 h) / dpm in Put at 0 h] X Mean[b] amounts of Put at 0-8 h.
3. % loss of Put (24 h to 72 h) = [(dpm in Put at 24 h - dpm in Put at 72 h) / dpm in Put at 24 h] X 100.

4. Specific activity of polyamines = dpm in polyamine g^{-1} FW / nmol polyamine g^{-1} FW at a given time.
5. Amount of Put converted into Spd in 8 h = [(dpm in Spd at 8 h - dpm in Spd at 0 h) / Mean[c] dpm in Put at 0-8 h] X Mean[b] amounts of Put at 0-8 h.

Where: Mean[a] Put = [nmol Put at 0 h + nmol Put at 2 h] / n; n = total number of samples;

Mean[b] Put = [nmol Put at 0 h + nmol Put at 2 h + nmol Put at 4 h + nmol Put at 8 h] / n; n = total number of samples

Mean[c] dpm in Put = [dpm Put at 0 h + dpm Put at 2 h + dpm Put at 4 h + dpm Put at 8 h] / n; n = total number of samples

Percent Recovery of Radioactivity in the PCA Extract and the Polyamines

The percentage recovery in the PCA extract was done at three steps from the amount of radioactivity present in the PCA fraction. A 20 μL aliquot of the PCA fraction (PCA I) was counted. The left over PCA was removed; the pellet was extracted with fresh 1 mL of 7.5% PCA and a 20 μL aliquot (PCA II) was counted again. This PCA was removed and then the pellet was once again extracted with fresh 1 mL of 7.5% PCA. A 20 μL aliquot was counted (PCA III) and the cell pellet was left re-suspended in the newest PCA. This analysis was done to see if most of the counts were recovered in the PCA extract in the first extraction.

Percent recovery at each step of dansylation was done by counting a fraction at each step (i.e. toluene and aqueous fractions from the dansylated PCA extract), (methanol fraction obtained from the dried toluene fraction), and respective polyamines on the TLC plate.

Putrescine Secretion into the Medium

Secretion of Put into the medium was studied at 0, 2, 4, 8 and 24 h using [U-^{14}C] Orn treated cells. The set up was the same as for studying Put turnover experiments. The cells were fed with 1 μCi 20 μL^{-1} radioactive substrate and washed once with label-free medium. One mL of

the medium was collected at the respective time periods and 92 μL of 60% PCA was added to achieve a final concentration of 5% PCA in the sample. Before the samples were dansylated, PCA counts were taken to determine the amount of radioactivity present in the medium.

Diamine oxidase Enzyme Characterization

Three to six day old cells were used to check the optimum conditions for measurement of the enzyme activity. Different fresh weights and incubation times were tested to check the linear range of the enzyme assay. The range of fresh weight tested was 10 mg to 300 mg. The incubation times tested ranged from 15 min to 120 min. The optimum pH for enzyme activity was checked at pH 6.0, 7.0 and 8.0 (Santanen, 2000). Live, frozen-thawed as well as homogenized cells were tested for enzyme activity (Minocha et al., 1999a). The cells were frozen-thawed three times. Cells (0.4 g) in 1.1mL of 0.1 M K^+ phosphate buffer, pH 8.0) were homogenized using a Polytron homogenizer (Brinkmann, Littau, Switzerland) for 90 sec at 20000 rpm. After homogenization, the samples were spun at 14000 X g for 5 min at 4^0C. The supernatant was used for the assay. This comparison was done after results for all the other parameters were obtained. This represented the same ratio as 0.2 g cells in 550 μL buffer used for frozen-thawed cells.

Diamine oxidase Enzyme Assay

A seven day study was conducted for the polyamine levels and the DAO activity. The cell lines tested were NT, 2E and NT-HP. Cells were collected by vacuum filtration and samples for both polyamine analysis and DAO activity were collected from the same cell pellet. For determining DAO activity, 200 mg of cells were suspended in 550 μL of 0.1 M potassium phosphate buffer, pH 8.0. The cells were placed in a 2 mL microfuge tube and frozen-thawed three times and vortexed prior to assay. Thawing was done on ice to prevent denaturation of the enzyme. To each tube, 50 μL of labeled substrate (0. 1 μCi of [1,4 ^{14}C] Put in 1.0 mM cold Put) were added, the tubes were vortexed briefly, and they were placed on a shaker (100 rpm)

horizontally at 37^{0}C. Following incubation, the reaction was terminated by adding 150 µL of saturated sodium carbonate. Labeled Δ^1-pyrroline was extracted immediately by partitioning with 500 µL of Photrex grade toluene. The samples were vortexed for 1 min and centrifuged at 10000 rpm for 5 min. Three hundred µL of the toluene layer was removed and counted for radioactivity in 10 mL of Scintilene (Fisher Scientific). The enzyme activity was expressed as nmol Δ^1-pyrroline. h^{-1}, g^{-1} FW. This experiment was done four times.

Metabolism of [^{14}C] Spermidine and Spermine

The experimental cultures were started by pooling and transferring 14 mL of 7 d old culture (instead of 7 mL) to 50 mL medium from two or three 7 d old flasks. Three day old NT and 2E cells in 125 mL flasks were allowed to settle for a few min and top 24 mL of the medium was decanted using a pipette. To the remaining suspension, 1µCi of [^{14}C] Spd. tri HCl (specific activity 112 mCi $mmol^{-1}$, Amersham Life Science; Cat # CFA.512) was added and the cells placed back on the shaker. Following 2 h incubation with radioactive substrate, the cells were transferred to sterile 50 mL polypropylene tubes and centrifuged at 1000 rpm for 2 min. The supernatant was decanted and the cells were washed once with 30 mL of label free MS medium. The cells were spun again, the supernatant decanted and the cells transferred to 50 mL of fresh MS medium in 125 mL Erlenmeyer flasks. The flasks were incubated on the shaker and samples were collected at different times after transfer of cells to fresh medium i.e. 0 h, 2 h, 4 h, 8 h, 24 h and 72 h. Five to eight mL aliquots were removed with a sterile pipette and filtered through Miracloth under vacuum to collect the cells.

The turnover of Spm was determined the same way using 1µCi of [^{14}C] Spm. tetra HCl (specific activity 110 mCi $mmol^{-1}$, Amersham Life Science; Cat #CFA.511). Following 2 h incubation, the cells were washed with label-free MS medium, transferred to label-free medium and samples were collected at time of transfer to fresh medium (i.e. 0 h), and subsequently at 4 h,

8 h, 24 h, 48 h and 72 h. In both the cases, the cells were mixed with 7.5% PCA (w/v) (0.5 g cells in 1 mL of PCA), and frozen at –20°C for polyamine analysis by TLC and HPLC. The loss of polyamines during dansylation was followed the same way as for Put. The PCA fraction (20 μL) was counted for determining the recovery of radioactivity during the processing of samples. For incubations with [^{14}C] Spm, the radioactivity bound to the pellet was also determined. This was done, as the radioactivity in the 2E cells did not decrease with time (discussed later). The pellet was washed twice with 1 mL of 5% PCA after removing all the remaining original PCA. The pellet was resuspended in 0.5 mL of 6N HCl and allowed to digest overnight at 80^{0}C. The samples were vortexed and then centrifuged. Fifty μL of the digested pellet was counted in 10 mL of Scinitiverse.

Calculation for % Back Conversion= dpm in polyamine/ dpm in toluene at time tested x 100.

Statistical Analysis

For all experiments involving quantitative analysis, 3 or 4 replicates were used for each treatment. Each experiment was repeated at least twice, most being repeated 3-4 times. The data were subjected to Analysis of Variance using SYSTAT version 7 or 9. Student t-test was used to determine significance at $p \leq 0.05$. Data for the turnover experiments are from three combined experiments. Data for all other experiments come from a single representative experiment unless otherwise specified.

Effect of Put overproduction on the *adc, Spd synthase, gad,* and *mouse odc* Expression

The expression levels of *adc*, *Spd synthase*, *gad* and mouse *odc* were tested on 1, 3, 4 and 6 d of the week. *G3PDH* was used as as internal control. The objective was to establish a baseline study of the RNA levels of these genes over the subculture cycle in the two cell lines, NT and 2E used throughout this study.

To achieve this objective, RNA from NT and 2E cells was isolated as described earlier from 1, 3, 4 and 6 d old cultures. Formaldehyde denaturing gels were run as explained earlier and the RNA was checked for integrity prior to blotting. Gels used for checking the quality of RNA were not used for blotting as staining reduced the transfer efficiency dramatically. The expression levels of the gene of study were determining by normalizing the data with the expression level of *G3PDH*. The amount of RNA loaded was 18 μg per sample in a total volume of 4.5 μL.

Expression level of gene on day X: Intensity/ Area of gene divided by the Intensity/ Area of *G3PDH* of the respective sample.

For these set of analysis, chemiluminiscent detection was done instead of colorimetric detection that was used earlier. The detection buffer used was the same as for colorimetric detection except that the buffer did not contain Mg^{++} in this case. The membrane was placed in detection buffer for 5 min. Five hundred microliter (20-30 drops) of CSPD (Disodium-3-(4-methoxyspiro{ 1,2-dioxetane-3,2'-[5' chloro] tricyclo[3.3.1.1$^{3/7}$] decan}-4-yl) phenyl phosphate solution (Roche Biochemicals, Cat # 1755633) was spread on the membrane, membrane was wrapped in Saran wrap, placed at room temperature for 5 min and incubated at 37^{0}C for 10 min. After this, excess CSPD solution was squeezed out and the membrane was wrapped tightly so that no air bubbles were present. The wells were then marked with fluorescent glow on the saran wrap and the film was exposed for 15-45 min to achieve optimal signal.

The probes used in this study were specific to poplar. Degenerate primers and primers from other plant species were used to perform a gradient PCR reaction. PCR products were sequenced twice (forward and reverse primer) and then used for making probes. Dr. Lee had cloned PCR products for the *adc* and *G3PDH* genes into TOPO vector and plasmid minipreps were done for these genes.

Plasmid Miniprep

Plasmids were isolated following the Promega Miniprep kit (Promega, Madison, WI; Cat # A7500;). The *E.coli* culture in 4 mL LB medium containing 100 μg mL^{-1} kanamycin was grown overnight and the cells were spun in a 1.5 mL centrifuge tube at 10000 rpm for 15-30 sec. The pellet was gently resuspended in 200 μL cell resuspension solution (50 mM Tris HCl, pH 7.5, 10 mM EDTA and 100 μg mL^{-1} RNAse). To this solution, 200 μL of cell lysis solution (0.2M NaOH and 1% SDS) was added and mixed by inversion. Then, 200 μL of ice-cold cell neutralization solution was added and mixed by inversion. The samples were spun at 14000 rpm for 10 min. Five hundred μL of the supernatant was transferred to a new tube using a 1 mL pipette with cut tips. To the supernatant, 500 μL of isopropanol was added, mixed by inversion and placed at -20^{0}C for 30 min. The samples were spun at 14000 rpm for 15 min at 4^{0}C. The supernatant was discarded; pellet was washed with 95% ethanol and dried in the SpeedVac. The pellet was resuspended in 50 μL water, left at 50^{0}C for 1 h for maximum elution and then quantified. Plasmid DNA for poplar *adc* and *G3PDH* were isolated using this protocol.

Primer Design

Program Megalign was used to align sequences for the gene of interest from three different species if the sequence of gene of interest was not available from *Populus* species. Degenerate primers were designed from conserved regions using OLIGO 4.0 (National Bioscience Inc., Plymouth, MA).

Primer Sequences

The sequences of the primers used were:

Actin (*Porphyra yezoensis*)

Forward primer (34): 5' TGT GAC AAT GGA ACA GGA ATG 3'

Reverse primer (1071): 5' CAT CTG CTG GAA GGT GCT GAG 3'

G3PDH (*Porphyra yezoensis*)

Forward primer (374): 5' TCS AAG GAY GCS CCN ATG TTY GT 3'

Reverse Primer (937): 5' TAN CCC CAY TCD TTD TCG TAC CA 3'

GAD (*Arabidopsis thaliana*)

Forward primer: 5' CTY CAG AAY CGM TGY GTR AAC ATK ATA 3'

Reverse Primer: 5' AAT ACC AGC ATA GAC MAG HCC ATA YTT GTG 3'

Spd synthase (*Datura stramonium)*

Forward primer (119): 5'CTG GGG AAG CAC ACT CAT TG 3'

Reverse primer (916): 5' TTC GCA AAA GAT GGC AAA CA 3'

ADC (*Lycopersicon esculentum*)

Forward primer (845): 5' GCA GGG CAA TTG TTT CTC ACC AT 3'

Reverse primer (1419): 5' ATA AGC CCC ACC CAA AAA CAT CC 3'

Sequences for the mouse *odc* gene are already given. The PCR profile used for *actin* was: denaturing step of 95^{0}C for 2 min, followed by 94^{0}C for 30 sec, annealing temperature of 63^{0}C for 30 sec and then elongation at 72^{0}C for 1 min. This was repeated for 30 cycles and then a final extension at 72^{0}C for 5 min. The profile for *G3PDH* as well as *Spd synthase* was the same as that of *actin* except that an annealing temperature of 68^{0}C and 54^{0}C, respectively was used. The template DNA used for amplification of *G3PDH* and *adc* was plasmid DNA, and *Spd synthase, gad* and *actin* were cDNA.

Gradient PCR

A gradient PCR was performed to determine the optimum annealing temperature for the degenerate primers with poplar single stranded cDNA as well as genomic DNA. Single stranded cDNA was made as described earlier. The gradient profile followed was the same as used for standard PCR except that the annealing temperature was changed depending on the primers. The

profile used for GAD amplification was: an initial denaturing step of 95^{0}C for 2 min, followed by 94^{0}C for 30 sec, annealing temperature of 54^{0}C with a gradient of 6^{0}C and then elongation at 72^{0}C for 1 min. This was repeated for 30 cycles and then a final extension at 72^{0}C for 5 min. The reaction was held at 4^{0}C in the PTC. The gradient tested for *gad* had an annealing temperature of 50^{0}C, 53^{0}C, 58^{0}C and 60^{0}C.

Sequencing Protocol

The PCR products so obtained from degenerate primers were sequenced to determine their identity. The following components were added: Sequencing reagent premix (DYEnamic ET Terminator, Amersham): 8 μL; DNA (0.1-0.2 pmole) and water: up to 11.5 μL. To this mix, (66-132 ng of DNA for a 1 Kb size fragment that is approximately 0.1-0.2 pmole) and forward or reverse primer (5 pmole): 0.5 μL (of 10 μM primer working stock) to a total of 20 μL was added. The reaction was run as follows: 95^{0}C for 20 sec, 50^{0}C for 15 sec and 60^{0}C for 1 min. The number of cycles can vary from 20 to 30, however, 30 cycles were used and the samples were held at 14^{0}C. In a sterile 1.5 mL centrifuge tube containing 2 μL of sodium acetate/ EDTA buffer, 20 μL of the sequencing mix was added. To this, 80 μL of 95% ethanol was added. The contents were mixed and placed on ice for 15 min. The samples were spun in a centrifuge at 4^{0}C at 13000 X g for 15 min and the supernatant was drawn off by aspirating with a pipette tip. The samples were washed by adding 400 μL of 70 % ethanol and centrifuged briefly. The supernatant was aspirated and the pellet was dried in the SpeedVac for 2 min. The samples were sent for sequencing and 4 μL of the loading dye was added to the sample before loading on the gel.

Probe Preparation

Non-radioactive (DIG labeled) probes for the respective genes were prepared from the PCR products (cDNA as template in PCR reactions) and quantified as described earlier. These probes were used for northern hybridization studies as mentioned earlier in this section.

RESULTS

Confirmation of Stable Transformation

Polymerase Chain Reaction (PCR) Analysis

Poplar cells transformed with plasmid pCW122 containing the *gus* gene and pCW122-*odc* containing a mouse *odc* cDNA (Fig. 5, 6) were used for molecular and biochemical analysis. The putative *odc* transgenic cell lines were selected on 100 mg mL^{-1} kanamycin. A PCR analysis using oligonucleotide primers to amplify the mouse *odc* and *npt* gene produced bands of size 1.3 Kb (Fig. 9A) and 1 Kb (Fig.10D), respectively. Genomic DNA from the putative transformed cell lines had the band for both the genes while the band was absent in the NT cell line (Fig. 9A). The cell lines tested were NT and three *odc* transformed cell lines: 2E, 2F and 8B.

Southern Hybridization of PCR product of mouse odc cDNA

The PCR product for the mouse *odc* gene was hybridized to the mouse *odc* specific probe and showed a hybridization signal for the transformed cell lines (Fig. 9B). These results confirmed that the PCR products could hybridize with the mouse *odc* probe and these putative transgenic cell lines did indeed contain the mouse *odc* gene.

Southern Hybridization of Genomic DNA

Southern hybridization of genomic DNA was carried out for confirming stable transformation as well as to determine the number of copies of the transgene integrated in the

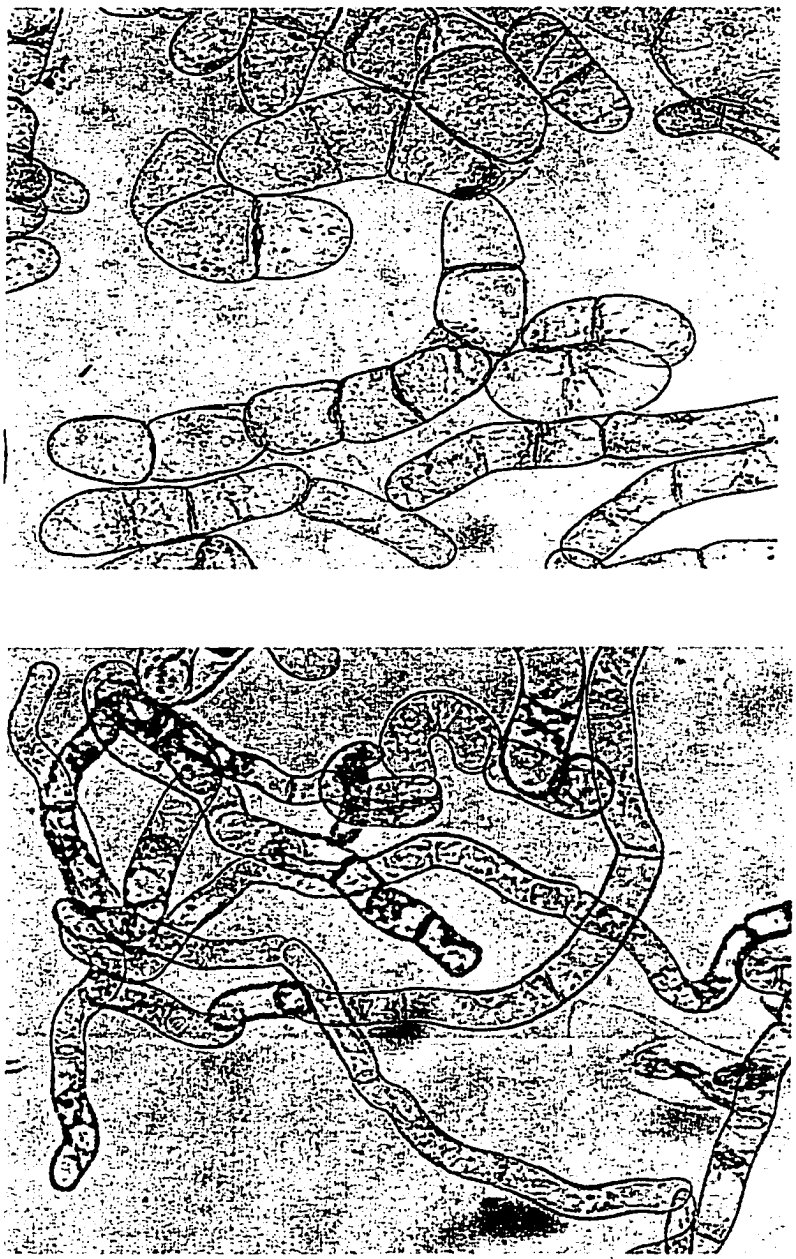


Fig. 8. Poplar cells: NT (A) and 2E (B) under the light microscope (200X) Magnification.

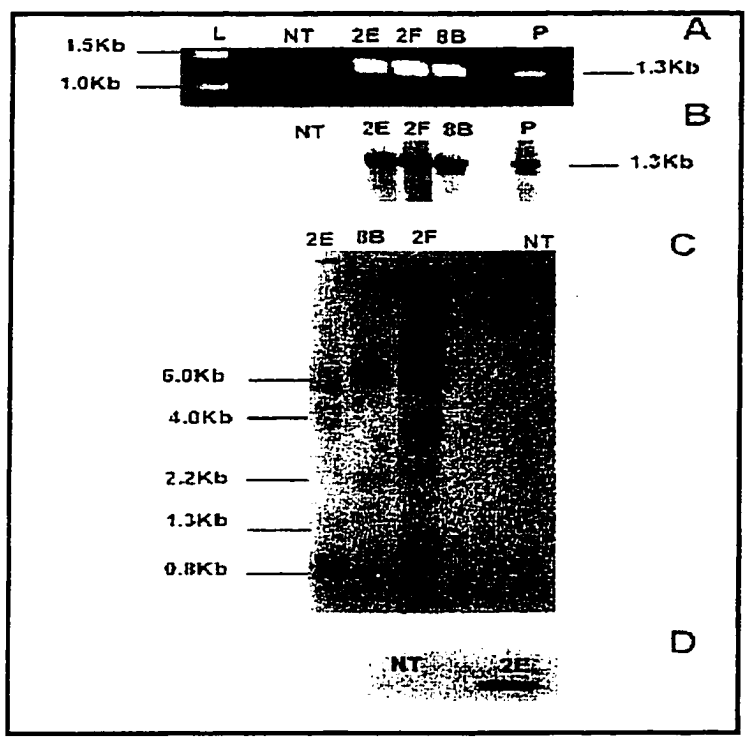


Fig. 9. Molecular analysis of transgenic cells. Amplification product of PCR using genomic DNA from different cell lines and mouse *odc*-specific primers (A); NT = non transgenic cells, 2E, 2F and 8B = different transgenic lines, P = control plasmid. Southern hybridization of PCR amplification products from Fig. 9A above using DIG-labeled probe made from mouse *odc*-cDNA (B); Southern hybridization of *Hind*III-restricted genomic DNA from different cell lines using DIG-labeled probe made from mouse *odc*-cDNA (C); Slot-blot northern hybridization of total RNA from and NT and 2E cells using DIG-labeled probe made from mouse *odc*-cDNA (D).

genome. The cell lines tested were NT and three *odc* transformed cell lines: 2E, 2F and 8B. Genomic DNA was digested separately with *Eco*RI and *Hind*III, processed for electrophoresis and transferred to nylon membrane for Southern hybridization. Genomic DNA digested with *Eco*RI gave the desired 2.2 Kb mouse *odc* band in all the transgenic cell lines (Fig. 10A). This size was obtained because *EcoR*I flanked the gene and some adjoining sequences. There were additional bands present (≥4.0Kb) with the *Eco*RI digest only in the transformed cell lines. *Hind*III cuts only once within the plasmid and will give fragments of different sizes depending on the site of integration of mouse *odc* cDNA in the poplar genome. Results on digestion with *Hind*III demonstrate that cell line 2E had multiple copies of the gene inserted in the genome and the gene existed in one or two copies in cell lines 2F and 8B (Fig. 9C). This indicates independent transformation event for each cell line. No hybridization signal was seen in the NT cell line.

Slot Blot and Northern Hybridization of Total RNA

Slot blot hybridization and northern blot hybridization of total RNA also showed no signal in the NT line (Fig. 9D, 10B). The cell line 2E had a strong signal on hybridization with a probe specific for mouse *odc* gene.

Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR) Analysis

This analysis was done as another approach to characterize the cell lines used in biochemical experiments. Figure 10C shows that the transformed cell lines had variable levels of gene expression. Cell line 2E showed a more intense band and the intensity was significantly lower in 8B and 2F.

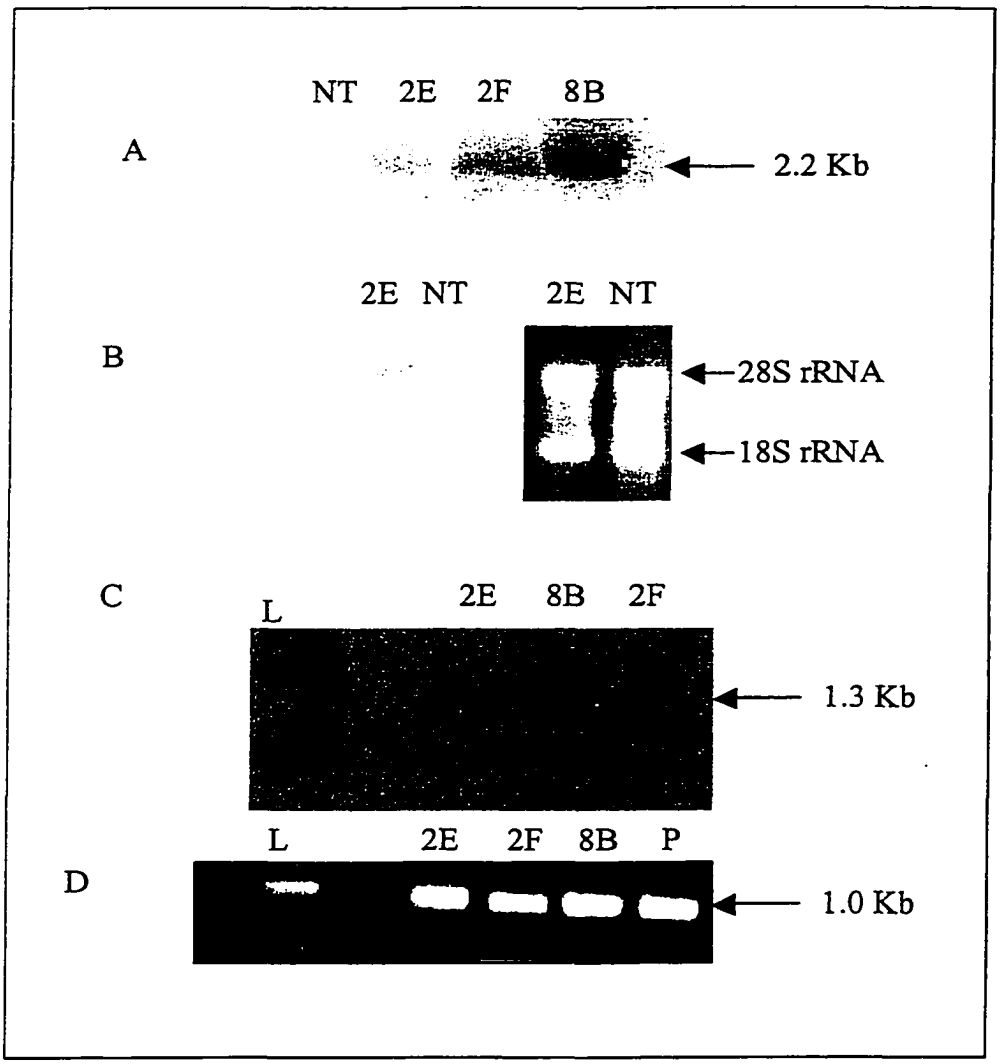


Fig. 10. Molecular analysis of transgenic cells. Southern hybridization of *Eco*RI restricted genomic DNA (12 μg) from different cell lines using DIG labeled probe made from mouse odc-cDNA. The DNA was resolved on a 1% agarose gel. The size of the band (in kilobase pairs) are indicated on the right (A). Northern hybridization of total RNA (20 μg) using DIG-labeled probe made from mouse odc-cDNA. RNA was isolated from 3 d old cells. The 28S and 18S rRNA bands show equal loading and quality of RNA of the two cell lines (B). RT-PCR analysis of mouse *odc* mRNA from transgenic cell lines (C). PCR product for the *npt* gene in mouse odc transformed cell lines (D). The lanes are: NT = non transgenic cells, 2E, 2F and 8B = different transgenic lines, P = control plasmid.

Neomycin Phosphotransferase (NPT-II) Enzyme Assay

The NPT-II protein was assayed using ELISA in the NT and 2E cell lines. The results showed that no NPT-II protein was present in the NT cell line whereas the transformed cell line had significantly higher amounts of the NPT protein (Fig. 11). It was clear from the results that the *nptII* gene was being expressed in the cell line 2E.

The NT and 2E cells used in this study were visualized under the light microscope (Courtesy: Dr. Charles Walker) at 200X magnification. The NT cells appeared bigger and more globose than the 2E cells (Fig. 8).

Ornithine Decarboxylase (ODC) and Arginine Decarboxylase (ADC) Enzyme Analysis

The rate of decarboxylation of [^{14}C] Orn and [^{14}C] Arg was determined using intact cells as described in Minocha et al. (1999a) and under Materials and Methods. Decarboxylation attributable to the mouse ODC activity in the transgenic cells can be distinguished from the plant ODC by its sensitivity to DFMO (DeScenzo and Minocha, 1993). The enzyme activity was determined in 3 d old cells. Substantial activity of mouse ODC was observed in *odc* transformed cell lines, 2E and 6B (Fig. 12A). This activity was almost completely inhibited by 2 mM DFMO. Cell line GUS7A also showed enzyme activity comparable to the NT cell line and was included in the assay as a positive control. No native ODC activity was observed in the transgenic and NT cells (data not shown). The enzyme activity was measured two times.

The activity of ADC was found to be either comparable in all cell lines (transgenic or NT), or was somewhat higher in the transgenic cells on some days (Fig. 12B). The activity of ADC was inhibited by DFMA by as much as 60-100%. The actual amount of enzyme activity varied in different transgenic cell lines and within the same cell line on different days of analysis.

In alternate experiments, 3 d old cells were incubated with [U-^{14}C] Orn for varying lengths of time (1 to 8 h) and the data were collected on the release of $^{14}CO_2$. The uptake and

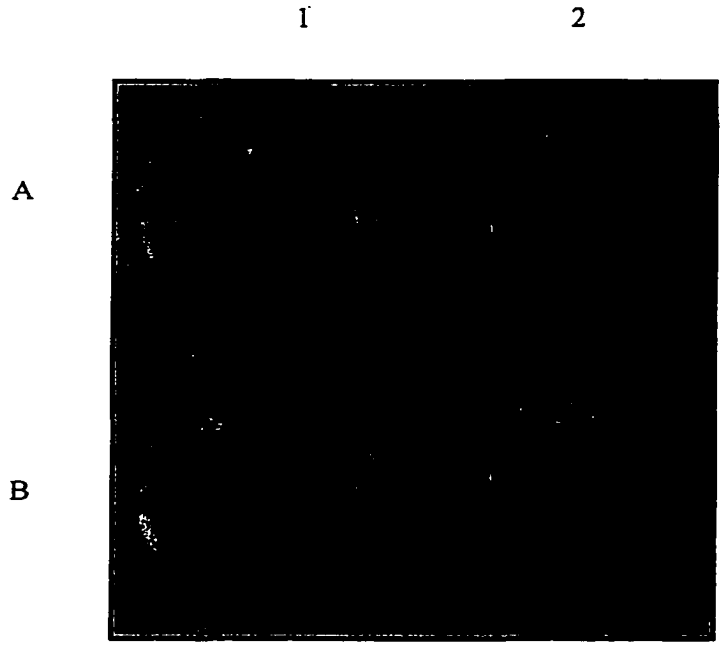


Fig. 11. A photographic representation of ELISA for the detection of neomycin phosphotransferase (NPT) in 2E cells (B1-B2) and NT cells (A1-A2).

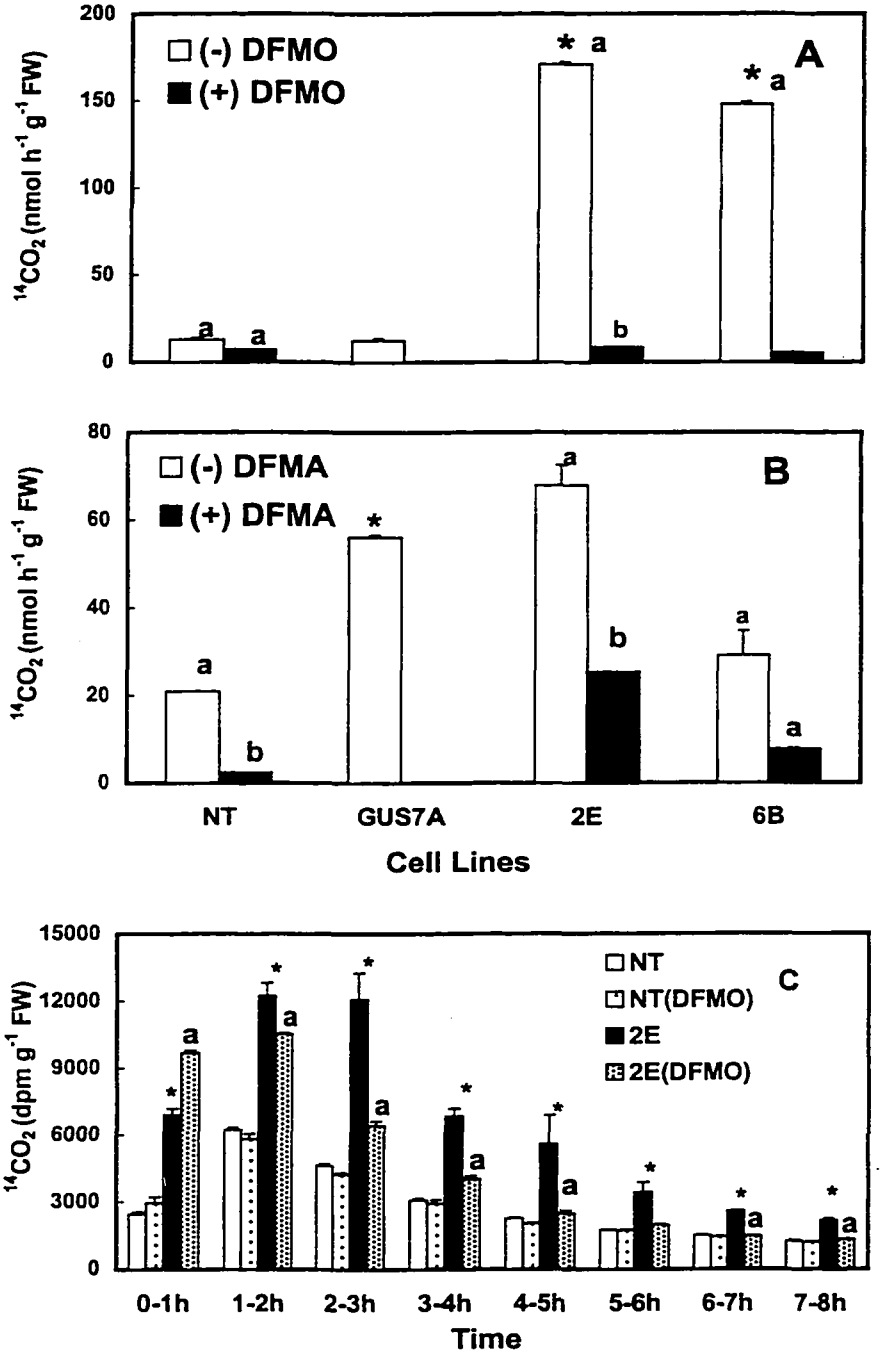


Fig. 12. The rate of $^{14}CO_2$ production from [1-^{14}C] Orn (A) and [1-^{14}C] Arg (B) by NT and *gus* (GUS7A) and *odc* (2E and 6B) transformed cells. Intact cells were used in the absence and presence of DFMO or DFMA. Intact cells were incubated with [U-^{14}C] Orn for 8 h and the production of $^{14}CO_2$ and the effect of DFMO analyzed at 1 h intervals (C). Data presented are mean ± SE of two replicates for (A) and (B) and three replicates for (C). An asterisk indicates that values for 2E are significantly different ($p \leq 0.05$) from NT cells at a given time. A letter indicates that the values are significantly different ($p \leq 0.05$) for the presence (+) and absence (-) of the inhibitor within the same cell line.

metabolism into polyamines of the cells was also quantified in these cells at the end of the incubation time, i.e. 8 h. The rates of $^{14}CO_2$ production from [U-^{14}C] Orn were typically 2-3 fold higher (statistically significant at $p \leq 0.05$) in the 2E cell line as compared to the NT cells during the entire 8 h period of incubation (Fig. 12C). Sources other than direct decarboxylation of [U-^{14}C] Orn contributing to the $^{14}CO_2$ production may be the breakdown of either radio-labeled Put or radio-labeled Arg, both being produced from labeled Orn. The effect of 2 mM DFMO was also studied on the rates of $^{14}CO_2$ production. Significant decrease in $^{14}CO_2$ production was seen in 2E cells treated with DFMO (Fig. 12C) at all time periods except at the end of 1 h. There was no effect of DFMO on the $^{14}CO_2$ production by the NT cells.

Polyamine Levels in Mouse *odc* Transformed Cell Lines

Figure 13 (A-C) shows the polyamine content in different *odc* transformed cell lines in comparison with the NT cell line. Putrescine contents were generally 2-5 fold higher in most of the *odc*-transgenic cell lines on any given day as compared to the NT cells. Typical data of Put content in several cell lines are shown in Fig. 13A. However, it must be pointed out that the content of Put in any given cell line varied on different days of analysis (Fig. 13D). Putrescine contents in the *gus*-transgenic cells (GUS7A: Put= 1478 ± 59.7 nmol g^{-1} FW) were either higher or comparable to NT cells on any given day. However, another control (a somaclonal variant) NT-HP (non-transformed-high Putrescine) had higher Put levels than NT (Put= 1794 ± 76.0 nmol g^{-1} FW) even though it tested negative for PCR with mouse *odc* specific primers and other molecular analysis. This cell line was not used as a control for experiments involving a comparison with the transgenic cells. Putrescine concentration in some of the transgenic cells was as high as 6500 nmol g^{-1} FW. Spermidine contents of transgenic cells were either similar to those in the NT cells or were slightly higher in the former on some days but not on others (Fig. 13B,E). Spermine in most transgenic cell lines was often lower than the NT cells (Fig. 13C,F). One cell line (2F) which showed a small increase in Spd as well as Spm on some days did not consistently

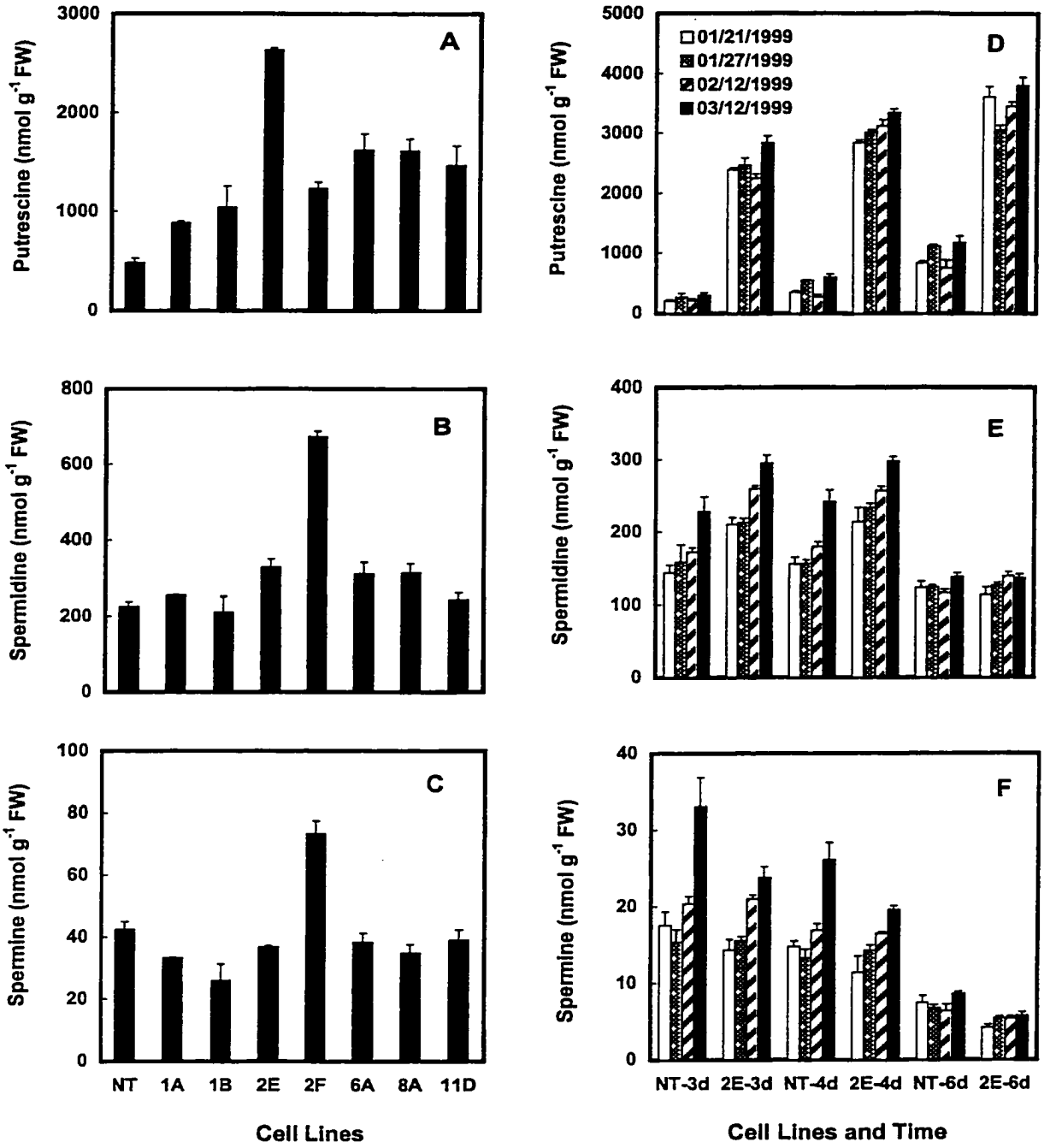


Fig. 13. Amounts of polyamines in non-transgenic (NT) and several transgenic cell line grown for 7 d on solid medium (A-C). Amounts of free polyamines in NT and a transgenic (2E) cell line grown in liquid medium for 3, 4 and 6 d (D-F). Data for D through F are from four different experiments conducted over a period of 2 months. Each bar represents mean ± SE of four replicates.

show a major increase in Put and was not followed up for further experimentation. The contents of other two polyamines in GUS7A (Spd= 488.12 ± 10.9 nmol g^{-1} FW; Spm= 47.5 ± 2.8 nmol g^{-1} FW) and NT-HP (Spd= 210.61 ± 11.5 nmol g^{-1} FW; Spm= 17.47 ± 1.4 nmol g^{-1} FW) were similar or slightly higher as compared to NT.

The *odc* transformed cell line 2E that was used for further detailed study always had two to ten fold higher Put content than the NT cells. The cellular content of Spd in the 2E cells was also generally higher than that in the NT cells, the overall amount of Spd (nmol g^{-1} FW) being lower than that of Put at any given time. Spermine, which constituted less than 10% of the total soluble polyamines at any given time for both types of cells, showed no differences between the two cell lines or was lower in the 2E cells than the NT cells. Figure 13 (D-F) shows cellular levels of the three polyamines in the NT and 2E cell lines on 3, 4 and 6 d of the culture cycle at different time periods. There were statistically significant differences in the cellular Put levels of the two cell lines on any given day (Fig. 13D). Spermidine and Spm levels in both the cell lines did not change much during the first four days of culture but declined somewhat at 6 d (Fig. 13E,F).

Metabolism of [^{14}C] Ornithine- Uptake

After the cells were incubated with the labeled substrate for varying periods of time, collected by vacuum filtration and mixed with 7.5% PCA, the PCA extracts were dansylated. The dansyl-polyamines were partitioned in the toluene fraction. An aliquot of 20 μL from the toluene fraction was counted for radioactivity to determine total polyamine formation from the labeled Orn. The radioactivity present in the 2E cells was significantly higher as compared to NT cells at all times tested (Fig. 14A). There was no further accumulation of label in the cells beyond the first 2 h. The effect of 2 mM DFMO was also studied on the uptake of radioactivity and metabolism into polyamines during the 1, 2 and 4 h time period. There was a substantial reduction in the label accumulated in the toluene fraction because of DFMO in the cell line 2E

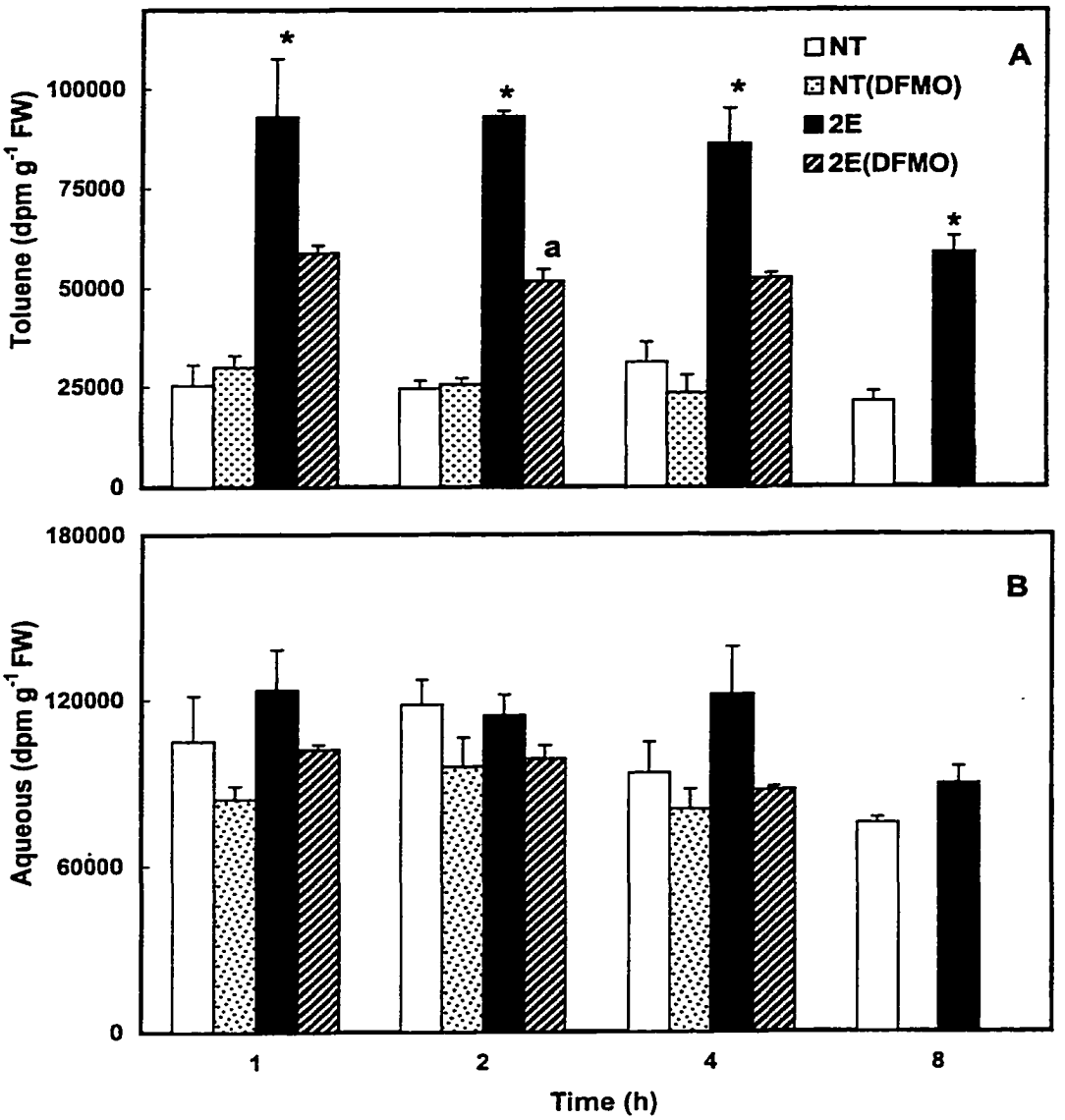


Fig. 14. Radioactivity present in the toluene (A) and aqueous (B) fractions after dansylation of PCA extracts of NT and 2E cells incubated with [U-^{14}C] Orn for 1, 2, 4 and 8 h. Total amount of radioactivity in each treatment was 0.2 μCi. Data presented are mean ± SE of two replicates. An asterisk indicates that values for 2E are significantly different ($p \leq 0.05$) from NT cells at a given time and a letter indicates values for inhibitor treated cells are significantly different ($p \leq 0.05$) from untreated cells.

