Metabolism of Polyamines in Transgenic Cells of Carrot Expressing a Mouse Ornithine Decarboxylase cDNA

Scott E. Andersen  
University of New Hampshire - Main Campus

Dhundy R. Bastola  
University of New Hampshire - Main Campus

Subhash C. Minocha  
University of New Hampshire - Main Campus, subhash.minocha@unh.edu

Follow this and additional works at: https://scholars.unh.edu/nhaes

Part of the Biochemistry Commons

Recommended Citation  

This Article is brought to you for free and open access by the Research Institutes, Centers and Programs at University of New Hampshire Scholars' Repository. It has been accepted for inclusion in New Hampshire Agricultural Experiment Station by an authorized administrator of University of New Hampshire Scholars' Repository. For more information, please contact nicole.hentz@unh.edu.
Metabolism of Polyamines in Transgenic Cells of Carrot Expressing a Mouse Ornithine Decarboxylase cDNA

Rights
Copyright © 1998 American Society of Plant Physiologists

This article is available at University of New Hampshire Scholars' Repository: https://scholars.unh.edu/nhaes/241
Metabolism of Polyamines in Transgenic Cells of Carrot Expressing a Mouse Ornithine Decarboxylase cDNA

Scott E. Andersen, Dhundy R. Bastola, and Subhash C. Minocha*

Department of Plant Biology, University of New Hampshire, Durham, New Hampshire 03824

The metabolisms of arginine (Arg), ornithine (Orn), and putrescine were compared in a nontransgenic and a transgenic cell line of carrot (Daucus carota L.) expressing a mouse Orn decarboxylase cDNA. [14C]Arg, [14C]Orn, and [14C]putrescine were fed to cells and their rates of decarboxylation, uptake, metabolism into polyamines, and incorporation into acid-insoluble material were determined. Transgenic cells showed higher decarboxylation rates for labeled Orn than the nontransgenic cells. This was correlated positively with higher amounts of labeled putrescine production from labeled Orn. With labeled Arg, both the transgenic and the nontransgenic cells exhibited similar rates of decarboxylation and conversion into labeled putrescine. When [14C]putrescine was fed, higher rates of degradation were observed in transgenic cells as compared with the nontransgenic cells. It is concluded that (a) increased production of putrescine via the Orn decarboxylase pathway has no compensatory effects on the Arg decarboxylase pathway, and (b) higher rates of putrescine production in the transgenic cells are accompanied by higher rates of putrescine conversion into spermidine and spermine as well as the catabolism of putrescine.

Polyamine metabolism has been implicated in regulating the level and composition of the soluble nitrogen pools in plant cells (Tabor and Tabor, 1984; Smith, 1985; Altman, 1989; Evans and Malmberg, 1989; Bagini and Pistocchi, 1990; Slocum, 1991). The three commonly occurring polyamines (putrescine, spermidine, and spermine) are synthesized largely from Orn and/or Arg. The rate-limiting step in the biosynthesis of putrescine in animals and most fungi is the decarboxylation of Orn by ODC (Fig. 1). In carrot (Daucus carota L.) cell cultures and many other plants, putrescine is synthesized primarily via the decarboxylation of Arg by ADC, followed by a series of intermediate steps (Pegg, 1986; Minocha and Minocha, 1995; Kumar et al., 1997; Walden et al., 1997). In some plants both ADC and ODC may be active, with their activity being either tissue specific or developmentally regulated. Putrescine is converted to spermidine with the addition of an aminopropyl group derived from decarboxylated SAM through the activity of spermidine synthase. The addition of another aminopropyl group to spermidine by spermine synthase gives rise to spermine. In most plants the cellular content of spermine is much lower than that of spermidine or putrescine (Slocum, 1991; Minocha and Minocha, 1995).

Polyamines are catabolized mostly by diamine and polyamine oxidases (Smith, 1985). Hormones, natural inhibitors, ozone, light, and polyamines have all been shown to influence diamine and polyamine oxidase activities (Federico and Angelini, 1991). Polyamine oxidases are possibly also involved in the production of uncommon polyamines, e.g. norspermidine, norspermine, caldoxypentamine, caldohexamine, homocaldopentamine, and homocaldohexamine (Kuehn et al., 1990).

A variety of external and internal stimuli and the location of the enzymes for putrescine biosynthesis may determine which pathway is functional in different plant tissues at different developmental stages. For example, it has been proposed that ODC may be active during cell proliferation, and that ADC may be required for growth by expansion and differentiation (Pandit and Ghosh, 1988). Also, it has been shown that the ADC pathway is generally involved in putrescine biosynthesis in response to stress (Smith, 1990). Robie and Minocha (1989) demonstrated that in the early stages of carrot somatic embryo development, free putrescine was derived entirely via ADC, whereas ODC was present only during the later stages of embryo development and plant growth.