(Fig. 14A). There was no DFMO effect on the NT cells. The radioactivity in the aqueous fraction was also counted and was found to be quite comparable in the two cell lines at all time periods. This fraction contains all the dansylated Orn, Arg as well as other amino acids but no polyamines. This demonstrates that the uptake rates of labeled Orn were similar in both the cell lines (Fig. 14B). DFMO caused a small reduction in the amount of label present in the aqueous fraction in both the cell lines.

TLC Separation of Radioactive Polyamines

The Thin Layer Chromatography (TLC) based separation of polyamines was optimized using the protocols of Flores and Galston (1982) and Slocum and Flores (1991) as modified by Andersen et al. (1998). The retention values of various polyamines were determined by running 10 mM of each dansyl-polyamine standard separately (Fig. 15A) and as a standard mix or in various combinations (Fig. 15B) on the TLC plate. The standards tested were Put, Spd, Spm, agmatine, cadaverine and GABA. The R_f values for various polyamines were: Put: 0.3; Agmatine: 0.32; Cadaverine: 0.5; Spd: 0.78; Spm: 0.85 and GABA: 0.90 from Fig. 15C. Figure 15C also shows the difference in the fluorescence of Put content of NT and 2E when the TLC plate was visualized under the UV light. Many fluorescent bands other than the three polyamines were observed under UV light on the TLC plates and in order to determine radioactivity bound in these fractions, the entire TLC plate was scraped in 1 cm sections from the origin to the solvent front and the silica powder from each band was counted separately (Fig. 16). Most of the radioactivity appeared in the Put fraction when cells were incubated with [U-^{14}C] Orn (Fig. 16A). Approximately, 10-15% of the total radioactivity recovered from the entire TLC plate was found in all the bands other than those of the polyamines (Fig. 16B).

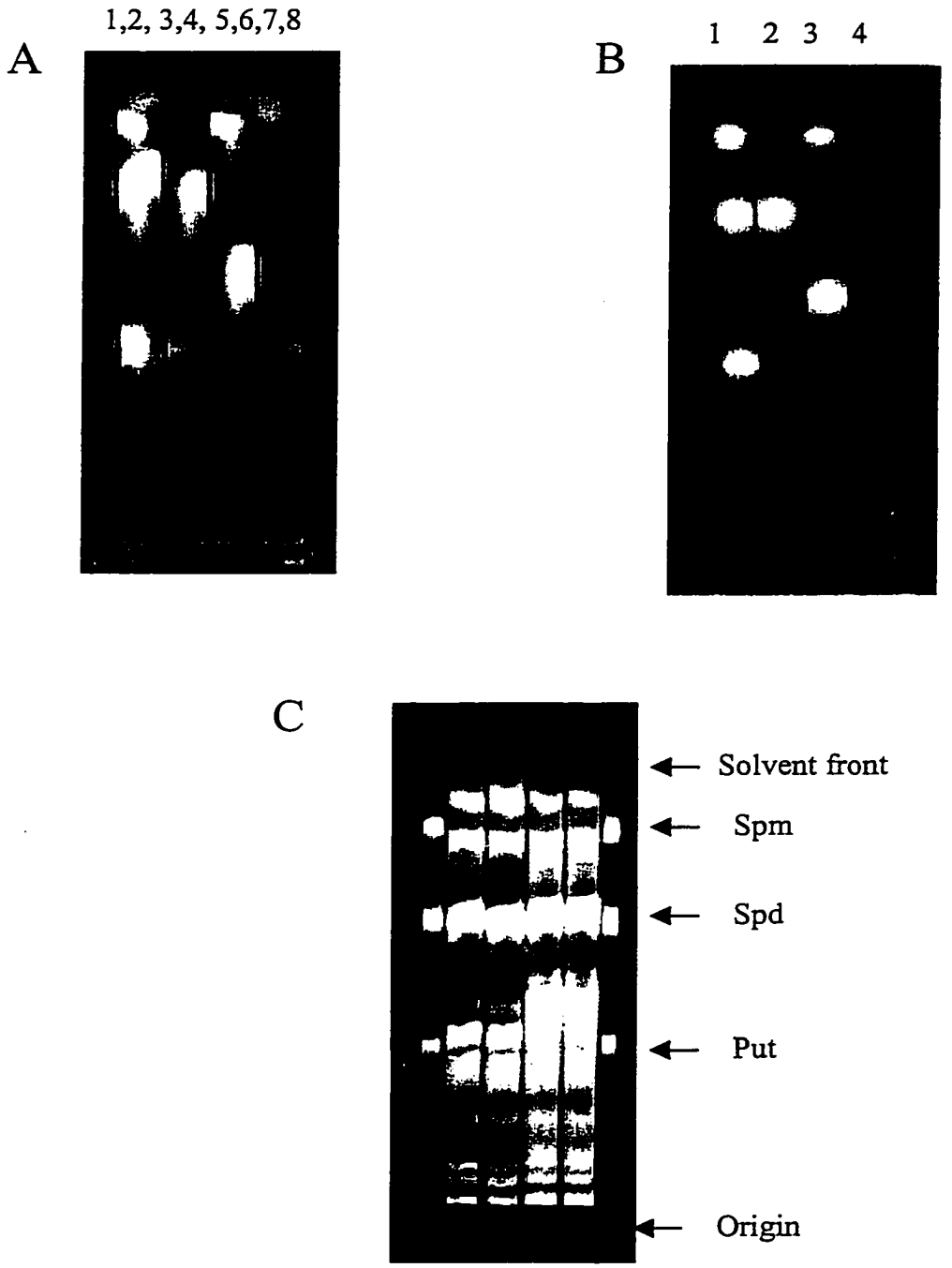


Fig. 15. Separation of dansyl-polyamines on TLC plate as visualized under UV light. Lanes are: (1 and 2) all three polyamines (3) Put, (4) Spd, (5) Spm, (6) cadaverine (7) GABA and (8) Agmatine (A); Lanes are: Mix of polyamines (1) all three polyamines (2) Put + Spd (3) Cadverine +Spm (4) Agmatine + GABA (B); Polyamine profile of NT and 2E cells incubated with [U-^{14}C] Orn on the TLC plate (C).

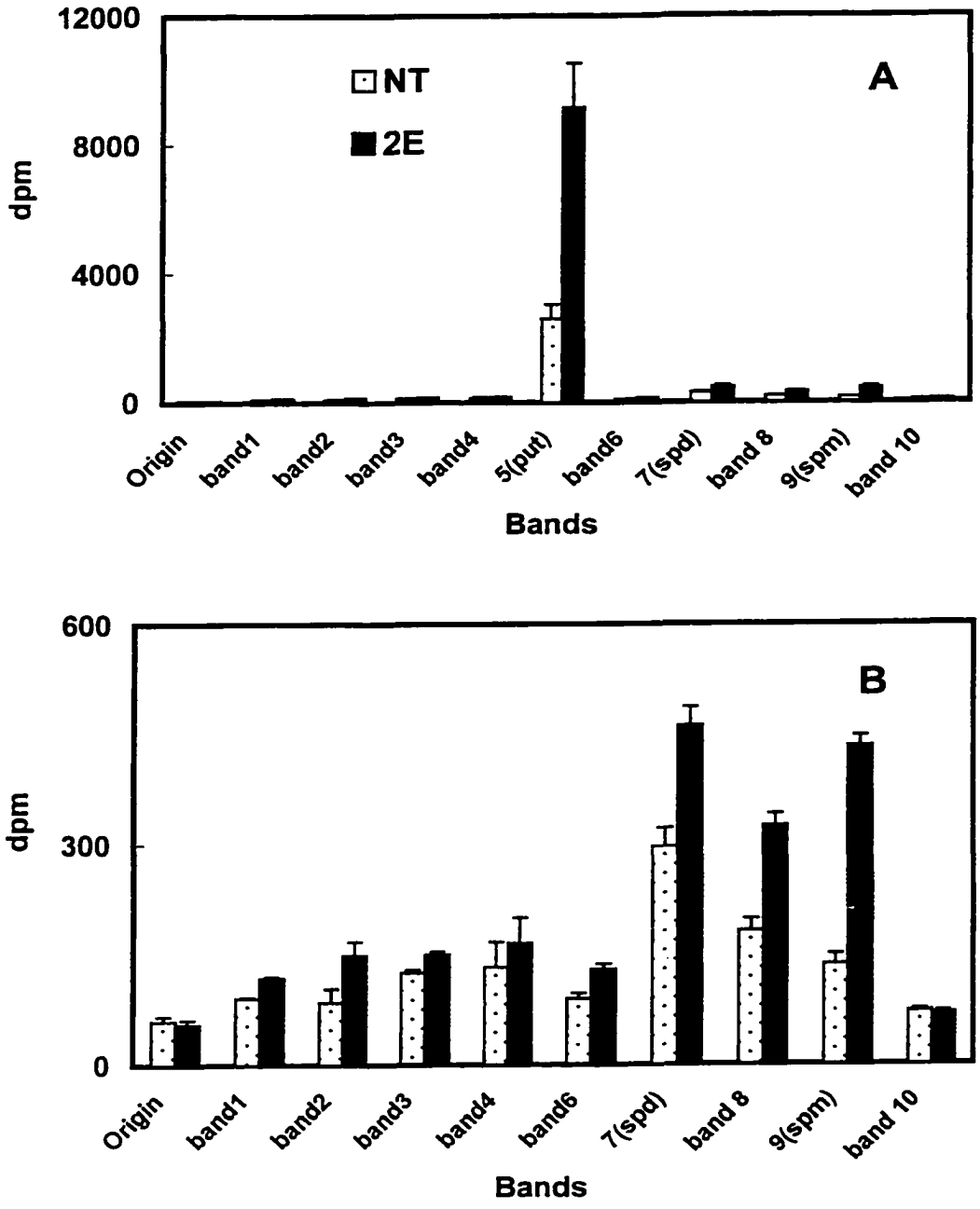


Fig. 16. The amount of radioactivity present on the TLC plate from the origin to the solvent front when incubated with [U-^{14}C] Orn (A). Radioactivity associated with all other fractions except Put (B).

Metabolism of [^{14}C] Ornithine- Incorporation

The dansyl-polyamines partitioned in the toluene fraction were placed in the SpeedVac to evaporate acetone from samples, reconstituted in methanol and separated on TLC plates. Figure 17 shows the amounts of radioactivity recovered in the three major polyamines in NT and 2E cells at different times of incubation with [U-^{14}C] Orn. The incorporation of label from [U-^{14}C] Orn into Put was significantly higher in the 2E cells than the NT cells (Fig. 17A). In the 2E cells, the amount of label in the Put fraction was seen to decline after the first 4 h of incubation while in the NT cells, the amount of [^{14}C] Put did not change much with time of incubation. The radioactivity in the Spd and the Spm fractions was also generally higher in the 2E cells as compared to the NT cells (Fig. 17B,C). The total amount of label recovered in Put was several-fold higher than that in the other two polyamines. Incorporation into all the three polyamines was significantly reduced as a result of DFMO treatment (Fig. 17A,B,C) in the 2E cells. However, incorporation in the NT cells was not affected. Intriguingly, during the first 1 h of incubation, DFMO caused an increase in the NT cells in both the $^{14}CO_2$ production (Fig. 12C) as well as incorporation from [U-^{14}C] Orn into Put (Fig. 17A,B).

Metabolism of [^{14}C] Glutamic Acid

Three day old cells were incubated with [U-^{14}C] Glu and the rate of $^{14}CO_2$ production was measured at the end of 1, 2, 3 and 4 h. The cell lines tested were NT, NT-HP and 2E. Cell line 2E produced significantly higher $^{14}CO_2$ as compared to the other two cell lines (Fig. 18A). There was a substantial increase (approximately six fold) in the amounts of $^{14}CO_2$ collected during the second h as compared to the first h. The $^{14}CO_2$ production dropped significantly at the end of 3 h and decreased further at 4 h. The trends were the same in all of the three cell lines; however, the changes were more dramatic in 2E than the other two cell lines. The $^{14}CO_2$ release was always higher in the 2E cells than the NT cells. The difference between the two NT cell lines was significant during the first hour of $^{14}CO_2$ collection but after that $^{14}CO_2$ release was comparable in

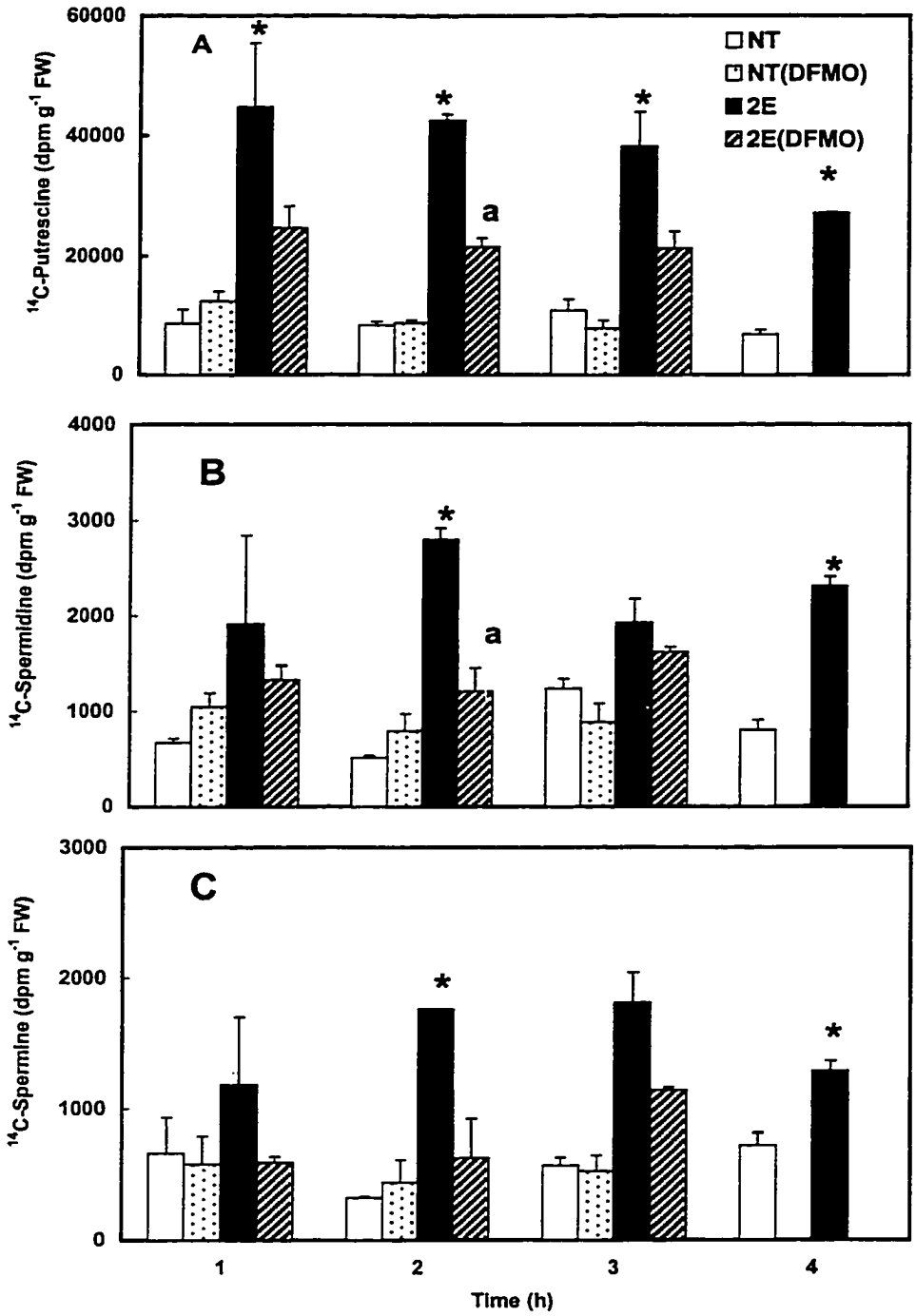


Fig. 17. Incorporation of radioactivity into free Put (A), Spd (B) and Spm (C) and the effect of DFMO in NT and 2E cells at different times of incubation. Three day old cells were incubated with 0.2 μCi of [U-^{14}C] Orn 10 mL^{-1}. Data presented are mean ± SE of two replicates. An asterisk indicates that values for 2E are significantly different ($p \leq 0.05$) from NT cells at a given time. A letter indicates that the values for the inhibitor treated cells are significantly different ($p \leq 0.05$) from untreated cells.

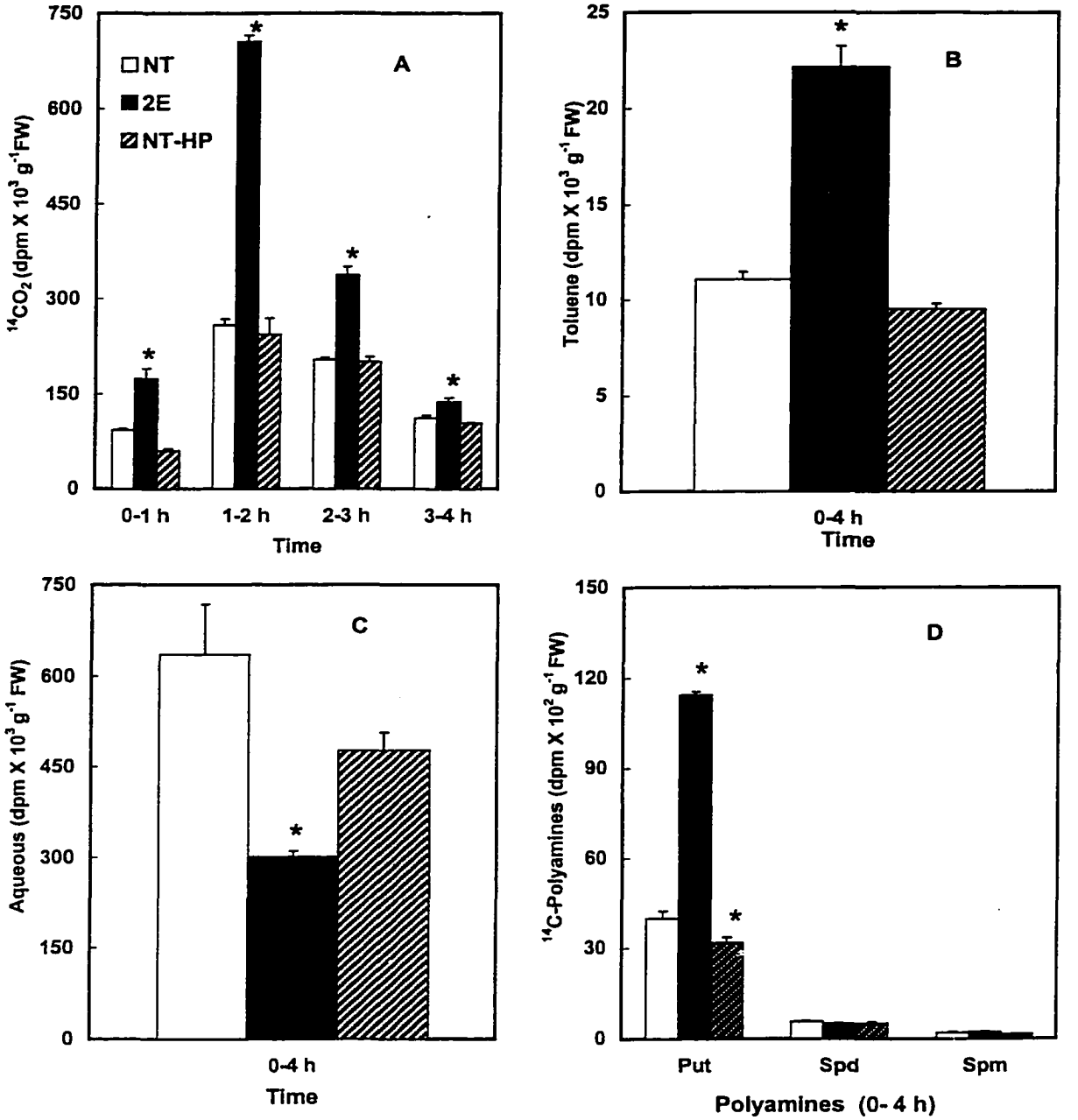


Fig. 18. The rate of $^{14}CO_2$ production up to 4 h (A) and radioactivity present in the toluene fraction (B), aqueous fraction (C) and Put, Spd and Spm at the end of 4 h (D). Three day old NT, 2E and NT-HP cells were incubated with 1 μCi of [U-^{14}C] Glu 10 mL^{-1} medium. Data presented are mean ± SE of three replicates. An asterisk indicates that values are significantly different ($p \leq 0.05$) from NT cells at a given time.

the two cell lines. The $^{14}CO_2$ produced represents the release from all decarboxylation reactions. Approximately 33% of the radioactivity fed to the cells was released as $^{14}CO_2$ in cell line 2E at the end of 1 h.

The cells treated with [U-^{14}C] Glu were collected for polyamine analysis at the end of 4 h incubation, as described earlier for [U-^{14}C] Orn incubations. Following dansylation, the radioactivity accumulated in the toluene fraction was found to be significantly higher in 2E cells as compared to the other two cell lines (Fig. 18B). There was either no difference in the NT and NT-HP cell lines or the NT-HP was slightly lower. The counts in the aqueous fraction were approximately 10-60 fold of those incorporated in the toluene fraction (Fig. 18C). This fraction presumably includes amino acids of the Glu family and all charged products as well as degradation products of the polyamines. The aqueous fraction had lower amount of radioactivity in the 2E cell line as compared to NT and NT-HP. Of the radioactivity present in the toluene fraction, most of the counts were incorporated in the Put fraction in all the three cell lines, the 2E showing significantly higher label than the other two cell lines. The incorporation in the Spd and Spm fractions was comparable in all the cell lines (Fig. 18D).

Effect of Inhibitors of ADC and ODC on the Cellular Polyamine Levels

The effect of 1 mM and 2 mM DFMA on cellular polyamine content was tested on 3 d old NT and 2E cells. Treatment with 2 mM DFMA was given for 4 and 8 h in addition to 24 and 72 h. Both the concentrations of DFMA used had significant effects on the Put levels at 24 and 72 h. The levels of Put declined in both the cell lines as a result of DFMA treatment and the effect was more pronounced at 72 h than at 24 h (Fig. 19A,D). However, there were no major effects on the cellular levels of Spd (Fig. 19B,E) and Spm (Fig. 19C,F) at most of the time periods tested in both the cell lines. The effects of DFMA were similar in both short term (1-4 h) and longer term (24 and 72 h) treatments.

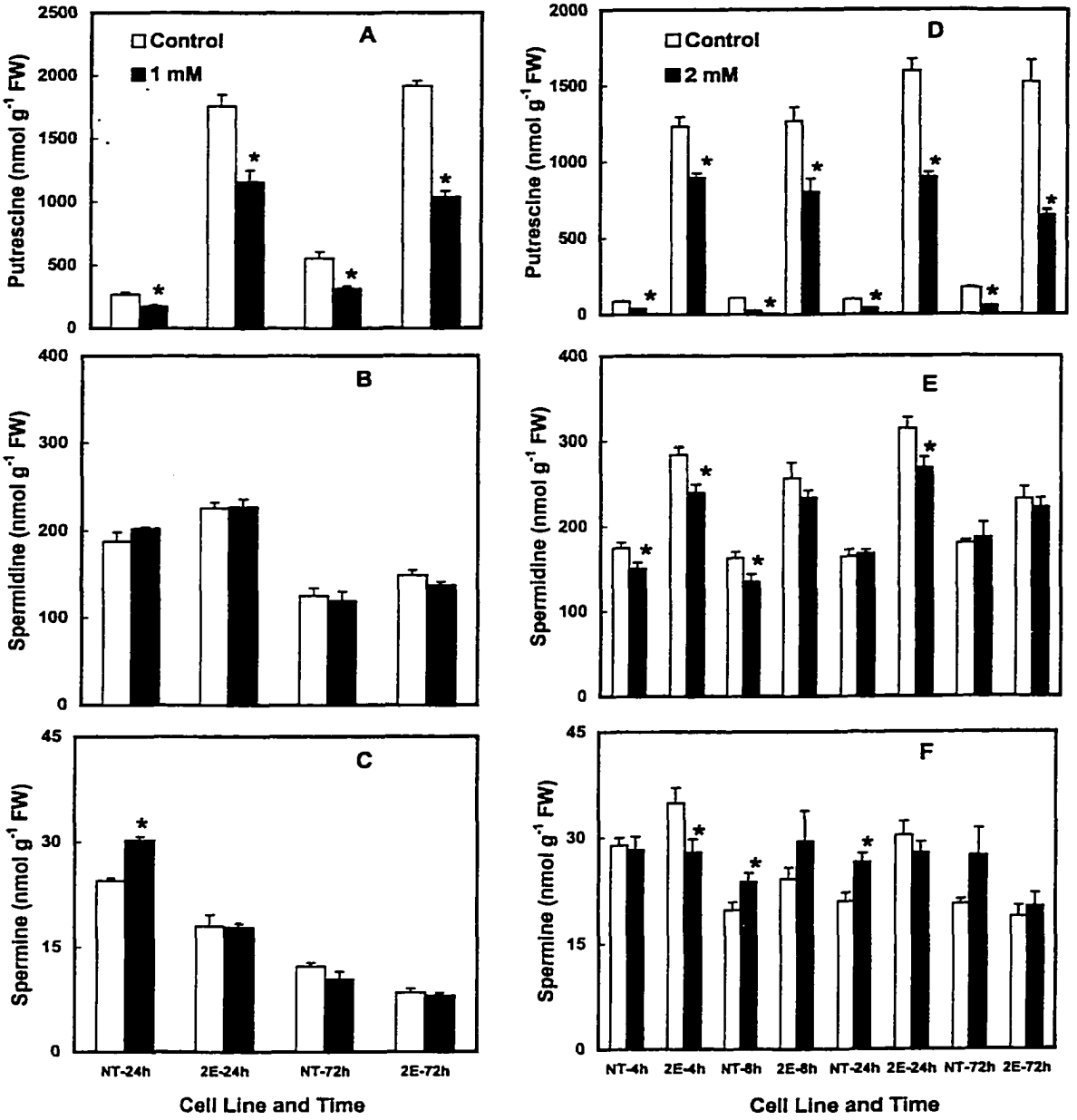


Fig. 19. The effect of DFMA on Put (A,D), Spd (B,E) and Spm (C,F) in 3 d old NT and 2E cells. Each bar represents mean ± SE of three or four replicates. An asterisk indicates that values for treated cells are significantly different ($p \leq 0.05$) from the untreated cells within the same cell line.

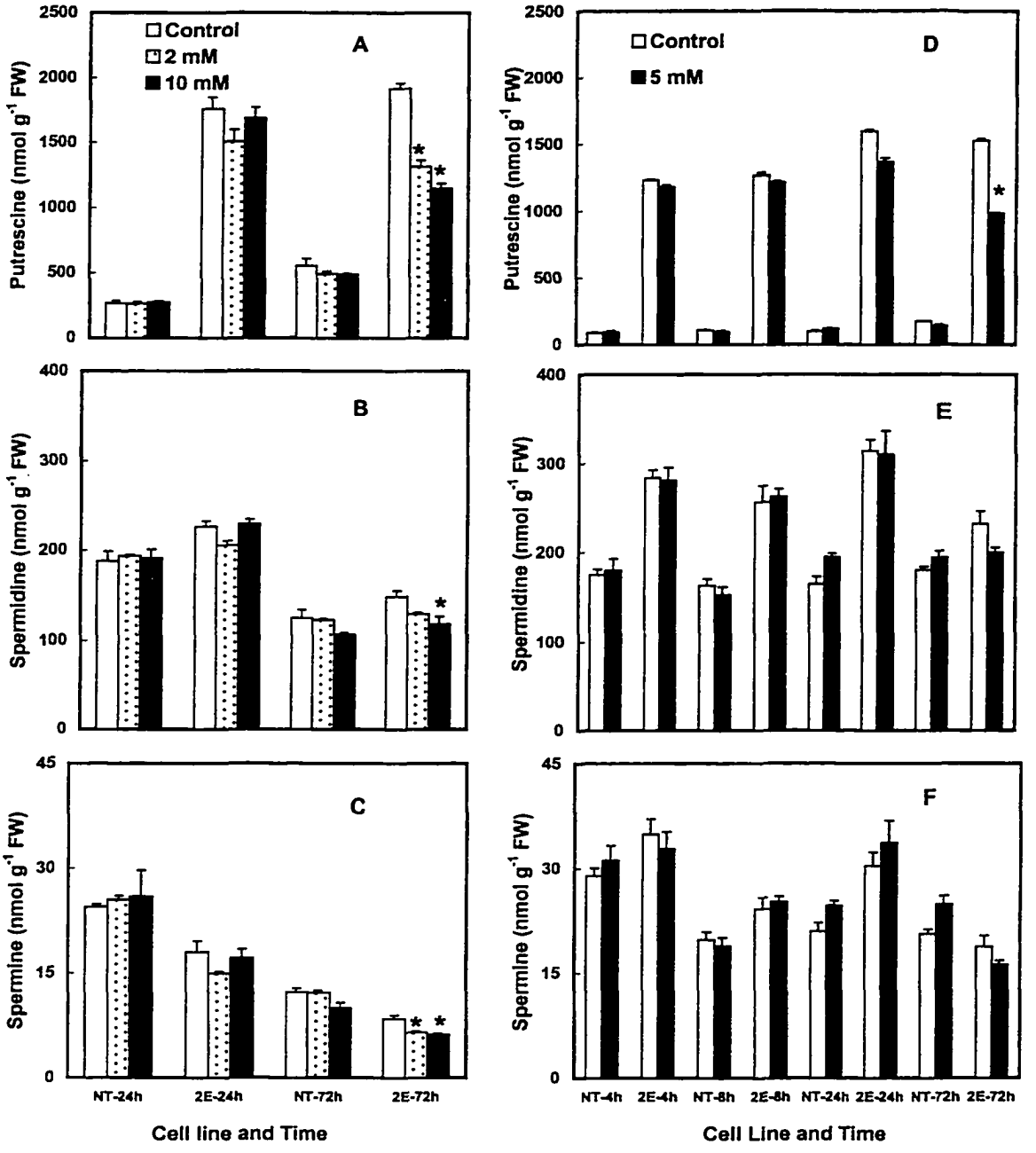


Fig. 20. The effect of different concentrations of DFMO on Put (A,D), Spd (B,E) and Spm (C,F) in 3 d old NT and 2E cells. Each bar represents mean ± SE of three or four replicates. An asterisk indicates that values for treated cells are significantly different ($p \leq 0.05$) from the untreated cells within the same cell line.

In a parallel set of experiments, three day old cells were incubated with DFMO (2, 5 and 10 mM) to test the effect of the inhibitor on polyamine levels. The effect of 5 mM DFMO was tested at 4 and 8 h as well. The 2E cells exhibited a significant decrease in cellular Put with increasing concentrations of DFMO at 72 h (Fig. 20A,D). The NT cells generally showed no pronounced effect of DFMO on Put (Fig. 20A,B,C). The effects on cellular Spd and Spm were not significant in most cases (Fig. 20B,C).

Supplementation with Precursors and Inhibitors of Polyamine Biosynthesis

Proline is largely synthesized from Glu. It can also be synthesized from Orn (Fig. 2). Gabaculine is an inhibitor of ornithine aminotransferase (OAT), an enzyme that regulates the conversion of Orn into Pro via Glu-γ-semialdehyde. Figure 21 shows that treatment with 10–100 μM gabaculine for up to 72 h had little effect on the cellular content of any of the three polyamines in either the NT or the 2E cells. A higher concentration of 1 mM increased Put and Spd production significantly in the 2E cell line at 72 h time period only while decreasing the Put cellular content significantly in the NT cells at the same time (Fig. 21A,B). There was a slight decline in the Spd and Spm concentrations at 24 h with 1 mM gabaculine in the NT cells, lower concentrations had little effect (Fig. 21B,C).

When 3 d old cells were treated with L-methionine sulfoximine (L-MSX), there was a significant decrease in the cellular levels of Put (Fig. 22A) as well as an increase in the pH of the medium from 4.0 to 7.0 with all concentrations of the inhibitor. The change in pH indicates the accumulation of NH_4^+ in the medium. L-methionine sulfoximine inhibits glutamine synthetase (GS), the enzyme responsible for assimilation of NH_4^+ into Gln. The decrease in Put was observed in both NT and 2E cells. It is known that the mouse ODC enzyme can use lysine as a substrate and produce cadaverine when Orn supply is limiting in the cells (Herminghaus et al., 1991; Bastola and Minocha, 1995). The same was observed with 2E cells in the present study. Figure 23 shows the HPLC profile of 2E cells showing the presence of a cadaverine peak when

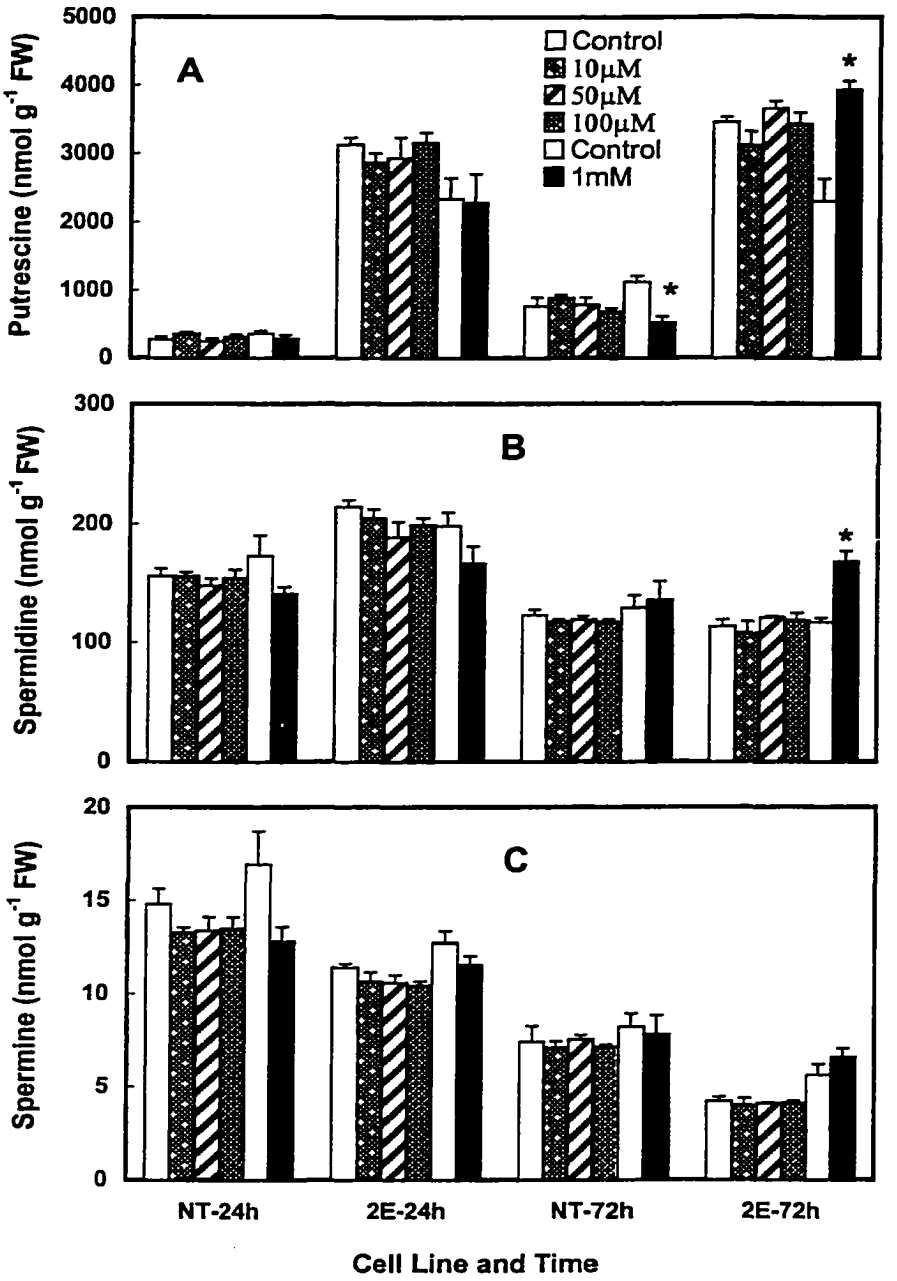


Fig. 21. The effect of different concentrations of gabaculine on cellular Put (A), Spd (B) and Spm (C) levels in 3 d old NT and 2E cells at 24 h and 72 h. Each bar represents mean ± SE of three replicates. Experiment for the effect of 1 mM was done separately from the other treatments. An asterisk indicates that values for treated cells are significantly different ($p \leq 0.05$) from the untreated cells within the same cell line at a given time.

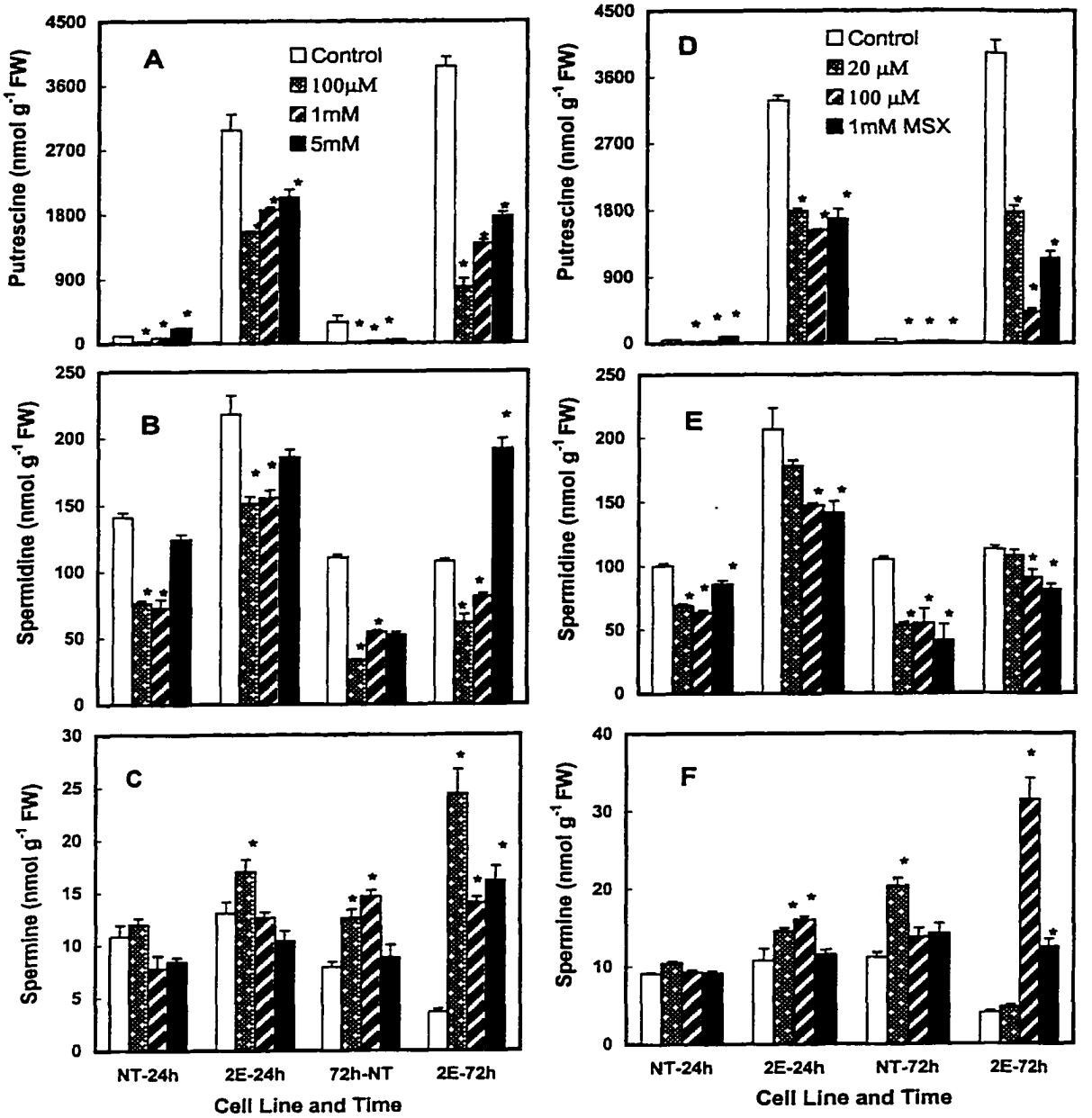


Fig. 22. The effect of different concentrations of L-MSX (methionine sulfoximine) on cellular Put (A,D), Spd (B,E) and Spm (C,F) levels in 3 d old NT and 2E cells at 24 h and 72 h. Each bar represents mean ± SE of three replicates. An asterisk indicates that values for treated cells are significantly different ($p \leq 0.05$) from the untreated cells within the same cell line.

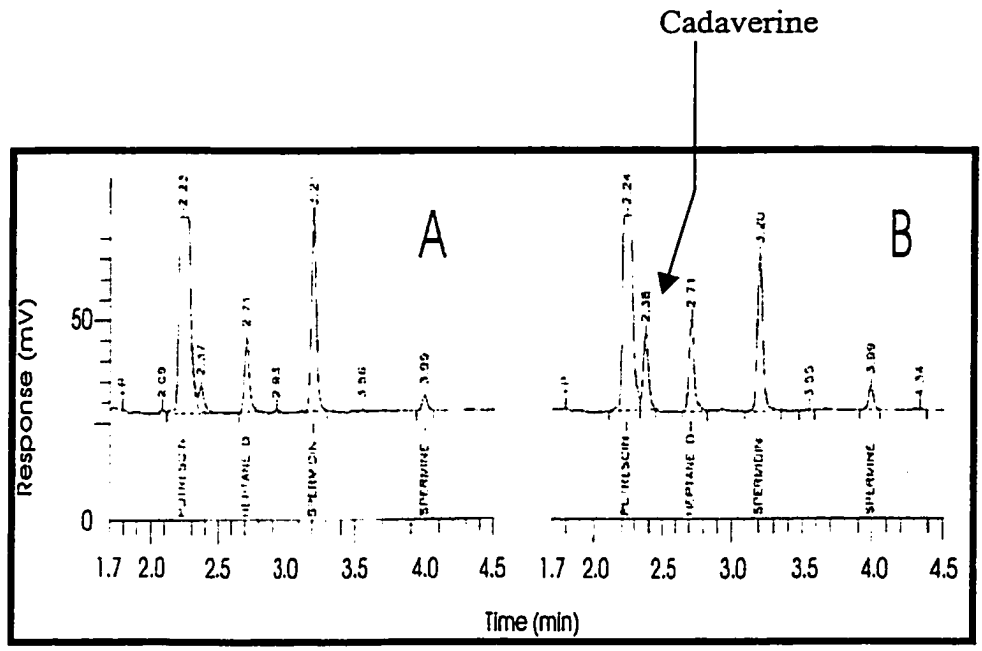


Fig. 23. HPLC Profile of dansyl-polyamine in control (no L-MSX (methionine sulfoximine) treatment) (A) and MSX treated transformed cell line 2E (B). The cadaverine peak appears at 2.38 min.

the cells were treated with L-MSX. No such peak was observed in the NT cell line. The lowest concentration of L-MSX (20 μM) was often more effective than the higher concentrations in reducing Put content in the cells. The effects were that cellular Put content was dramatically decreased as a result of the inhibitor (Fig. 22A) in both the cell lines. The levels of Put are barely detectable in the NT cells when treated with L-MSX. The cellular content of Spd was also significantly lower in the MSX-treated cells in both cell lines (Fig. 22B,E). Spermine levels either increased significantly or were unaffected in response to MSX treatment (Fig. 22C,F).

Cells were also treated with various non-labeled precursors of the polyamine pathway and the effects on Put levels with these precursors are shown as follows: Arg (Fig. 24A), Orn (Fig. 24B), urea (low concentrations) (Fig. 24C) and Gln (Fig. 24D). The concentrations used for all the precursors are given in Table IV in Materials and Methods section. Data presented in Fig. 24A show that 250-500 μM Arg caused a small increase in the Put content of NT cells but not the 2E cells. At higher concentrations (2-10 mM), however, there was a significant decrease in Put content in both cell lines, the effect being more pronounced in NT cells. Addition of Orn, particularly at 2 and 10 mM, resulted in a significant increase in Put at both 24 and 72 h in both cell lines (Fig. 24B). Lower concentrations of Orn had a smaller effect. No further increase in Put content was seen in either of the cell line at 24 or 72 h in response to the addition of up to 250 μM urea (Fig. 24C) or 1 mM of the Gln (Fig. 24D).

Effects of exogenous Arg (Fig. 25A,B), Orn (Fig. 26A,B), urea (low concentrations) (Fig. 27A,B) and Gln (low concentrations) (Fig. 28A,B) on Spd and Spm content were also studied in addition to Put. While lower concentrations of Arg had no effect, Spd levels declined as a result of 10 mM Arg in both the cell lines at 24 h, however, the levels increased at 72 h in the 2E cells (Fig. 25A). Conversely, 10 mM Arg was promotory to Spm levels at both 24 and 72 h in both the cell lines (Fig. 25B). Ornithine had no significant effect on Spd levels at both higher and lower concentrations (Fig. 26A). Likewise, cellular Spm levels in both the cell lines were not affected by Orn at either of the time period tested (Fig. 26B).

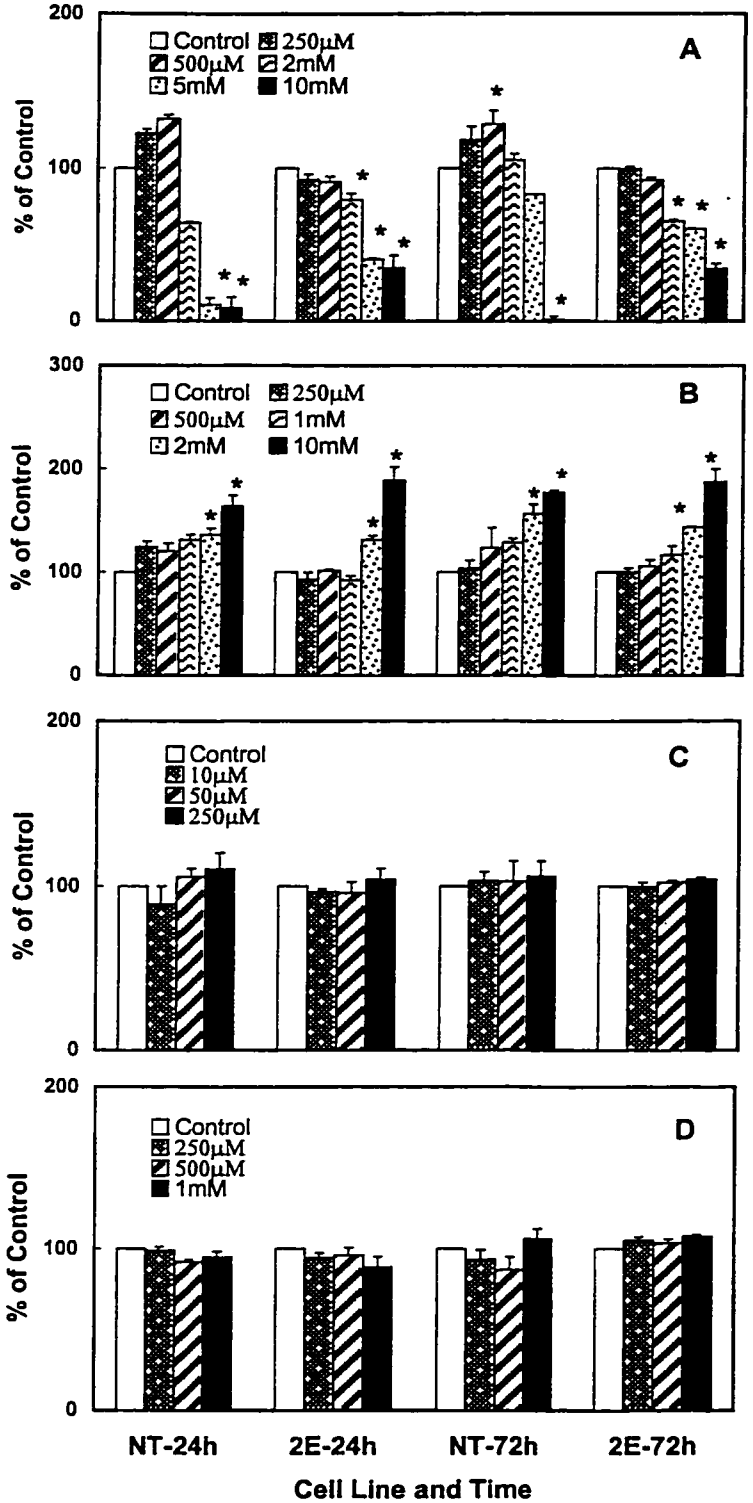


Fig. 24. The effect of different concentrations of L-Arg (A), L-Orn (B), Urea (C) and L-Gln (D) on cellular Put levels in 3 d old NT and 2E cells at 24 h and 72 h. Data are expressed as percentage of control for the respective cell lines. Each bar represents mean ± SE of three (for 2 and 10 mM) or four (all other treatments) replicates. An asterisk indicates that values for treated cells are significantly different ($p \leq 0.05$) from the untreated cells within the same cell line.

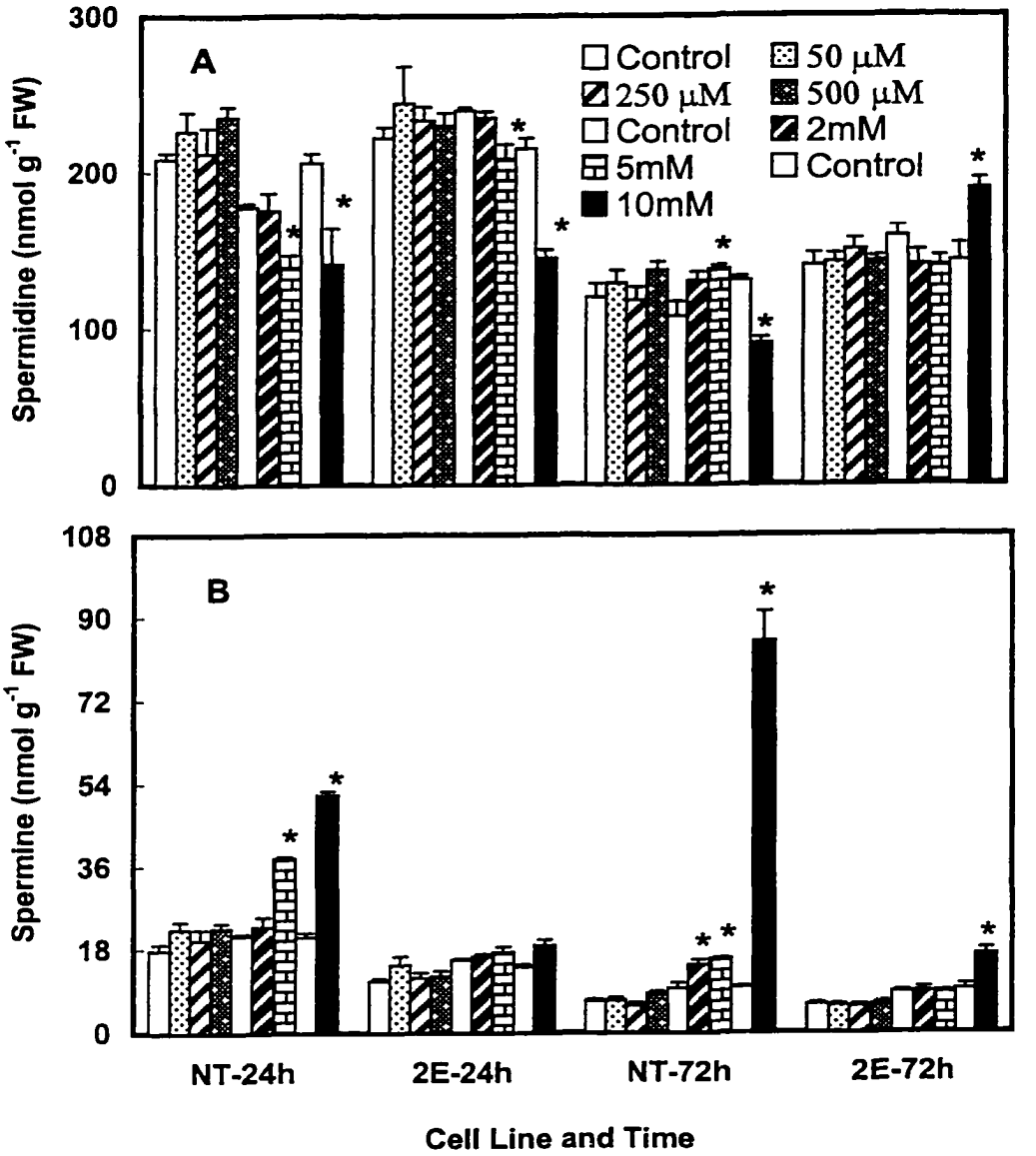


Fig. 25. The effect of different concentrations of L-Arg on cellular Spd (A) and Spm (B) levels in 3 d old NT and 2E cells at 24 h and 72 h. Each bar represents mean ± SE of four replicates. An asterisk indicates that the values for treated cells are significantly different ($p \leq 0.05$) from untreated cells at a given time within the same cell line.

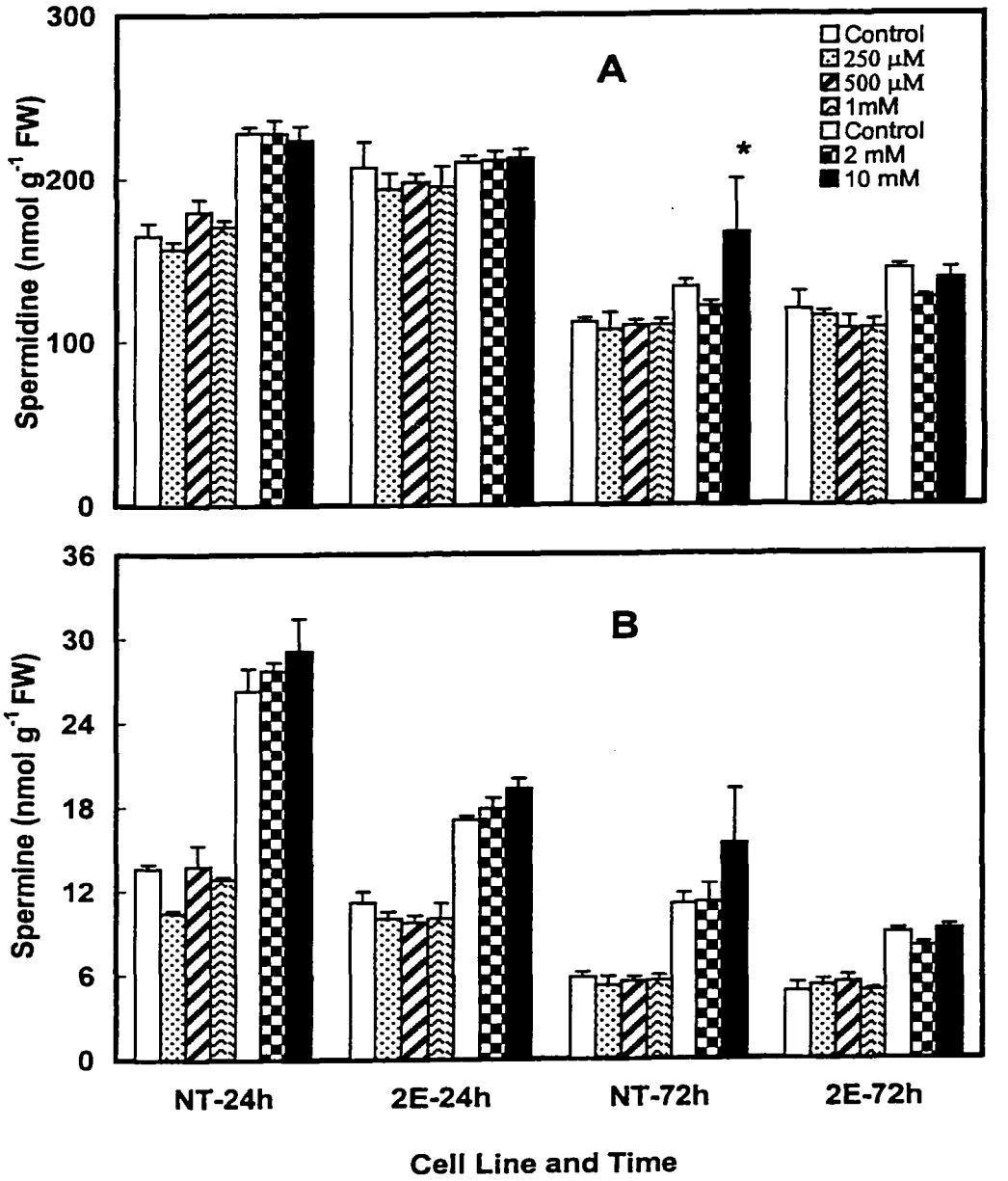


Fig. 26. The effect of different concentrations of L-Orn on cellular Spd (A) and Spm (B) levels in 3 d old NT and 2E cells at 24 h and 72 h. Each bar represents mean ± SE of four replicates. An asterisk indicates that the values for treated cells are significantly different ($p \leq 0.05$) from untreated cells at a given time within the same cell line.

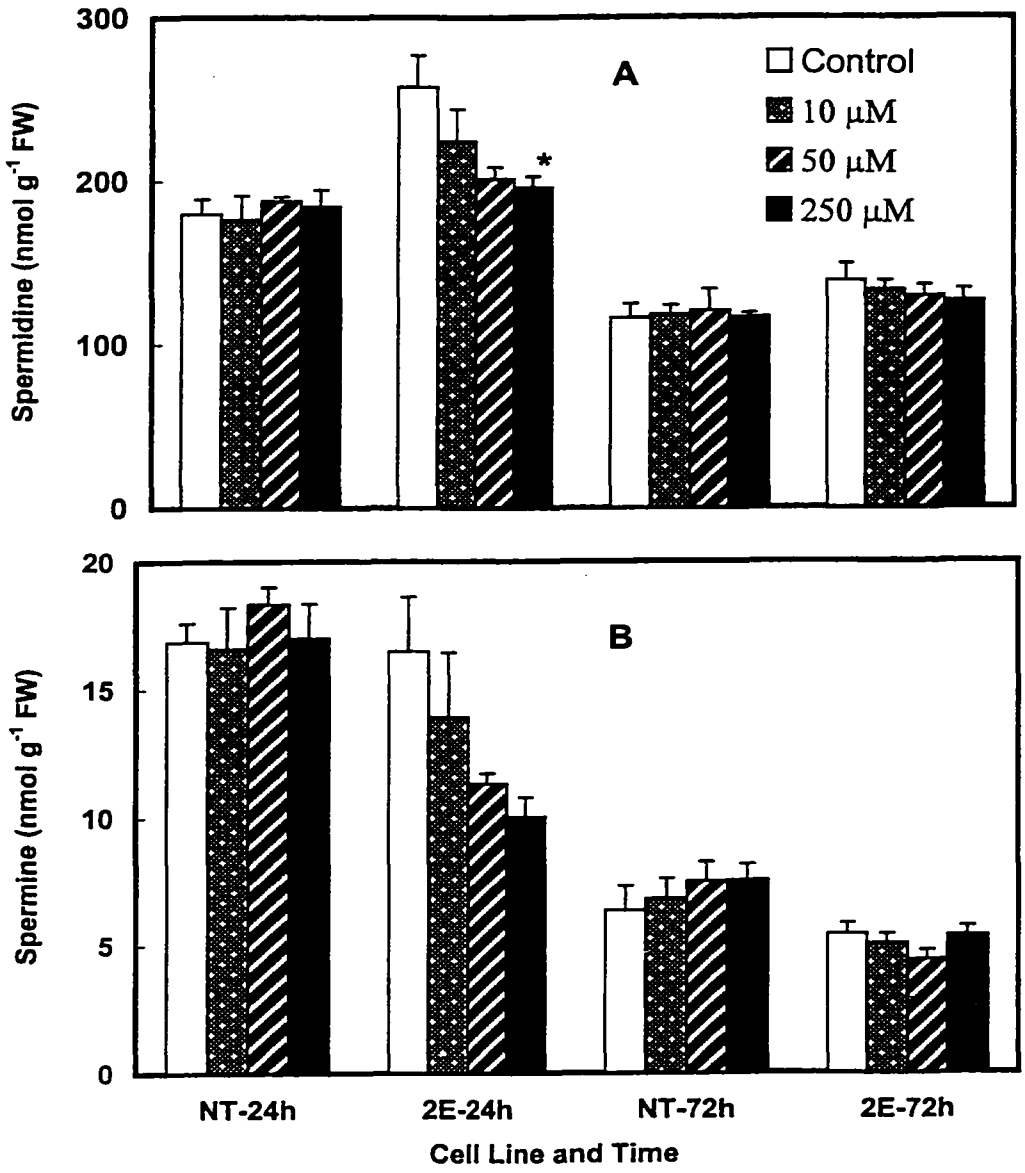


Fig. 27. The effect of different concentrations of urea on cellular Spd (A) and Spm (B) levels in 3 d old NT and 2E cells at 24 h and 72 h. Each bar represents mean ± SE of four replicates. An asterisk indicates that the values for treated cells are significantly different ($p \leq 0.05$) from untreated cells at a given time within the same cell line.

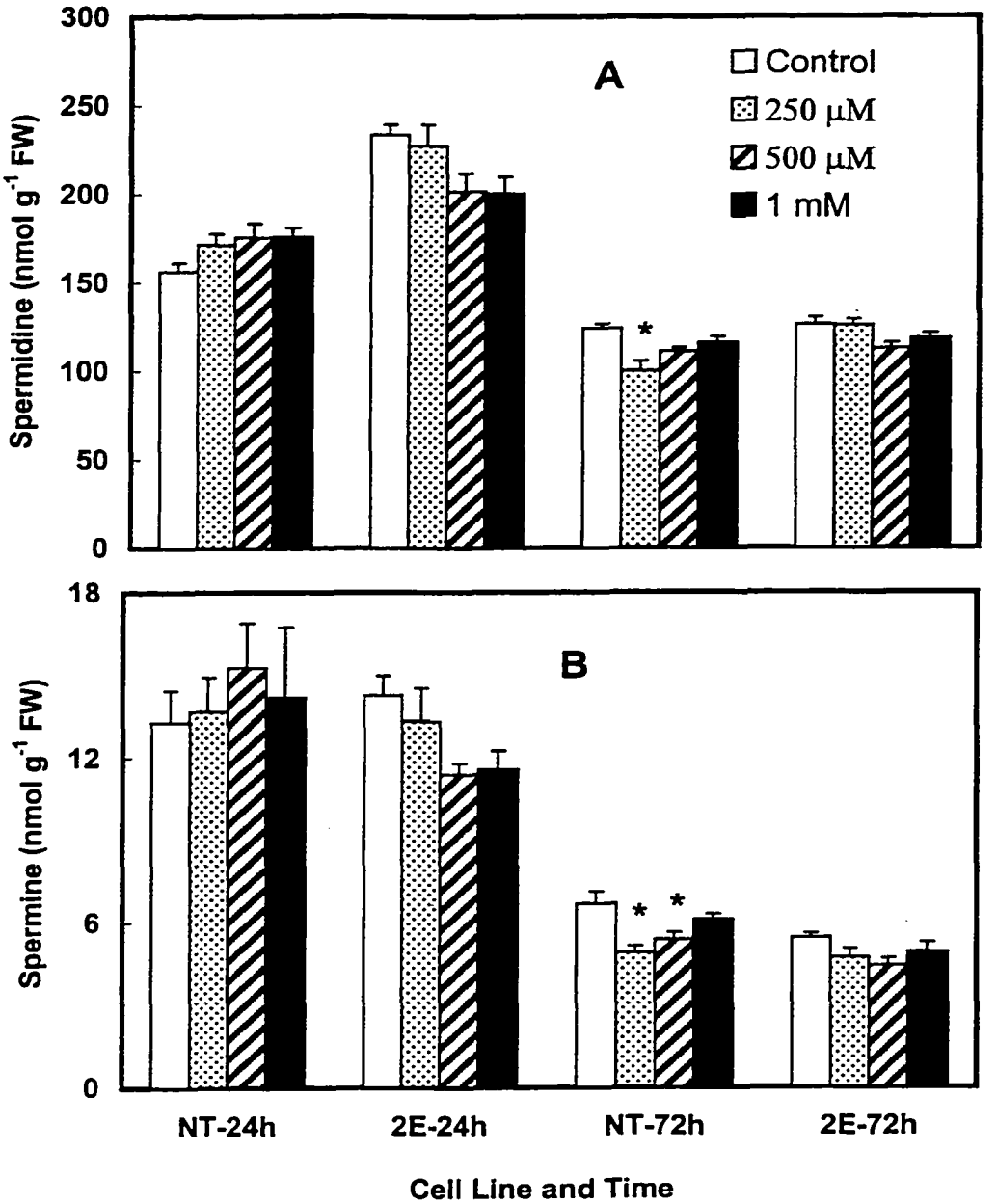


Fig. 28. The effect of different concentrations of L-Gln on cellular Spd (A) and Spm (B) levels in 3 d old NT and 2E cells at 24 h and 72 h. Each bar represents mean ± SE of four replicates. An asterisk indicates that the values for treated cells are significantly different ($p \leq 0.05$) from untreated cells at a given time within the same cell line.

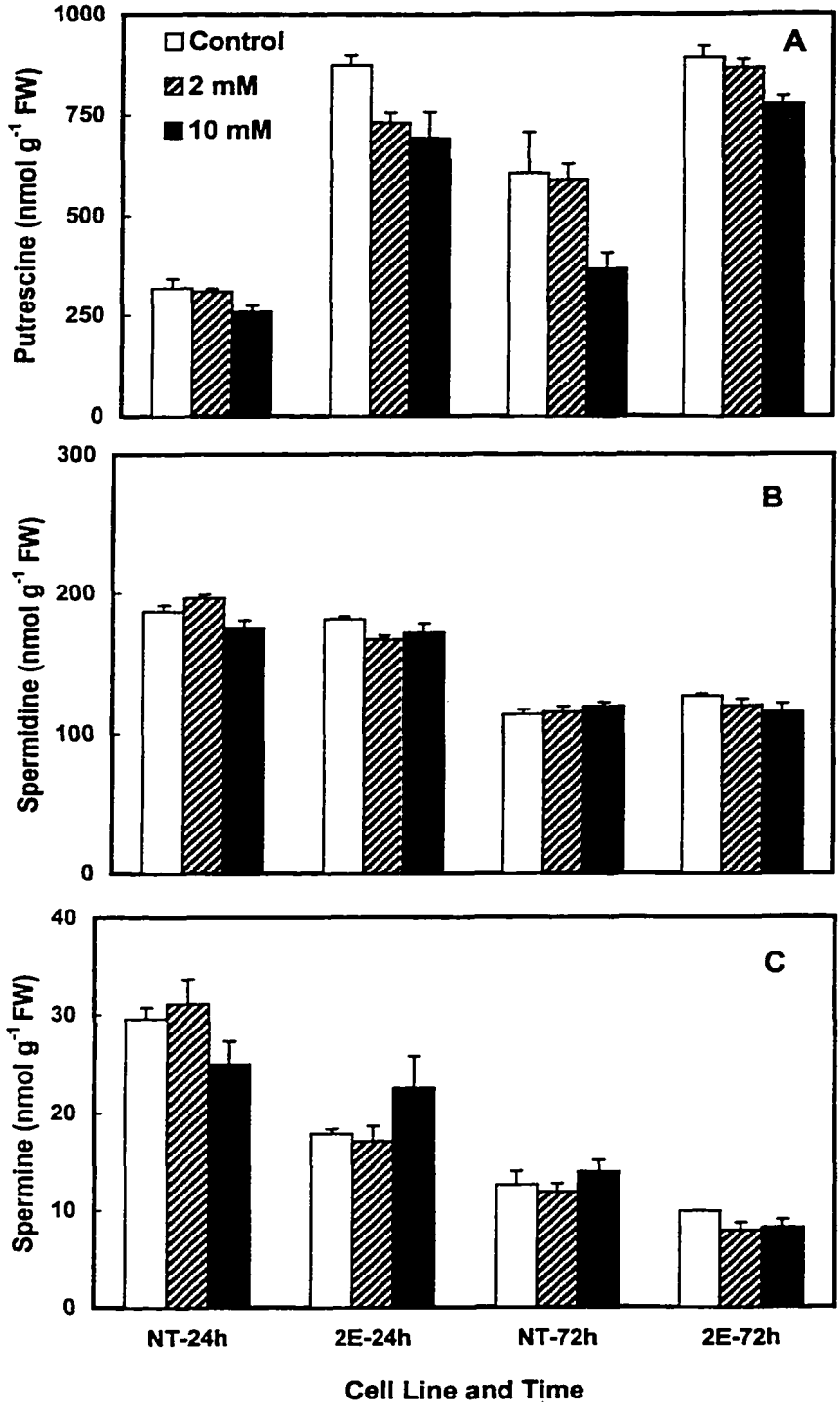


Fig. 29. The effect of different concentrations of urea on cellular Put (A), Spd (B) and Spm (C) levels in 3 d old NT and 2E cells at 24 h and 72 h. Each bar represents mean ± SE of three replicates. An asterisk indicates that values for treated cells are significantly different ($p \leq 0.05$) from the untreated cells within the same cell line at a given time.

Urea, when used as an additional source of N, at low concentrations had no effect on the cellular content of Put as shown earlier (Fig. 24C), however, high concentrations, namely, 2 and 10 mM caused a small decline in cellular Put in both the cell lines (Fig. 29A). Spermidine and Spm levels were generally not affected as a result of both high (Fig. 29B,C) and low (Fig. 27A,B) concentrations of urea except a small decrease in the cellular content of both the polyamines in 2E cells with 250 μM urea at 24 h (Fig. 27A,B).

Precursors for the amino acids Orn and Arg, namely, Gln and Glu were also tested for their effect on polyamines. Both low (250-1000 μM) (Fig. 24D; Fig. 28A,B) and high (2-10 mM) (Fig. 30A,B,C) concentrations of Gln supplied to the cells had no significant effect on the cellular content of the polyamines. However, Glu caused a significant reduction in the Put content in both cell lines (Fig. 31A). The decline in Put was concentration dependent, i.e., higher the concentration of Glu, greater was the reduction in Put. Maximum reduction in Put was seen at 10 mM. There was no significant effect of any concentration of Glu on the higher polyamines, Spd and Spm (Fig. 31B,C).

The effect of treating 3 d old cells with exogenous lysine was tested after 24 h and 48 h. The objective was to see if Put synthesis was affected as a result of exogenous lysine in the NT and 2E cell lines. There was no effect of 2 mM lysine on Put levels in NT cells at both the time periods whereas both 2 mM and 10 mM lysine caused a significant drop in cellular Put levels in the 2E cells at 48 h (Fig. 32A), indicating indirectly that lysine may be competing with Orn in these cells. There was no significant effect of lysine on Spd levels (Fig. 32B), however, Spm levels increased slightly in the NT cells at 48 h (Fig. 32C).

The effect of 1 mM GABA was studied to see if there was any feedback effect on the accumulation of cellular Put in the 2E cells. There was no significant effect of 1 mM GABA on any of the polyamines in either of the cell lines (Fig. 33A,B,C). This experiment was done only once.

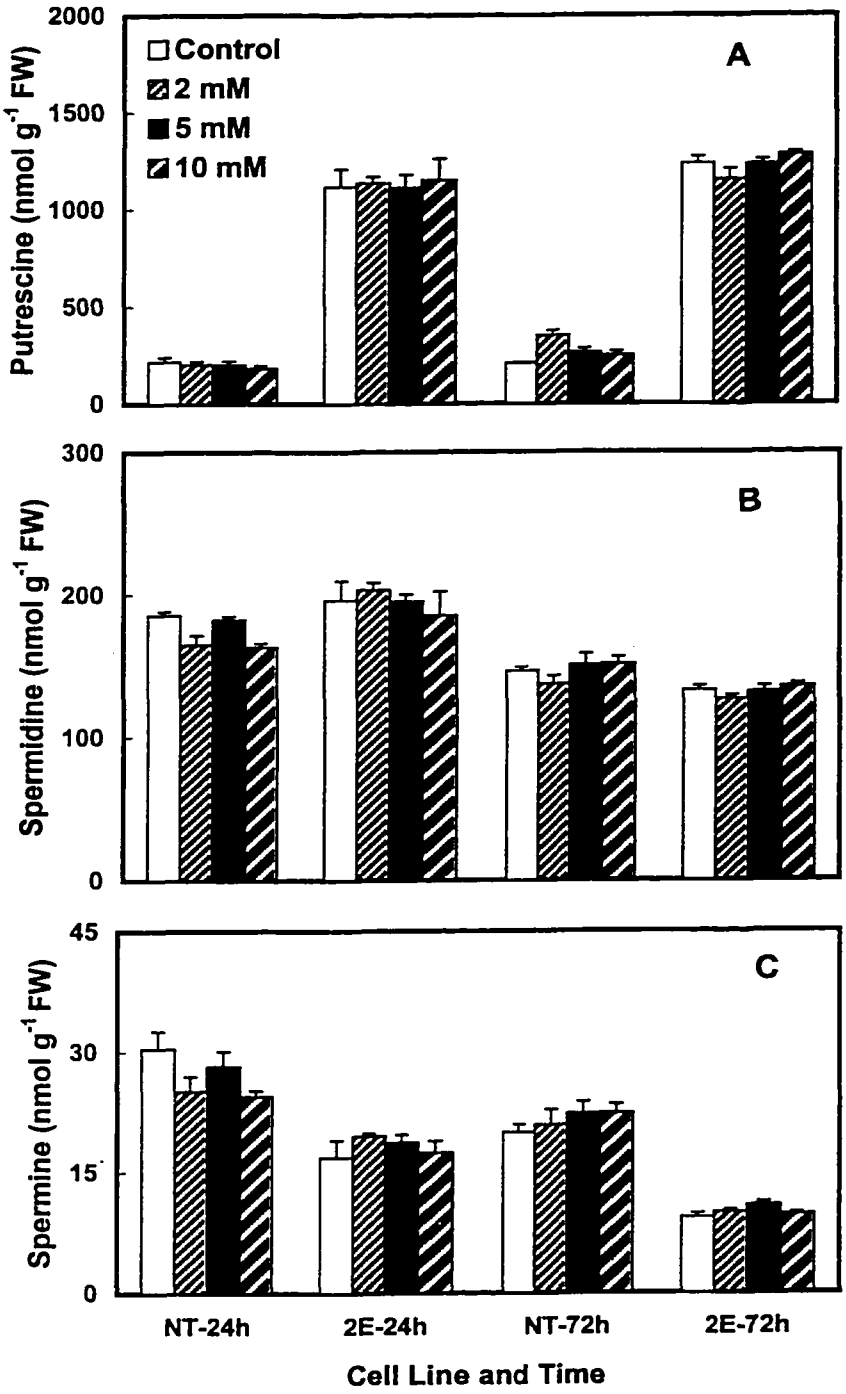


Fig. 30. The effect of higher concentrations of L-Gln on cellular Put (A), Spd (B) and Spm (C) levels in 3 d old NT and 2E cells at 24 h and 72 h. Each bar represents mean ± SE of three replicates.

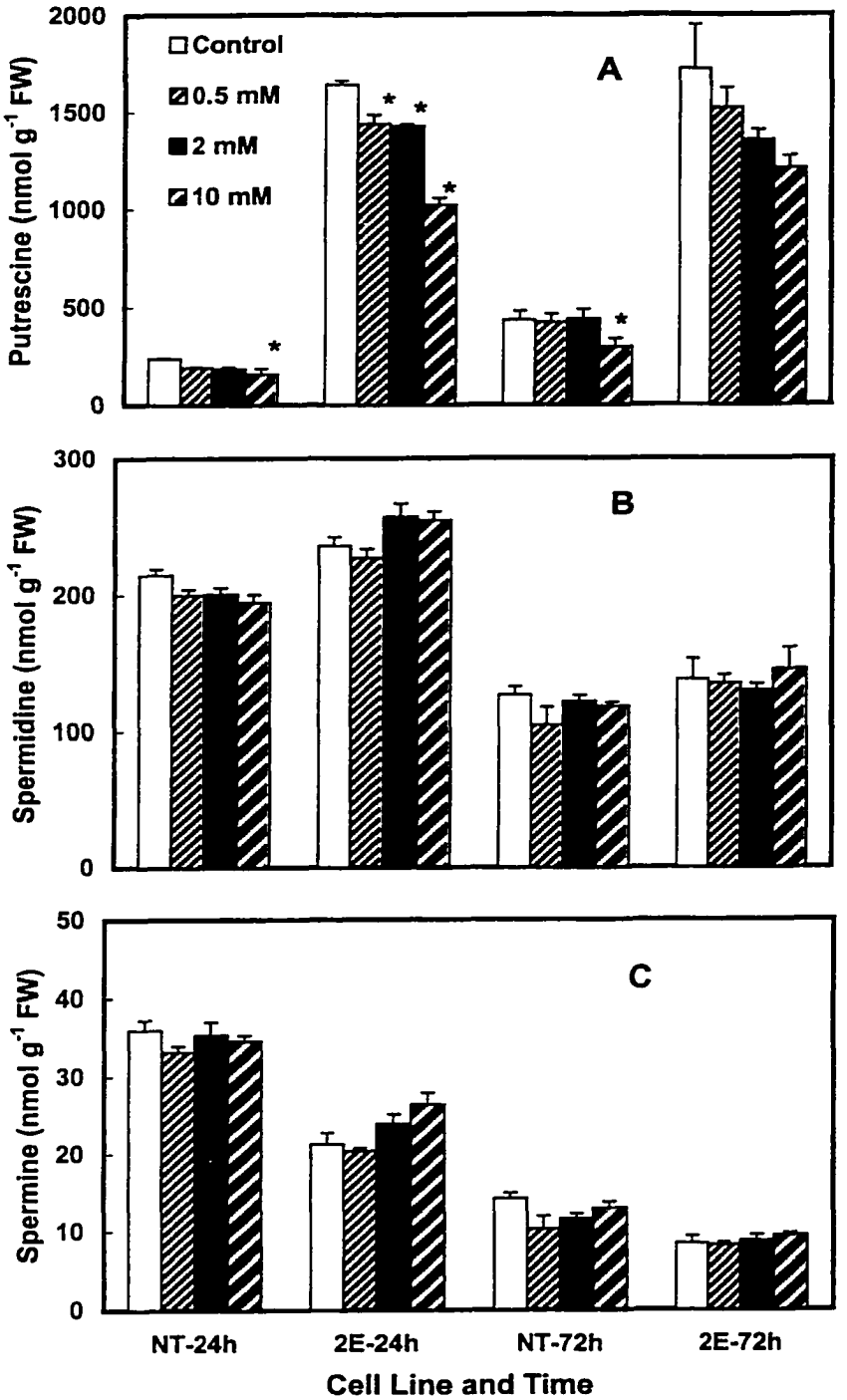


Fig. 31. The effect of different concentrations of L-Glu on cellular Put (A), Spd (B) and Spm (C) levels in 3 d old NT and 2E cells at 24 h and 72 h. Each bar represents mean ± SE of three replicates for all treatments. An asterisk indicates that values for treated cells are significantly different ($p \leq 0.05$) from the untreated cells within the same cell line.

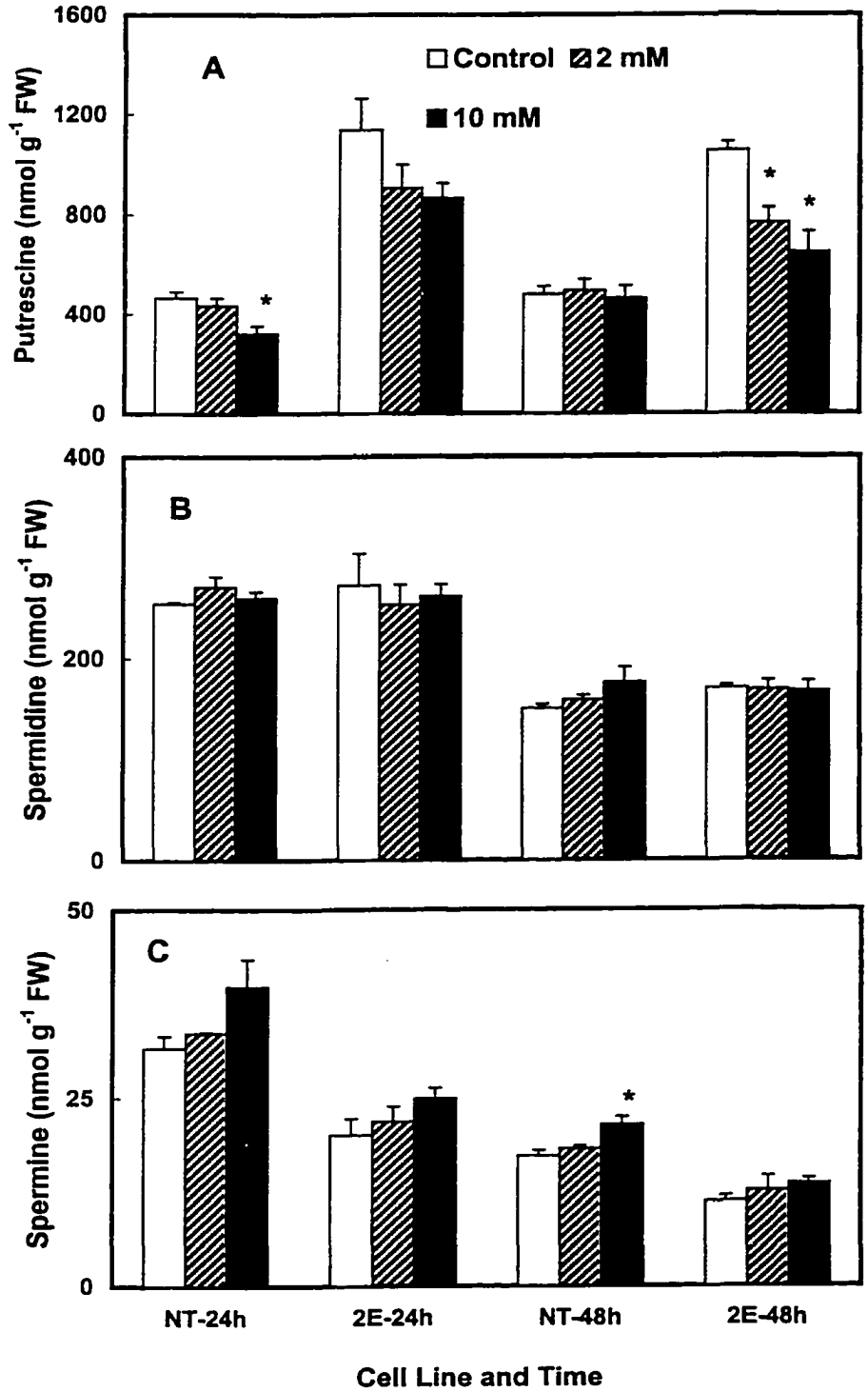


Fig. 32. The effect of different concentrations of L-lysine on cellular Put (A), Spd (B) and Spm (C) levels in 3 d old NT and 2E cells at 24 h and 48 h. Each bar represents mean ± SE of three replicates. An asterisk indicates that values for treated cells are significantly different ($p \leq 0.05$) from the untreated cells within the same cell line.

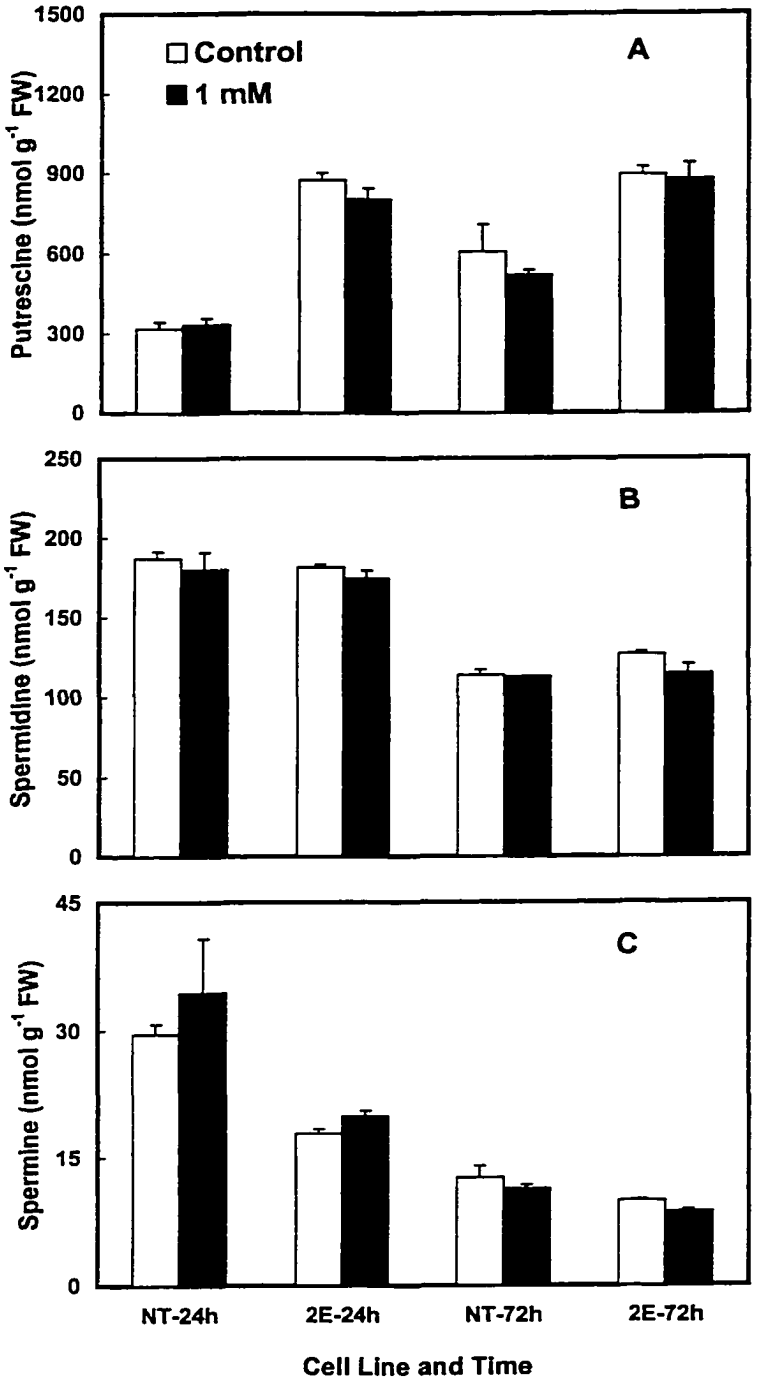


Fig. 33. The effect of 1 mM GABA on cellular Put (A), Spd (B) and Spm (C) levels in 3 d old NT and 2E cells at 24 h and 72 h. Each bar represents mean ± SE of three replicates.

The effect of agmatine was tested to study if there was an increase in Put production as a result of higher agmatine availability. Agmatine is formed from Arg through the enzyme ADC as an intermediate for Put production. Agmatine itself often does not accumulate in the cells. All concentrations of agmatine tested (1, 2, 5 and 10 mM) had significant promotory effects on Put levels in both NT and 2E cells (Fig. 34A). There was no major effect on Spd and Spm (Fig. 34B,C).

Citrulline is an intermediate in the pathway for Arg biosynthesis from Orn and/ or Glu. Citrulline had no effect on Spd (except at 72 h in the NT cell line) and Spm levels (Fig. 35B,C) whereas Put levels increased at 10 mM concentration of citrulline in the NT cells and the 2E cells at 24 h (Fig. 35A).

Since MSX caused a significant drop in the cellular polyamine levels, inhibitor treated cells were provided with different substrates exogenously to study if they could cause a reversal of the inhibitor effect. The reversal was studied by treating inhibited cells with Orn, Arg and Gln. Both Arg (Fig. 36D-F) and Gln (Fig. 37A-C) were largely ineffective in reversing the MSX effects on polyamines in either of the cell lines. The addition of 1 mM Orn in the presence of MSX caused a substantial (but never complete) reversal of the effect of this inhibitor on cellular Put in the transgenic cells and a partial reversal of Spd in both the cell lines. However, the MSX effects on Spm were not reversed (Fig. 36A-C). The cells were also treated with the substrate alone to show the specificity of the substrate in causing a reversal of the inhibitor.

Effects of Reduced Nitrogen Content

Since the transgenic cells utilize large amounts of Orn, which is produced from Glu, it was decided to test if the amounts of nitrogen (N) in the medium could be limiting for the production of Orn. Furthermore, treatments using reduced levels of N in the medium were also used to see their effects on cellular polyamines in the two cell lines. For this series of experiments, 3 d old cells were washed with NH_4NO_3-free medium and incubated without

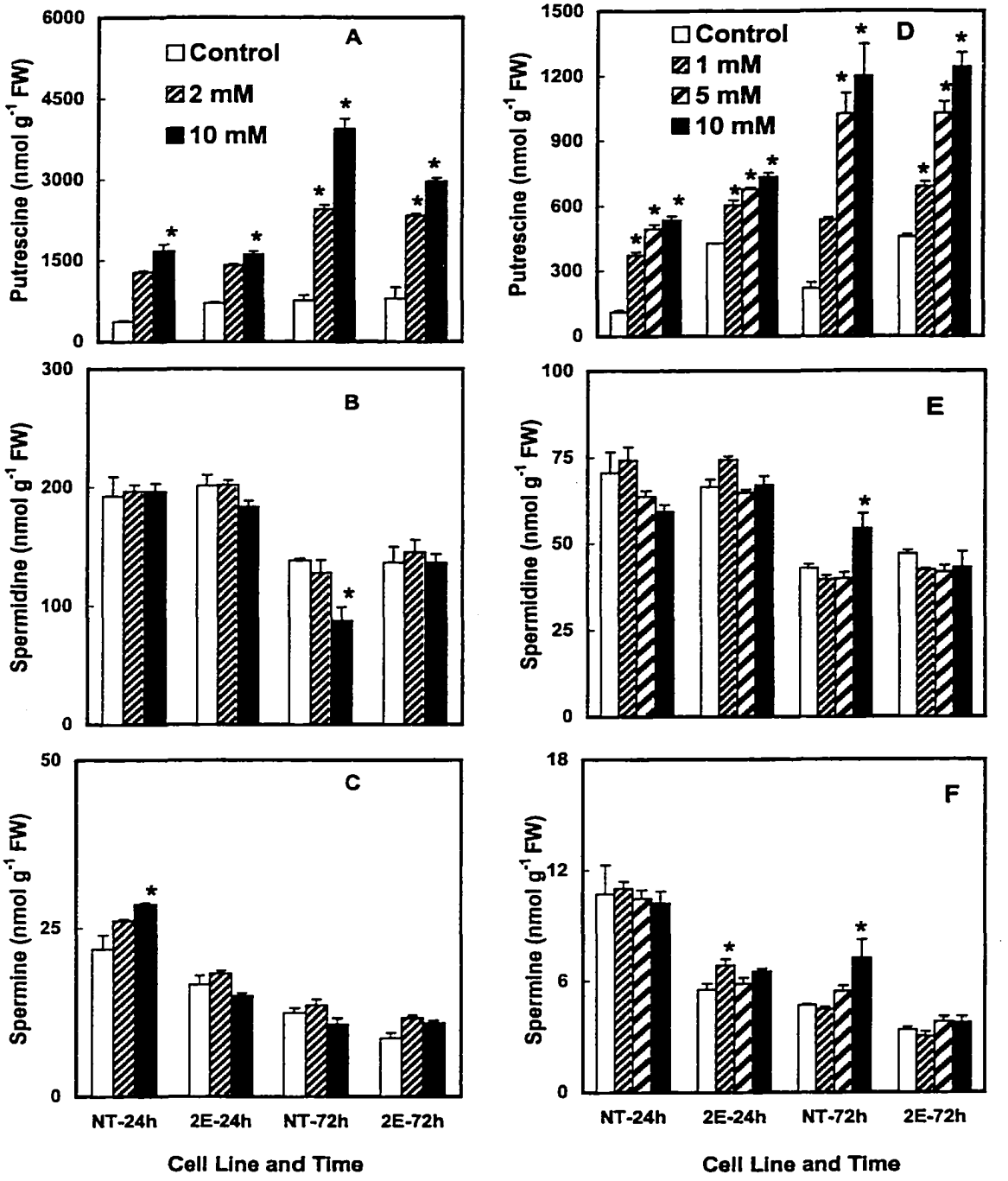


Fig. 34. The effect of different concentrations of agmatine on cellular Put (A,D), Spd (B,E) and Spm (C,F) levels in 3 d old NT and 2E cells at 24 h and 72 h. Each bar represents mean ± SE of three replicates. An asterisk indicates that values for treated cells are significantly different ($p \leq 0.05$) from the untreated cells within the same cell line.