Previous studies of polyamine biosynthesis in relation to somatic embryogenesis in our laboratory have focused on the role of auxin and a potential competition between polyamine and ethylene biosynthesis for SAM, which acts as a precursor for both pathways (see Minocha and Minocha, 1995, and refs. therein). These studies have shown that (a) a high cellular content of polyamines during somatic embryogenesis correlates with the increased activities of ADC and SAMDC; (b) the ODC activity increases only when mature, green somatic embryos are produced; (c) the inhibition of ADC by DL-α-difluoromethylarginine and of SAMDC by methylglyoxal bis(guanylhydrzone) inhibits polyamine biosynthesis as well as somatic embryogenesis; and (d) with the addition of DL-α-difluoromethylornithine there is a promotion of ADC and

* Corresponding author; e-mail sminocha@christa.unh.edu; fax 1–603–862–3784.

1 This is scientific contribution no. 1959 from the New Hampshire Agricultural Experiment Station.
2 Present address: Gene Discovery and Expression, Monsanto, St. Louis, MO 63042.
3 Present address: Department of Biochemistry, School of Medicine, University of Nevada, Reno, NV 89577.
4 Corresponding author; e-mail sminocha@christa.unh.edu; fax 1–603–862–3784.

Abbreviations: ADC, Arg decarboxylase; ODC, Orn decarboxylase; PCA, perchloric acid; SAM, S-adenosylmethionine; SAMDC, S-adenosylmethionine decarboxylase.
polyamines, an inhibition of ethylene, and a promotion in the development of somatic embryos in the absence of 2,4-D. It has been suggested that increased polyamine biosynthesis may promote somatic embryogenesis via a reduction in ethylene production (Minocha and Minocha, 1995).

More recently, transgenic carrot cell lines overexpressing a mouse ODC cDNA have been produced (Bastola, 1994; Bastola and Minocha, 1995). When the transgenic cells were placed on embryogenic medium, somatic embryos formed earlier than in the control cells. This was correlated with higher levels of putrescine in the transgenic cells (Bastola and Minocha, 1995). The transgenic cells were able to grow and produce somatic embryos in the presence of Minocha and Minocha, 1995). The transgenic cells were able to grow and produce somatic embryos in the presence of dl-α-difluoromethylarginine (a strong inhibitor of ADC), showing that the mouse ODC was providing sufficient quantities of putrescine to the cells when the ADC pathway was inhibited. Exogenously supplied polyamines actually inhibited somatic embryogenesis. It is therefore believed that increased somatic embryogenesis is related to the increased rates of polyamine biosynthesis and their fast turnover, and not merely to the presence of high concentrations of polyamines in the cells (Robie and Minocha, 1989; Minocha and Minocha, 1995). This increased metabolism of polyamines, in turn, may affect the metabolism of ethylene, ammonia, or both.

The present study was aimed at (a) a detailed examination of the metabolism of Orn, Arg, and putrescine in nontransformed and in transgenic cells of carrot expressing a mouse ODC cDNA, and (b) determination of the compensatory effects of increased putrescine production via ODC on the native ADC pathway. The results show that increased production of putrescine via the mouse ODC in the transgenic cells was accompanied by an increased catabolism of putrescine. Furthermore, the ADC pathway was not significantly affected by the increased production of putrescine via mouse ODC.

Figure 1. Polyamine biosynthetic pathway in plants (Slocum, 1991).

### MATERIALS AND METHODS

#### Carrot Cell Cultures

Cell cultures of carrot (*Daucus carota* L.) were maintained in B5 medium (Gamborg et al., 1968) supplemented with 2% Suc and 2.3 μM 2,4-D. The medium was prepared from premixed powder (catalog no. G-5893, Sigma) and adjusted to a pH of 5.5 before autoclaving. The medium was distributed to 500-mL Erlenmeyer flasks at 200 mL per flask. Cultures were routinely transferred at 7-d intervals. To maintain relatively small cell clumps, the cultures were sieved every other week through a 250-μm sieve. Stock cultures were also maintained on solid media (0.8% agar) that were transferred at 4-week intervals. Whereas the stock cultures of transgenic cells were grown in media containing 300 mg/L kanamycin sulfate, the experimental media did not contain kanamycin (Bastola and Minocha, 1995). The stock cell suspensions were subcultured using a 10-mL-wide-bore Komagome pipette (Iwaki Glass Co. Ltd, Tokyo, Japan), placing 10 mL of the 7-d-old cell suspension into 200 mL of the fresh B5 medium. All liquid cultures were kept on a gyratory shaker at 155 rpm under 16 h of fluorescent light (80 ± 10 μE m⁻² s⁻¹) at 25 ± 2°C.

#### Experimental Setup

To prepare the experimental cultures, 7-d-old cell suspension was centrifuged at 500g for 1 min in a 50-mL conical centrifuge tube. The cells were washed three times by centrifugation with fresh B5 medium without 2,4-D or kanamycin, and resuspended in 100 mL of B5 medium. After 3 d the cell suspensions were centrifuged at 500g for 1 min in 50-mL conical centrifuge tubes, approximately one-half of the medium was discarded, and the cells were resuspended in the remaining medium. Suspensions from several flasks were pooled together in a round flask and dispensed with a Komagome pipette in 15-mL aliquots into 50-mL Erlenmeyer flasks. Each experimental flask contained about 3.0 g of cells in 15 mL of medium. To each flask, 100 μL of radioactive substrate (0.2 μCi) was added. To measure decarboxylation, the flask was capped with a rubber stopper holding a polypropylene well containing a 1.5- × 2-cm piece of Whatman 3MM filter paper soaked with 50 μL of Scintigast (Fisher Scientific) to adsorb 14CO₂. The flasks were incubated at 25°C and sampled at 1, 2, and 4 h for the short-term experiments and at 4, 8, and 24 h for the long-term experiments. The filter paper was removed from the well and counted for radioactivity in 10 mL of Scintiverse (Fisher Scientific) for 10 min in a liquid-scintillation counter (model LS 6000, Beckman). The radio-active precursors used in this study were [1,4-14C]putrescine (NEN; specific activity 117 mCi/mmol); l-[U-14C]Arg (Moravek, Brea, CA; specific activity 270 mCi/mmol); l-[U-14C]Orn (Amersham; specific activity 257 mCi/mmol).

#### Collection of Cell Samples

At sampling times, 5-mL aliquots of the cell suspension were removed and transferred to a vacuum-filtration unit...
fitted with Miracloth (Calbiochem) to collect the cells. The cells were washed once with 1 mL of 5 mM unlabeled substrate and twice with 4 mL each of distilled H₂O. For polyamine analysis, 1 g fresh weight of cells was added to 3 mL of ice-cold 5% PCA in a chilled 16-× 100-mm disposable polypropylene tube. The cells were frozen (−20°C) and thawed at room temperature three times to extract PCA-soluble metabolites (Minocha et al., 1994).

Dansylation of Polyamines for TLC

The procedure for dansylation and quantitation of polyamines was modified from Minocha et al. (1994) to suit TLC separation using larger quantities of tissue. The tubes containing frozen and thawed tissue in PCA were centrifuged at 500g for 5 min. From the tubes, 1 mL of PCA extract was removed and placed in a 5-mL glass Pyrex reaction tube with a screw cap lined with a Teflon septum. A 1-mL sample of 0.4 mM standard polyamine mixture (putrescine, spermidine, and spermine) was dansylated parallel with the samples. An equal amount of saturated sodium carbonate was added to the reaction tube followed by 1 mL of dansyl chloride solution (20 mg/mL in acetone). The vials were capped, vortexed for 15 s, and incubated in a 60°C water bath for 1 h. The samples were then placed in a SpeedVac (Savant Instruments, Farmingdale, NY) with a 60°C water bath for 1 h. The samples were then placed in the SpeedVac for 12 min to reduce the volume to 3 mL of ice-cold 5% PCA in a chilled 16-× 100-mm disposable polypropylene tube. The cells were frozen (−20°C) and thawed at room temperature three times to extract PCA-soluble metabolites (Minocha et al., 1994).

Acid-Insoluble Fraction

The pellet from the PCA-extracted material was washed three times with 5% PCA by centrifugation. One milliliter of 1 N NaOH was added to the tube, and the tube was incubated at 90°C for 1 h. From the tube, a 100-μL aliquot was counted for radioactivity. This fraction presumably contains the protein and cell wall-bound label from the precursor and the metabolites.

Calculations, Statistical Analysis, and Data Presentation

After each incubation period, one-third of the radioactive activity was removed with the 5-mL aliquot of the cell suspension and a new filter paper was placed in the well. Thus, the data for 14CO₂ evolution experiments are presented as a function of the time interval of incubation. On the other hand, incorporation into the polyamines was calculated over the entire length of the incubation period and the data are presented as total dpm h⁻¹ g⁻¹ fresh weight. Three replicate samples were collected at each time and each experiment was repeated at least three times. The three repeat experiments yielded similar results and data from a single representative experiment are presented here. Since the endogenous concentrations of metabolites were not measured in these experiments, the data are presented directly as dpm and pertain only to the metabolism of exogenously supplied radioactive substrate.

The data were analyzed using a Student’s t-test in the general linear model for one-way analysis of variance in Systat for Windows version 5.0 (SYSTAT Inc., Evanston, IL). In most cases, statistical comparisons were made between nontransgenic and transgenic cells only at a given time.