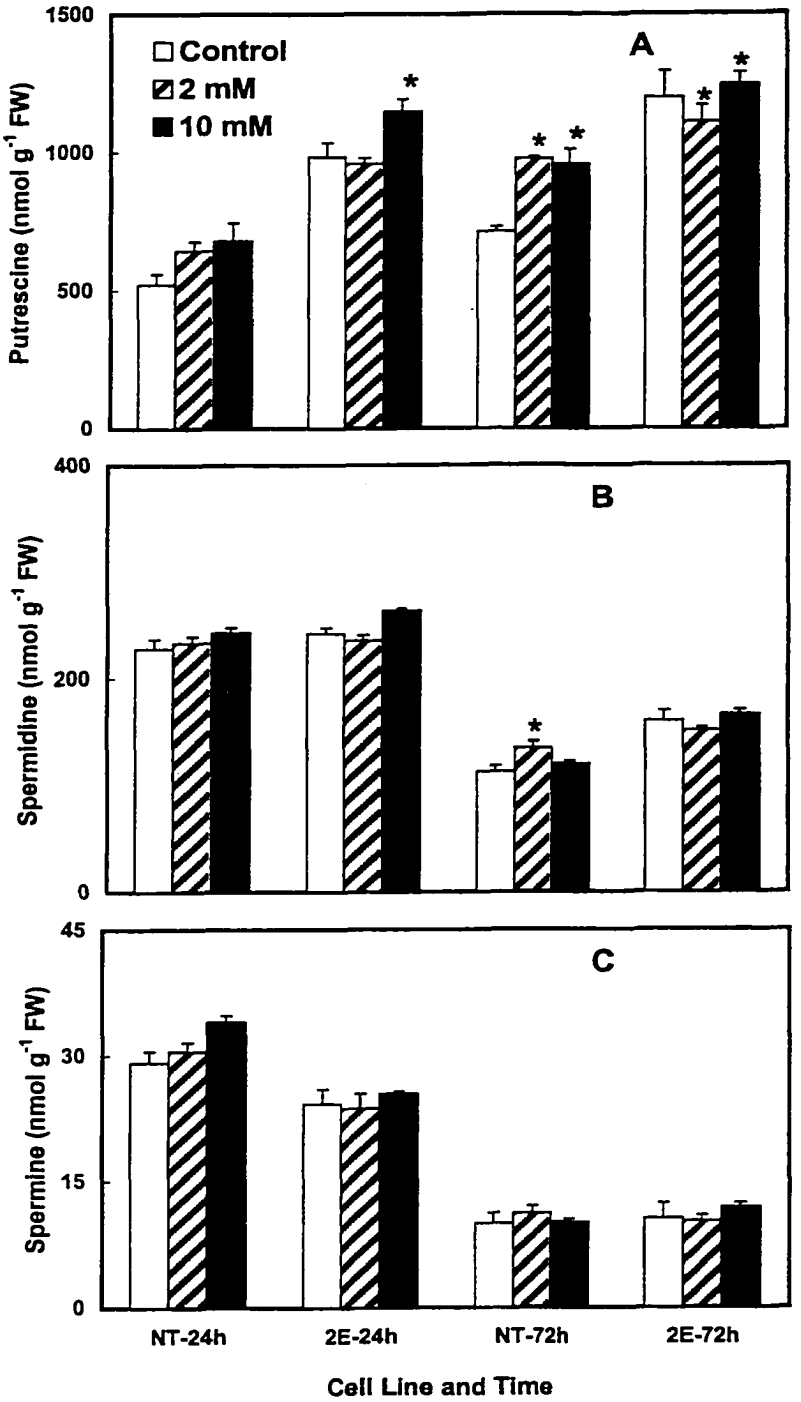


Fig. 35. The effect of different concentrations of L-citrulline on cellular Put (A), Spd (B) and Spm (C) levels in 3 d old NT and 2E cells at 24 h and 72 h. Each bar represents mean ± SE of three replicates. An asterisk indicates that values for treated cells are significantly different (p 0.05) from the untreated cells within the same cell line.

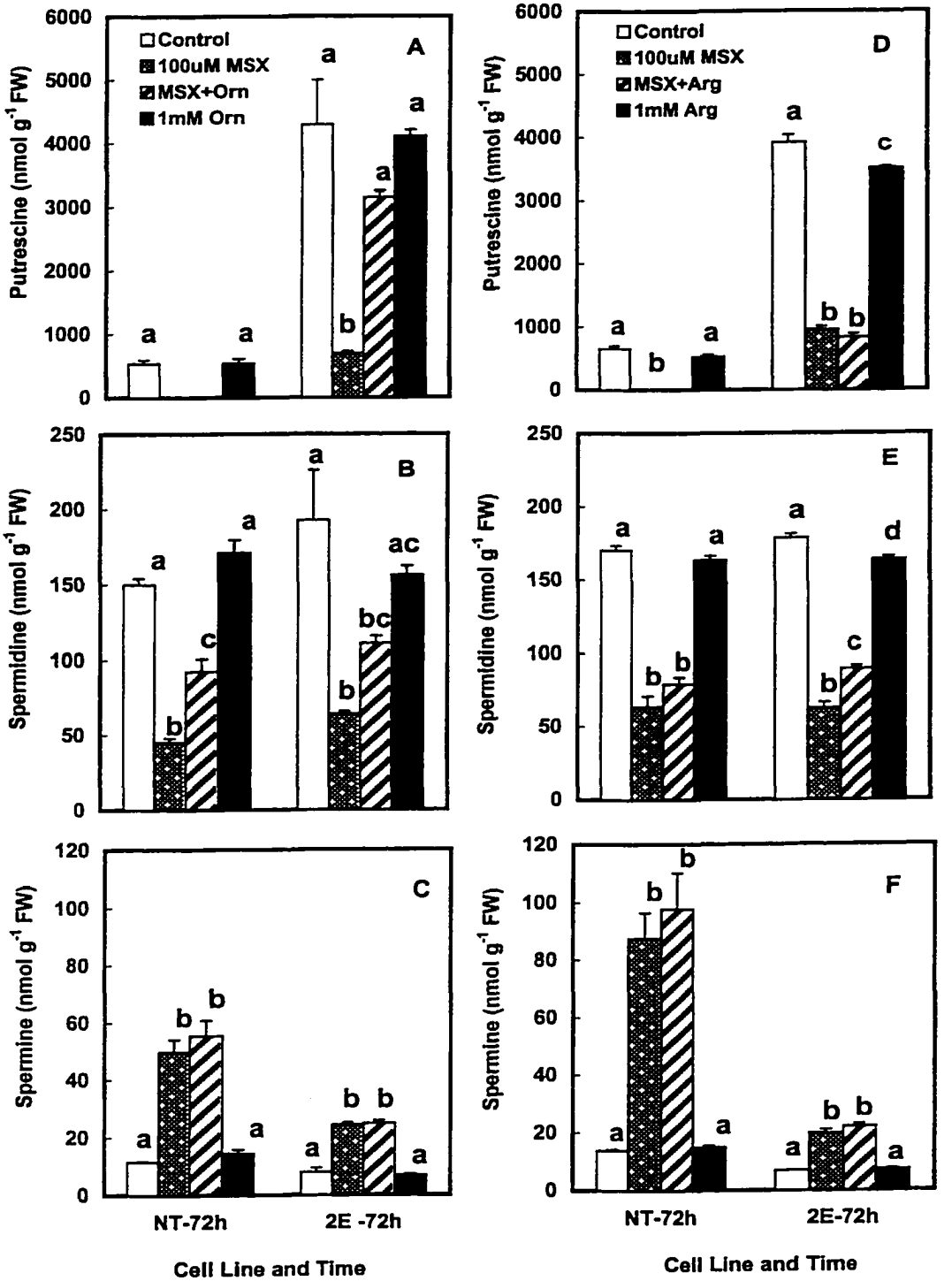


Fig. 36. The effect of 100 μM L-MSX on cellular Put (A,D), Spd (B,E) and Spm (C,F) in 3 d old NT and 2E cells and its reversal by Orn (A-C) or Arg (D-F). Treatments were given for 72 h. Data presented are mean ± SE of four replicates. Different letters above the bars indicate that values are significantly different ($p \leq 0.05$) from each other for the same cell line.

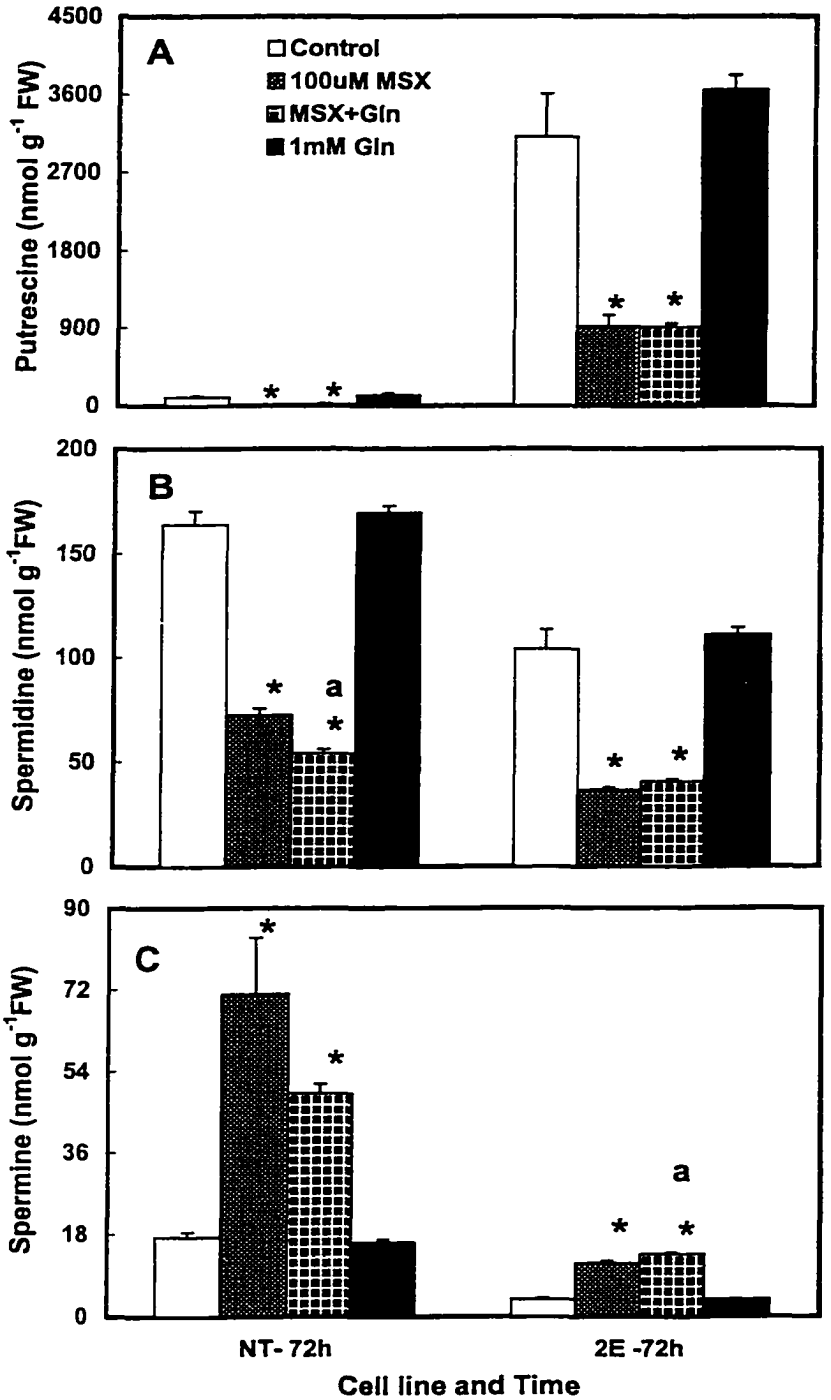


Fig. 37. The effect of 100 μM L-MSX on cellular Put (A), Spd (B) and Spm (C) in 3 d old NT and 2E cells and its reversal by Gln. Treatments were given for 72 h. Data presented are mean ± SE of four replicates. An asterisk indicates that values for different treatments are significantly different ($p \leq 0.05$) from control within the same cell line and a letter indicates significant differences of MSX+Gln treated cells from MSX treated cells.

NH_4NO_3, two different concentrations of NH_4NO_3, and one concentration of NH_4Cl. Unwashed cells were allowed to grow in the same medium and collected as controls. The cells were collected at 24 and 72 h after the experimental treatments. In an alternate set up, the cells were washed with completely N-free medium (i.e. no NH_4 NO_3, no KNO_3) and allowed to grow without N for 96 h. Samples were collected at different times for polyamine analysis. Similar experiments with elevated levels of NH_4NO_3 or KNO_3 were done in parallel by Dr. R. Minocha's laboratory. Removal of NH_4NO_3 from the medium caused a rapid reduction in the Put content of both the NT and the 2E cells (Fig. 38A). At 24 h, the percent loss of Put was higher in the NT cells while the absolute amount of Put loss was greater in the 2E cells. The effect of NH_4NO_3 removal was more pronounced at 72 h of treatment - both cell lines having lost more than 80% of Put by this time. The addition of NH_4NO_3 to washed cells resulted in a significant recovery of Put content in both cell lines. Even 20% of the normal strength of NH_4NO_3 in the medium was as effective as 100% of the normal strength at 24 h. Again; the response of the two types of cells was similar. The recovery in Put content was visible at both 24 and 72 h. The recovery was dependent upon the presence of NO_3^- as a counter ion along with NH_4^+, the addition of NH_4Cl was not effective. Also, a reduction in Put content was seen in the washed cells regardless of whether or not NH_4NO_3 was added back into the medium.

With respect to Spd, there was only a small (less than 20%) loss at 24 h on removal of NH_4NO_3 from the medium, there being no significant decrease at 72 h in either of the cell line (Fig. 38B). The addition of NH_4NO_3 at full strength or at 20% of the normal strength were equally effective in maintaining Spd at control levels, NH_4Cl causing a greater loss than even the absence of NH_4NO_3. At 72 h, the Spd content in both the cell lines was higher than the respective controls when normal amounts of NH_4NO_3 were added back. A similar increase was seen in the NT cells with 20% of the normal amount of NH_4NO_3 being added back.

The effect of removal of NH_4NO_3 on cellular Spm content was even less than that seen for Spd (Fig. 38C). There was no change in Spm at 24 h and an increase in Spm at 72 h on

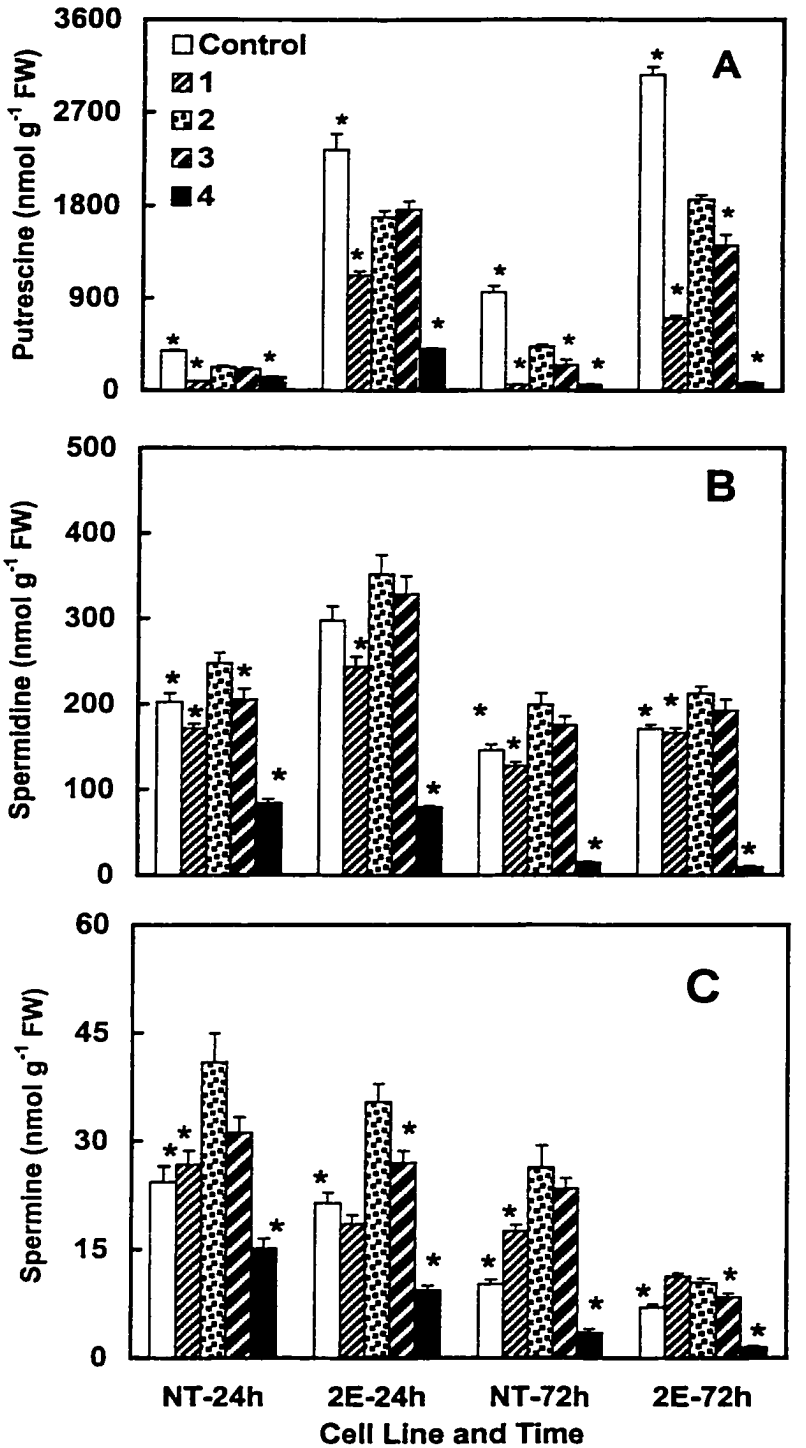


Fig. 38. Effect of addition of different concentrations of NH_4NO_3 to 3 d old cells on the levels of cellular Put (A), Spd (B) and Spm (C). Treatments are unwashed control (Control); Washed with no added NH_4NO_3 (1); Washed control having normal medium strength (20.6 mM) NH_4NO_3 (2); washed with one fifth normal medium strength (4.12 mM) NH_4NO_3 (3) and 20 mM NH_4Cl (4). Data are mean ± SE of six replicates that represent two experiments. An asterisk indicates significant differences ($p \leq 0.05$) from washed control (treatment 2) for the same cell line at a given time period.

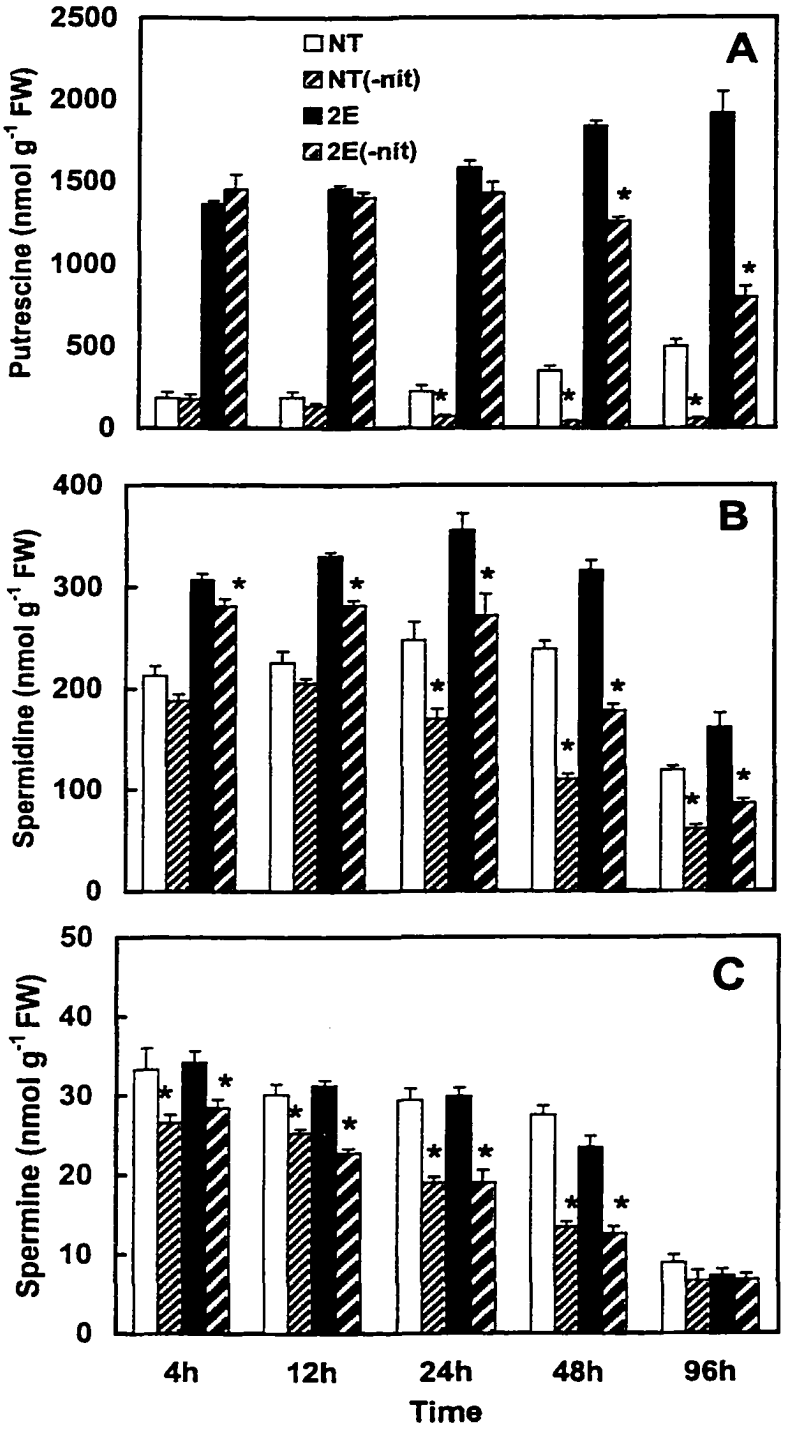


Fig. 39. Cellular content of Put (A), Spd (B) and Spm (C) in the NT and 2E cells after transfer of cells to nitrogen free medium. Three day old cells were transferred to nitrogen free medium for indicated time period. Data presented are mean ± SE of six replicates that represent two experiments. An asterisk indicates significant differences ($p \leq 0.05$) between control and treatment for the same cell line at a given time period.

washing and removal of NH_4NO_3 from the medium. Further increases in Spm were seen by the re-addition of NH_4NO_3 at either 20% or 100% of the normal strength. Ammonium chloride again caused a significant reduction in the Spm content, the effects being similar in the two cell lines.

On complete removal of N from the medium, a time-dependent loss in Put content was observed for NT cells, starting as early as 4 h and continuing up to 96 h of incubation (Fig. 39A). In the 2E cells, however, a decrease was not seen until 24 or 48 h following the removal of N. While the NT cells lost as much as 80-90% of Put by 96 h, the maximum losses in the 2E cells were only 50-60%. A much smaller reduction in Spd as compared to Put was seen in the NT cells on incubation in the N-free medium, the reduction becoming visible at 12-24 h and continuing up to 96 h (Fig. 39B). The greatest reduction reached 50% at any time. Similar trends of changes in Spd were seen for 2E cells, these cells always maintaining higher levels of Spd than the NT cells at any given time of analysis. Changes in the cellular content of Spm followed a trend similar to that seen for Spd in that its levels decreased with time in both cell lines up to 48 h, and the maximum loss was about 50% (Fig. 39C).

In experiments involving supplementation with higher amounts of NH_4NO_3 or KNO_3, a reduction in Put content was observed and the effect on Spd and Spm were relatively small in both cell lines (Minocha et al., submitted).

Percent Increase in Fresh Weight

The increases in fresh weight of the two cell lines at 3, 4 and 6 d of culture were studied. Four experiments were selected at random for measurement of FW yield per flask. The fresh weight of the cells in 10 mL medium was recorded on day zero, i.e. the day of subculture. The increase in fresh weight is expressed as percent increase over day zero. It was observed that in most experiments the percent increase in fresh weight was significantly higher in 2E cells than the NT cells. The growth showed a progressive increase as expected at least up to 6 d of culture in

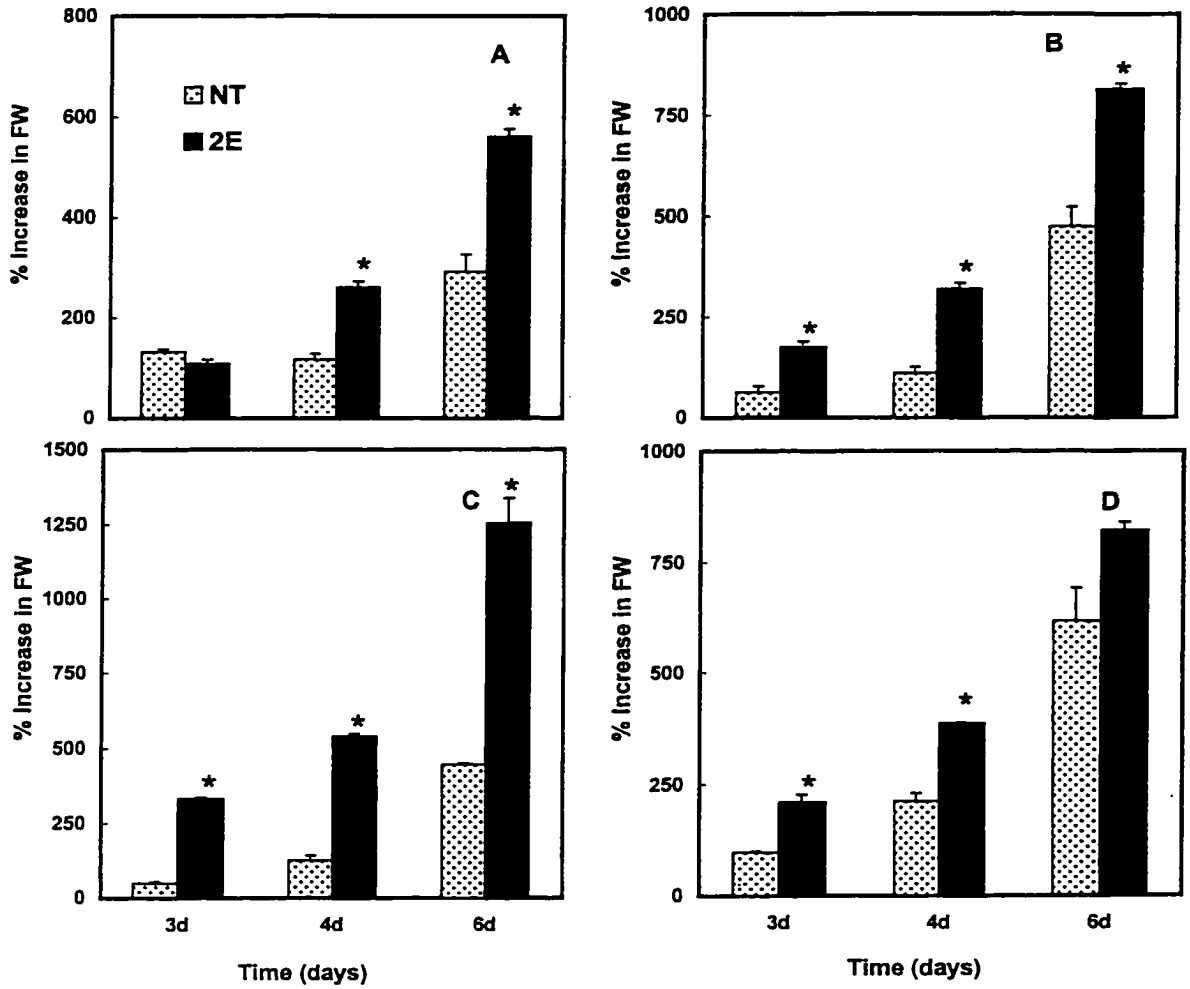


Fig. 40. The percentage increase in fresh weight of NT and 2E cells at 3, 4 and 6 d of analysis (A-D). Data presented are mean ± SE of three replicates. Each graph represents a separate experiment. An asterisk indicates that values for 2E are significantly different ($p \leq 0.05$) from NT cells at a given day.

both the cell lines (reaching up to a 1200% increase by 6 d) (Fig. 40). Data shown are from four separate experiments. The dry weight of the cells from the two cell lines was found to be quite similar (data not shown).

Glutamine synthetase Activity

Since GS is a key enzyme in NH_4^+ assimilation, its activity would be expected to change in response to higher utilization of NH_4^+ for Orn biosynthesis. Therefore, the GS activity was analyzed on 3, 4 and 5 d of culture when the cells are in the log phase of growth. A standard curve of γ-hydroxyGlu versus absorbance was generated to determine the amount of Gln made in the reaction as a result of enzyme activity of GS (Fig. 41A). The enzyme activity was substantially higher in the NT cell line as compared to the 2E cells on all days the analysis was done (Fig. 41B). Small variation in GS activity on different days of culture was observed in both the cell lines. The experiment was done two times and data from a representative experiment are shown here.

Effect of Cycloheximide on Mouse ODC

Mouse ODC is known to have a rapid turnover (approximately 30 min). This experiment was aimed at analyzing the half-life of the enzyme in the 2E cell line. The mouse *odc*-cDNA used for transformation (see Fig. 6) is a truncated sequence from which PEST amino acid domain that is responsible for the rapid turnover of the mouse ODC protein was excluded. This imparts the protein higher stability. Three day old 2E cells were treated with [U-^{14}C] Orn and the rate of $^{14}CO_2$ produced was analyzed for 4 h in the presence or absence of the protein synthesis inhibitor, cycloheximide in order to determine the half-life of mouse ODC. The inhibitor-treated cells had significantly lower $^{14}CO_2$ production within the first 30 min as compared to the untreated cells. The $^{14}CO_2$ produced increased only slightly in the first 30 min (both with and without inhibitor

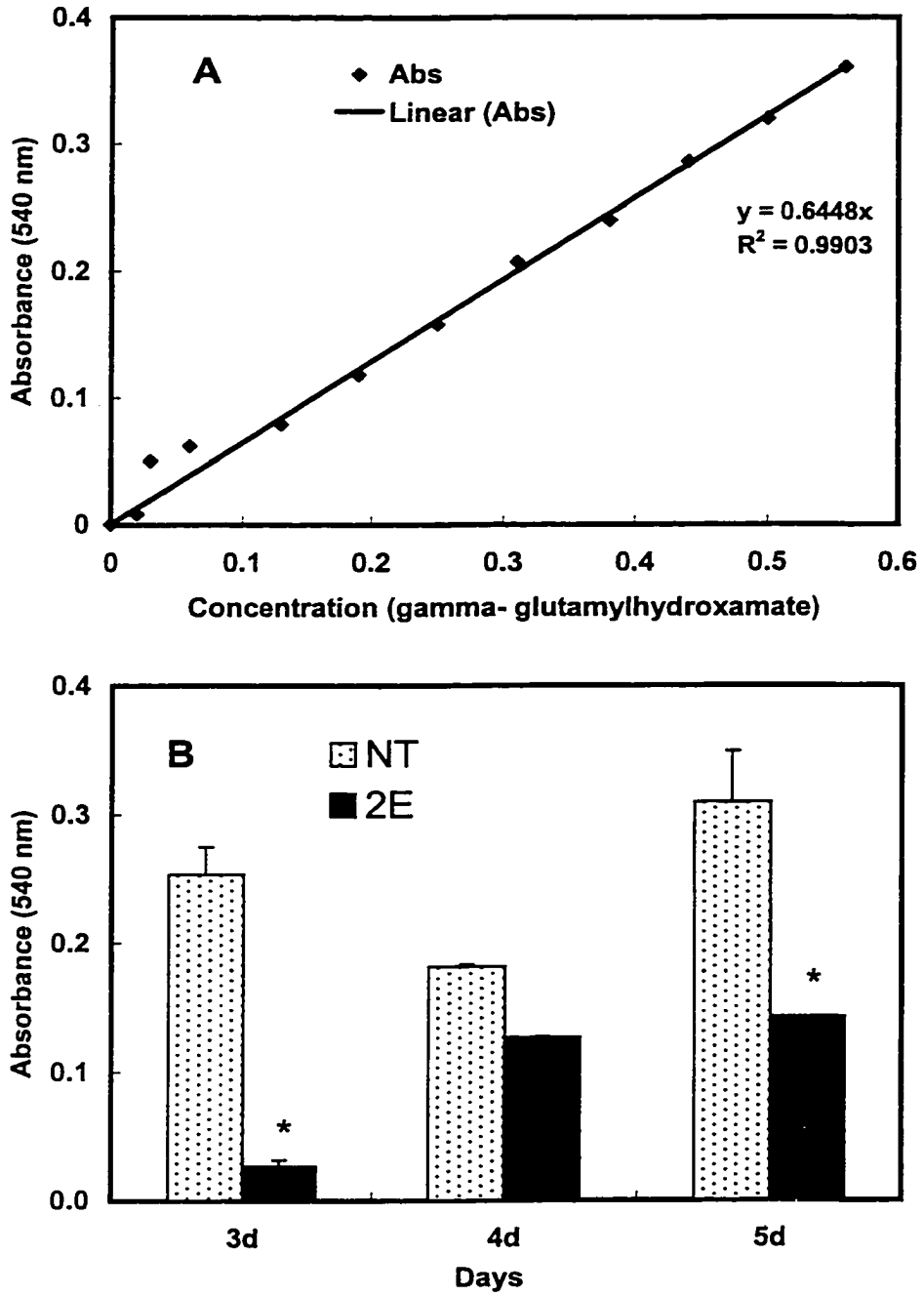


Fig. 41. Glutamine synthetase (GS) assay of 3, 4 and 5 d old NT and 2E cell lines. Standard curve for the assay (A). Absorbance after 20 min (B). An asterisk indicates that values for 2E are significantly different ($p \leq 0.05$) from NT cells at a given day.

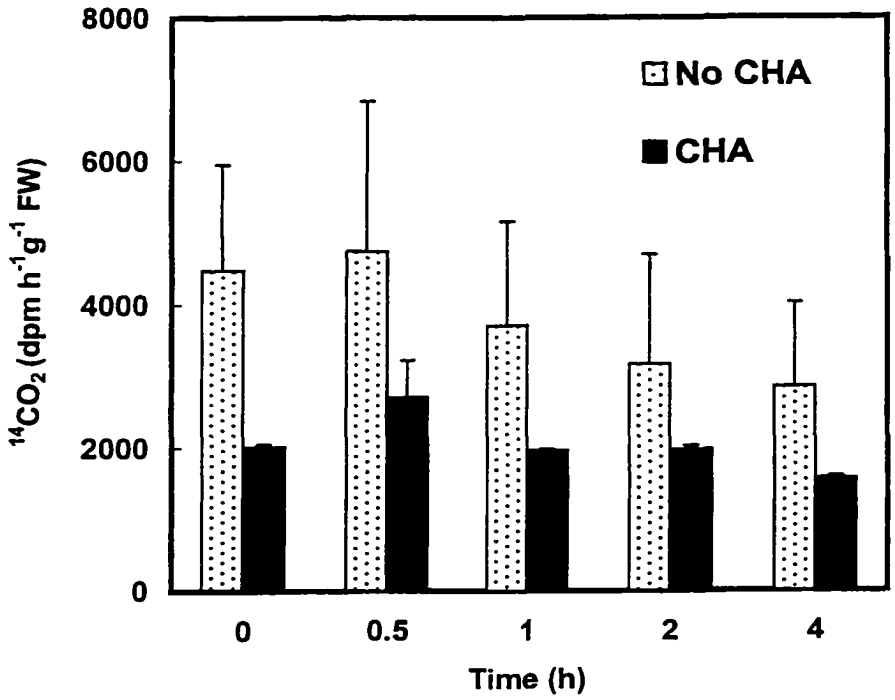


Fig. 42. The rate of $^{14}CO_2$ production from 2E cells in the presence (+) and absence (-) of cycloheximide (CHA) to determine the half-life of mouse ODC. Three day old cells were incubated with 0.1 μCi of [U-^{14}C] Orn for varying lengths of time. Data presented are mean ± SE of four replicates.

treated cells) and then progressively declined in the 4 h study period (Fig. 42). Longer term incubations would be required to calculate the half-life of the enzyme since the truncated version of the mouse ODC is expected to be quite stable. The results show that the ODC protein in the 2E cells is more stable than the full-length ODC proteins.

Effect of Jasmonic Acid (JA) and Salicylic Acid (SA) on Polyamine Levels

Jasmonic acid and salicylic acid are components of the signaling pathways implicated in stress responses of plants. They are known to have an inhibitory effect on ethylene, whose biosynthetic pathway may compete with the polyamine biosynthetic pathway. The objective was to study if providing these compounds exogenously in cell cultures had any effect on the cellular polyamine content.

Three day old cultures were treated with 2, 10 and 50 μM JA and analyzed for polyamine content at 24 h and 72 h. All concentrations of JA caused a small decline in the cellular Put levels at 24 h in the 2E cell line, the two higher concentrations being inhibitory in the NT cells as well (Fig. 43A). At 72 h, only 50 μM JA caused a decrease in Put in both cell lines, lower concentrations resulting in a small increase in Put. Exogenous JA had variable effects on Spd in that it was promotory at higher concentrations at 72 h while the levels declined at 24 h (Fig. 43B). There was generally little effect on Spm levels at either 24 h or 72 h (Fig. 43C).

Three day old cells treated with 0.2, 1 and 5 mM SA exhibited a dramatic decline in the levels of Put at 24 h as well as 72 h in both the NT and 2E cells (Fig. 44A). Higher the concentration, higher was the inhibitory effect of SA. The higher the concentration (5 mM SA) was inhibitory to the cells for Spd and Spm as well (Fig. 44B,C).

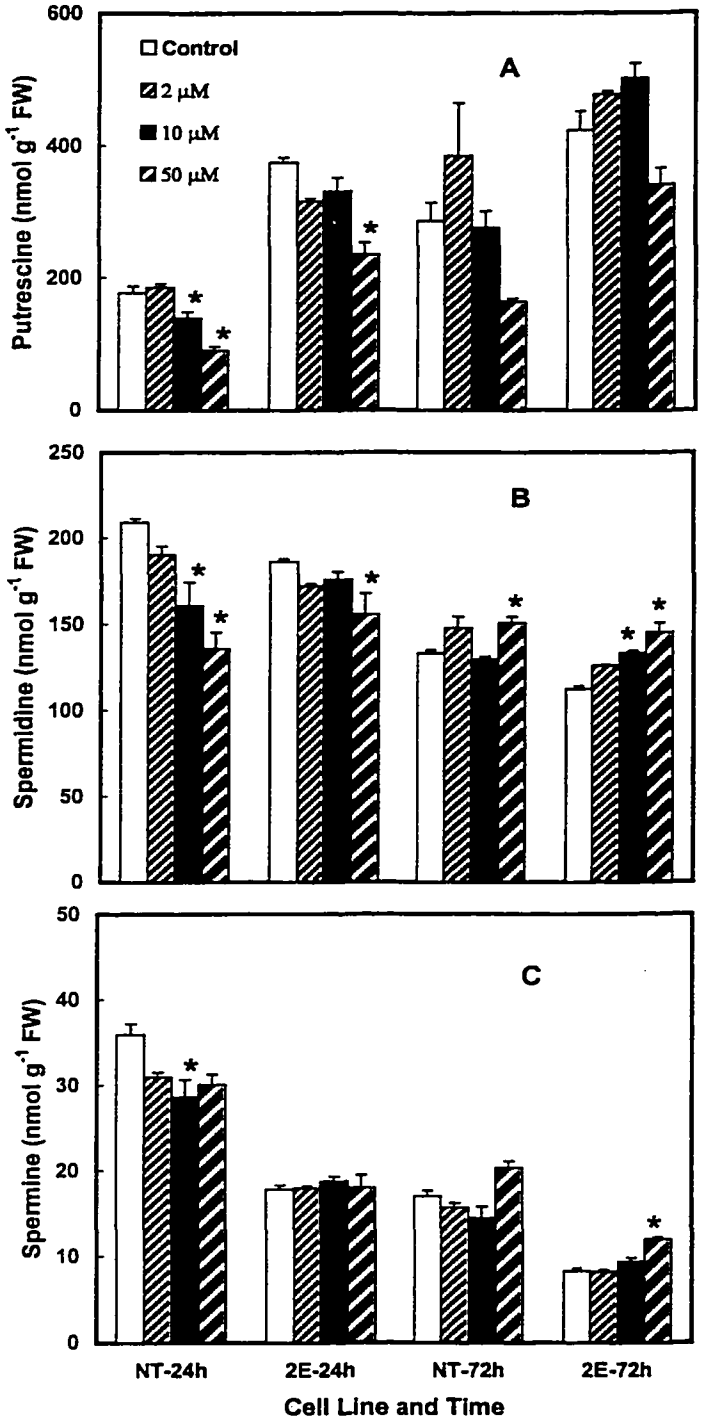


Fig. 43. The effect of different concentrations of jasmonic acid (JA) on cellular Put (A), Spd (B) and Spm (C) levels in 3 d old NT and 2E cells at 24 h and 72 h. Each bar represents mean ± SE of three replicates. An asterisk indicates that values for treated cells are significantly different ($p \leq 0.05$) from the untreated cells within the same cell line at a given time.

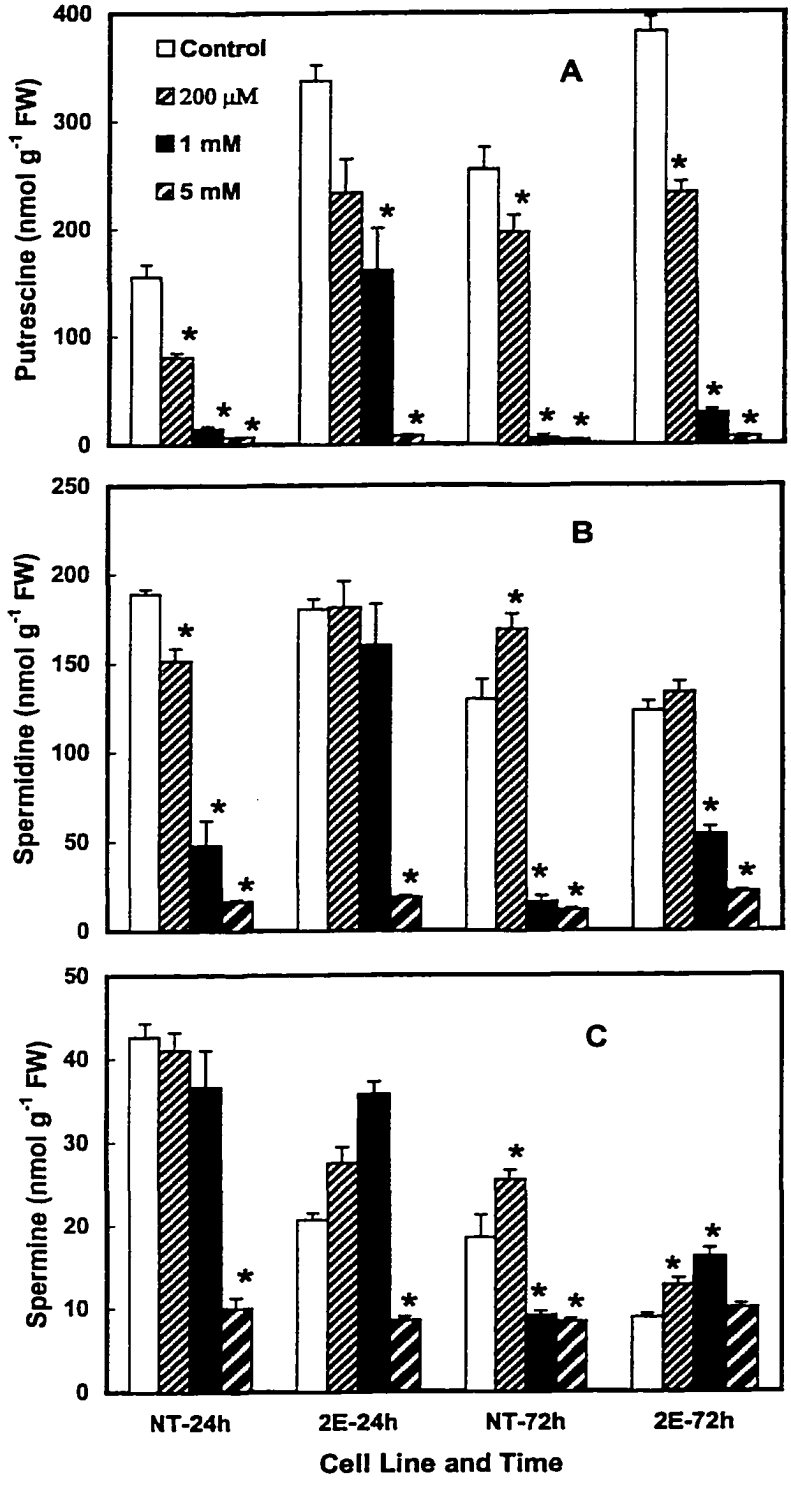


Fig. 44. The effect of different concentrations of salicylic acid (SA) on cellular Put (A), Spd (B) and Spm (C) levels in 3 d old NT and 2E cells at 24 h and 72 h. Each bar represents mean ± SE of three replicates. An asterisk indicates that values for treated cells are significantly different ($p \leq 0.05$) from the untreated cells within the same cell line at a given time.

Putrescine Turnover in Transgenic and Non-transgenic Cells

The turnover of cellular Put in the two cell lines was investigated in two different ways: (1) labeling endogenous Put in the cells by feeding them with [^{14}C] Orn and analyzing the loss of label from the Put fraction in the cells with time after trandferring to label-free medium; and (2) allowing the cells to accumulate exogenously supplied [^{14}C] Put and following its loss with time after being transferred to label-free medium. The cells were incubated either with [U-^{14}C] Orn or with [1,4-^{14}C] Put for 2 h, washed with label-free medium, and then transferred to label-free fresh medium. Following transfer to the label-free medium, samples were collected at different times for analysis of their [^{14}C] polyamine content by Thin Layer Chromatography (TLC) and the total polyamine content by High Performance Liquid Chromatography (HPLC).

Cellular Content of Polyamines

Since precise calculations of the specific radioactivity of the three major polyamines were planned, it was deemed necessary to measure the total amounts of the PCA soluble polyamines in the same cell samples that were used for turnover studies. The data presented in Table V show cellular polyamines in the two cell lines during the entire period of study at different times when samples were collected for analysis of labeled polyamines. Although the overall contents of polyamines in both cell lines were generally higher during the period of this study, the 2E cells had 2-3 fold higher Put content and higher Spd content than the NT cells; the Spm levels were either comparable or lower in the NT cells. Also, as expected, Spm was always less than 5% of the total polyamines in the cells. These data were used for calculation of the specific activity of the three polyamines in combination with the data on labeled polyamines.

Recovery of Label During Dansylation and Processing of Polyamines

The efficiency of extraction of radioactivity from the cells into PCA by freeze-thawing, and the recovery of label during various steps of the dansylation procedure and TLC separation

Table V. Cellular content of PCA soluble polyamines (nmol g^{-1} FW) in the non transgenic (NT) and transgenic (2E) cell lines of poplar at different time intervals after transfer of cells treated with [1,4-^{14}C] Put to label free medium. Data presented are mean ± SE of six replicates from two experiments. An asterisk indicates that the values for 2E cells were significantly different from the NT cells at a given time ($p \leq 0.05$).

Cell Line	Time	Put	Spd	Spm
NT	0 h	1181.74 ± 105.2	419.2 ± 17.9	73.3 ± 5.5
2E		3609.32 ± 171.0*	573.2 ± 35.5*	80.0 ± 11.3
NT	2 h	1313.9 ± 208.0	453.4 ± 54.0	69.3 ± 15.0
2E		3180.2 ± 153.4*	564.5 ± 33.4	74.0 ± 5.9
NT	4 h	1070.6 ± 145.3	476.4 ± 34.5	80.6 ± 12.0
2E		3481.3 ± 244.6*	632.3 ± 70.3	76.5 ± 15.5
NT	8 h	932.2 ± 111.4	417.1 ± 23.7	59.1 ± 7.8
2E		3314.6 ± 335.9*	715.5 ± 95.4*	97.0 ± 18.1
NT	24 h	1193.2 ± 123.8	520.0 ± 26.0	62.3 ± 10.5
2E		3296.1 ± 237.8*	715.8 ± 90.0	81.6 ± 11.8
NT	72 h	1755.2 ± 346.4	339.9 ± 34.4	31.6 ± 6.9
2E		3940.9 ± 443.2	522.5 ± 76.6	41.66 ± 8.8
NT	84 h	2341.9 ± 442.0	339.9 ± 34.4	21.5 ± 2.9
2E		3159.2 ± 315.2	276.2 ± 26.1	13.8 ± 2.0

Table VI. Recovery of label at various steps during dansylation and TLC separation of PCA extract of cells. Cells were incubated in [1,4 -^{14}C] Put for 2 h and extracted with 5% PCA by freezing and thawing (3X). One milliliter of the first PCA extract was dansylated, the dansyl-polyamines were extracted in 1 mL toluene, of which 800 µL was dried under vacuum and reconstituted in 50 µL methanol. Two microliter of the methanol fraction was counted for radioactivity and 40 µL was plated on TLC. The percentage recovery from previous step is adjusted for total volumes. Data presented are mean ± SE of six replicates.

Step	Fraction	DPM mL^{-1} PCA	% of previous step
1	PCA Extract I	122258 ± 4379	100
1A	PCA Extract II (discard)	9679 ± 206	7
1B	PCA Extract III (discard)	1343 ± 115	1
2	Toluene	86559 ± 3580	71
2A	Aqueous (discard)	40558 ± 3067	33
3	Methanol	65779 ± 2362	76
4	TLC Plating (Total Polyamines)	43920 ± 1812	67
4A	Putrescine	40543 ± 1798	92
4B	Spermidine	2899 ± 148	7
4C	Spermine	477 ± 75	1

were followed carefully in order to assess the extent of loss of labeled metabolites during the processing of samples. The supernatant fraction from 3X freezing and thawing process contained more than 90% of the total radioactivity present in the cells incubated with [^{14}C] Put for 2 h, i.e. at the time of transfer to label-free medium (Table VI). The second and the third PCA extractions contained about 8% and 1% of the remaining radioactivity, respectively. Less than 1% of the total radioactivity was found in the PCA-insoluble fraction. Data presented in Table VI further show that during the various steps of dansylation, no losses of radioactivity were incurred; all of the [^{14}C] being recovered in the toluene or the aqueous fractions. The toluene fraction contained morethan 70% of radioactivity from the PCA extract. The steps of vacuum-drying of the toluene fraction and the reconstitution of dansyl-polyamines in methanol resulted in about 25% loss of radioactivity. Of the total amount of radioactivity loaded on to the TLC plates in the methanol fraction, 67% was present in the three polyamine fractions.

Assuming that losses for the three polyamines in both cell lines were proportionate to their amounts present at each step, the data on specific activities of polyamines, and the calculations of amounts of Put loss and its conversion into Spd represent reliable and realistic measures of actual cellular contents of these metabolites. The information obtained from this experiment on the percent loss of radioactivity from the PCA extract to the recovery in the various polyamine fractions on TLC plates was used to calculate the values presented in Fig. 45-51 for both [^{14}C] Orn and [^{14}C] Put feeding experiments.

Labeling with [U-^{14}C] Orn

As shown in Table VII, the 2E cells labeled for 2 h with [^{14}C] Orn (i.e. at time zero of transfer to label-free medium) contained almost twice the amount of PCA extractable radioactivity as compared to the NT cells showing that the 2E cells absorbed twice the amount of [^{14}C] Orn than the NT cells. In both cell lines, the total amount of [^{14}C] in the cells (PCA soluble fraction) declined with time following their transfer to label-free medium. At 8 h after transfer to

Table VII. The loss of PCA soluble radioactivity from the cells at 8 and 24 h following their transfer from labeled substrates to label-free medium. Numbers in parentheses represent percent loss from counts at time zero. Data presented are mean ± SE of three replicates of one representative experiment.

Substrate	Time	Cell Line (dpm g^{-1} FW)	
		NT	2E
[U-^{14}C] Orn	0 h	163122 ± 467	348444 ± 5801
	8 h	112335 ± 2210 [31.1%]	218692 ± 1954 [37.2%]
	24 h	87991 ± 930 [46.1%]	168278 ± 7685 [51.7%]
[1,4-^{14}C] Put	0 h	475700 ± 15165	455914 ± 10899
	8 h	244759 ± 13600 [48.5%]	251448 ± 6755 [44.8%]
	24 h	157524 ± 4937 [66.9%]	161247 ± 5486 [64.6%]

label-free medium, the NT cells had lost about 31% of the total radioactivity and the 2E cells lost about 37% (Table VII). By 24 h, the corresponding losses in the NT and the 2E cells were 46% and 52%, respectively. Table VIII shows the amount of radioactivity recovered in the toluene fraction (all three polyamines combined) at different times of analysis. This is the fraction that was vacuum dried, redissolved in methanol and used for separation of the three polyamines on TLC plates. When the dansylated [^{14}C] polyamines were analyzed, it was found that at time zero, in the 2E cells, about 57% of the radioactivity in the PCA extract was present in the Put fraction, while in the NT cells, [^{14}C] Put constituted only about 27% of the total radioactivity (Table VII, Fig. 45A). This indicates that [^{14}C] Orn taken up by the 2E cells was being rapidly converted into Put. Radioactivity in the Put fraction also declined in the two cell lines with time, reaching very low levels (to <5% of that at time zero) by 96 h (Fig. 45A). The decline in radioactivity in the two cells lines followed different kinetics, the 2E cells losing radioactive Put at a slower rate than the NT cells during the first 8 h (Fig. 45A, inset). At all times during the experimental period, the content of [^{14}C] Put remained significantly higher in the 2E cells than the NT cells.

When changes in the amount of radioactive Put were converted into actual amounts of Put lost using information on cellular Put content during this period of study (Table V) (see Materials and Methods for calculations), it was found that during the first 2 h, the NT cells lost about 130 nmol Put g^{-1} FW as compared to 500 nmol g^{-1} FW loss by the 2E cells (Table IX). By 8 h, the amounts of Put lost by the 2E cells were about three-fold higher than for the NT cells, amounts reasonably proportionate to the cellular contents of Put in the two cell lines (Table V). Table X shows that the amount of Put lost from 24 h-96 h was also about 3.5 fold higher from the 2E cells than the NT cell line. This represents a 77% loss from the 2E cells during the 24 to 96 h time period as opposed to about 45% from the NT cells.

When the data on the loss of radioactivity during the first 8 h period were processed for linear regression and plotted to determine the half-life (L_{50} - loss of 50% radioactivity) of [^{14}C]

Table VIII. The radioactivity in toluene fraction in the NT and 2E cells when incubated with [U-^{14}C] Orn for 2 h and cells collected at different time periods following transfer to label-free fresh medium. Data presented are mean ± SE of nine replicates (from three separate experiments). An asterisk indicates that the values for 2E are significantly different ($p \leq 0.05$) from the NT cells at a given time.

Time (h)	NT	2E
0 h	57433.37 ± 7515	215936.05 ± 18766*
2 h	51395.77 ± 6695	187118.29 ± 18843*
4 h	37505.39 ± 5228	151739.73 ± 16151*
8 h	30777.74 ± 4364	133447.72 ± 15956*
24 h	27066.32 ± 2617	100253.28 ± 9237*
72 h	14633.25 ± 2354	36579.46 ± 3650*
96 h	10916.73 ± 2133	25380.63 ± 3348*

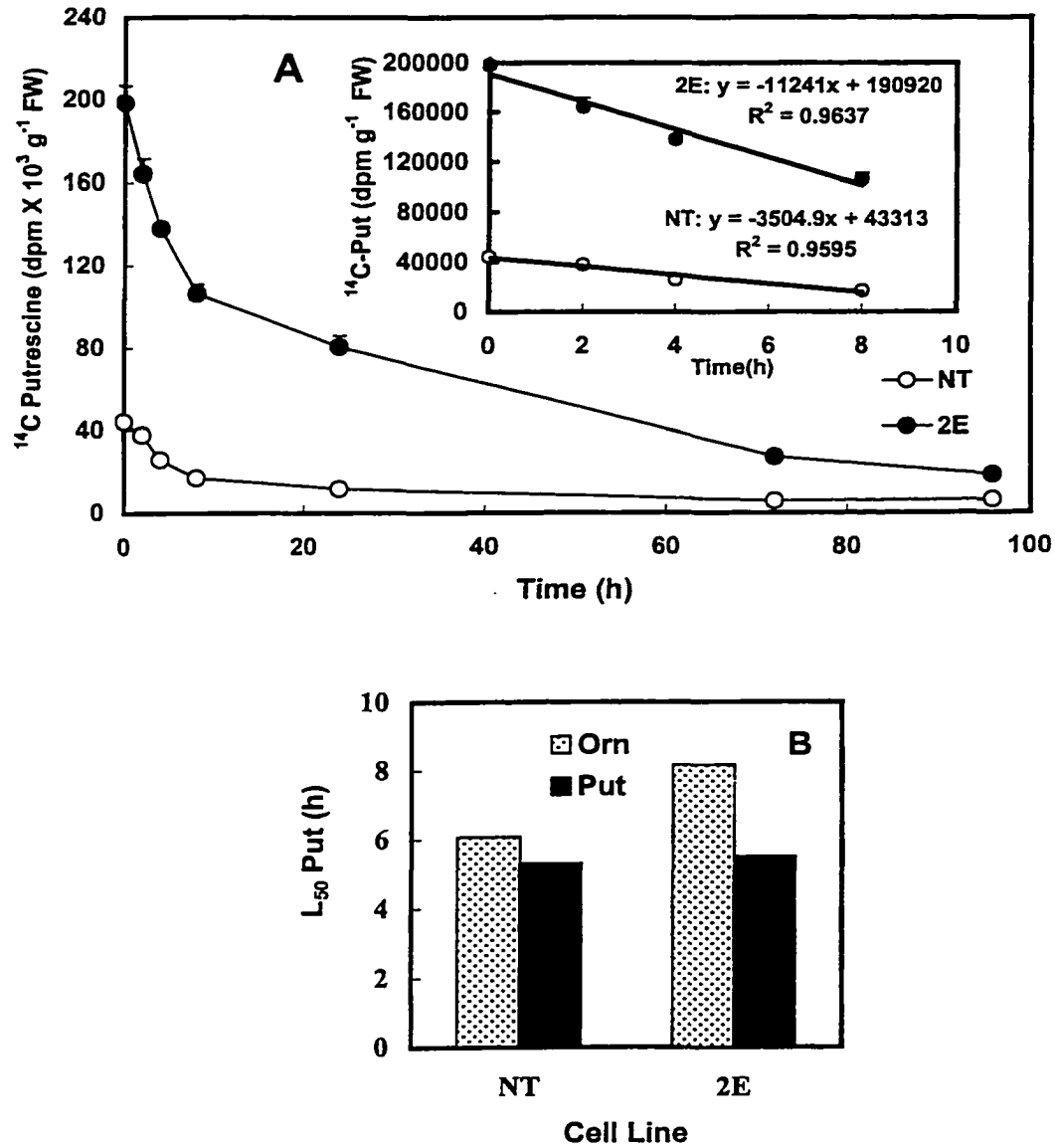


Fig. 45. Changes in the amount of [^{14}C] Put (A) derived from [U-^{14}C] Orn in the NT and 2E cells at different times following transfer of cells to label-free medium. 45A inset: regression curve for the loss of [^{14}C] Put over the 8 h period following transfer of labeled cells to label-free medium. Data presented are mean ± SE of nine replicates. Calculated half-life (L_{50}) of [^{14}C] Put in NT and 2E cells treated with [U-^{14}C] Orn or [1,4-^{14}C] Put (B). Each bar represents the mean L_{50} value of combined data from three (for [^{14}C] Orn) or two (for [^{14}C] Put) experiments, each with three replicates. The L_{50} was calculated by using data on the loss of [^{14}C] Put at various times during the first 8 h period after transfer of cells to label-free medium. Values in Fig. 45A have been adjusted for losses during dansylation procedure.

Table IX. The amount of total Put lost during the 2 h and 8 h period and the rate of conversion of Put to Spd during the 8 h period following transfer of cells from labeled substrates to label-free medium. Values for total polyamines used for calculations (nmol g^{-1} FW) for [1,4-^{14}C] Put are from Table V. The radioactive polyamine data (dpm g^{-1} FW) for [^{14}C] Orn feeding experiment are given in Fig. 45A and for [^{14}C] Put feeding experiment are given in Fig. 50A. The calculations were done as shown in Materials and Methods. Data presented are mean of 9 replicates for [^{14}C] Orn-feeding experiments and 6 replicates for [^{14}C] Put-feeding experiments. All values for 2E were significantly different from the NT cells at a given time ($p \leq 0.05$).

Substrate	Cell Line	Put Loss (nmol g^{-1} FW)		Put to Spd conversion (nmol g^{-1} FW)
		2 h	8 h	8 h
[U-^{14}C] Orn	NT	129.85	487.94	
	2E	500.26	1217.52	
[1,4-^{14}C] Put	NT	172.65	789.59	99.07
	2E	1031.40	1996.33	290.54

Table X. The percentage of Put lost and the amount of total Put lost during the 24 to 96 h period when cells were incubated with for [U-^{14}C] Orn for 2 h and then transferred to label free medium and 24 to 84 h period when cells were incubated with for [1,4-^{14}C] Put for 2 h and then transferred to label free medium. Data presented are mean ± SE of nine replicates for [U-^{14}C] Orn and six replicates for [1,4-^{14}C] Put.

Substrate	Cell Line	% Put Loss	Put Loss (nmol g^{-1} FW)
[U-^{14}C] Orn	NT	45.84	747.16
	2E	77.20	2691.09
[1,4-^{14}C] Put	NT	85.68	1659.89
	2E	80.82	2702.65

Put in the cells (Fig. 45A, inset), it was calculated to be 6.07 h for the NT cells and 8.16 h for the 2E cells (Fig. 45B), the two numbers being significantly ($p \leq 0.05$) different from each other. It should be pointed out that the half-life calculations were done using only radioactivity data. Under these conditions, the observed loss of radioactive Put in the 2E cells is likely to be an underestimate because of the continued production of [^{14}C] Put by the mouse ODC during this period from [^{14}C] Orn remaining in the cells (Fig. 46C). This would not be the case in the NT cells because these cells have very low rates of conversion of [^{14}C] Orn into [^{14}C] Put.

The amount of label from [^{14}C] Orn in the Spd fraction increased with time up to 24 h and declined thereafter in both cell lines (Fig. 46A). The trends of changes in the two cell lines were similar; however, the amount of labeled Spd was significantly higher in the 2E cells than the NT cells at any given time. The total amount of Spd (as determined by HPLC) in the two cell lines showed only small changes during the period of study but was higher in the 2E cells than the NT cells (Table V). The specific radioactivity of Spd (calculated as dpm [^{14}C] Spd per nmol of total soluble Spd) increased during the first 8 h following transfer to the label-free medium, there being only small differences between the two cell lines (Fig. 46B). The specific activity of Spd also fell after 8 h in both cell lines. The loss of [^{14}C] Spd between 24 and 96 h was proportionate to the amount of radioactivity present in this fraction in the two cell lines (Fig. 46A).

The amount of [^{14}C] Spm was often very small (less than 5% of the total [^{14}C] in polyamines). The radioactivity in Spm was three fold higher in the 2E as compared to the NT cells at time zero (Fig. 47A). This number was almost two fold higher at 2, 4 and 8 h time periods. There was a decrease in the amount of label in the 2E cells with time but the label in the Spm fraction did not change much for the NT cells. After a general decline in radioactive Spm content up to 8 h, there was an increase in both the cell lines at 24 h. This was followed by a decrease at both 72 and 96 h. The [^{14}C] Spm content was significantly different in the two cell lines till 24 h (Fig. 47A). The specific activity of Put (Fig. 48A) was slightly higher in the 2E

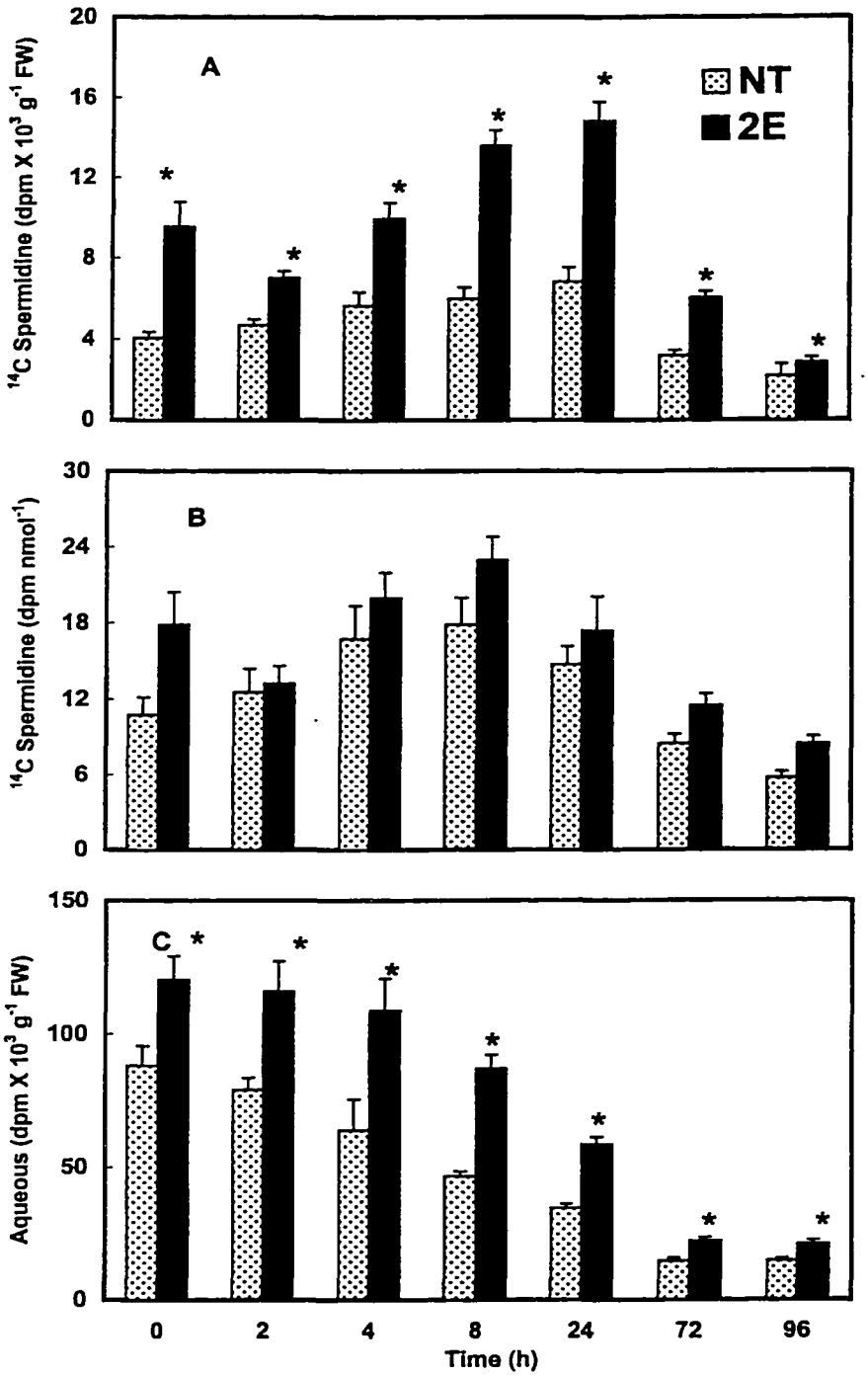


Fig. 46. Changes in the amount of [^{14}C] Spd derived from [U-^{14}C] Orn in the NT and 2E cells at different times following transfer of cells to label-free medium (A). Specific radioactivity of [^{14}C] Spd derived from [U-^{14}C] Orn in NT and 2E cells at different times following transfer of cells to label-free medium (B). The amount of label present in the aqueous fraction of dansylated PCA extract of NT and 2E cells at different times of analysis after treatment with [U-^{14}C] Orn for 2 h and transfer to label-free medium (C). Data presented are mean ± SE of nine replicates. An asterisk indicates that the values for 2E cells are significantly different ($p \leq 0.05$) from NT cells at a given time. Values have been adjusted for losses during dansylation procedure for 46A,B.

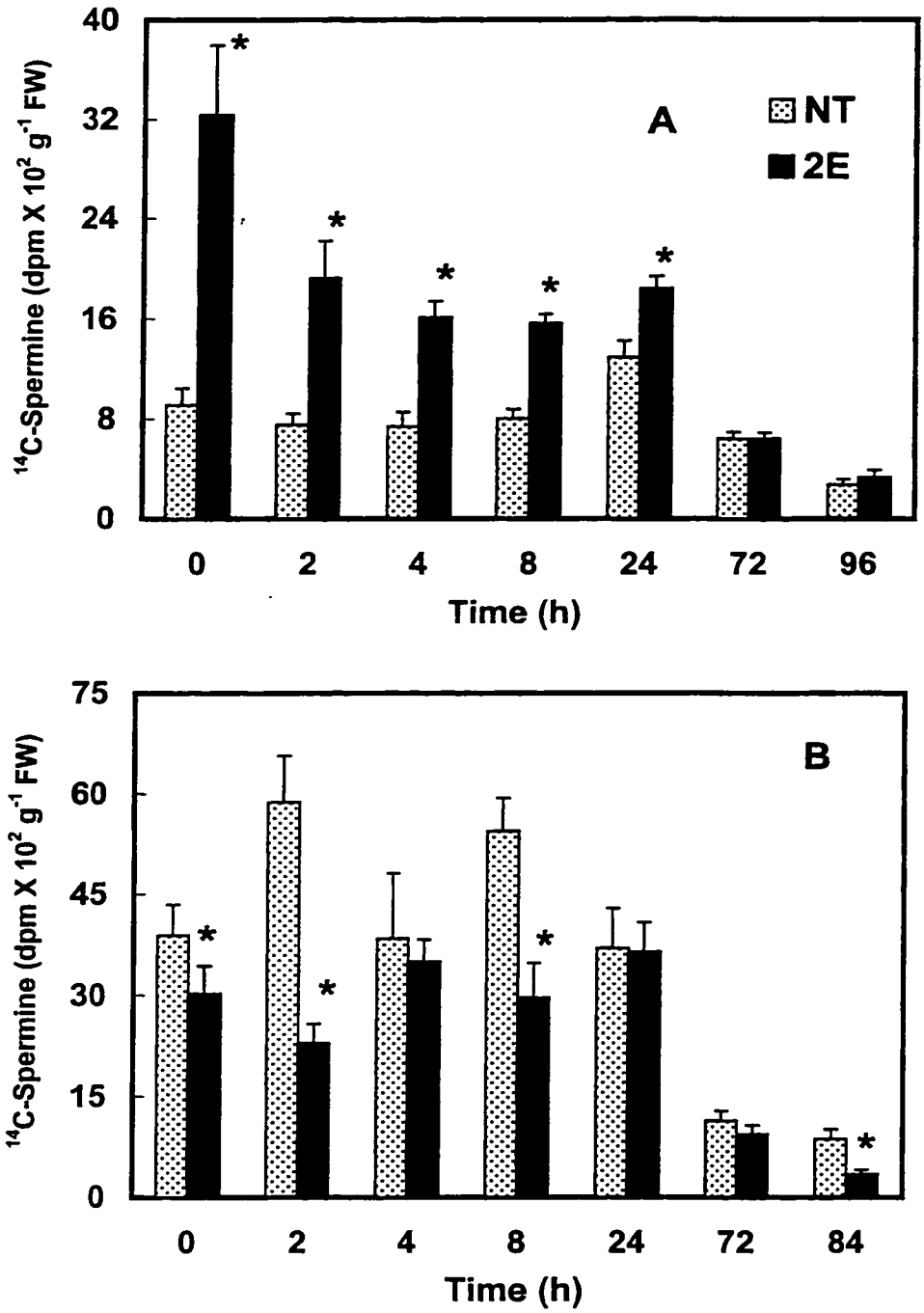


Fig. 47. Changes in the amount of [^{14}C] Spm derived from [U-^{14}C] Orn (A) and [1,4-^{14}C] Put (B) in the NT and 2E cells at different times of analysis following treatment with substrate for 2 h and transfer of cells to label-free medium. Data presented for (A) are mean ± SE of nine replicates representing three separate experiments and (B) are mean ±SE of six replicates representing two separate experiments. An asterisk indicates that the values for 2E cells are significantly different ($p \leq 0.05$) from NT cells at a given time. Values have been adjusted for losses during dansylation procedure.

cells than the NT cells; there being a steady decline in Put specific activity in both cell lines with time. Specific activity of Spm however, did not exhibit any clear trend (Fig. 48B).

Following dansylation and partitioning of dansyl-polyamines into toluene, radioactivity in the remaining aqueous fraction was also counted. The radioactivity in this fraction represents mostly the unused [^{14}C] Orn taken up by the cells, plus the amino acids derived from [^{14}C] Orn, and the charged products (at pH $\leq$ 9.88) of [^{14}C] Put degradation. The total amount of [^{14}C] present in the aqueous fraction was higher in the 2E cells at most of the times (Fig. 46C). For NT cells, the aqueous fraction represented more than 54% of the total label in the cells, whereas the corresponding amount for 2E cells was less than 35%. The loss of label from this fraction in the 2E cells was quite slow during the first 8 h. This [^{14}C] Orn would allow some [^{14}C] Put to be synthesized during the first few hours of analysis in the 2E cells, thus contributing to the [^{14}C] Put pool, and causing an underestimate of the loss of radioactivity in the Put fraction, and an overestimate of the L_{50} of Put in these cells. Overall, in both cell lines, radioactivity in the aqueous fraction declined with time.

Labeling with [1,4-^{14}C] Put

In another set of experiments similar to those involving [^{14}C] Orn feeding, the cells were incubated with [1,4 ^{14}C] Put for 2 h to load them with exogenous Put, and then analyzed for changes in the content of labeled and total polyamines at different times following transfer to label-free medium. Unlike the situation with [^{14}C] Orn feeding experiments, the total amount of radioactivity present at time zero in the PCA extract (Table VII), toluene fraction (Fig. 49A), the aqueous fraction (Fig. 49B) and in the Put fraction (Fig. 50A) in the two cell lines was quite comparable. This shows that the uptake of [^{14}C] Put in the two cell lines was similar even though the 2E cells contained several-fold higher amounts of endogenous Put. The loss of PCA-extractable label with time followed similar kinetics in the two cell lines; both losing 45-48% of radioactivity within 8 h and 65-70% of radioactivity within 24 h of transfer to label-free medium

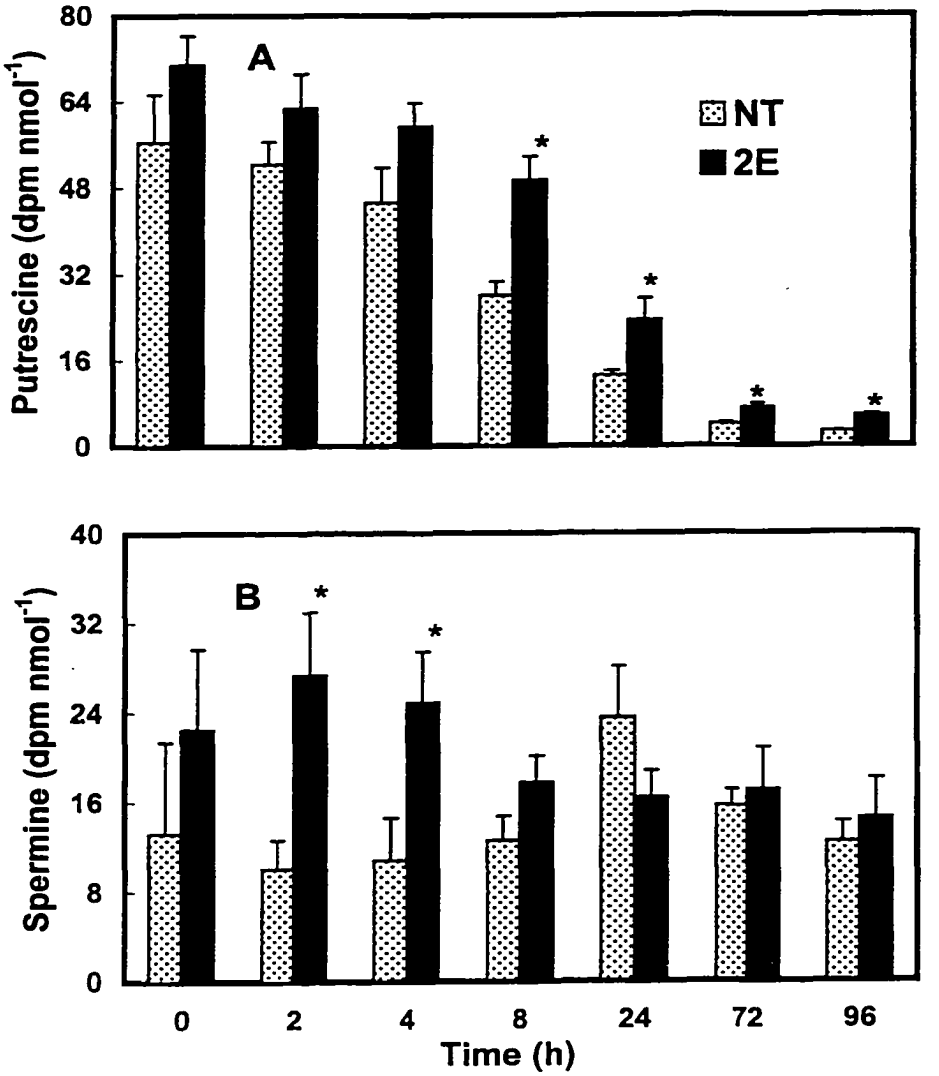


Fig. 48. Specific radioactivity of [^{14}C] Put (A) and [^{14}C] Spm (B) derived from [U-^{14}C] Orn in NT and 2E cells at different times following transfer of cells to label-free medium. Data presented are mean ± SE of nine (from three experiments) replicates. An asterisk indicates that values for 2E are significantly different ($p \leq 0.05$) from NT cells at a given time. Values have been adjusted for losses during dansylation procedure.

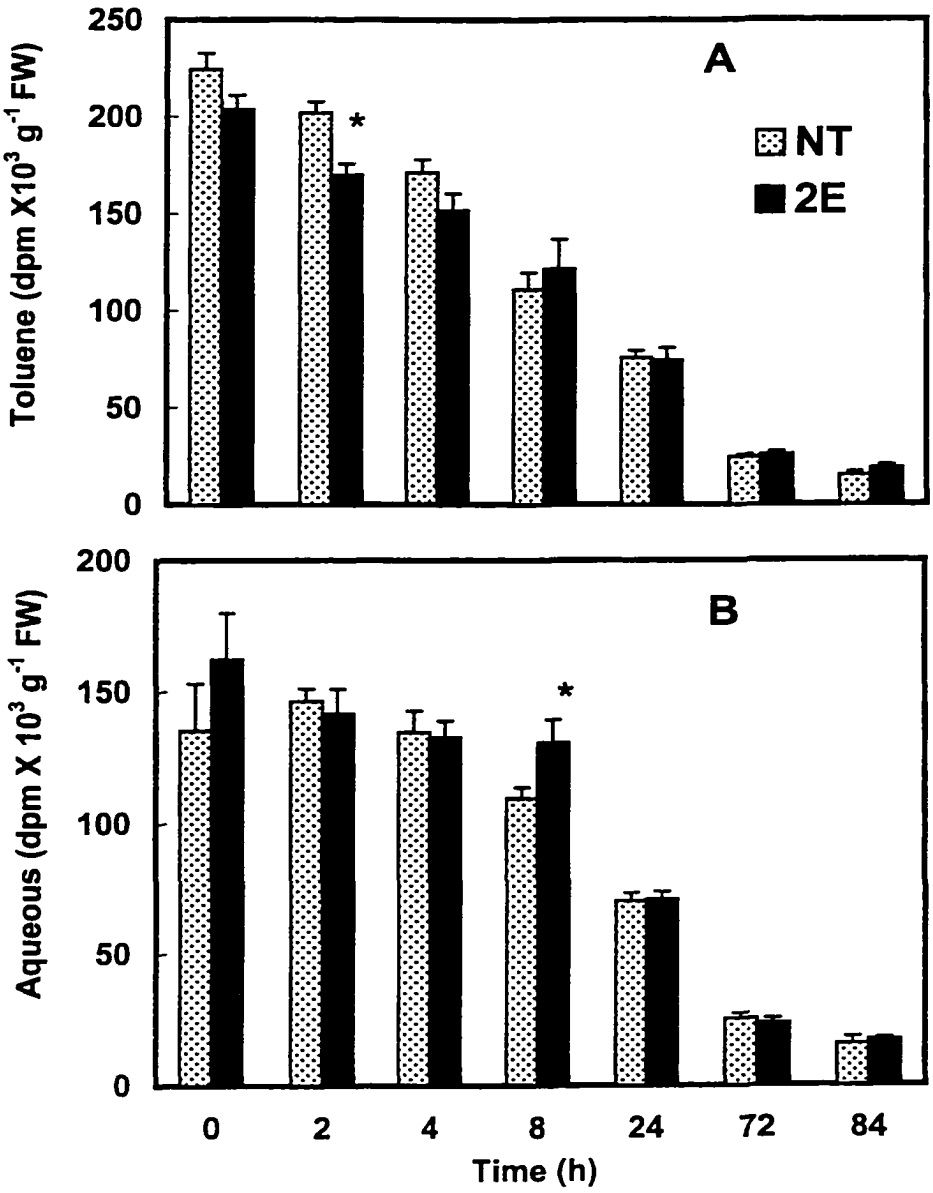


Fig. 49. Radioactivity present in the toluene (A) and aqueous (B) fractions after dansylation of PCA extracts of NT and 2E cells incubated with [1,4-^{14}C] Put for 2 h, washed with label free medium and transferred to label free fresh medium. Cells were collected at different times of analysis and total amount of radioactivity in each treatment was 1 μCi. Data presented are mean ± SE of six replicates (from two experiments). An asterisk indicates that values for 2E are significantly different ($p \leq 0.05$) from NT cells at a given time.

(Table VII). A comparison of the relative losses of PCA-extractable radioactivity accumulated from [^{14}C] Put and [^{14}C] Orn shows that for both cell lines, the radioactivity losses were significantly higher for the former at both 8 h as well as 24 h. While only 31 and 37% of the label from [^{14}C] Orn was lost during the first 8 h for the NT and 2E cells, respectively, the corresponding losses from [^{14}C] Put were 48 and 45% in the two cell lines. Likewise, by 24 h, 25-45% more radioactivity was lost from cells fed with [^{14}C] Put than the cells fed with [^{14}C] Orn (Table VII). The counts in the aqueous fraction also declined with time (Fig. 49B).

The amount of radioactivity in the Put fraction decreased with time in both cell lines on transfer to fresh medium (Fig. 50A). While the profile of [^{14}C] Put loss was similar in the two cell lines, the 2E cells retained slightly higher amounts of label at all times from 8 to 84 h. Because of the different amounts of non-radioactive Put in the two types of cells (Table V), the specific radioactivity of Put (dpm [^{14}C] Put per nmol of total soluble Put) in the two cell lines differed significantly; the NT cells having higher specific activity of Put than the 2E cells during the first 24 h of the experiment (Fig. 50B). The specific activity of Put also declined with time after transfer of cells to label-free medium, showing the continued production of new unlabeled Put in the cells. On the basis of nmol of Put lost over the first two hours, the 2E cells lost about six times as much Put as the NT cells, the amounts lost being 1031 and 173 nmol g^{-1} FW, respectively (Table IX). About three-fold differences of Put loss between the 2E and the NT cells were seen for the 8 h period. Between 24 and 72 h, both cell lines showed a further loss of 70-80% of radioactivity in the Put fraction. Although the percentage of Put lost from the 2E cells (80%) was lower than NT cells (85%) in the 24-84 h period (Table X), the total amount of Put lost (2702 nmol g^{-1} FW) was higher than NT (1659 nmol g^{-1} FW). The L_{50} for [^{14}C] Put turnover during the first 8 h was calculated to be 5.30 h for the NT cells and 5.51 h for the 2E cell (Fig. 50A inset and Fig. 45B), there being no significant difference between the two cell lines in this case.

As with the experiments using [^{14}C] Orn, in [^{14}C] Put feeding experiments also, the amount of labeled Spd fraction increased during the first 8 h in both the NT and the 2E cells,

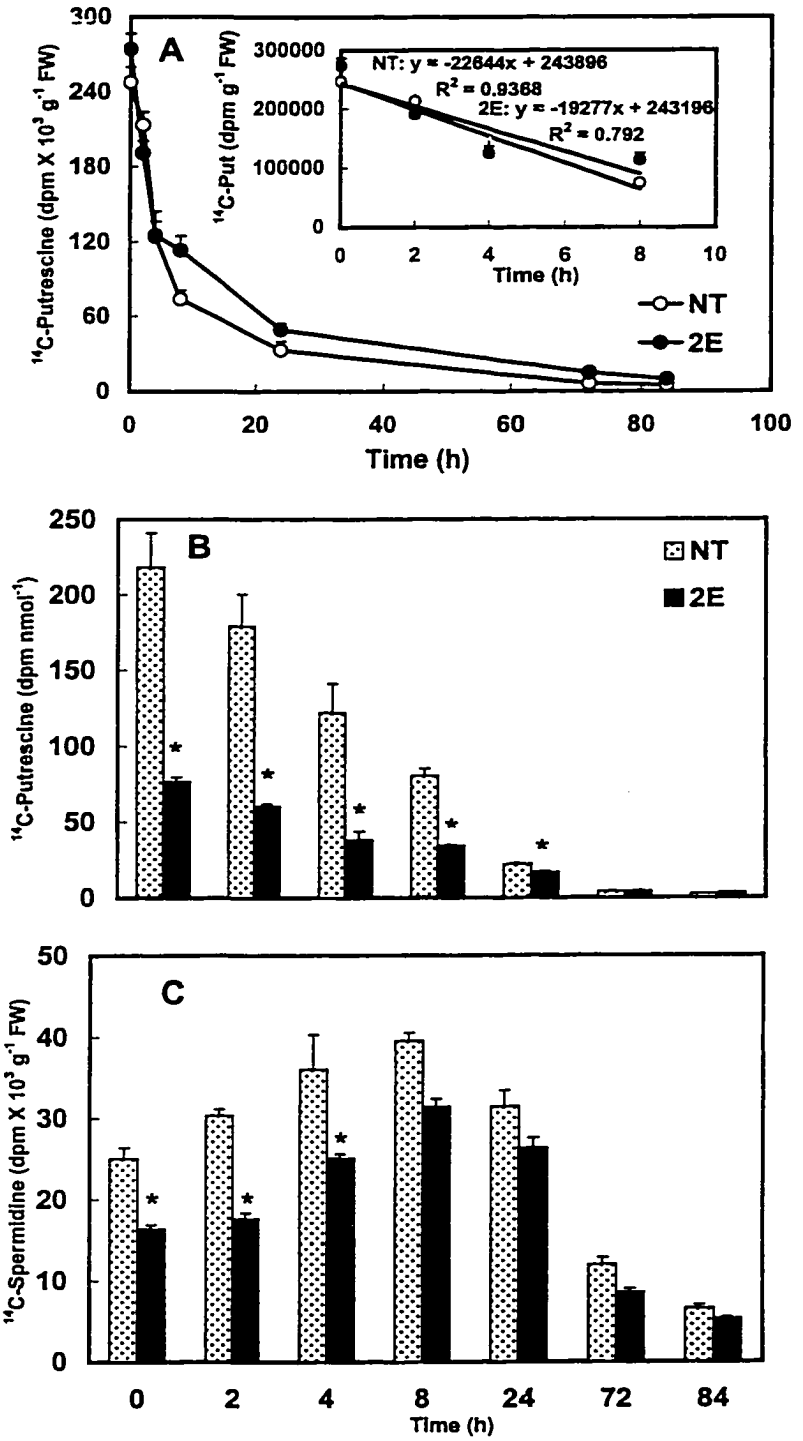


Fig. 50. Changes in the amount of [^{14}C] Put (A), specific radioactivity of [^{14}C] Put (B) and amount of [^{14}C] Spd (C) in the NT and 2E cells at different times following transfer of cells treated with [1,4-^{14}C] Put for 2 h to label-free medium. Data presented are mean ± six replicates from two separate experiments. An asterisk indicates that the values for 2E cells are significantly different ($p \leq 0.05$) from NT cells at a given time. Values have been adjusted for losses during dansylation procedure.

followed by a gradual decline in both cases (Fig. 50C). Labeled Spd was always higher in the NT cells than the 2E cells at any given time, the differences being significant for up to 4 h only. This is probably due to higher specific activity (ratio of labeled to unlabeled Put) of [^{14}C] Put in the NT cells, even though higher rates of conversion were seen in the 2E cells. When the conversion of Put into Spd was calculated as nmol g^{-1} FW, taking into account the cellular Put contents (Table V) and the specific radioactivity of Put, it was found to be about three-fold higher in the 2E cells than the NT cells (Table VIII). This difference is greater than the difference in the cellular contents of Spd in the two cell lines during this period (Table V).

Once again, the conversion of labeled Put into labeled Spm was quite low (less than 5% of the total label was present in Spm), and there were no major differences between the two cell lines (except at 0, 2, and 8 h) nor was there a clear trend in changes of radioactive Spm on transfer to label-free medium (Fig. 47B). While the NT cells showed an increase at 2 h and then a decrease at 4 h and an increase again at 8 h followed by a decline at 24 h, the trends were reversed for the 2E cell lines. The [^{14}C] Spm content was generally higher in the NT cells than the 2E cells till 8 h. The specific activity of Spd and Spm was also determined. Specific activity of Spd increased up to 8 h in both the cell lines and then declined (Fig. 51A). The trends for the specific activity of Spm were similar (Spd increased till 8 h but Spm did not) to the amount accumulated as [^{14}C] Spm from [^{14}C] Put (Fig. 51B). Specific activity of both Spd and Spm was generally higher in the NT cells than the 2E cells.

Putrescine Secretion into the medium

Three day old cells were incubated with [U-^{14}C] Orn and the secretion of Put from the cells into the medium was analyzed. The amount of radioactivity lost from the cells into the medium was five fold higher in the 2E than the NT cells at time zero of analysis (Fig. 52). This loss probably represents immediate washout of the radioisotope from the cells. The amount of radioactivity lost continued to be high in the 2E cells for the first 4 h (about two fold at this time

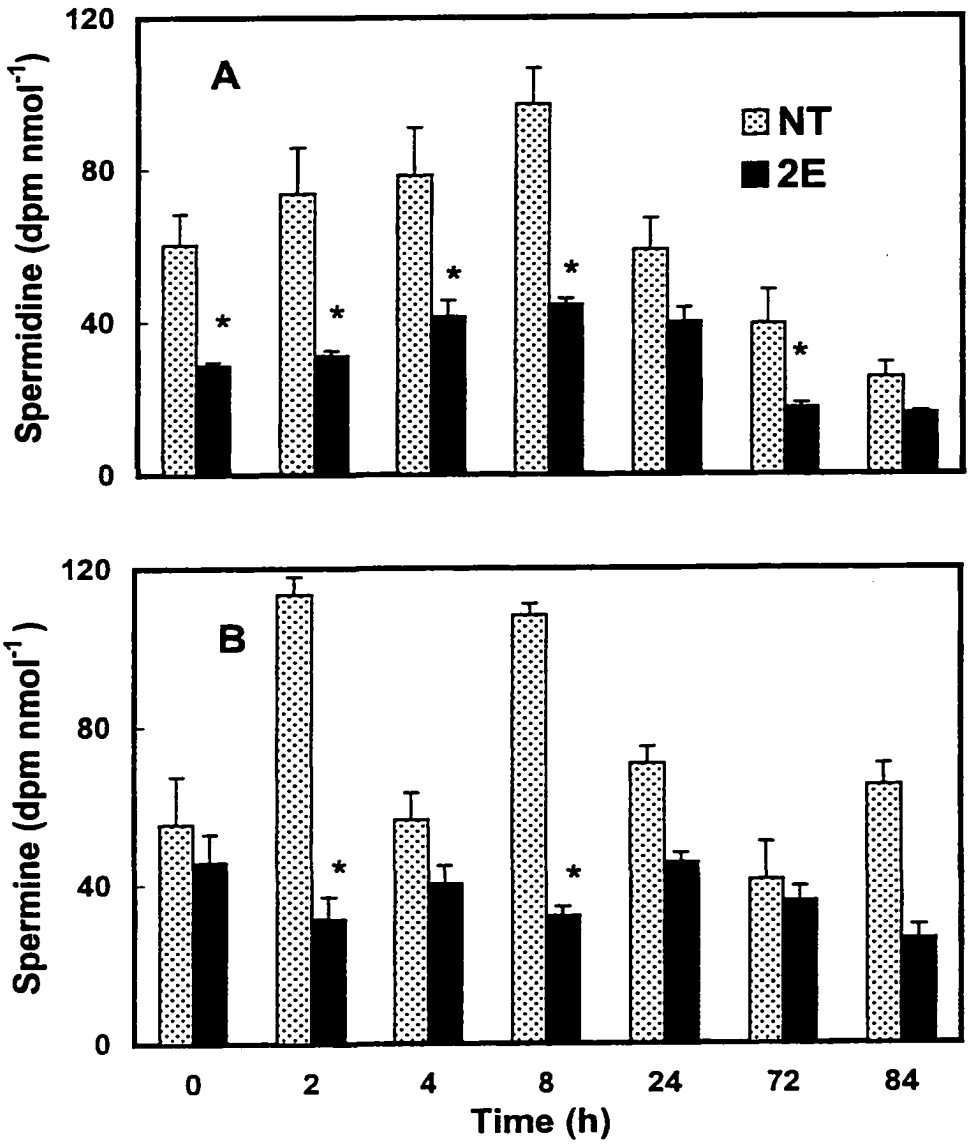


Fig. 51. Specific radioactivity of [^{14}C] Spd (A) and [^{14}C] Spm (B) derived from [1,4-^{14}C] Put in NT and 2E cells at different times following transfer of cells to label-free medium. Data presented are mean ± SE of nine (from three experiments) replicates. An asterisk indicates that values for 2E are significantly different ($p \leq 0.05$) from NT cells at a given time.

period) and became comparable at 8 h between the two cell lines (Fig. 52). This radioactivity secreted into the medium is a measure of all labeled products, which includes amino acids and polyamines. A more accurate analysis of the cells throwing out Put would be by using [1,4-^{14}C] Put as the substrate and then determining the Put loss. Nevertheless, these results give us an indication that the total radioactivity loss is higher from the 2E cells as compared to the NT cells.

Activity of Diamine Oxidase

As described earlier, Put is catabolized primarily by the enzyme DAO, the product of the reaction being Δ^1-pyrroline, which eventually enters the TCA cycle via GABA and succinic acid (Fig. 3). Since the 2E cells show several-fold higher rate of Put catabolism, one can ask the question: Is the increased catabolism of Put in 2E cells accompanied by increased DAO activity? In order to test this, the activities of DAO in the two cell lines over the 7 d culture period were compared. Prior to measurement of DAO activity, the protocol for enzyme assay in poplar cells was optimized by modification of the procedure of Santanen and Simola (1994) and Santanen (2000). A major change was that the assays were conducted using frozen-thawed cells without homogenization. The enzyme activity in the frozen-thawed cells was first compared with the supernatant and the pellet fractions of homogenized cells. The data presented in Fig. 53A show that: (a) about two-thirds of the DAO activity in the cells was present in the pellet and about one-third in the supernatant fraction of the homogenate, and (b) the total DAO activity in the frozen-thawed cells was comparable to the combined activity in the pellet and the supernatant fraction of the homogenate. Following this determination, the DAO activity in the frozen-thawed cells was analyzed at three different pH values, at various incubation periods, and six different quantities of cells per tube. For frozen-thawed cells, the rate of [^{14}C] Δ^1-pyrroline formation was proportionate to the amount of cells for up to 200 mg FW per tube (Fig. 53B), the optimum pH was 8.0 (Fig. 53C), and the reaction was linear for at least up to 120 min (Fig. 53D).

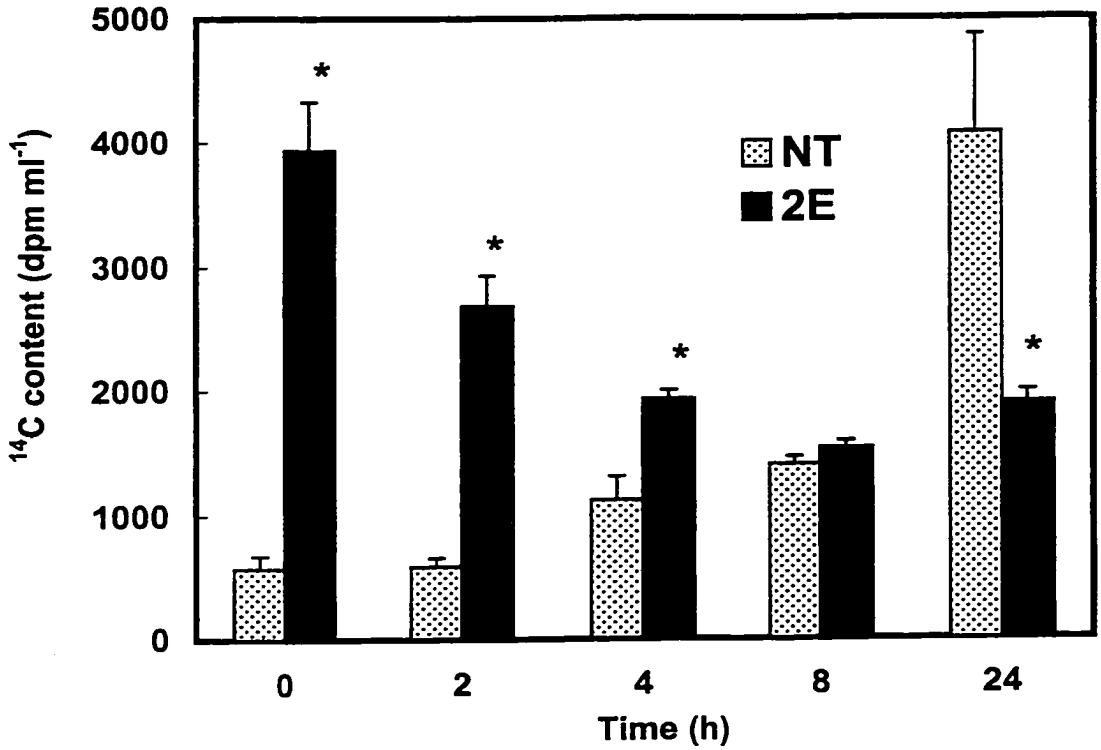


Fig. 52. The secretion of [^{14}C]-compounds from the cells into the medium when 3 d old cells were incubated with [U-^{14}C] Orn. Data presented are mean ± SE of three replicates. An asterisk indicates that the values for 2E are significantly different ($p \leq 0.05$) from the NT cells at a given time. The experiment was done once.

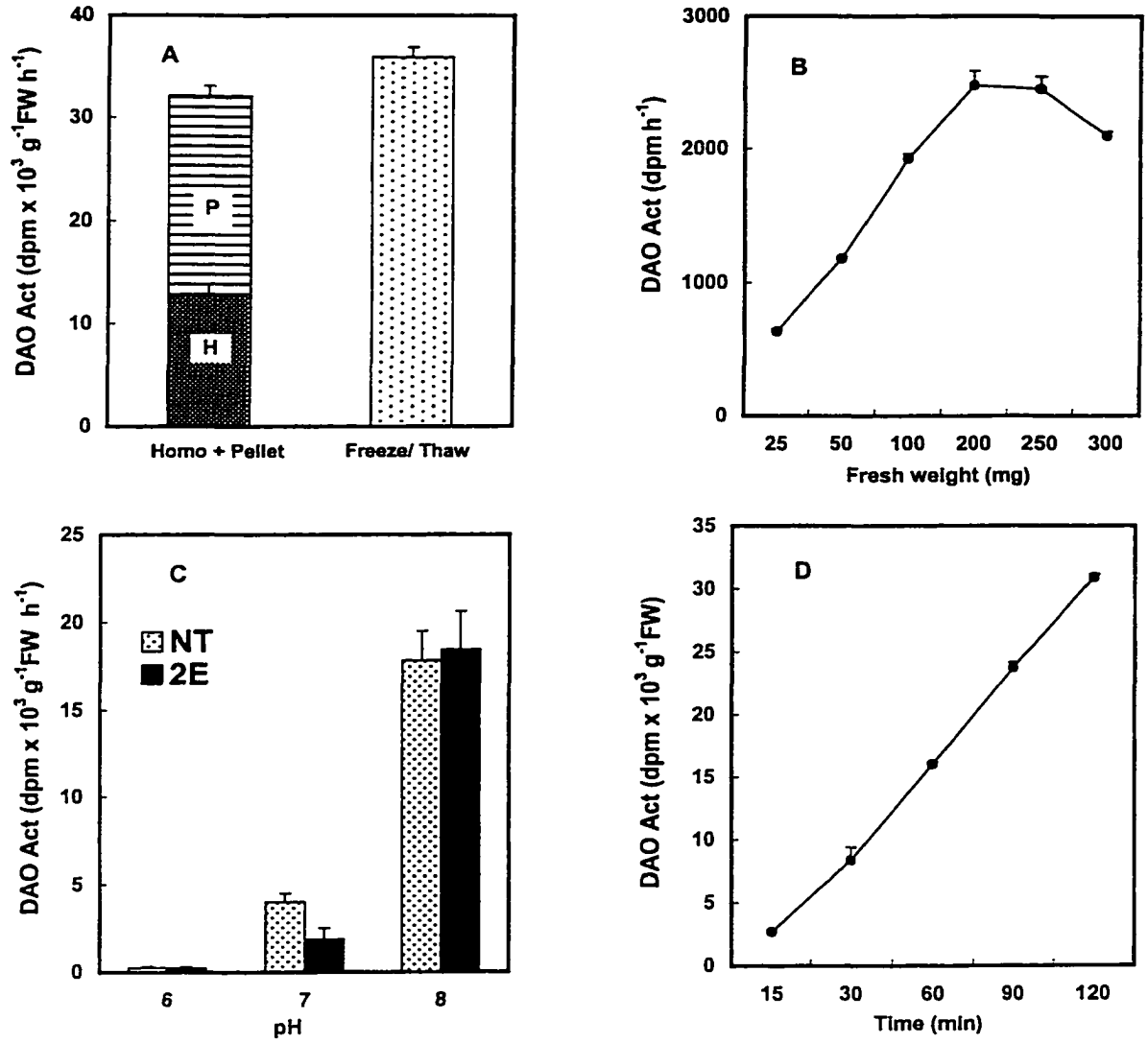


Fig. 53. Characterization of DAO activity in poplar cells with respect to its extractability by freeze-thawing vs. homogenization of cells (A), linearity with the amount of cells used (B), the effect of pH (C), and the effect of time of incubation (D). For A, C and D, 200 mg FW of frozen-thawed cells were used per tube; for 53A, 53B and 53D, the reaction was run at pH 8.0. The data are mean ± SE of three replicate assays from a single representative experiment.

Based upon the above characterization, DAO activity was measured on different days of culture in the two cell lines using 200 mg FW of cells that were frozen and thawed three times. The reaction was run at pH 8.0 for a period of 60 min. Data presented in Fig. 54 show that the enzyme activity g^{-1} FW of cells varied considerably on different days of culture in both the cell lines. It was the lowest at the time of subculture (0 d in Fig. 54) and increased several-fold during the next two days in both cell lines. The enzyme activity declined significantly after 4 d of culture, reaching the lowest level by day 7 (the same as 0 d in Fig. 54). The trend of changes in DAO activity during the 7 d culture period was similar in the two cell lines. Except for 6 and 7 d, when the 2E cells had somewhat higher DAO activity, the enzyme activity in the two cell lines was either comparable, or it was lower in the 2E cells than the NT cells.

Metabolism of ^{14}C-Spermidine

Polyamine levels in the 2E and NT cells

During the period of this study, the free cellular Put content showed a two-fold difference in the two cell lines with the 2E being higher than the NT (Fig. 55A). The Put levels did not change significantly from 0 h to 24 h but increased during 72 h of analysis in both the cell lines. The cellular contents of Spd and Spm although slightly higher in the 2E cells than the NT cells, at any time of analysis, were not significantly different between the two cell lines (Fig. 55B,C).

Recovery of label during separation of polyamines (Incubation with ^{14}C-Spd)

Data presented in Table XI show the percent radioactivity associated with various stages of processing of the samples, such as, the extraction of radioactivity from cells into PCA, dansylation, and TLC separation of polyamines.

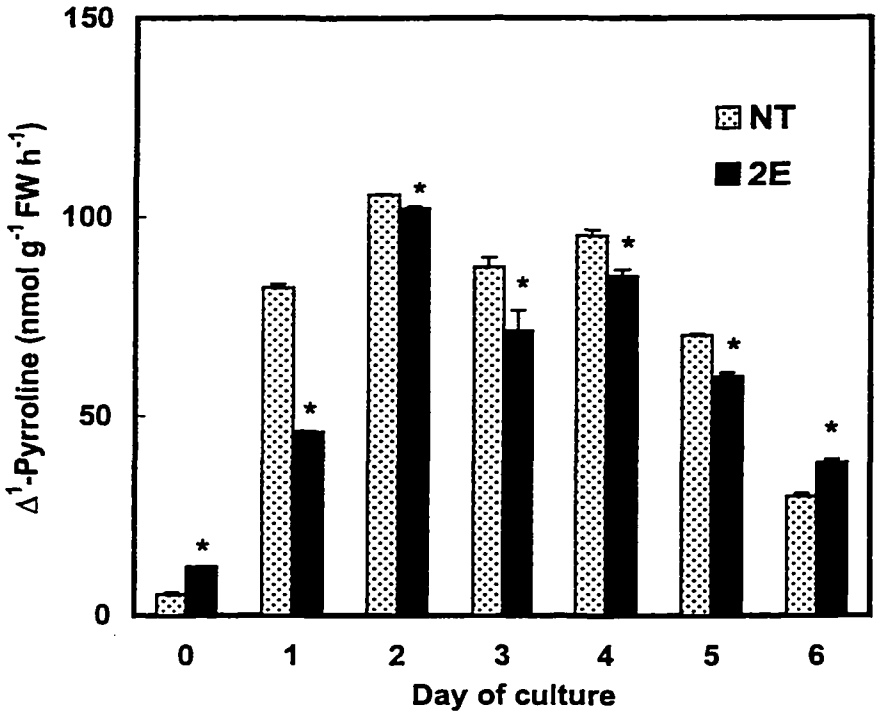


Fig. 54. The activity of DAO (amount of Δ^1-[^{14}C]-Pyrroline formed) in NT and 2E cells over a seven day time course. The cells (200 mg) were collected in 0.1M potassium phosphate buffer, frozen-thawed three times, and analyzed for the formation of Δ^1-[^{14}C] Pyrroline from [1,4-^{14}C] Put over a period of 60 min at 37^{0}C. Each bar represents mean ± SE of three replicate assays from a representative experiment. An asterisk indicates that the values for 2E cells are significantly different ($p \leq 0.05$) from NT cells at a given time.

This information permits the assessment of losses incurred during the processing of samples. Approximately 90% of the total radioactivity incorporated in the cells during the 2 h incubation period was extracted in the first round of PCA extraction. The remaining radioactivity: 9% and 1% were solubilized in the second and third rounds of PCA extraction, respectively. Of this, all the radioactivity was recovered in the toluene and aqueous fractions combined, demonstrating, once again that negligible losses were incurred during the various steps of dansylation. The toluene fraction contained 94% of the radioactivity recovered in the PCA extract. This was as expected on partitioning of the dansyl-polyamines in the toluene phase. Samples were vacuum dried to evaporate toluene and the dansyl-polyamines were reconstituted in methanol. About 66% of the radioactivity evaporated from the toluene fraction was present in the methanol fraction. There was 75% radioactivity recovered in the three polyamines of the total radioactivity loaded on the TLC plate. The radioactivity associated with the Spd fraction was the highest while that in the Put and Spm fraction was very low and comparable between the two polyamines.

Spermidine turnover in the transgenic and non transgenic cells

After incubating the cells for 2 h with [^{14}C] Spd, washing with label-free medium and transferring to label-free fresh medium, the samples were collected at different times of analysis. The cells were analyzed for their [^{14}C] polyamine content by TLC and total polyamine content by HPLC.

Since 2 h incubations were found to be optimum with Put feeding experiments (steady state levels of [^{14}C] Put were achieved within the first 2 h), the same 2 h incubations were used to accumulate [^{14}C] Spd also. In addition, biosynthesis of [^{14}C] Spd from [U-^{14}C] Orn had high incorporation of the label within the first 2 h (Fig. 17B). The amount of label accumulated from [^{14}C] Spd feeding that was present in the toluene fraction was always higher (about 1.7 fold) in the NT cells as compared to 2E cells (Fig. 56A). Significantly higher radioactivity at time zero in

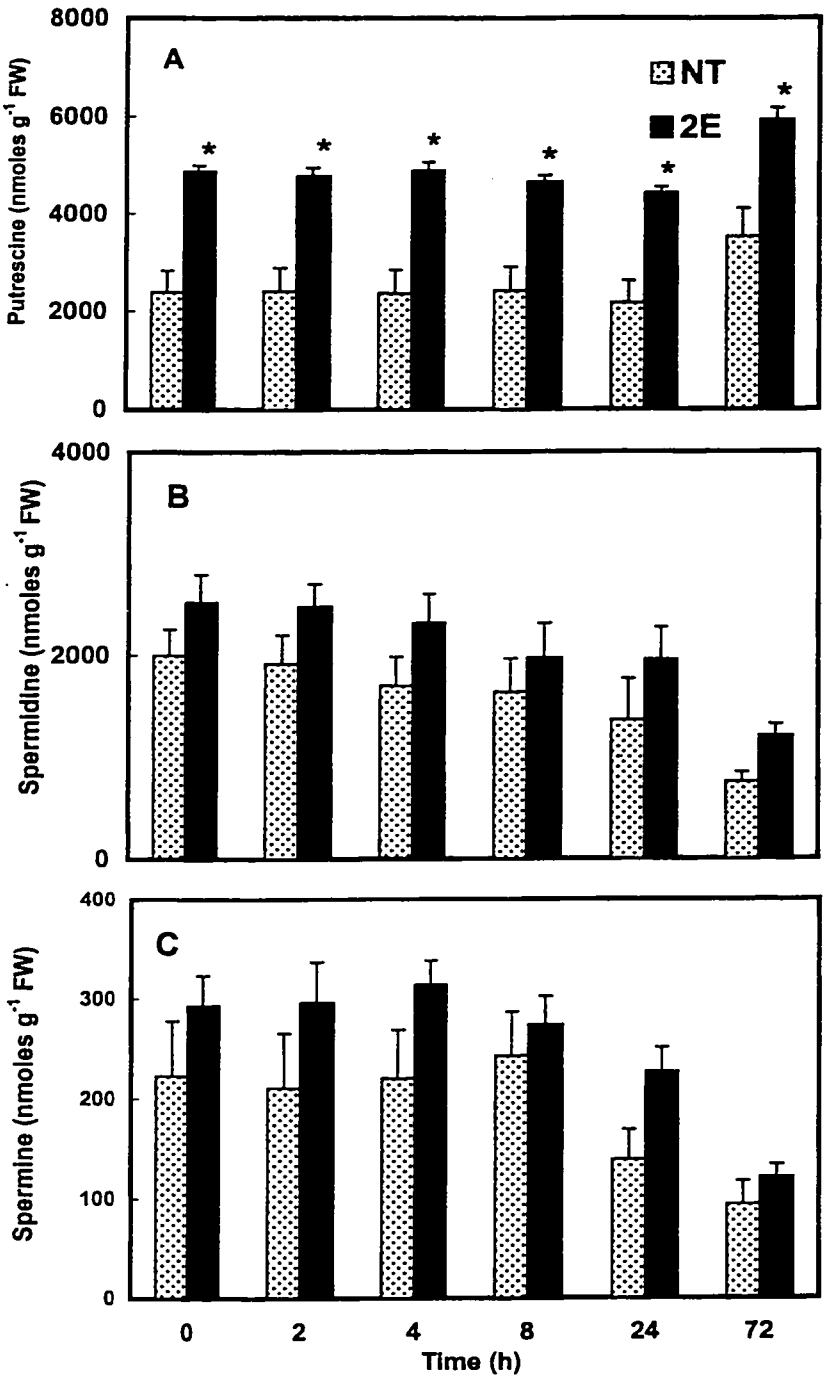


Fig. 55. Cellular content of PCA soluble polyamines (nmol g^{-1} FW) in the NT and 2E cell lines at different time intervals after transfer of cells treated with [^{14}C] Spd to label-free medium. Data are mean ± SE of nine (from three experiments) replicates. An asterisk indicates that values for 2E are significantly different ($p \leq 0.05$) from NT cells at a given time.

Table XI. Recovery of label at various steps during dansylation and TLC separation of PCA extract of cells. Cells were incubated in [^{14}C] Spd for 2 h and extracted with 5% PCA by freezing and thawing (3X). One milliliter of the PCA fraction was dansylated, the dansyl-polyamines were extracted in 1 mL toluene, of which 800 μL was dried under vacuum and reconstituted in 50 μL methanol. Two microliter of methanol fraction was counted for radioactivity and 40 μL was plated on TLC. The percentage recovery from previous step is adjusted for total volumes. Values are mean ± SE for six replicates at 0 h time period for NT and 2E cells, i.e., immediately at the end of 2 h of incubation with [^{14}C] Spd. Data presented are from one representative experiment.

Step		Fraction	DPM mL^{-1} PCA	% of previous step
1		PCA Extract I	94445 ± 11736	100
	1A	PCA Extract II (discard)	9026 ± 888	9
	1B	PCA Extract III (discard)	1391 ± 211	1
2		Toluene + Aqueous	109971 ± 17825	117
	2A	Toluene	86870 ± 13247	94
	2B	Aqueous (discard)	23100 ± 6545	23
3		Methanol	58684 ± 10383	66
4		TLC Plating (Total Polyamines)	43027 ± 6866	75
	4A	Putrescine	1227 ± 159	3
	4B	Spermidine	40575 ± 6483	94
	4C	Spermine	1224 ± 228	3

the toluene fraction of NT cells represented higher [^{14}C] Spd uptake in this cell line as compared to the 2E cells. There was a decline in the radioactivity associated with the toluene fraction in both the cell lines after 24 h from both the cell lines. The counts in aqueous fraction were less than 25% of the total at any given time (except at time zero) and showed no significant trend with time. These counts represent the charged catabolic products of Spd (Fig. 56B).

The counts in all the three polyamines were higher in the NT cells than the 2E cells. The [^{14}C] Spd fraction exhibited a decline in both the cell lines with time (Fig. 57A). The kinetics of losses as seen in Fig. 57A were different in the two cell lines. The initial losses from the NT cells (up to 24 h) were more pronounced than the 2E. Linear regression was done on the data for the first 24 h to determine the initial half-life of Spd (L_{50}- loss of 50% radioactivity) as described earlier for Put (Fig. 57A, inset). The L_{50} of [^{14}C] Spd was calculated to be 21.61 h and 32.61 h for NT and 2E cells, respectively, the two values being significantly different (Fig. 58). The total amount of Spd exhibited a small decline in both the cell lines with time and was generally higher in the 2E than NT cells. When the radioactive Spd data were converted to the actual amount of Spd lost as well as the amount of Spd converted to Spm and Put in 8 h (see materials and methods for details), it was observed that the NT cells lost 441 nmol g^{-1} FW and the 2E cells lost 223 nmol g^{-1} FW Spd. However, the amount of Spd converted to Spm, and Put, respectively, was comparable in both the cell lines (Table XII). Studies from Put turnover described earlier demonstrate that the rate of conversion of Put to Spd was three times higher in the 2E cell line than the NT even though the cellular amounts of Spd in the two cell lines were not significantly different. The amount of [^{14}C] Put produced from [^{14}C] Spd was approximately two fold higher in the NT than the 2E at all time periods tested (Fig. 57B). [^{14}C] Putrescine amount continued to increase up to 4 h after which it declined at 8-24 h. This decline could be explained by the half-life of Put in the two cell lines determined to be 5-6 h.

Table XII. The amount of total Spd lost (nmol g^{-1} FW) (A) during the 8h period and the rate of conversion of Spd to Spm (nmol g^{-1} FW) (B) and Spd to Put (nmol g^{-1} FW) (C) during the 8 h period following transfer of cells from labeled substrate to label-free medium. Values for total polyamines used for calculations (nmol g^{-1} FW) for [^{14}C] Spd are from Fig. 55. The radioactive polyamine data (dpm g^{-1} FW) for Spd feeding experiment are given in Fig. 57. The calculations were done as shown in Materials and Methods.

Cell Line	Spd Loss (nmol g^{-1} FW)	Spd to Spm conversion (nmol g^{-1} FW)	Spd to Put conversion (nmol g^{-1} FW)
NT	441.64	92.81	24.68
2E	223.44	93.37	27.09

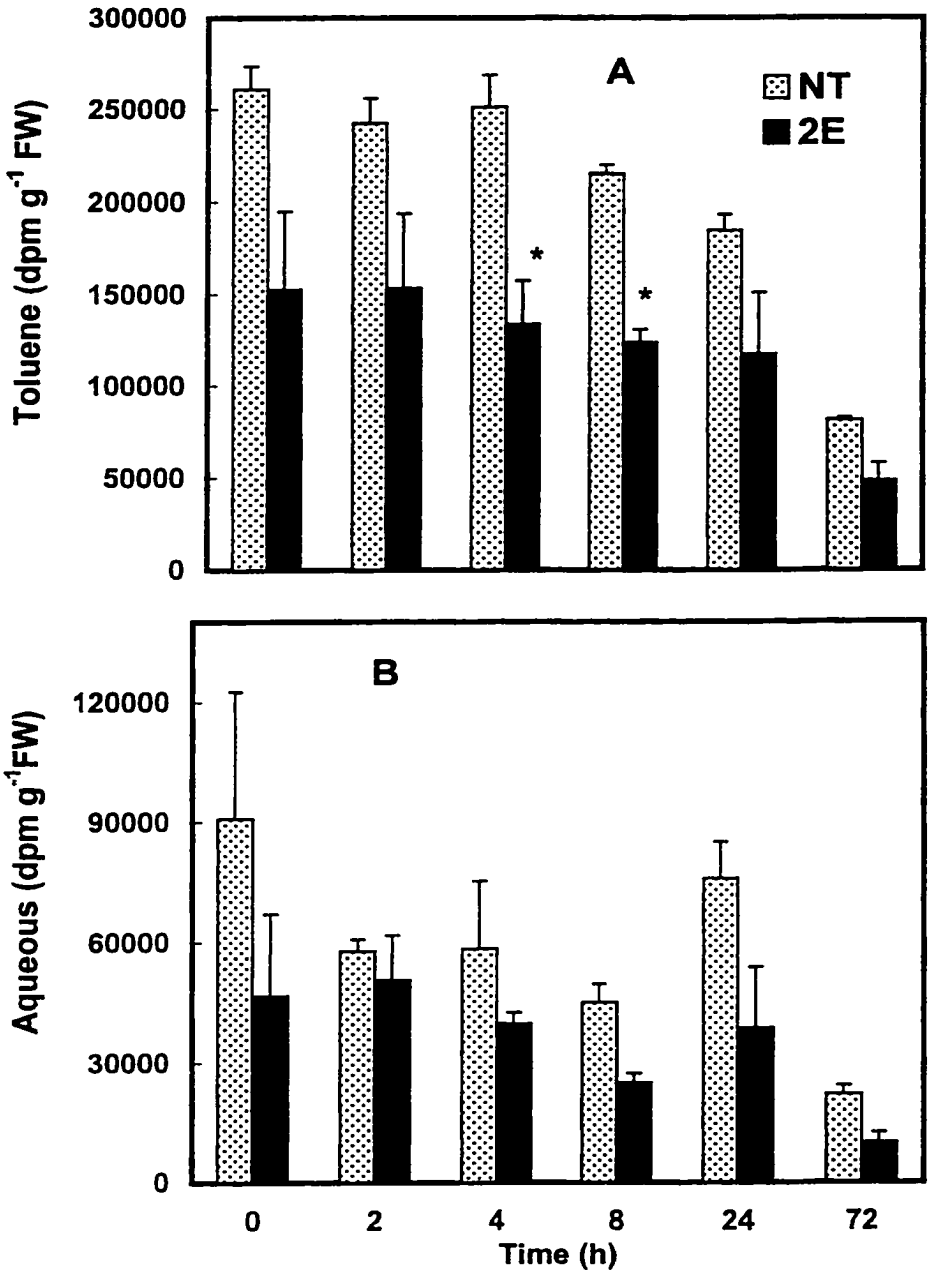


Fig. 56. Radioactivity present in the toluene (A) and aqueous (B) fractions after dansylation of PCA extracts of NT and 2E cells incubated with [^{14}C] Spd for 2 h, washed with label-free medium and transferred to label free fresh medium. Cells were collected at different times of analysis and total amount of radioactivity in each treatment was 1 μCi. Data presented are mean ± SE of nine replicates (from three experiments). An asterisk indicates that values for 2E are significantly different ($p \leq 0.05$) from NT cells at a given time.

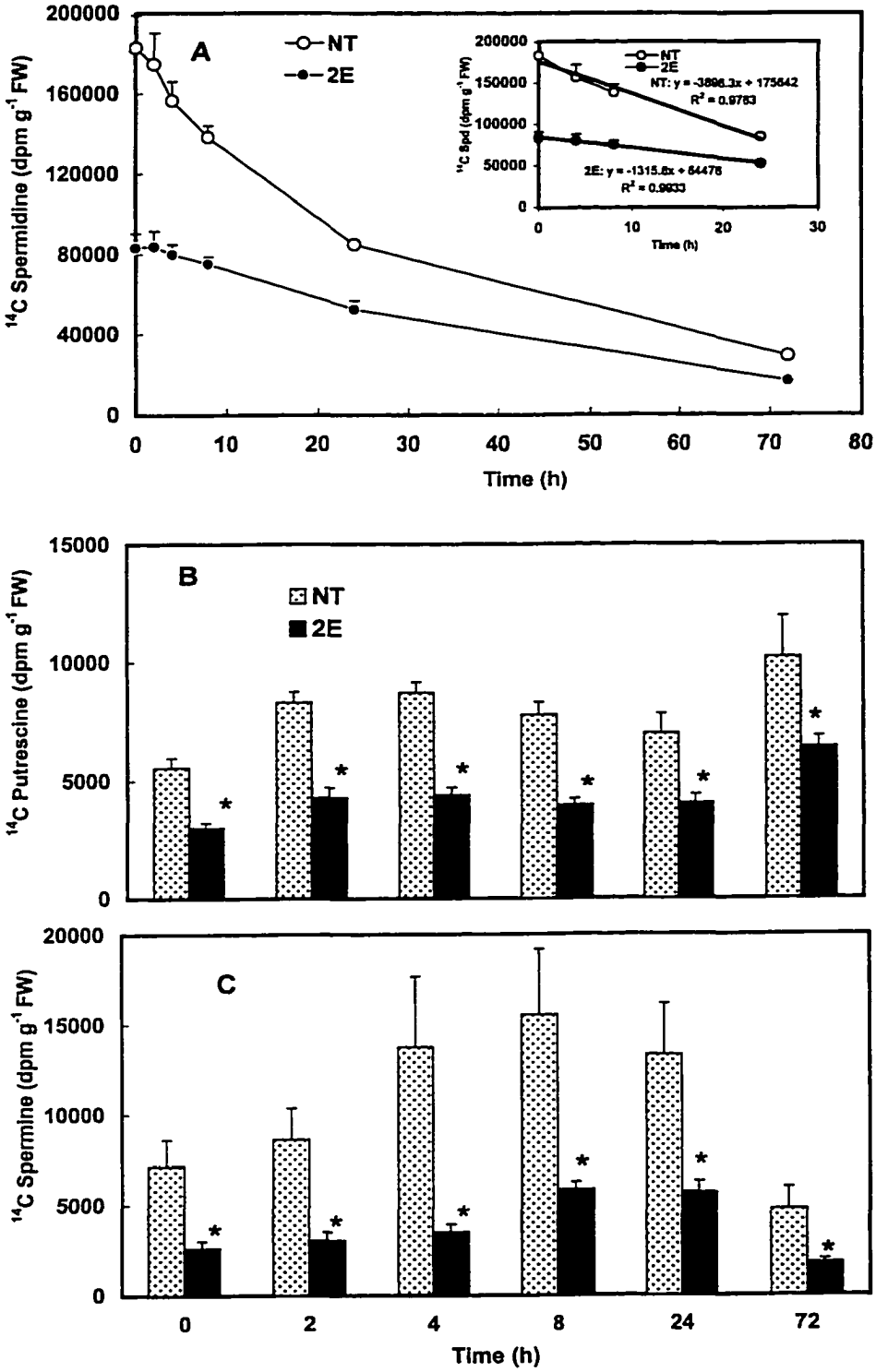


Fig. 57. Changes in the amount of [^{14}C] Spd (A), [^{14}C] Put (B) and [^{14}C] Spm (C) derived from [^{14}C] Spd in the NT and 2E cells at different times following transfer of cells to label-free medium. 57A inset: regression curve for the loss of [^{14}C] Spd over the 24 h period following transfer of labeled cells to label-free medium. Data presented are mean ± SE of nine (from three experiments) replicates. An asterisk indicates that values for 2E are significantly different ($p \leq 0.05$) from NT cells at a given time.

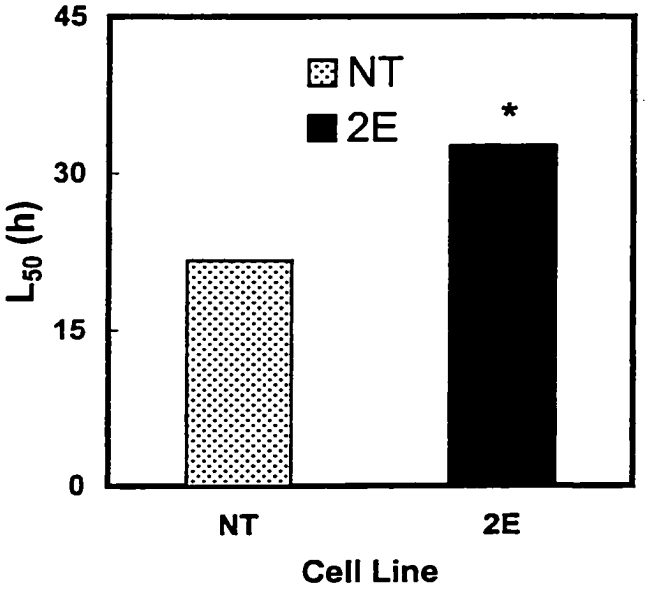


Fig. 58. Calculated initial half-life (L_{50}) of [^{14}C] Spd in NT and 2E cells treated with [^{14}C] Spd. Each bar represents mean ± SE of nine replicates (from three different experiments). The L_{50} was calculated by using data on the loss of [^{14}C] Spd at various times during the 24 h period after transfer of cells to label-free medium. An asterisk indicates that values for 2E are significantly different ($p \leq 0.05$) from NT cells.

The turnover of [^{14}C] Put starts to occur rapidly leading to a loss of the label from this fraction. At 72 h again, there was an increase in the radioactivity incorporated in the Put fraction in both the cell lines (Fig. 57B). At 2 h of analysis after transfer to fresh medium, there was an increase in the radioactive Put in both the cell lines. A short -term study was done at time periods of 0 h, 30 min, 60 min, 90 min and 2 h. The results show that high amounts of [^{14}C] Spd were converted into Put within the first 30 min with little further accumulation with time (Fig. 59A). The counts in Spd fraction in this experiment declined only after 60 min of incubation in the label-free medium (Fig. 59B). Accumulation of [^{14}C] Spm produced from [^{14}C] Spd remained low, the radioactivity in Spd being higher in the NT cells from time zero to the end of the experiment (120 min) (Fig. 59C).

Counts from [^{14}C] Spd found in the Spm and Put fractions represented 3% (after incorporation of recovery factor) of the total radioactivity in the toluene fraction at time zero. The [^{14}C] Spm content (Fig. 57C) increased up to 8 h in both the cell lines and then showed a decline. The trend observed in the 2E were the same as NT cells, however, the changes were not very dramatic. In the short term (0-120 min) study also, [^{14}C] Spm showed no particular trend, the label being maintained at a low level in both the cell lines (Fig. 59C).

Overall, the trends of changes in radioactivity in all the three polyamines in the two cell lines were similar, the label in the NT being higher than 2E. The specific radioactivity of all the three polyamines was always higher in the NT as compared to the 2E cells. The specific activity of Spd did not change in either of the two cell lines up to 24 h of analysis followed by a major decline at 72 h (Fig. 60A). The specific activity of Spm showed similar trends in both the cell lines with time with a major increase in the radioactive Spm at 24 h in the NT cells. In the 2E cells, the changes were generally small (Fig. 60B). The specific activity of [^{14}C] Put derived from [^{14}C] Spd feeding experiments was found to be very low and changed only slightly with time through the entire period of study in both types of cells (Fig. 60C). The specific activity of Put was very low for both cell lines because of low [^{14}C] counts and high Put amounts in the cells.

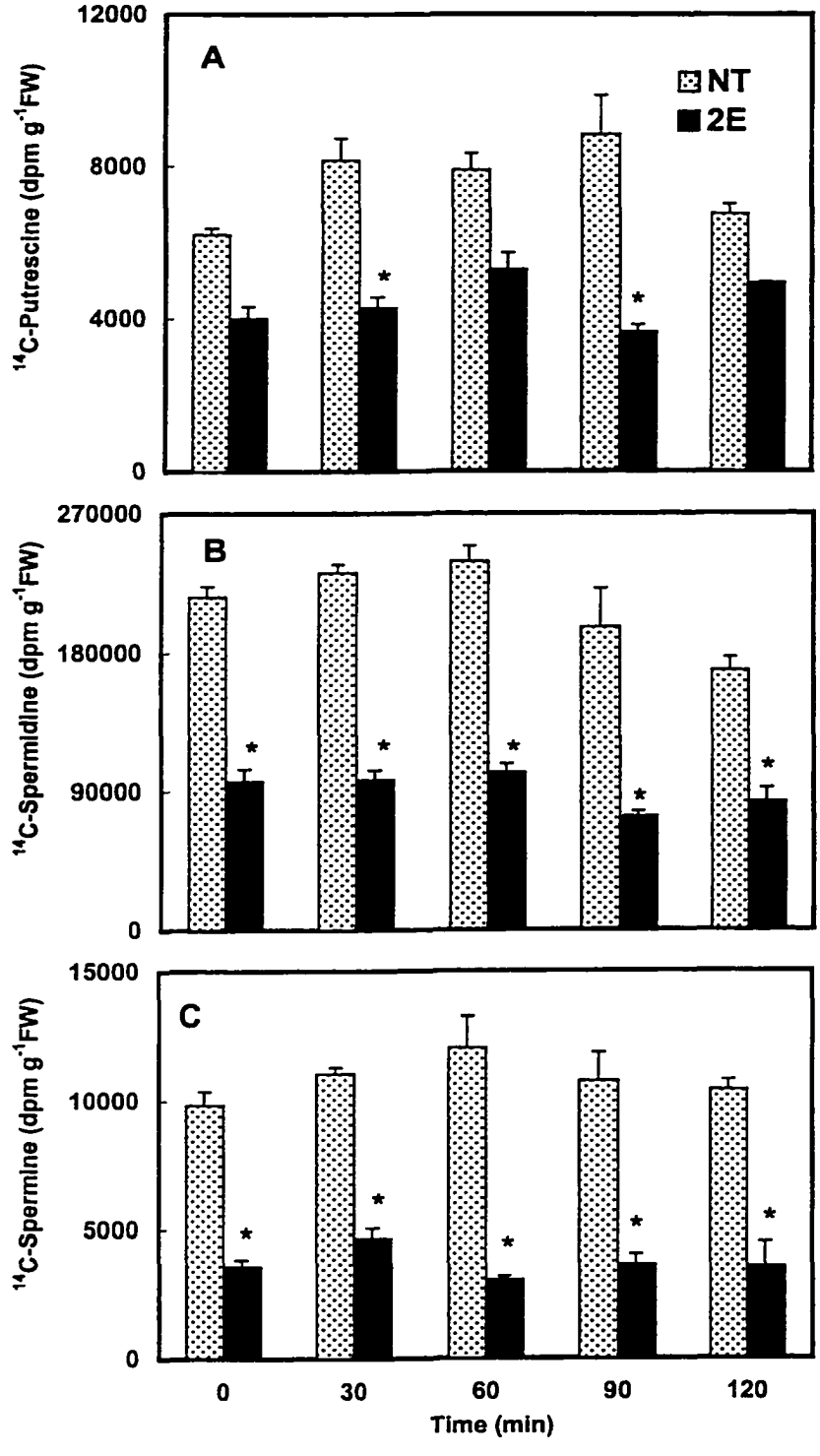


Fig. 59. Changes in the amount of [^{14}C] Put (A), [^{14}C] Spd (B) and [^{14}C] Spm (C) derived from [^{14}C] Spd in the NT and 2E cells at different times following transfer of cells to label-free medium. Data presented are mean ± SE of three replicates. An asterisk indicates that values for 2E are significantly different ($p \leq 0.05$) from NT cells at a given time.

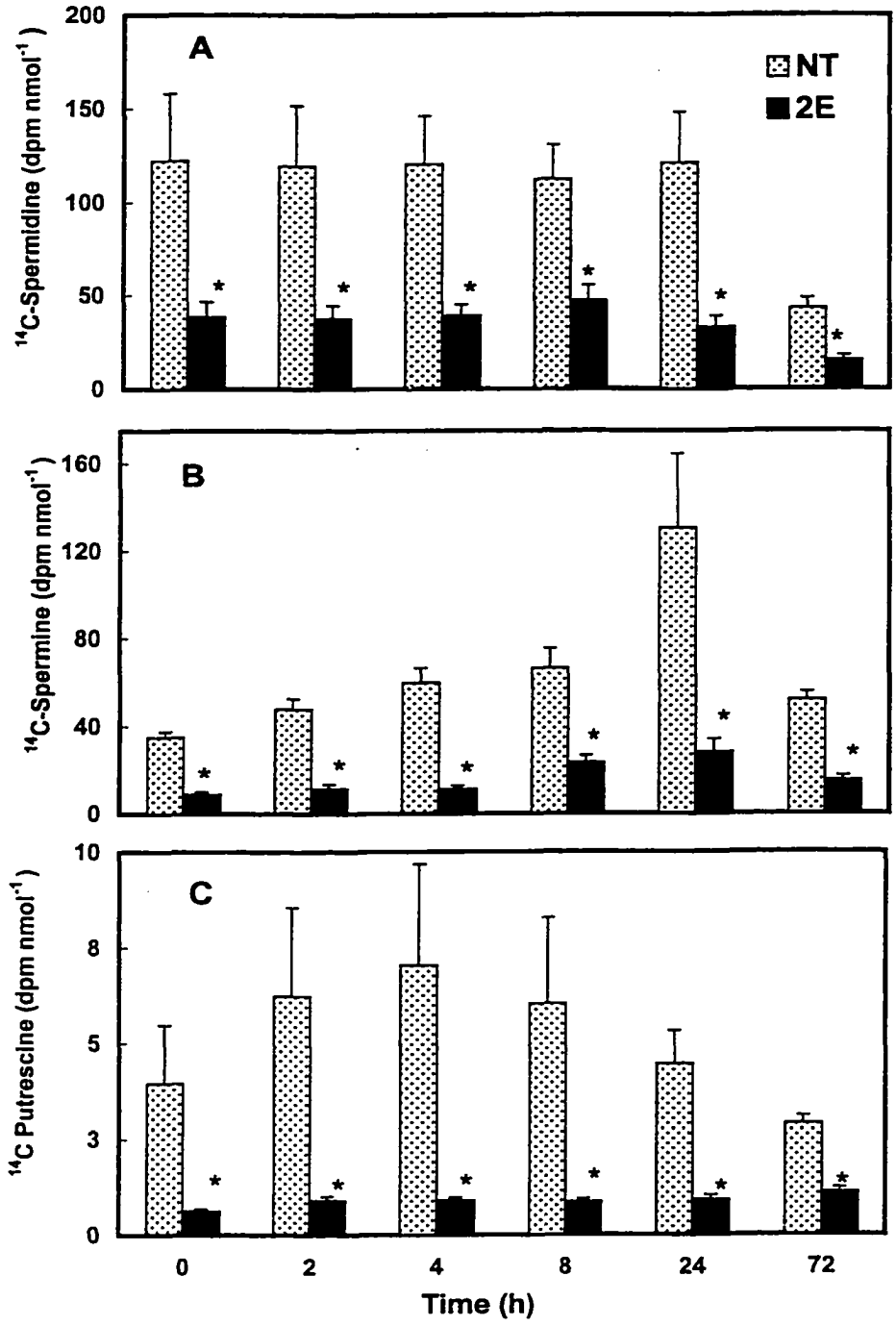


Fig. 60. Specific radioactivity of [^{14}C] Spd (A), [^{14}C] Spm (B) and [^{14}C] Put (C) derived from [^{14}C] Spd in NT and 2E cells at different times following transfer of cells to label-free medium. Data presented are mean ± SE of nine (from three experiments) replicates. An asterisk indicates that values for 2E are significantly different ($p \leq 0.05$) from NT cells at a given time.

Metabolism of ^{14}C-Spermine

Spermine Turnover in the transgenic and non transgenic cells

A similar experimental plan was followed for studying the [^{14}C] Spm turnover as for [^{14}C] Spd turnover in the two cell lines. Following 2 h of labeling and transferring the cells to label-free medium, the samples were collected at 0 h, 4 h, 8 h, 24 h, 48 h and 72 h. A time period of 48 h was included in this study as opposed to 2 h as longer-term changes in profiles were expected with [^{14}C] Spm incubation after studying the Spd turnover.

In contrast to the results with [^{14}C] Put and [^{14}C] Spd experiments, the counts in the toluene fraction with [^{14}C] Spm incubation either did not change or actually increased with time (Fig. 61A). This was intriguing since the cells had been washed off to remove excess exogenous radioactivity and were transferred to label free medium. The label in the toluene fraction increased in the 2E cell line and peaked at 8 h. There was no increase in the NT cells but neither was there any loss of [^{14}C] Spm, the levels being very similar throughout the period of the study. The amount of radioactivity in the 2E cells was always higher than the NT in the toluene fraction reaching two fold higher values at certain time periods, e.g., 24 h (Fig. 61A). The aqueous fraction represented 9 % (NT cells) and 12% (2E cells) of the radioactivity extractable in the PCA fraction since most of the counts were partitioned into the toluene phase as expected. As with the toluene fraction, in the aqueous fraction also, an increase in radioactivity with the time of incubation up to 24 h in the 2E cell line and 8 h in the NT cells was seen (Fig. 61B). This indicates the cell pellet may possibly be releasing bound radioactivity at these initial time periods. To test if the cell pellet was actually releasing bound radioactivity, the pellet was digested with 6N HCl and the extract was counted to see if there was any decline in this fraction to compensate for the increase in the PCA extract and subsequent processing steps. The data in Fig. 62A show that the total PCA extractable radioactivity also increased with time for up to 24 h in both cell lines.

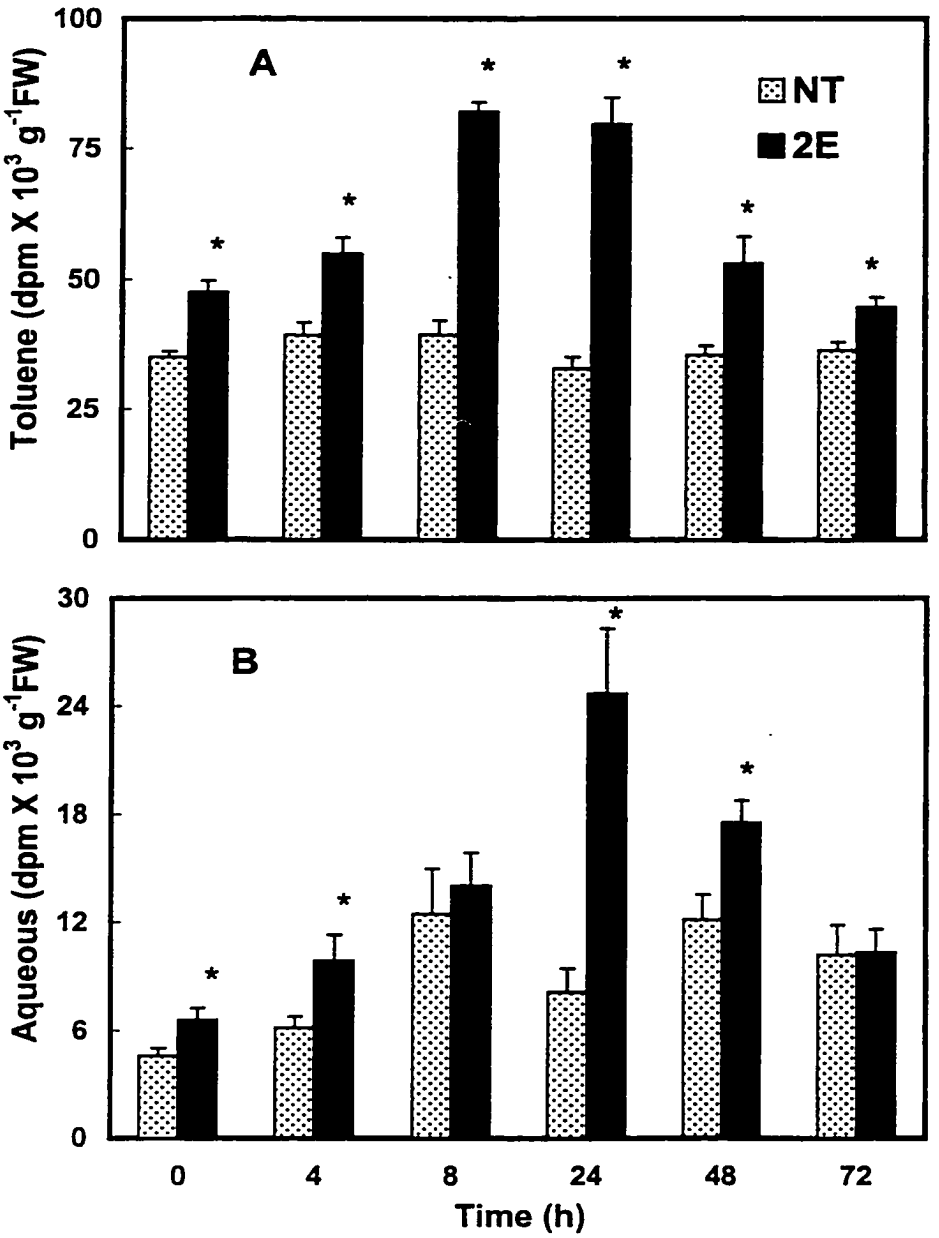


Fig. 61. Radioactivity present in the toluene (A) and aqueous (B) fractions after dansylation of PCA extracts of NT and 2E cells incubated with [^{14}C] Spm for 2 h, washed with label free medium and transferred to label free fresh medium. Cells were collected at different times of analysis and total amount of radioactivity in each treatment was 1 μCi. Data presented are mean ± SE of nine replicates (from three experiments). An asterisk indicates that values for 2E are significantly different ($p \leq 0.05$) from NT cells at a given time.

Thereafter, an increase in this fraction was also observed. However, Fig. 62B shows that the amount of remaining radioactivity in the pellet did not change appreciably with time. The amount of radioactivity was similar in pellets from the two cell lines at any given time and constituted only a small fraction (<20%) of the total radioactivity in the cells.

It was expected that at time zero most of the counts partitioned in the toluene fraction would be present in the Spm fraction and it was found to be the case in both the cell lines (Fig. 63A). The label in Spm fraction increased at 8 h in the 2E, then declined at 24-72 h. In the NT cells, there was a slow and steady decline in the [^{14}C] Spm with time throughout the 72 h of incubation. There were always higher counts present in the 2E than NT except at 48 h and 72 h.

When the counts accumulated in the Spd fraction were analyzed, it was observed that the radioactivity in the 2E cell line increased up to 24 h, peaking at 24 h, there being a statistically significant difference in the 8 h and 24 h counts (Fig. 63B). Again, the increase in the Spd fraction was more pronounced in the 2E cell line. This was followed by a decrease in the label progressively at 48 h and then 72 h. However, in the NT cell line, the label continued to accumulate gradually up to 72 h (Fig. 63B).

The [^{14}C] Put produced from [^{14}C] Spm also exhibited a gradual increase with time in both the cell lines (Fig. 63C), a major increase being seen at 48 h and 72 h in the 2E cells, a time coincident with the loss of counts in the [^{14}C] Spd fraction. The increase in [^{14}C] Put in the NT cells was much smaller. The 2E cells incorporated more radioactivity in the [^{14}C] Put fraction than NT cells at all time periods. There was a 5.5 fold increase in the Put fraction in the 2E cells from 0 h to 72 h, whereas a 2.5 fold increase was seen in the NT cells (Fig. 63C). By 24-48 h, almost all of the counts lost from the Spm fraction could be accounted for in Spd and Put fractions.

The cellular content of polyamines in the cells during the period of [^{14}C] Spm metabolism study is given in Fig. 64 and the specific activity of the three polyamines when they are incubated

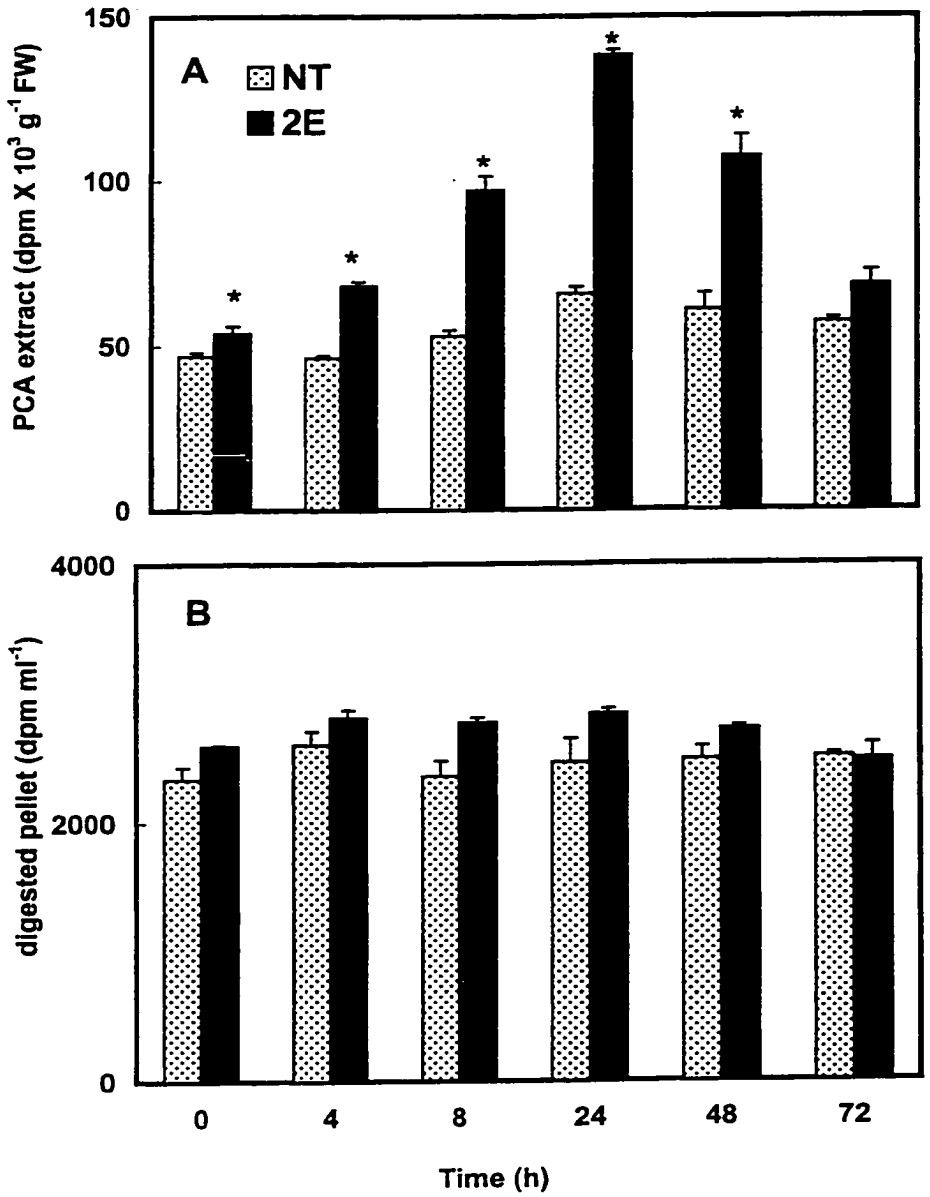


Fig. 62. Radioactivity present in the PCA extract (A) and pellet (B) digested with 6N HCl of NT and 2E cells incubated with [^{14}C] Spm for 2 h, washed with label-free medium and transferred to label-free fresh medium. Cells were collected at different times of analysis. Total amount of radioactivity in each treatment was 1 μCi. Data presented are mean ± SE of three replicates (from one experiment). An asterisk indicates that values for 2E are significantly different ($p \leq 0.05$) from NT cells at a given time.

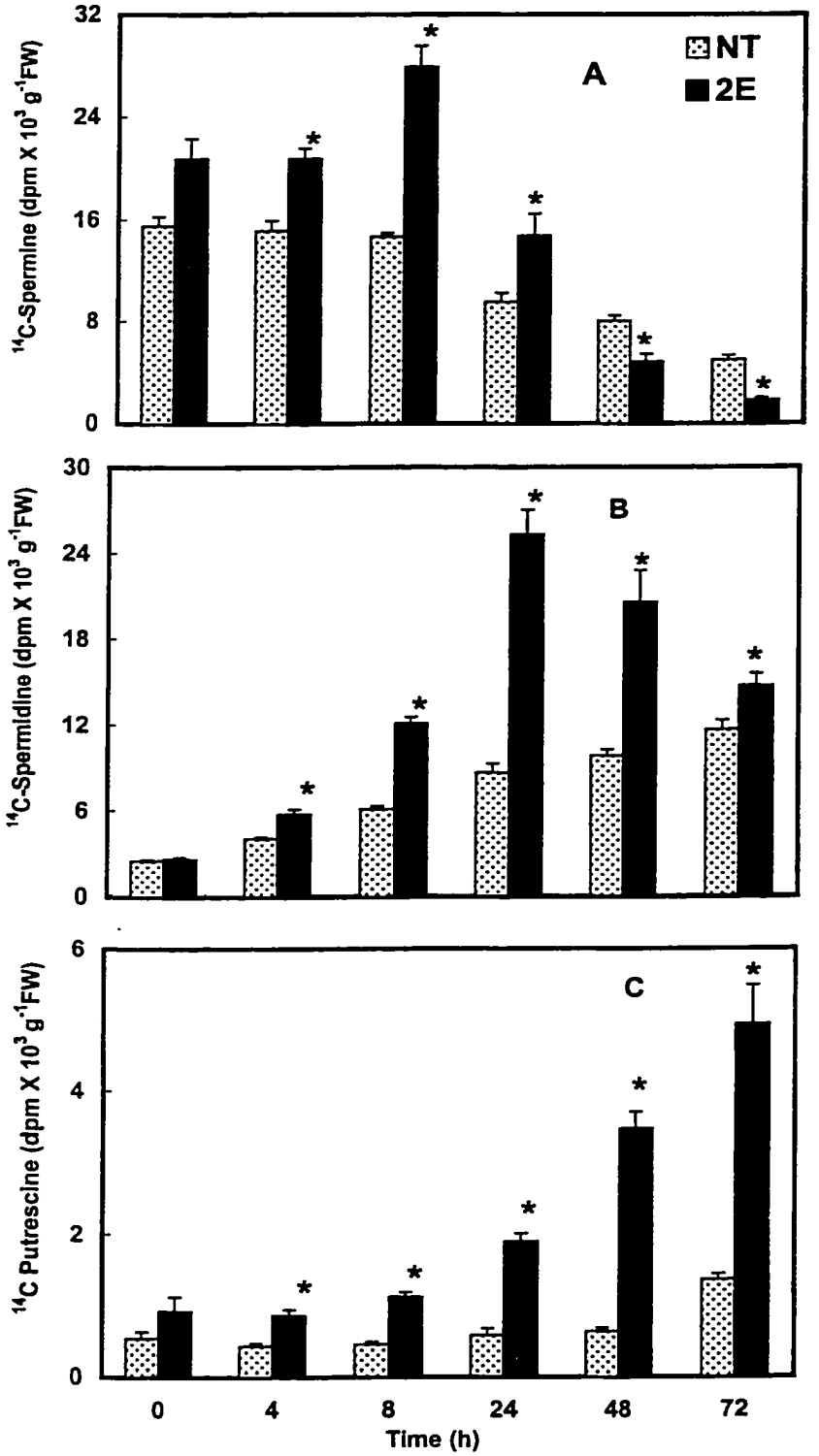


Fig. 63. Changes in the amount of [^{14}C] Spm (A), [^{14}C] Spd (B) and [^{14}C] Put (C) derived from [^{14}C] Spm in the NT and 2E cells at different times following transfer of cells to label-free medium. Data presented are mean ± SE of nine (from three experiments) replicates. An asterisk indicates that values for 2E are significantly different ($p \leq 0.05$) from NT cells at a given time.

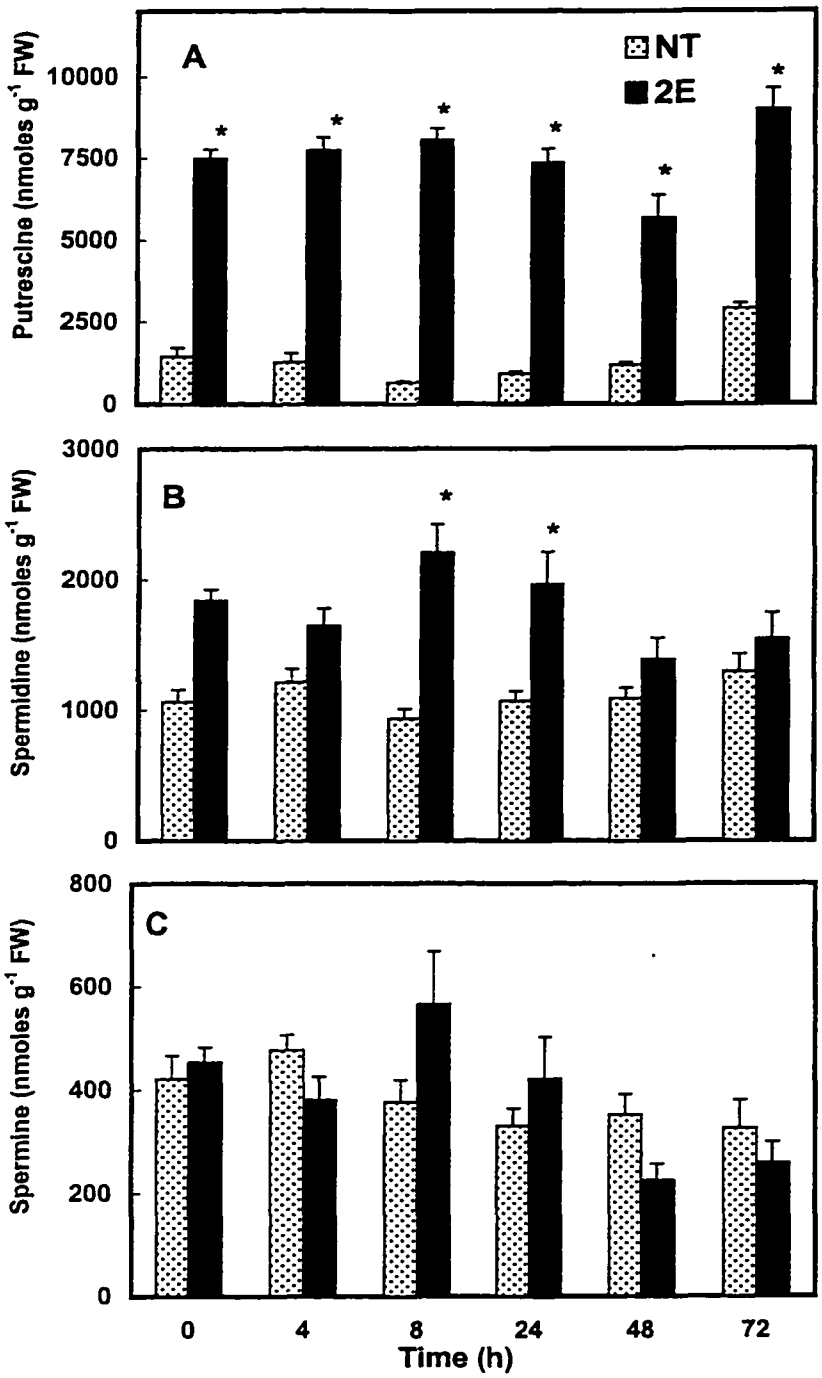


Fig. 64. Cellular content of PCA soluble polyamines (nmol g^{-1} FW) in the NT and 2E cell lines at different time intervals after transfer of cells treated with [^{14}C] Spm to label-free medium. Data are mean ± SE of nine (from three experiments) replicates. An asterisk indicates that values for 2E are significantly different ($p \leq 0.05$) from NT cells at a given time.

with [^{14}C] Spm is shown in Fig. 65. As expected, the cellular Put in the 2E cells was several fold higher than the NT cells and the amount did not change appreciably with time (Fig. 64A).

Likewise, Spd content of 2E cells was also significantly higher than the NT cells and did not change much with time (Fig. 64B). The Spm content of the two cell lines was quite comparable at all times (Fig. 64C). The specific activity of [^{14}C] Spm declined consistently after 4 or 8 h of incubation in the [^{14}C] free medium in the two cell lines (Fig. 65A). The trend of increase in specific activity of [^{14}C] Spd was similar in the two cell lines (Fig. 65B). With regard to [^{14}C] Put its specific activity was higher in the NT cells up to 24 h, thereafter the two cell lines had similar [^{14}C] Put specific activity (Fig. 65C). While [^{14}C] Put specific activity increased substantially in the 2E cells for 2- 48 h, in the NT cells the change was not well pronounced. Table XIII shows the percentage of back conversion of Spd into Put and Spm into Spd and Put at different time periods in these cell lines. Data presented show that the percentage of [^{14}C] Spd that was converted into Put in the two cell lines was quite comparable and increased with time from about 3.3% at time zero to over 20% at 72 h. The percentage of [^{14}C] Spd appearing as [^{14}C] Spm was much higher in the NT than the 2E cells. The maximum amount of [^{14}C] Spd converted into Spm was always less than 10%. For [^{14}C] Spm conversion into Put and Spd, the results were quite different in that 2E cells converted a higher percentage of Spm into Put than the NT cells, the maximum amount being about 11% at 72 h. The conversion of [^{14}C] Spm into [^{14}C] Spd was much greater than that converted into Put. However, the percent of [^{14}C] Spm appearing as Spd was similar in the two cell lines at any given time.

The total amount of [^{14}C] Spd lost (turned over) during the first 8 h of transfer to fresh medium in the two cell lines was significantly different; the NT cells losing twice as much Spd as the 2E cells (Table XII). The conversion of [^{14}C] Spd into [^{14}C] Spm as well as [^{14}C] Put was comparable in the two cell lines for the first 8 h period. The amount converted into Spm was about three fold higher than that converted into Put.

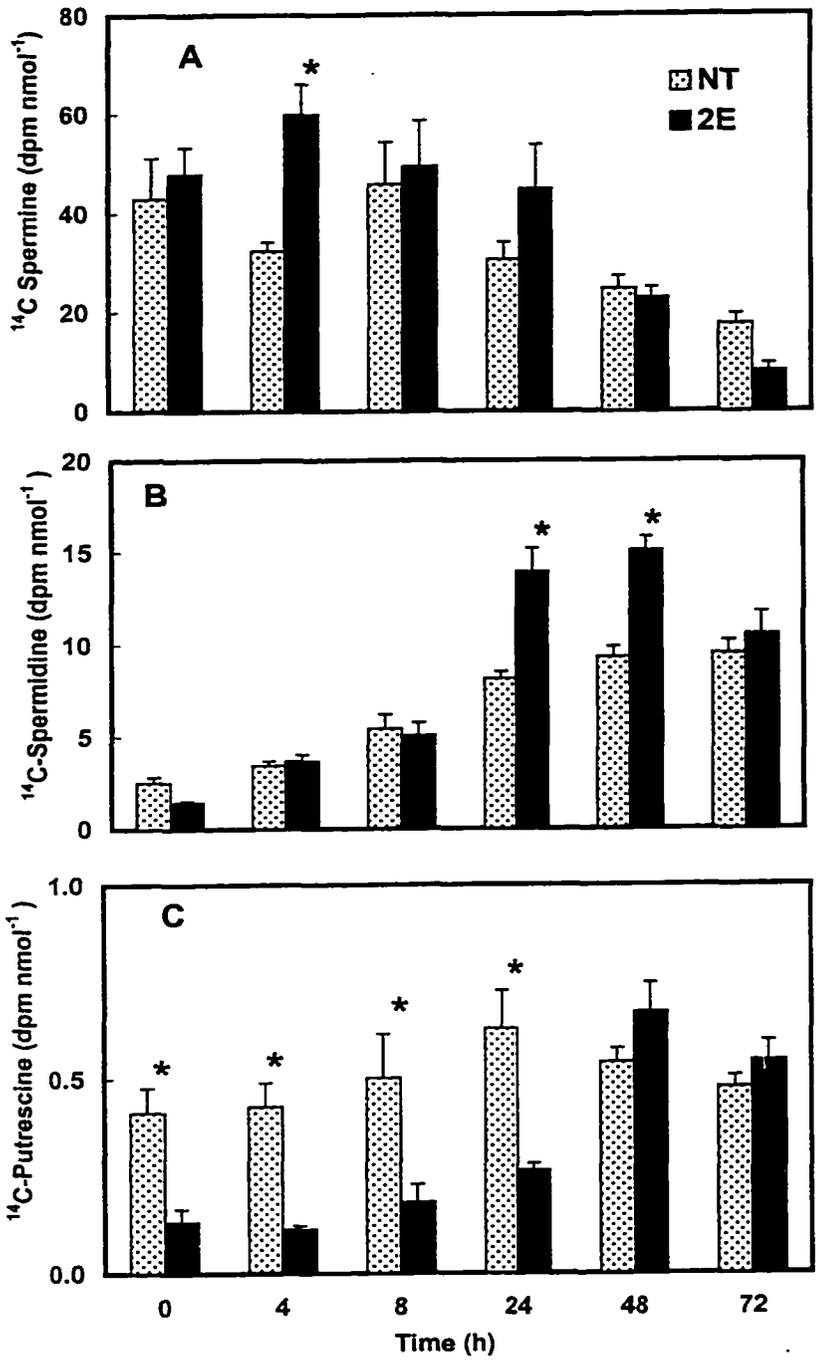


Fig. 65. Specific radioactivity of [^{14}C] Spm (A), [^{14}C] Spd (B) and [^{14}C] Put (C) derived from [^{14}C] Spm in NT and 2E cells at different times following transfer of cells to label-free medium. Data presented are mean ± SE of nine (from three experiments) replicates. An asterisk indicates that values for 2E are significantly different ($p \leq 0.05$) from NT cells at a given time.

Table XIII. The percentage of radioactivity accumulated in the polyamines as a fraction of the toluene content present at that time period when cells were labeled with [^{14}C] Spd and [^{14}C] Spm for 2 h and then transferred to label free medium and collected at different time periods.

		% of Toluene fraction incorporated					
Substrate	**Time (h)**	**Put**		**Spd**		**Spm**	
		NT	2E	NT	2E	NT	2E
[^{14}C] Spd	0	3.35	3.28			4.32	2.82
	8	4.63	3.52			9.24	5.20
	72	18.54	20.90			8.64	5.77
[^{14}C] Spm	0	1.53	1.92	7.07	5.47		
	24	1.74	2.37	26.30	31.75		
	72	3.75	11.05	32.09	32.97		

Effect of Put overproduction on the *adc, Spd synthase, gad* and mouse *odc* expression

Source of Probes

A 570 bp band was obtained for the Glu decarboxylase (GAD) PCR product using degenerate primers and poplar cDNA made from RNA of 3 d old cells. Two bands of size 800 bp and 1Kb were obtained with *gad* primers using genomic DNA as template. The bands were gel purified and sequencing was done using both the forward and reverse primers for the cDNA as well as genomic PCR products. The bands were sequenced and showed homology to the *gad* gene. Two bands of sizes 817 bp and 300 bp were obtained using *Datura* spermidine synthase (Spd synthase) primers. The 817 bp band was gel purified, sequenced and showed homology to *Spd synthase*. A 1037 bp PCR product obtained using primers specific for *Porphyra actin* was sequenced and showed homology to the *actin* gene. Table XIV shows the percent homology of the PCR products of the above genes to the sequences from different plant species in the Genbank. Poplar *adc* PCR product obtained using ADCF2-R2 primers gave the desired band of 520 bp. Restriction digestion was performed on the Topo plasmid containing an *adc* cDNA using *Eco*RI to obtain the desired band of 520 bp. Glycerol stocks for *adc* and *G3PDH* were made by Dr. J.S. Lee in the laboratory.

Northern Analysis for *adc*, *gad*, mouse *odc* and *Spd synthase* genes

The northern hybridization blots of total RNA with the four genes are shown in Fig. 66A-E. The results on the blots are interpreted as a ratio of intensity of the band after normalizing to *G3PDH*. The intensity/ area value obtained for the respective gene is divided by the intensity/ area of *G3PDH*.

Table XIV. Characteristics of probes used for northern hybridization.

Gene	Source of Primers	Template DNA used	Size (bp)	Genbank Accession Number	Match Overlap (bp)	% identity	Blast-X E-value	Homology	Organism
GAD	*Arabidopsis thaliana*	cDNA	570	AAK18620	144	82	6e-71	GAD isozyme 3	*Nicotiana tabacum*
		Genomic	800	7436483	54	89	1e-23	GAD2 calmodulin binding isozyme 3	*Nicotiana tabacum*
Spd Synthase	*Datura stramonium*	cDNA	817	6094335	121	91	7e-62	Spd Synthase	*Coffea arabica*
Actin	*Porphyra yezoensis*	cDNA	1037	11276968	165	93	4e-91	Actin	*Picea rubens*

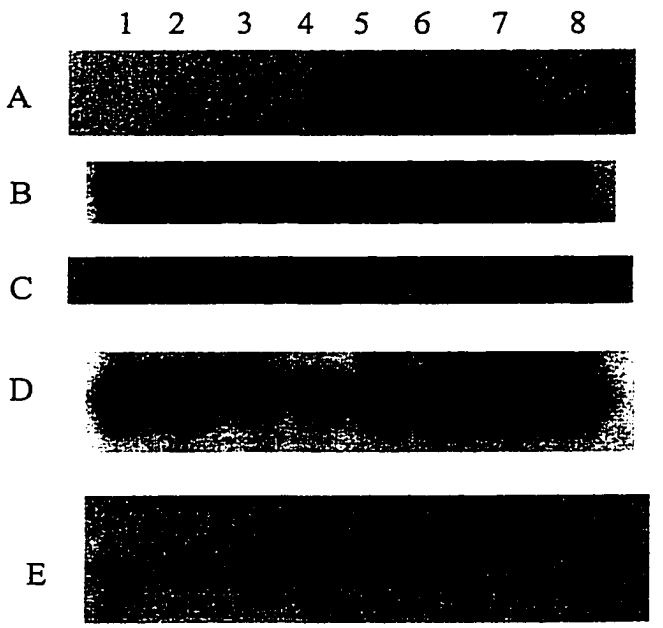


Fig. 66. Northern hybridization showing total RNA hybridized with mouse ODC (A); Glu decarboxylase (GAD) (B); Spd Synthase (C); ADC (D) and G3PDH (E). The lanes are: NT 1 d (1); NT 3 d (2); NT 4 d (3); NT 6 d (4); 2E 1 d (5); 2E 3 d (6); 2E 4 d (7) and 2E 6 d (8). 18 μg of total RNA was loaded in each case.

The ratios are presented in Fig. 67 and 68. There was no signal seen for the expression of mouse *odc* in the NT cell line as would be expected (Fig. 66A). The expression of the transgene was low on 1 d of the culture and then increased significantly at 3 d, after which there was a slight decline at 4 d and then very low levels of expression were seen at 6 d (Fig. 66A; 67A,B). The expression levels of *adc* (chief source of Put production in the NT cells) were also determined. The *adc* mRNA levels were either comparable between the two cell lines or were slightly higher in the 2E cells (Fig. 66E; 67C,D). This was consistent with the observation that the ADC enzyme acitivity was not feedback inhibited (i.e. suppressed) as a result of Put overproduction in the 2E cells.

After having determined that the rates of Put biosynthesis and catabolism were higher in the 2E cells as compared to NT cells, and that there was three fold higher production of Spd from the 2E cells, the expression of the enzyme involved in the production of Spd from Put, i.e. Spd synthase was determined. The expression of the gene in the NT cell line did not change much during 1,3 and 4 d of culture but the transcript levels lowered at 6 d (Fig. 66C). In the 2E cells, there was a peak of mRNA levels at 3 d and the mRNA levels were comparable on all the other days tested (Fig. 66C; 68C). The ratios of the *Spd synthase* gene after normalizing to *G3PDH* were slightly different than the actual expression of the gene due to slight numerical differences in the quantification of the *G3PDH* gene at 1 d of the NT cell line (Fig. 68D).

Glutamate decarboxylase produces GABA from Glu in a single step reaction. Alternatively, GABA is a chief catabolic product of Put. It was deemed interesting to study if the expression levels of *gad* were lower in the 2E cells as a result of Put overproduction. The *gad* gene expression was higher in the NT cell line at all days tested except day 1 (Fig. 66B; 68A). Once again, the ratio of the *gad* gene expression was slightly different than the actual expression of the gene due to slight numerical differences in the quantification of the *G3PDH* gene at 1 d of the NT cell line (Fig. 68B). The levels of *G3PDH* were generally comparable between the two

cell lines at all days. (Fig. 66E) and also show that the loading of RNA in different lanes was generally similar.

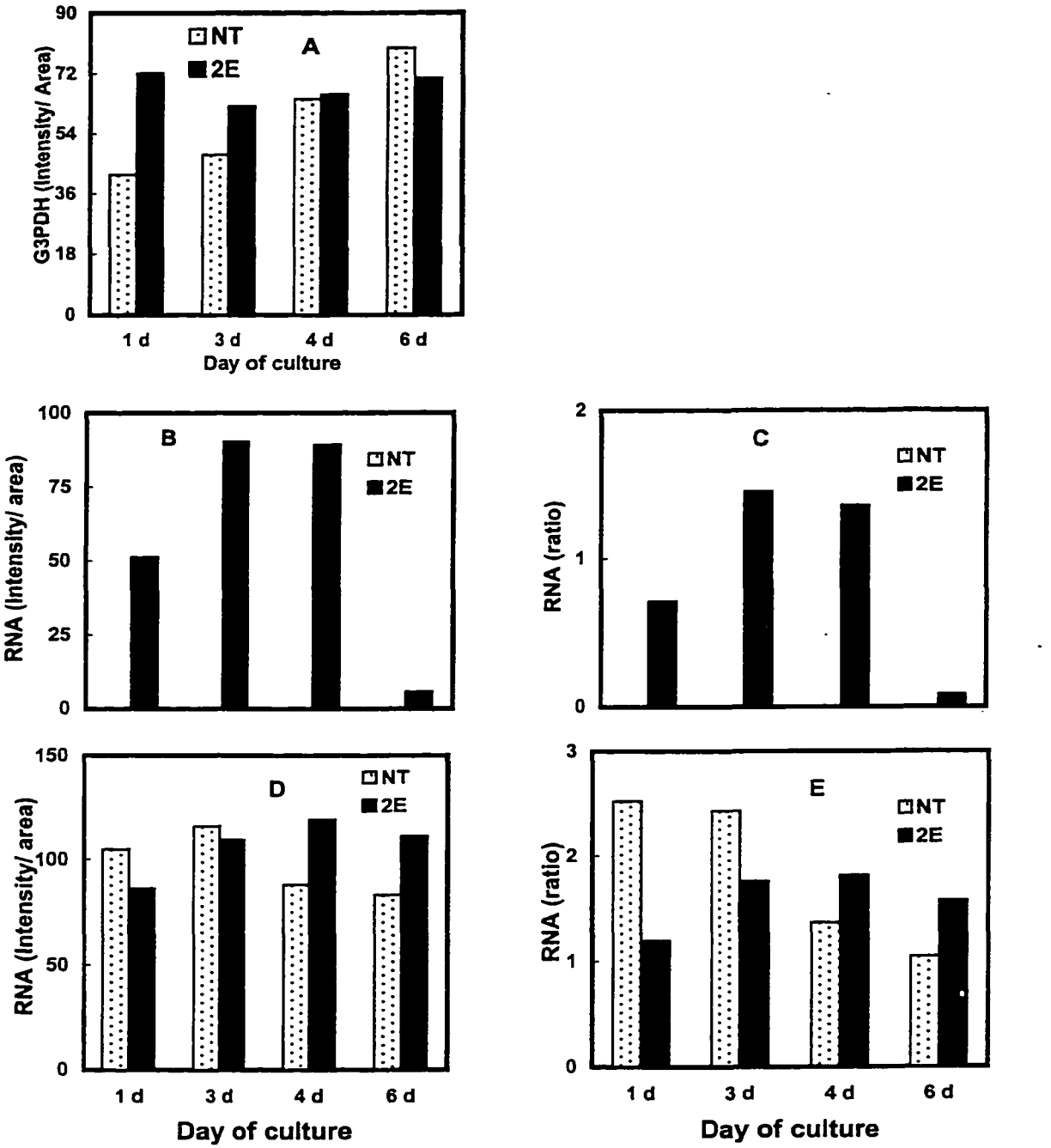


Fig. 67. A comparison of densitometry results in northern hybridization, expressed as intensity/ area of two cell lines on different days of the subculture cycle probed with *G3PDH* (A), mouse *odc* (B) and *adc* (D). The intensity/ area values normalized to *G3PDH* for mouse *odc* (C) and for *adc* (E).

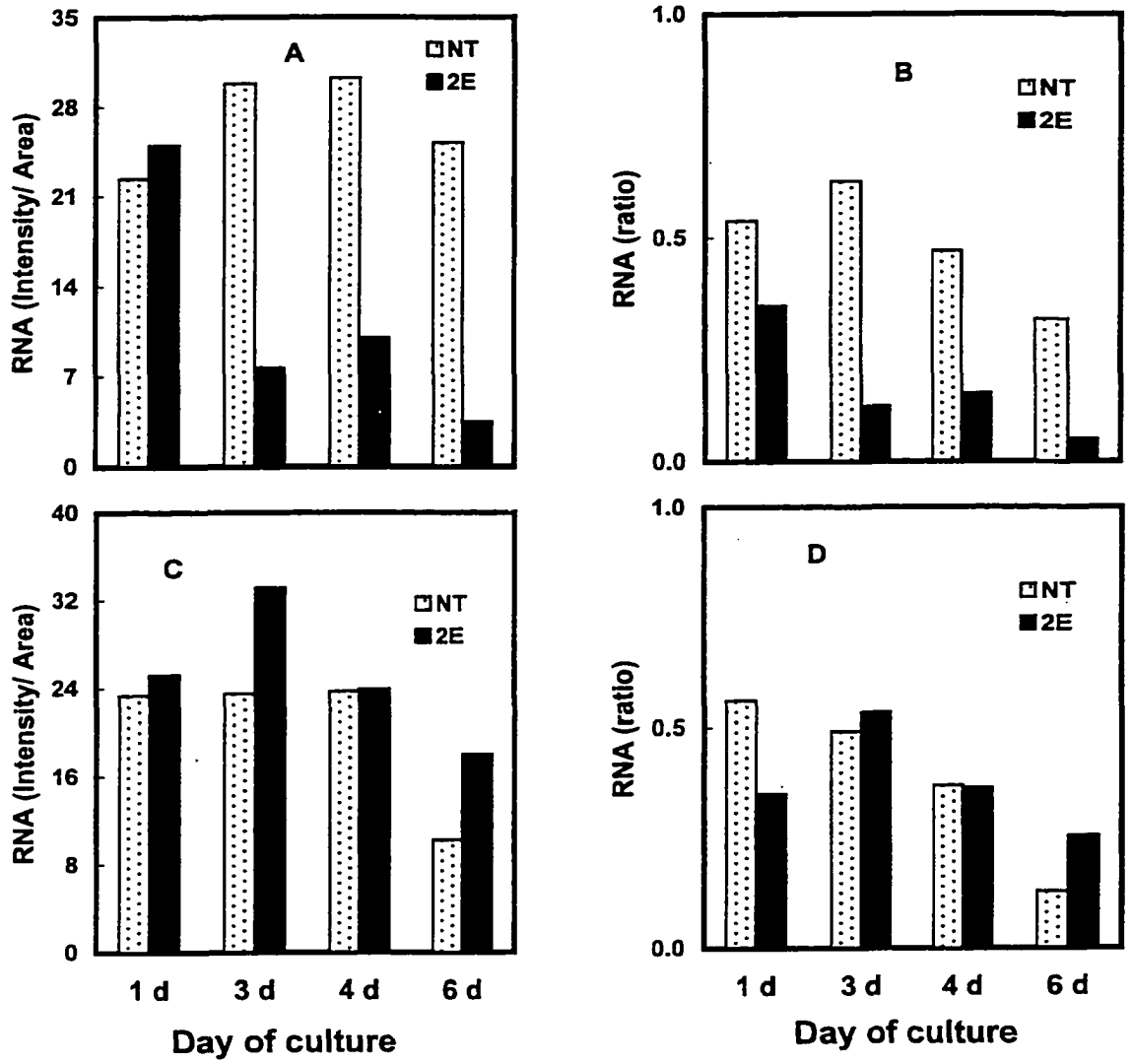


Fig. 68. A comparison of densitometry results in northern hybridization, expressed as intensity/ area of two cell lines on different days of the subculture cycle probed with *gad* (A) and *Spd synthase* (C). The intensity/ area values normalized to *G3PDH* for *gad* (B) and for *Spd synthase* (D).

DISCUSSION

The transgenic approach to the study of cellular metabolism of plants can sometimes reveal mechanisms of metabolic regulation that are difficult to demonstrate through inhibitor studies or mutant analysis. The effect of manipulation of a single step in one pathway can be studied on the related pathways as well. The polyamine pathway, in addition to being ubiquitous in all living organisms, is relatively short, all the genes have been cloned, and the pathway serves as a fine link between the carbon and nitrogen (N) metabolic pathways. In the present study, poplar cells were transformed with mouse *odc* gene and a cell line (2E) overproducing Put was selected. The impact of overproducing Put was studied on the polyamine catabolism, turnover, and on related pathways, such as, N assimilation pathway.

Mouse *odc* cDNA Used for Transformation

The roles of the 5' and the 3' UTR (untranslated regions) in the regulation of mammalian *odc* (which has some of the longest UTRs seen in animal mRNAs) have been variously discussed (Grens and Scheffler, 1990; Wallstrom and Persson, 1999). Previous studies from our laboratory (DeScenzo and Minocha, 1993; Bastola and Minocha, 1995) showed that the presence of only 59 bp at the 3' end of the 737 bp long 5' UTR was sufficient for expression of the mammalian *odc* cDNA in plants. The results presented here clearly demonstrate that the expression of mouse *odc* in plants does not require any part of either the 5' or the 3' UTRs (See also, Wallstrom and Persson, 1999). Likewise, the PEST amino acid domain at the C-terminus of the ODC protein is not essential for the activity of this enzyme. In fact, the expression of mammalian cDNA containing sequence for the 3' PEST region of 37 amino acids caused a much smaller increase in ODC activity in transgenic tobacco than the truncated version without this sequence (DeScenzo

and Minocha, 1993). This could be attributed to a rapid turnover of the full-length mouse enzyme protein in the transgenic cells. These results are consistent with the reported properties of mammalian ODC and with studies on the transgenic expression of mammalian *odc* in animal cells (Halmekytö et al., 1991; Kauppinen and Alhonen, 1995).

Polyamines in Transgenic Cells

A number of studies on transgenic expression of homologous and heterologous sequences of *odc* in animals and *odc* as well as *adc* in plants have been reported (reviewed in Kumar and Minocha, 1998; Table I). In transgenic mice overexpressing human *odc* gene, most tissues (except brain and testis) showed a homeostatic response to increased ODC activity, i.e. normal levels of Put were maintained in these tissues (Halmekytö et al., 1991). However, the brain and the testis did accumulate Put in higher amounts without significant changes in either Spd or Spm. The maintenance of near normal levels of Put in most transgenic tissues was later shown to be the result of increased export/excretion of Put from these tissues (Halmekytö et al., 1993; Seiler et al., 1996). In several studies with plants, including the present one, significant increases in the content of Put in transgenic cells have been observed with the expression of either *odc* or *adc* cDNAs (see Kumar and Minocha, 1998 for review; Table I). In most cases, however, little or no increase in Spd or Spm was observed. Also, in studies where significant changes in Put content were observed during normal development or in response to a variety of abiotic stress factors, only a little or no concomitant change in Spd or Spm content was observed (Zhou et al., 1995; Minocha et al., 1993, 1996, 1999b; Bouchereau et al., 1999). The situation with transgenic poplar cells overexpressing the mouse *odc* cDNA seems to be consistent with these reports. Davis et al. (1992) have proposed that animal cells, while able to tolerate high concentrations of Put are unable to tolerate high concentrations of Spd and Spm. If true, this would require a tight metabolic regulation of Spd and Spm content within the cells. This probably is achieved by the regulation of key enzymes needed for their biosynthesis, namely SAMDC and Spd/Spm

synthases, and/or increased turnover or secretion of Put from the cells as mentioned above. Studies to date reveal that increased Spd and Spm biosynthesis in animal cells is often accompanied by increased catabolic breakdown of these compounds through induction of Spd/Spm acetyltransferase (SSAT) and PAO activities (Cohen, 1998). Whether or not in plants also the levels of Spd and Spm are regulated by similar compensatory mechanisms involving increase in their catabolic turnover by PAO, is presently unknown.

In plants, in addition to serving as a precursor for Spd biosynthesis, Put can be conjugated with a variety of phenolic acids, and in some cases, converted to secondary metabolites, e.g. alkaloids (Walden et al., 1997). Genetic manipulation of Put biosynthesis to modulate nicotine content in tobacco has been attempted by using a yeast *odc* cDNA (Hamill et al., 1990). Since an early catabolic product of Put in plants is γ-aminobutyric acid (GABA), which may play a variety of important roles in plant development (Shelp et al., 1999), increased Put production in transgenic plant cells may also have far-reaching implications for their physiological responses involving this compound. Neither the conjugation of Put with phenolic compounds nor the cellular content of GABA has yet been analyzed in transgenic plant cells overproducing Put. However, studies on short term incorporation of [^{14}C] Orn and [^{14}C] Put show that little if any incorporation of Put into bound forms occurs in the transgenic poplar cells. Almost all of the radioactivity in the cells was extractable into PCA at the end of 2 h of incubation (Table VI and VII). A small decrease in Spm content often seen in the *odc* transgenic cells has no obvious explanation at present and may be physiologically insignificant, since Spm constitutes only a small proportion (less than 5%) of the total polyamines.

Are the substrates for ODC and ADC Limiting?

It is presently unknown as to how the cellular levels of Put are regulated in plant cells, and what factors determine the upper limits of Put accumulation in the transgenic poplar cells overexpressing the mouse *odc* cDNA. However, we do know that poplar cells can actually

tolerate and maintain much higher levels of Put than they normally contain, as shown by the levels of Put in transgenic and the NT-HP cells compared to the normal NT cells. For NT cells, some possibilities for regulation of cellular Put content include: (1) limitation of the substrate Arg since these cells primarily use ADC and do not possess much ODC activity, (2) limitation of the enzyme ADC, (3) feedback regulation (inhibition of enzyme activity or suppression of gene activity) of ADC activity by Put, and (4) increased Put catabolism. For transgenic cells, it can be hypothesized that a constitutive overexpression of the mouse *odc* cDNA could possibly lead to a depletion of their Orn pools since this amino acid is being used at a high rate by the mouse ODC. The depletion of Orn could in turn reduce the availability of Arg (for ADC) since it is also the precursor of Arg (Fig. 2, steps 10, 11). This would then limit the amount of Put that can be synthesized in these cells via ADC. In order to test this hypothesis, the cells were exogenously supplied with Orn or Arg and analyzed for their polyamine contents. Based upon the data presented here, it can be argued that: (1) concomitant with increased utilization of Orn, its biosynthesis is also enhanced in the transgenic cells, without affecting its cellular pools, (2) this enhancement is still insufficient to saturate the available ODC enzyme in these cells since exogenous Orn causes an increase in Put content of transgenic cells, and (3) exogenous Orn can be converted into Arg in NT cells providing additional substrate for ADC and causing increased Put production. This indicates the existence of a homeostatic regulatory mechanism, which induces increased Orn production concomitant with its increased utilization. The observed inhibition of Put accumulation by high concentrations of Arg in both cell lines is difficult to explain at present. Although Arg metabolism has been extensively studied (Wu and Morris, 1998), relatively little is known about homeostatic regulation of Orn pools in plant cells.

Mammalian ODC is known to be regulated by feedback mechanisms that operate both at transcriptional and translational levels (Kanamoto et al., 1986, 1993; Glass and Gerner, 1986; Nilsson et al., 1997; Cohen, 1998). Also, the turnover of ODC in animals is promoted by excess polyamines via the induction of an antizyme protein which is mechanistically involved in ODC

degradation (Nishiyama et al., 1989; Hayashi and Murakami, 1995). The existence of similar controls for ODC and ADC in plants has not been demonstrated. Primikirios and Roubelakis-Angelakis (1999) have hinted at the existence of a feedback regulation of the amounts of ADC enzyme by exogenous Put in *Vitis vinifera*. The data presented here on transgenic *Populus* cells, and also the results published earlier from our laboratory with transgenic tobacco (DeScenzo and Minocha, 1993) and carrot cells (Andersen et al., 1998), clearly show that at least in these species there is no evidence of a feedback regulation of ADC either by Put or by total polyamine levels in the cells. The transgenic cells exhibit as much (or more) ADC activity as the NT cells even though the former contain several-fold higher amounts of Put. This lack of feedback inhibition is evident both at transcriptional and post transcriptional levels. Although the subcellular location of ADC in poplar cells is not known, it is conceivable that cellular ADC may be compartmentalized away from the increased Put produced by mouse ODC, which is presumably present in the cytoplasm.

What is the Source of Ornithine?

Ornithine biosynthesis in plants occurs largely from Gln/Glu using several enzymes (Fig. 2; also see Davis, 1986; Ireland, 1997). Alternatively, Orn can be produced from Arg by arginase as a part of the urea cycle (Fig. 2, step 12). Assuming that Orn levels in transgenic cells were limiting (see argument above), and the urea cycle pathway was an important source of Orn in the transgenic cells, one would expect a depletion of Arg in these cells. This would, in turn make it a limiting factor for Put production via ADC also. Consequently, exogenous supply of Arg should promote both the ADC-produced Put, and the amount of Orn available to ODC, resulting in an increase in Put levels in both the NT and the transgenic cells. However, exogenous Arg supplied to transgenic cells did not cause increased Put production, nor was the conversion of [^{14}C] Arg into Put altered in the transgenic cells (see also Andersen et al., 1998 for similar results with carrot cells). Therefore, it can be argued that most of the Orn used for Put production in poplar

cells comes from Glu and not from Arg. This explanation is consistent with an overall low activity of arginase and its mitochondrial location in plant cells (Jenkinson et al., 1996). The above conclusion is further supported by the results of MSX treatment, which inhibits NH_3 assimilation into Gln and Glu (Leason et al., 1982; Florencio and Vega, 1983), thus limiting the amounts of Glu available for Orn production. The effects of MSX were partially reversed by the addition of exogenous Orn but not Arg.

The apparent lack of an effect of exogenous Gln on polyamines in the transgenic cells leads us to postulate that the production of Gln/Glu from NO_3^- and NH_4^+ in the medium is keeping pace with its increased utilization for Orn production, and that N in the medium is not a limiting factor for this pathway. This argument is further supported by the results from urea addition to the medium, which also had no effect on polyamine levels in either the NT or the transgenic cells. It can thus be concluded that as long as a source of inorganic N is available to the cells, its conversion into Gln/Glu and, subsequently into Orn, is not a limiting factor for polyamine biosynthesis. In other words, the primary regulation of Put biosynthesis is achieved by ODC or ADC activities and not by substrate levels in the cells.

Both Put and Pro accumulate in plants under conditions of abiotic stress (Bouchereau et al., 1999). Gabaculine is a strong inhibitor of Orn aminotransferase (OAT), an enzyme which channels Orn towards Pro biosynthesis (Davis, 1986; Ireland, 1997). If there was a competition between Pro and Put biosynthesis for utilization of Orn, less Orn may become available to ODC for Put production when Pro production is high. Therefore, the inhibition of Pro biosynthesis from Orn would be expected to increase the availability of Orn for mouse ODC. Gabaculine had no significant effect on cellular Put in either the NT or the transgenic cells (at concentrations up to 100 μM), indicating that there probably is little competition between ODC and OAT for the utilization of Orn as a substrate by these two enzymes. This argument is compatible with the conclusion stated above that the rates of Orn biosynthesis are adjusted according to its overall consumption in the polyamine biosynthetic pathway. Thus, a stimulation of Gln/Glu biosynthesis,

Orn biosynthesis and its consumption in Put production, and Pro biosynthesis must all be part of a coordinated response to stress in plants. An enhancement of this pathway may also be important for the regulation of free NH_3 in the cells, as well as for inhibition of ethylene production, since the latter utilizes the same substrate (SAM) as the higher polyamines and the two pathways presumably compete with each other. In addition, increased catabolism of Put via DAO could result in increased GABA production, thus making polyamines important players in stress response of plants in more than one way, i.e. through effects on Pro, ethylene as well as GABA production (Bouchereau et al., 1999).

Inhibitors

The ADC inhibitor, DFMA, was able to significantly lower cellular Put production in both the cell lines. This observation is quite consistent with the earlier studies from our laboratory in carrot (Robie and Minocha, 1989; Bastola and Minocha, 1994), and the data presented here showing that ADC is the predominant pathway for Put production in poplar NT cells, where its inhibition causes a reduction in Put levels. There is barely detectable activity of the poplar ODC enzyme to produce Put. In the 2E cells, the inhibition of cellular Put by DFMA can be explained in two ways: (1) since ADC activity was found to be comparable between the two cell lines, this pathway is also operational in these cells and is not feedback regulated, (2) some DFMA may be getting converted to DFMO thus inhibiting mouse ODC activity, and causing Put levels to drop. DFMA can be converted to DFMO by arginase as shown by Slocum et al. (1988). So, the combined effect of DFMA and DFMO may be causing lower Put production in cell line 2E. There was no effect of DFMO on cellular Put levels in the NT cells confirming that the inhibitor is highly specific for mouse ODC. The effect of DFMO on all the three polyamines in the 2E cells at 72 h was concentration dependent.

Gabaculine, at a higher concentration of 1 mM, at 72 h increased Put production in the 2E cells. There was no effect at 24 h, indicating, that in addition to providing higher concentration of

the inhibitor, a longer exposure time was required as well to affect the channeling of Orn away from Pro production. The observations (i.e. higher Put production) based on supplementing cultures with Orn suggested that Orn maybe limiting in these cells. A higher concentration of gabaculine may allow the channeling of more Orn into Put.

Nitrogenous Supplements to the Medium

Addition of urea at higher concentrations caused a decline in cellular Put in a concentration dependent manner. Additional N sources, such as Glu and lysine also caused a decline in Put levels in the 2E cells. Excessive N content in the medium can lead to toxicity. Lysine is utilized by LDC to produce cadaverine; the mouse ODC can also use lysine as a substrate under Orn limiting conditions to produce cadaverine (Cohen, 1998). This uncommon polyamine was also detected when the cells were treated with MSX, causing Orn levels to drop and causing NH_3 toxicity. A stimulation of Glu/Gln biosynthesis was seen in the 2E cells (as explained earlier) and when cultures were exogenously supplied with Glu (substrate for Gln, Orn, Pro and Arg and GABA), the Put levels declined in these cells. This inhibition may be indicative of the fact that the excess Glu given exogenously may be toxic to the cells or inhibitory to N assimilation. Agmatine, unlike Glu and lysine, had a promotory effect on the cellular Put levels in both the cell lines, and also, citrulline promoted Put production in the NT cells. The reason is obvious as agmatine, a metabolite of Arg via ADC, promoted Put by fueling the pathway from N-carbamoyl Put to Put. Although similar concentrations of Arg (10 mM) were highly inhibitory to the cells, the results with agmatine suggest that different mechanisms of control are operational. There is not much information available on the regulation of agmatine in plants, although Nam et al. (1997) demonstrated that ADC is very sensitive to agmatine or Put but not to Spd or Spm. In the presence of 0.5 mM agmatine (or Put), the enzyme activity was inhibited by 70%. This inhibition by Put was not seen in carrot cells (Andersen et al., 1998) nor observed in poplar cells in the present study. A recent report indicates that citrulline may contribute to oxidative stress

tolerance under drought conditions in water melon as a novel hydroxyl radical scavenger (Akashi et al., 2001). Citrulline is an intermediate in the pathway from Orn to Arg. Higher availability of citrulline probably results in increasing the amount of Arg available to the NT cells for Put production.

Recent reports show that treatment with jasmonates increased the accumulation of polyamines conjugated to hydroxy cinnamic acids (Lee et al., 1997; Biondi et al., 2000). Biondi et al. (2001) and Walters et al. (2002) reported that the gene expression and activities of ADC, ODC, SAMDC, DAO and phenyl ammonia lyase were all strongly upregulated by methyl jasmonate. Lower concentrations of jasmonic acid (JA) had no effect on Put levels in the two poplar cell lines studied here. Increase in levels of soluble Put and Spd conjugates were observed in barley seedlings on treatment with methyl jasmonate. Since, biosynthetic activities were also stimulated, conjugation may help to regulate the size of the free amine pool (Biondi et al., 2001). The reason of JA inhibition of free cellular polyamine pools in poplar cells is not clear. The effect may be due to the difference in tissue used, i.e. cell cultures versus whole plants.

Li et al. (1992) studied the regulation of wound inducible ACC synthase gene expression in tomato fruit. The transcript increased upon wounding the fruit as well as during fruit ripening. Salicylic acid (SA), an inhibitor of wound-responsive genes in tomato, inhibited the wound-induced accumulation of the ACC synthase transcript. Also, inhibition of ACC synthase was observed by the polyamines, Spm being more effective than Put or Spd. Their data suggest that SA and polyamines may specifically regulate ethylene biosynthesis at the level of ACC synthase transcript accumulation. Our results show that the polyamines decreased on exogenous SA addition to the medium in a concentration dependent manner. Higher concentrations (5 mM) were toxic to cells as also seen by the drop in fresh weight of cells over the collection period. Once again, this may be specific to the cell cultures.

Effect of Cycloheximide on the Mouse ODC Enzyme

Mouse ODC has an unexpectedly short half-life of 12-20 min (Cohen, 1998). The protein is turned over rapidly by combining with an antizyme protein and its degradation via a 26S proteasome. Polyamines generally promote this degradation while some inhibitors of polyamine synthesis, such as, MGBG, prolong the half-life of mouse ODC (Hibasami et al., 1988). The presence of a PEST amino acid sequence in the carboxy terminus of the ODC protein has been shown to be responsible for the instability of the protein. Truncation of the 37 residues, which constitute the PEST region, resulted in a stable protein (Ghoda et al., 1992). The half-life of mouse ODC enzyme is expected to be longer in the 2E cells since the PEST coding sequences were deleted from the cDNA. The results of cycloheximide treatment to 2E cells show that the enzyme is stable atleast for several hours.

Regulation of Cellular Putrescine in Poplar Cells

The transgenic cells maintained a significantly higher threshold of Put throughout the 7 d culture period. Two important questions that can be asked of these cells are: (a) Why does Put in the NT cells plateau at a lower level than in the transgenic cells? and (b) Why do the transgenic cells even have a plateau of Put when the biosynthetic enzyme (ODC in this case) is being constitutively produced? Three possible answers for the first question are: (a) since the NT cells use only the ADC pathway for Put biosynthesis, the amount of Arg in the cells may become limiting; (b) there is a feedback inhibition of ADC by Put; and (c) an equilibrium is established between the rates of Put biosynthesis and its degradation. The results presented here show that the addition of either Orn (a precursor of Arg biosynthesis) or Arg to the medium does cause increased accumulation of Put in the NT cells, showing that these precursors may be limiting in the cells. Additional support for this explanation is provided. It is apparent that the exogenous [^{14}C] Orn is metabolized quite rapidly in poplar cells; as much as two-thirds of the label in the 2E cells appeared as [^{14}C] Put within 2 h of [^{14}C] Orn feeding. Studies also showed that there was no

significant feed-back inhibition of ADC by Put in these cells. Thus it can be argued that a steady state equilibrium between biosynthesis and degradation of Put is established, the former being largely responsible for regulating the amounts of Put present at a given time.

If the same arguments were applied to the transgenic cells possessing a heterologous ODC pathway for Put production in addition to the native ADC, it would be expected that the cellular Put content is again the net product of its biosynthesis and degradation with only small amounts being converted into Spd or secreted into the medium. It is clear that Put biosynthesis in the transgenic cells is increased several-fold due to the constitutive presence of the mouse ODC without adverse effects on the ADC pathway. Consequently, in the transgenic cells, the ODC pathway is able to continuously produce Put, in turn, causing a steady rise in its cellular concentration. However, there apparently is a mechanism to achieve an increased degradation of this diamine in these cells so that a plateau is established, albeit at higher levels, for Put.

Regardless of whether Put is synthesized in the cells from exogenously supplied Orn, or it is directly taken up as Put from outside, its loss is proportionate to its amount present in the cells, i.e. 2E cells with higher amount of Put lose it at a higher rate than NT cells with low levels of Put. The relative importance of the three components of Put loss (i.e. conversion into Spd, excretion/secretion into the medium, and catabolic breakdown) can be estimated from the data presented here. First of all the amount of Put converted into Spd constitutes only a small fraction of the total amount of Put in the cells. For radioactive Put produced either from [^{14}C] Orn or taken up from the medium as [^{14}C] Put, only 6-10% is converted into Spd in the first 8 h of feeding the label in either cell line. For [^{14}C] Put feeding experiments, this amount is similar in the two cell lines in terms of dpm g^{-1} FW but almost three-fold higher in the 2E cells compared to the NT cells in terms of nmol g^{-1} FW. The second component of Put loss, i.e. by secretion from the cells is also relatively small (less than 10% of the Put pool in the cells) but again proportionate to the amount of Put in the two cell lines. The observation that a higher proportion of radioactivity is lost to the medium from cells fed with [^{14}C] Put than those fed with [^{14}C] Orn indicates that some of the

[^{14}C] Put may be present in the apoplast at the time of transfer, and thus washed out easily. Therefore, it can be argued that most of the Put loss from the cellular pool is by catabolic breakdown into other products which appear mostly in the aqueous fraction following dansylation, and/or as $^{14}CO_2$. That is probably why the radioactivity in the aqueous fraction is higher in the 2E cells than the NT cells at all times following the transfer of cells to the label-free medium.

The first step in Put breakdown is catalyzed by DAO. The question then is: Is the catabolic mechanism (i.e. the enzyme DAO) that handles extra Put already present in the cells or is it induced by a higher threshold of this diamine in the transgenic cells? The observation that increased catabolism of Put in the 2E cells occurs without an increase in the DAO activity leads us to conclude that the former situation is probably responsible for the increased degradation of Put in the transgenic cells. In other words, when Put degradation in poplar cells is increased concomitant with the rates of its biosynthesis, this increase happens without induction of the catabolic enzyme DAO. The data presented in Fig. 54 show that the measurable amounts of DAO activity in both the NT and the transgenic cells were quite comparable (or smaller in the 2E than the NT cells) at most of the times during the 7 d culture period, except when the cells were in a stationary phase of growth, i.e. on the 6th and 7th day of culture. A possible explanation for the same amount of DAO to catalyze higher rates of Put degradation is that in the wild type cells the enzyme is either functioning at a non-saturating level (i.e. high K_m of the enzyme but low amounts of available substrate) or the enzyme is present in non-saturating amounts (i.e. excess amount of the enzyme). Thus any additional Put in the cells will be degraded at a rate proportionate to its availability up to the level of saturation.

The above explanation is consistent with the earlier reports showing that DAO may be constitutively present in quantities that are sufficient to catabolize several-fold higher amount of Put than normally found in the cells (Burtin and Michael, 1997). In a situation reminiscent of what we have observed for the relationship of Put with its degradative enzyme DAO, Forlani et

al. (2000) detected no relationship between the cellular contents of free Pro and δ^1-pyrroline-5-carboxylate (P5C) dehydrogenase, a key degradative enzyme for Pro catabolism in potato tubers. Likewise, Hua et al. (1997) observed no positive correlation between cellular Pro and P5C reductase, another key enzyme involved in Pro catabolism. The above examples illustrate that the synthesis of these enzymes is not regulated by the amounts of the respective metabolites that serve as their substrates in the cells. In other words, the catabolism of these compounds is a function of their presence at non-saturating concentrations. Based upon the above arguments, I believe that a similar situation exists for Put catabolism in poplar cells. The higher threshold of Put in the transgenic cells is thus the net result of increased biosynthesis (via ADC and ODC) and a constitutively high rate of degradation, the former being the regulatory step responsible for the overall metabolic flux of this diamine.

Effect of Reduced Nitrogen Content in the Medium

The data presented here highlight two important points: (1) Total N content of the MS medium is not limiting for optimal production of Put, Spd and Spm in both the NT and the 2E cells, and (2) removal of NH_4NO_3 or total N from the medium causes a rapid reduction in cellular content of Put. On complete removal of N from the medium, a decrease in the cellular content of Put could be seen as early as 12 h in the NT cells. By 24 h, these cells had lost more than 60% of their Put and by 48 h, the decrease was greater than 80%. The results indicate that continued supply of N is required to sustain high levels of Put in these cells. In the absence of this, the Put levels decrease as a result of continued catabolism and reduced production. On the contrary, the loss of Put content from the 2E cells was not seen until 24 h, and the effect was much smaller than the NT cells even at 48 and 93 h after complete removal of N from the medium. As discussed earlier, rate of catabolism of Put is directly proportional to its rate of biosynthesis in these cells. Thus a reduction in the supply of N probably causes a reduction in Put biosynthesis, which in turn causes a reduction in its catabolism, leading to a homeostatic maintenance of higher

threshold of Put in the 2E cells. Concomitant with the loss of Put, there was a decrease in cellular Spd as well as Spm in both the cell lines when N was completely removed from the medium, the effect increasing with time. It is notable, however, that the loss of Spd was much slower than that of Put. This observation is consistent with the longer calculated half-life of Spd as compared with Put. The observation that the removal of NH_4NO_3 alone from the medium (keeping normal concentration of KNO_3) caused a more pronounced loss of Put in the 2E cells is consistent with the preferential utilization of NH_4 by plant cells in general (Forde and Clarkson, 1999).

The activity of GS was however found to be lower on all days tested in the 2E cells than the NT cells. Glutamine synthetase should be constitutively expressed in the poplar cells due to the vital role of being the first step in primary ammonia assimilation. Lower enzyme activity is difficult to explain in light of the expected higher rates of N assimilation in these cells. Labeled Glu had significantly higher $^{14}CO_2$ release from 2E cells at all time periods tested as compared to NT and NT-HP cells indicating a faster metabolism of Glu into all the downstream reactions in this cell line. The counts in the aqueous fraction were relatively lower in the 2E cells mainly because of the rapid utilization of labeled Glu. A stimulation of the Gln/Glu biosynthesis to support increased Orn biosynthesis is seen in the 2E cells (data supported by experiments involving the addition of N sources). The label in the toluene fraction being the highest in 2E amongst the three cell lines tested can be explained by the fact that mouse *odc* gene is able to convert available radioactive Orn (made from labeled Glu) to Put.

Existence of One or Two Pools of Putrescine

Since plant cells often possess and utilize both ADC and ODC pathways for Put biosynthesis, the relative contribution of the two pathways to cellular Put pools has been variously debated (Cohen, 1998). It has been suggested that Put produced by the two pathways may exist in separate pools and/or may be involved in different physiological functions. For example, ADC may play a greater role in stress-induced Put production while ODC may be

important for cell division and development. While no direct evidence for the existence of multiple pools of Put has been forthcoming, the idea has persisted for many years. Transgenic approach using cells in which a single endogenous pathway for Put biosynthesis is supplemented by an alternate pathway, and in which the metabolism of [^{14}C] Put produced endogenously (from [^{14}C] Orn), versus that given exogenously (as [^{14}C] Put) can be studied, provides an excellent opportunity to test these ideas.

The observation that the loss of labeled Put in the NT cells using two different sources of label (i.e. [^{14}C] Orn or [^{14}C] Put) follows similar kinetics (i.e. similar L_{50}) indicates that there probably is only a single pool of Put in these cells. The reasoning for this argument is as follows: If exogenously supplied Put was degraded differently from that produced endogenously, its degradation rate would be either higher (if it was preferentially degraded) or lower (if it was sequestered into a separate compartment). This, however, will not be the case if either there was only one pool of Put in the cells, or both pools were equally accessible to the catabolic enzyme(s). Since a significant proportion of DAO is believed to be wall-bound (Angelini et al., 1993; Møller and McPherson, 1998; Wisniewski et al., 2000; also Fig. 53A), one would expect that the exogenous Put will be catabolized faster than the intracellular Put. The results presented here however show that the profiles of [^{14}C] Put loss are similar in the two cell lines and the half-life (L_{50}) of Put remains unchanged, independent of the source of Put, indicating an equal access of the endogenous as well as exogenous Put to the catabolic pathway.

The results of this set of experiments also lead us to postulate that in these cells DAO may not be all cell-wall bound. If this were the case, then most of the degradation product of Put that is Δ^1-pyrroline would have to be reabsorbed into the cell for further degradation. Otherwise, it will be lost into the growth medium. Only a small fraction of radioactivity was being released into the medium. Direct measurement of DAO in the buffer soluble versus pelleted material from the cells shows that about 63% of the DAO was not extractable. However, no distinction was

made in this experiment between wall-bound or other pelleted organelles/membranes for retention of DAO activity.

The approach of using two different means of labeling cellular Put provides further insight into the metabolism of this diamine with regard to its turnover rates. Using a combination of these two approaches, the half life (L_{50}) of Put loss in both the NT and the 2E poplar cells was determined to be about 5-6 h, although the calculated L_{50} of Put in the 2E cells using [^{14}C] Orn as the precursor was found to be somewhat longer than that calculated from experiments using [^{14}C] Put (Fig. 45B). The apparent discrepancy is due to an underestimate of the real loss of this diamine in the 2E cells. The rationale is that during the first few hours after transfer of cells from [^{14}C] Orn medium to label-free medium, the cells still contain a relatively large amount of [^{14}C] Orn (Fig. 46C) that can be converted into [^{14}C] Put by the mouse ODC in the 2E cells, thus replenishing some of the radioactive Put lost by the cells. In [^{14}C] Put feeding experiments, no such replenishment of the label in Put fraction can occur. Since the calculations of L_{50} are based only upon the loss of radioactivity from the Put fraction at different times during the first 8 h period, the 2E cells add more radioactivity to the Put pool from [^{14}C] Orn than the NT cells. This is the first report on direct estimate of the rates of turnover and half-life of Put in plants.

Importance of Putrescine Degradation

From the published literature (Smith 1985; Cohen 1998; Bagni and Tassoni, 2001) it is clear that the catabolic degradation of Put by DAO is not simply a means to regulate the cellular content of this diamine. Putrescine catabolism provides intermediates that play important roles in the growth and development of plants as well as in the response of plants to various forms of abiotic stress. For example, GABA has been implicated to play a key role in signal transduction pathways during stress response of many plants (Ramputh and Bown, 1996; Bown and Shelp, 1997; Penel, 1997; Shelp et al., 1999) and also in the development of roots (Hausman et al., 1997a,b). Likewise, Put catabolites serve as precursors of important alkaloids in several plants

(Cohen, 1998; Hartmann, 1999). The degradation of Put also provides a metabolically crucial link between the polyamines and the TCA cycle, resulting in recycling of N as well as the carbon skeleton of Put. In addition, Put catabolism results in H_2O_2 production which is a substrate for peroxidation of lignin precursors in the cell wall (Federico and Angelini, 1988; Angelini et al., 1993). A specific role for DAO in cross-linking of amines to proteins as an alternative pathway to transglutaminases has also been suggested (Chiarello et al., 1996).

Whether or not increased catabolism of Put in the transgenic poplar cells has a physiological function beyond the removal of excess Put has not been investigated yet. Recycling of the carbon skeleton of polyamines would certainly allow the continued production of Glu, whose consumption is increased for Orn production to keep pace with its excessive utilization by the transgenic ODC. This would then create a futile cycle of Put biosynthesis and degradation in response to the presence of transgenic ODC and prevent the deleterious effects of excessive utilization of Orn and Glu, both of which are key metabolites serving as precursors of Arg, Pro and several other amino acids. If a similar enhanced flux cycle was to function under conditions of abiotic stresses that cause overproduction of Put, one of its roles would be to continuously recycle the NH_3 often produced under stress-induced physiological reactions, thus minimizing its toxicity (Kronzucker et al., 2001).

Nanjo et al. (1999) have discussed the existence of such futile cycles that limit the accumulation of a product in genetically engineered cells overexpressing a biosynthetic enzyme gene. For example, the biosynthesis of Pro from Glu by the enzyme P5C synthetase and its reconversion into Glu by a two-step process involving Pro dehydrogenase and P5C dehydrogenase (Verbruggen et al., 1996) constitutes a similar futile cycle that prevents over-accumulation of Pro in transgenic cells overexpressing a P5C synthetase gene. Under normal conditions, the cycle of Pro degradation is prevented by subcellular compartmentation of the catabolic enzymes in the mitochondria (Brandriss and Magasanik, 1981; Hare and Cress, 1997). Under hyper-osmotic stress conditions, the production of Pro dehydrogenase is inhibited, thus

allowing the maintenance of higher homeostatic levels of Pro in the cells. The importance of similar futile cycles in the regulation of sucrose metabolism, and sucrose loading and unloading in plants has been discussed by Nguyen-Quoc and Foyer (2001).

Spermidine and Spermine Metabolism in the Transgenic Cells

Spermidine plays a variety of important roles in cellular metabolism and function in all organisms (Davis et al., 1992; Ober et al., 2000; Graser and Hartmann, 2000). *E.coli* mutants lacking Spd synthase and SAMDC produced Put but no Spd and Spm, and showed a small decrease in growth rate but were markedly sensitive to paraquat (Minton et al., 1990). Yeast mutants that were unable to produce Spd and Spm due to a deletion in the SAMDC gene lost the ability to grow under anaerobic conditions (Balasundaram et al., 1991). These studies in microorganisms demonstrate the importance of these higher polyamines under stress conditions. Studies from our laboratory (Quan et al., 2002) with the same cell lines as used here (i.e. NT and 2E) have demonstrated that there is little or no competition for the substrate SAM between the ethylene and the polyamine pathways, thereby, indicating ample availability of SAM to produce dcSAM. In the present study, Spd levels were generally higher, though not significantly different, in the 2E cells from the NT cells. The reasons for the limited increases in Spd levels (even though Put is not limiting) in the 2E cells could then be that (1) the enzyme Spd synthase is limiting, or (2) there is an increased catabolism (turnover) of Spd in these cells concomitant with its increased production. In order to determine the effect of Put overproduction on the metabolism of higher polyamines, the Spd turnover was studied in the two cell lines. It is known that there is a tight regulation of Spd and Spm biosynthesis and that Spd/Spm levels are regulated by complex regulatory mechanisms. It is also known from the present study that the production of Spd and Spm in the 2E cells is several fold higher than the NT cells.

One type of polyamine catabolism, called the interconversion pathway, is seen in animals where polyamines are degraded by N^1-acetylation and oxidative splitting of the acetyl derivatives

(Fig. 4). The other route is said to be terminal type of polyamine catabolism, as the compounds formed cannot be reconverted into polyamines or each other. This is because the terminal amino groups of polyamines are removed (Seiler et al., 1985). The former mode of degradation is common in animals as shown by many reports based upon the administration of labeled Put, Spd or Spm resulting in the accumulation of label in the two other amines. However, this has not been studied in detail in prokaryotes, fungi, yeast and plants. Cellular acetylpolyamine concentrations in animals are often quite low and it seems acetylpolyamines only serve the role of intermediates in polyamine interconversions. It seems obvious that in the interconversion type of polyamine catabolism, Spd and Put formed from Spm and Spd, respectively, could be reutilized as a source for polyamines in the cells. The presence of 1-N-acetylSpd was reported in maize seedlings by deAgazio et al. (1996) but this compound was not involved in the Spd to Put interconversion pathway, and exogenous Spd could not act as a substrate for the acetylation process. However, Spd to Put interconversion has been suggested to occur in tobacco thin layer explants (Torrigiani et al., 1993) and chloroplasts of *Helianthus tuberosus* (Del Duca et al., 1995). On addition of exogenous Spd to maize seedlings, endogenous Put content contained 44% of the total [^{14}C] detected in the dansylated fraction (deAgazio et al., 1996). The results presented here with [^{14}C] Spd incubation show that the endogenous [^{14}C] Put content increased with time (0-72 h) from 3% to 20% of that observed in the toluene fraction, while Spm accumulating to only 8% in the NT and 5% in the 2E cells. The percentage of interconversions was higher at later time periods of the study as compared to earlier, e.g. Put accumulating up to 20% with [^{14}C] Spd as the substrate at 72 h, and the Spd content accumulating to 32% in both the cell lines when [^{14}C] Spm was used as a substrate. The low accumulation of these polyamines at earlier time periods may be basically due to the rapid turnover of Put (L_{50} of 5-6 h in both cell lines) and Spd (20-35 h in the two cell lines). The biochemical steps of this interconversion, however, remain unknown. The conversion of Spd to Spm being four fold (at 8 h) higher in these cells as compared to the conversion of Spd into Put indicates that although there is some back conversion, it is not a significant source of Put

in these cells. Thus the primary source of Put in the cells is via its biosynthesis from Orn/ Arg. In a recent report, in Spd treated *Arabidopsis*, it was seen that in most of the plant organs, exogenously supplied Spd was converted into Put (Tassoni et al., 2000). This report suggests the presence of an interconversion pathway presumably mediated by PAO.

In addition to observing some conversion into Put, the amount of [^{14}C] Spd lost was nearly two fold higher from the NT cells than the 2E cells. This was consistent with the NT cells having a faster turnover rate of Spd (i.e. shorter L_{50} of 21 h) than the 2E cells (L_{50} of 32 h). The apparently longer L_{50} of Spd in the 2E cells as measured by [^{14}C] Spd loss may be related to the observation of several fold higher rates of Spd biosynthesis in these cells. This will dilute (lower the specific activity) [^{14}C] Spd in these cells thus causing lower apparent loss of [^{14}C] Spd. The uptake of [^{14}C] Spd was found to be higher in the NT cells, indicating that that the mechanism for Spd uptake is different from that of Put (in that cellular Put levels affect Spd uptake). The aqueous fraction did not contain significant radioactivity when [^{14}C] Spd was used as a substrate in either of the two cell lines, suggesting that Spd is not a significant source of charged aqueous derivatives, such as amino acids etc.

Unlike Put and Spd, it was difficult to determine the half-life of Spm due to the increase in the radioactivity accumulating in the dansylated fraction at 24 h in the 2E cells. The results are perplexing, and not easy to explain although repeatable. But, the data presented here from radioactive Spm experiments do show the changes in [^{14}C] polyamine content with time. The trend seen in [^{14}C] Spm uptake was similar to [^{14}C] Orn for studying Put turnover (i.e. both having higher label accumulated in the 2E cells at all time periods) and, unlike Spd. Bagni and Pistocchi (1991) showed that for Spd uptake, there was a saturable component as well as a linear one while Spm had the same pattern for uptake like Put. Since Spm constitutes less than 5% of the polyamine content in the cells under normal conditions, it is not a significant source of either Spd or Put in the cells. Also, the homeostatic regulatory mechanisms for Spm are still unknown.

As with Spd, the conversion of [^{14}C] Spm into [^{14}C] Put as well as [^{14}C] Spd was observed, the products accumulating with time. This conversion was, however, a small proportion of the [^{14}C] Spm taken up by the cells. In animals, it is not known as yet if Spd and Spm synthases compete for the same pool of dcSAM or whether separate pools exist. Alfalfa Spd synthase was highly specific for Put as an initial substrate but an extended enzymatic reaction produced Spm and several other uncommon polyamines besides Spd (Bagga et al., 1997). These authors suggest the existence of an enzyme complex with multiple functions to explain the formation of other minor polyamines.

Plant PAOs show restricted substrate specificity, oxidizing Spd and Spm equally well in most cases. Plant PAOs are localized in the apoplast and are believed to be the source of H_2O_2 needed in lignin biosynthesis in the cell wall (Šebela et al., 2001). Diaminopropane, a catabolic product of PAO is also a metabolic precursor for uncommon polyamines, such as, caldine, thermine and caldopentamine (Kuehn et al., 1990). Diaminopropane is also found to be important in retarding senescence by inhibiting the rise of protease activity and the loss of chlorophyll (Tiburcio et al., 1994; Bouchereau et al., 1999). The terminal fate of these catabolic products is not known i.e. (1) whether the catabolic compounds are broken down further and become part of vital metabolic cycles, or (2) are sequestered and released under certain conditions, or (3) are excreted from the cells. The situation may be different in whole plant versus cell culture conditions.

Reports from animal studies suggest that proportional increases in ODC and SSAT are a means for increasing the turnover rate of polyamines without changing the equilibrium concentrations. The Spd turnover rates are directly correlated with the equilibrium concentrations of Put. In the mouse brain, it was observed that inhibition of ODC by DFMO caused a decrease in Put concentration, which was followed by a concomitant decrease in Spd turnover rate (Seiler and Bolkenius, 1985). Many reports suggest that SSAT activity is induced when cells need to get rid of excess polyamines where excess could mean polyamines that are present in amounts above

what is physiologically needed. Induction of SSAT and ODC will enhance the formation of Put and in turn activate the formation of Spd and Spm. The net result being that the polyamine turnover will be enhanced. To achieve a decrease in cellular polyamine concentrations, there has to be an elimination of polyamines via transport as well as catabolism into terminal products. Thus it can be expected that elevated SSAT activity is coupled with polyamine transport whereas uptake is linked with ODC activity and cell growth (Seiler, 1996). The basal polyamine turnover is ensured at very low ODC activity in animals, as Spd is continuously converted back to Put which can be reutilized for Spd formation. High ODC activity ensures activation of SAMDC in these systems, which lead to a high Spd/Spm amounts.

The determination of turnover rates will shed light on the regulatory mechanisms that exist in normal and transformed plant systems. This will ultimately help in understanding the elusive role of polyamines in plants under various growth and stress conditions.

Relevance of Cell Culture Studies to Whole Organisms

The poplar cell culture system used in the present study while serving as an excellent experimental model for metabolic studies, lacks the natural features of transport in the whole plant. Poplar cell lines are comprised of non regenerating, filamentous suspensions with cells that lack chloroplasts (Fig. 8). The cells themselves act as the sites of all biosynthetic as well as catabolic reactions. While these cells can (and do) excrete Put into the medium, they do so only to a limited extent (less than 10% of the pool) and lack the features of intercellular transport. How much role does the intercellular transport of polyamines play in the homeostatic regulation of their cellular levels in plants has not yet been studied. Based upon the limited knowledge about cellular content of polyamines in transportation fluids (xylem and phloem saps - Friedman et al., 1986) in plants, only a minor role for polyamine transport in this process has been envisioned. Since leaves and roots of plants do possess polyamine biosynthetic as well as catabolic enzymes, it can be argued that cellular levels of polyamines in these organs are a function of local reactions

based upon the availability of precursors and the enzymes. The cell line being used in the present study is not capable of regeneration into whole plants, therefore the 2E cells can not be used to address the question of long distance transport. Parallel work on transformation of poplar cells capable of regeneration into whole plants is currently underway in our laboratory.

The results obtained so far with poplar cells are quite consistent with the published work on transgenic manipulation of polyamines in animals, and advance our knowledge about the metabolism of polyamines in plants. In the testis of transgenic mice overexpressing a human *odc* gene, Halmekytö et al. (1991) observed a significant increase in not only the activity of ODC but also the activities of SAMDC, Spd synthase and Spm synthase. The catabolic enzymes Spd/Spm acetyltransferase and PAO were not affected. Other tissues (spleen, heart, kidney and liver) only showed an increase in ODC and did not produce extra Spd and Spm (Halmekytö et al., 1993). Only testis and brain showed an increase in Put and Spd levels, the former increasing several-fold and the latter only slightly. A noteworthy observation in animal studies was that excess Put was readily secreted into the blood stream by the transgenic tissues except the brain and the testis. In this regard, the poplar cells differ in that the amount of Put secreted into the medium is relatively small.

Expression of *adc*, *Spd synthase*, *mouse odc* and *gad* in Poplar Cells

Production of functional proteins from a transgene in the cell includes two classes of active participants: those that are contained within the transgene sequence and those that are contributed by the host cells (Minocha, 2000). In transgene expression studies, cDNAs have been preferred over genomic DNA because they lack introns, and complications of intron removal and exon splicing to put together a functional mRNA are avoided (Schuler, 1998; Guo and Sherman, 1996). The type of the mouse *odc* cDNA used in this study has been discussed earlier. As mentioned previously, the transgene in this system is under the control of a 2X 35S CaMV promoter and being, a constitutive promoter it is presumed to be active at all times. However,

constitutive promoters may show unregulated variable expression in different stages in different cell types and at different times (Williamson et al., 1989; Lemmetyinen et al., 1998). Similar observation was made in this study, where amounts of the mouse *odc* mRNA over 1, 3, 4 and 6 d of culture cycle showed marked changes in the 2E cell line. The changes in the mRNA levels, broadly speaking, may be attributed to the physiological status of the cells as well as the age of cells in culture. A few possible causes for the differences in expression are discussed. It is unlikely that the foreign gene is feedback inhibited by Put at the transcriptional level since it is not under the control of developmentally or chemically regulated promoter. It is known that the native gene in the mammalian cells is under transcriptional and post transcriptional regulation. The mouse ODC enzyme is fairly stable in this cell line (as discussed earlier, i.e., because of the absence of the PEST sequences). It is known, however, that selective degradation of mRNA is often used to regulate cellular polypeptide levels by the cells and the presence of ODC protein and high Put in this case could be regulating the mRNA levels. The absence of an RNA signal when probed with mouse *odc* at 6 d of culture may thus be related to mRNA instability in response to high Put at that time period. However, in subsequent experiments with RNA isolated from another week, the RNA signal at 6 d was similar to that observed on other days tested when probed with mouse *odc*. Alternatively, the mRNA may be degraded in relation to reduced cell divisions in 6 d old cultures. Unlike the differences in mRNA levels, the Put levels on 6 d were similar to those observed on 3 d and 4 d of the cell culture cycle (see Fig. 13D). In animal cells also, changes in ODC activity *in vivo* in response to polyamine depletion or repletion were not necessarily accompanied by changes in mRNA levels (Persson et al., 1986; Kahana and Nathans, 1985). The transcription initiation depends on the presence of trans-acting factors as well as the accessibility of the cDNA sequence (Zhou, 1999). As a result, there could be a competiton between the native and the foreign gene in the animal cells. However, in the present case, there cannot be a competition between the transgene message and the native message for the factors involved. This statement is based on the observation that there is no detectable enzyme activity of

ODC in these cells. The presence of a poplar *odc* transcript, and if present, changes associated with poplar *odc* expression levels have not been studied.

The levels of *adc* expression were comparable between the two cell lines and the changes over the entire subculture cycle were not very significant. The results are consistent with the observed activity of ADC that was also found to be comparable or slightly higher in the 2E cells than the NT cells. Rastogi et al. (1993) suggest that changes in *adc* mRNA alone cannot account for the differences in ADC activity in tomato and that other regulatory mechanisms must be operating. On the contrary, Nam et al. (1997) suggest that changes in the content of *adc* mRNA have an important role in the regulation of ADC enzyme activity during early development, tissue-specific activity and acid stress in soybean. Expression of ADC in pea is high in young developing tissues but lower in fully expanded leaflets and roots, while it is developmentally regulated in the ovary and fruit (Perez-Amador et al., 1995). It is clear that in poplar, increased Put production and accumulation do not appear to cause feedback inhibition of the ADC pathway in the 2E cells either at the level of the *adc* transcript or ADC enzyme activity.

The expression of *Spd synthase* was higher at 3 d and 6 d of the culture as compared to 1 and 4 d. The enzyme is responsible for the conversion of Put to Spd in a single step reaction. As discussed earlier, the rate of Put biosynthesis as well as the amount of Put converted to Spd was higher in the 2E cells. The expression of the gene being higher in the 2E cells seems consistent with this observation. No information is currently available on the regulation of Spd synthase expression by Put in plants. Changes in the profile of Spd synthase enzyme activity in the 2E cells may help us understand what type of regulation of this enzyme is operational. No changes in gene expression on certain days, such as, 1 and 4 d could also be related to the physiological status of the cells, i.e. cells just transferred to fresh medium (1 d) and in log phase of growth (4 d). There were two additional distinct transcripts seen in the *Spd synthase* northern blots hybridized with *Spd synthase* probe, one of a smaller and the other of a larger size. This band could be the result of cross reactivity of the probe with a Spm synthase that produces Spm from Spd or an alternative

splicing of the message could be responsible for different sized transcripts of *Spd synthase* itself Sequencing of additional bands should clarify this situation.

Glutamate decarboxylase catalyzes the reaction from Glu to GABA. The *gad* gene expression was higher in the NT cell line than the 2E cells at all time periods, except, day 1. The levels dropped drmatically in the 2E cell line after day 1 whereas it increased up to 4 d and then declined in the NT cells. Akama et al. (2001) observed that there were two *gad* genes (*gad*1 and *gad*2 (two different isoforms of GAD) and the two genes were differentially expressed in rice roots and maturing seeds. In my study, it is not clear as to which *gad* gene is being expressed in the poplar cells. The GAD enzyme activity was higher on all seven days of the week in 2E cells (Michelle Serapiglia, personal communication), the activity reaching its maximum on 2 d in the 2E cells and 3 d in the NT. These time periods are in the log phase of growth of cells. Thus, lower RNA levels for *gad* in the 2E cells are in contrast to higher or equal GAD activity in these cells. If anything, one would have expected lower GAD activity in the 2E cells because increased turnover of Put could result in increased GABA production in these cells. A high degree of stability of the DAO enzyme, or increased turnover of *gad* mRNA in the presence of high Put, or high turnover rates of GABA resulting in no additional accumulation of GABA could explain the observed results.

Genetic Manipulation of a Metabolic Pathway - Implications of Modulating One Step

The present study clearly illustrates the need for a critical examination of the metabolic effects of genetically manipulating a metabolite that: (a) accumulates in large quantities, (b) is not an end product but is a substrate for other enzymes that produce physiologically important metabolites, and (c) is actively secreted as well as degraded in the cells (see also, Kinney, 1998; Della Penna, 2001). This is in contrast to the transgenic expression of genes whose products are novel proteins that do not have a metabolic function, e.g. Bt protein or virus coat proteins. The Put biosynthetic pathway represents an excellent example of a model pathway for analysis of its

regulation through genetic manipulation. The results obtained thus far using this approach show that genetic manipulation of a single step in the polyamine biosynthetic pathway has pleiotropic effects on both the downstream as well as the upstream reactions in which the substrate of the transgene is used. Elevated levels of Put in the cells are not only accompanied by increased biosynthesis of Orn, the substrate, but also by an increase in subsequent biosynthetic (Spd biosynthesis) and degradative reactions. It is notable that the introduction of the transgenic ODC pathway does not adversely affect the native pathway for Put biosynthesis, i.e. via ADC. While it is not known at present as to how an increase in Spd biosynthesis is brought about (i.e. is there a corresponding induction of SAMDC and Spd synthase?), the degradation of Put occurs by a constitutive mechanism without involving increased production of the diamine oxidase. It is envisioned that there most likely are further downstream effects on Spd and Spm metabolism that contribute to the maintenance of their homeostasis in the cells. It is also expected that there will be additional effects on pathways that interact with the polyamine biosynthetic pathway, e.g., excessive utilization of Glu could affect the biosynthesis of other amino acids as well as the assimilation of NH_3 by the cells. These aspects of the metabolic effect of Put overproduction are currently being investigated.

Limitations of Transgenic Manipulation

One of the inherent limitations of enhancing a metabolic step through transgenic expression of the enzyme involved is that, while it may lead to an expected change in the product it may be insufficient to modulate the entire pathway. This can be attributed to the complex regulatory mechanisms involved, and that the control provided by an enzyme of the pathway can vary under different developmental and environmental conditions. While the nature of the problem persists, it has not been a stumbling block for research involving transgenic manipulation of plant cells. This is largely because while new research has strengthened the findings of existing reports, it has also helped reveal novel regulatory mechanisms.

CONCLUSIONS

Polyamine metabolism has been studied by using inhibitors to block a specific step in the pathway or by the use of mutants. More recently used, the genetic manipulation of a pathway provides the opportunity to study the effects of not only the suppression of a particular step but also overproduction of a compound in the cells and whole plants. The present study was aimed at analyzing the effects of genetically manipulated polyamine metabolism on the polyamine and related pathways in transformed poplar 2E cells overexpressing a mouse *odc* cDNA under the control of 2X 35S CaMV promoter.

The following conclusions can be drawn from the data presented here:

1. Transgenic expression of a heterologous *odc* gene can be used to modulate polyamine metabolism in plant cells. Poplar 2E cells have several fold higher putrescine content than NT cells due to the overexpression of mouse *odc* gene.
2. Overproduction of putrescine and its accumulation in high amounts does not affect the native ADC activity and its contribution to putrescine production.
3. The source of ornithine in poplar cells is primarily by biosynthesis from glutamine/glutamate and not from catabolic breakdown of arginine.
4. Ornithine biosynthesis may become a limiting factor for putrescine production in the *odc* transgenic cells.
5. There is no effect of inhibiting proline biosynthesis on ornithine availability to ODC or arginine availability to ADC in these cells.
6. Rate of putrescine catabolism is proportional to the rate of its biosynthesis.
7. Rate of spermidine turnover is slower in the transformed cells than the NT cells.
8. The expression of genes of enzymes involved in the polyamine (*adc* and *spd synthase*) and related pathways (*gad*) are altered as a result of putrescine production.

FUTURE PERSPECTIVES

Polyamines are present in millimolar quantities in the cells and they are considered to play an important role in plant development and stress responses. Polyamine levels have been known to change under these conditions. The present research provides a system where overproduction of Put is achieved and the effect on related pathways, such as N and ethylene have been studied. In addition, the turnover of polyamines as well as the expression levels of some genes under normal culture conditions have been determined. The expression of genes up or down regulated under developmental or stress conditions can be studied using these cell lines.

The expression levels of other genes, such as, those for enzymes involved in the N assimilation and related metabolism and the ethylene biosynthetic pathway, e.g. GS, GOGAT, arginase, DAO, ACC synthase, ACC oxidase and OAT will help reveal mechanisms of gene expression and activity under normal and high Put conditions in these cell lines. A global impact of high Put in the transgenic cells can be studied with more advance techniques of functional genomics, such as, microarrays.

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