RESULTS

The transgenic cell line (ODC N14) used in this study was chosen because of its high mouse ODC enzyme activity and high cellular putrescine content (Bastola and Minocha, 1995). In previous studies (Bastola and Minocha, 1995), the transgenic nature of this cell line was established with respect to (a) presence of the mouse ODC gene, (b) its expression at the transcriptional and translational levels, (c) increased mouse-specific ODC activity, (d) increased putrescine production, and (e) increased somatic embryogenesis on transfer to 2,4-D-free medium. These cells were maintained under the same conditions as the nontransgenic cells except that kanamycin was added to maintain selection pressure in the stock cultures of transgenic cells, as stated in “Materials and Methods.” Kanamycin was not present in the experimental media. The transgenic cells grew in the presence of kanamycin at rates comparable to those in its absence, and there were no significant effects of kanamycin on the cellular polyamine content (data not presented here).

Polyamine Biosynthesis from Orn versus Arg

It is known that the only pathway for putrescine production in carrot cells grown in culture is via ADC (Montague et al., 1978; Robie and Minocha, 1989). These conclusions are based primarily on measurements of ADC and ODC activity in cell extracts and not on the basis of decarboxylation of labeled Arg or Orn or their incorporation into polyamines by intact cells. In a series of experiments, decarboxylation of [U-14C]Arg and [U-14C]Orn were com-
pared for the two cell lines for a period of 24 h. Consistent with the results with studies using cell extracts, the rates of decarboxylation of [U-14C]Orn were found to be severalfold higher in the transgenic cells as compared with nontransgenic cells (Fig. 2A). Significant differences were visible within the 1st h of incubation and persisted through h 24 of experimentation (data presented here only for the first 4-h period). On the other hand, the rates of decarboxylation of [14C]Arg were not significantly different for the two cell lines at any time except during the 1st h of incubation (Fig. 2B).

In both short-term and long-term experiments, the accumulation of labeled putrescine derived from [U-14C]Orn was severalfold higher in the transgenic cells than in the nontransgenic cells (Fig. 3, A and B). Labeled spermidine (Fig. 3, C and D) and spermine amounts (Fig. 3, E and F) were also significantly higher in the transgenic cells as compared with the nontransgenic cells. The amounts of label present in all three polyamines increased with time in the transgenic cells but remained low in the nontransgenic cells throughout the 24-h period. Among the three polyamines, radiolabeled putrescine was the highest at any time followed by the amounts of labeled spermidine and spermine in that order.

The amounts of labeled polyamines derived from [U-14C]Arg were not significantly different in the two cell lines at most of the times tested (Fig. 4). In the nontransgenic cells radioactivity in all three polyamines increased with time up to 4 h. Thereafter, labeled putrescine levels showed a steady decline, whereas the label in the other two polyamines remained high. In the transgenic cells, on the other hand, labeled putrescine decreased with time soon after the 1st h, whereas spermidine and spermine showed a steady accumulation of label in these fractions up to the 24 h.

The total amount of labeled polyamines derived from [U-14C]Orn was severalfold higher in the transgenic cells as compared with the nontransformed cells at all times tested (Table I). For [U-14C]Arg, on the other hand, the contents of labeled polyamines were quite comparable in the two cell lines. For both [U-14C]Orn and [U-14C]Arg, the highest amount of label was generally present in putrescine. In the transgenic cells treated with [U-14C]Orn, the amount of radioactivity in spermidine was always less than 7% of that in total polyamines before 8 h. In contrast, almost 30% of the total label in polyamines was present as spermidine in the nontransgenic cells. At 24 h of treatment with [U-14C]Arg, labeled spermidine was significantly greater in the transgenic cells as compared to the nontransgenic cells. Moreover, the fraction of labeled spermidine in the former was as high as 50% of the total label in polyamines at 24 h of incubation. The relative proportion of labeled spermine derived from [U-14C]Orn was generally higher in the nontransgenic cells than the transgenic cells. This, however, was not the case for spermine derived from [U-14C]Arg.

**Uptake of Orn and Arg**

The cellular pools of unused [14C]Orn and [14C]Arg were estimated from the aqueous fraction after dansylated poly-
Amines had been extracted into toluene. All polar metabolites and amino acids stay in this fraction while the dansyl-polyamines partition into the toluene fraction. There was no significant difference between the two cell lines in the amount of labeled Orn present in the aqueous fraction at any time during the 4-h incubation period (Fig. 5A). Moreover, this fraction remained steady during the entire incubation period. The amount of labeled Arg was comparable in the two cell lines and it also remained unchanged during the 4-h incubation period (Fig. 5B).

Incorporation of \([14C]Orn\) and \([14C]Arg\) into Acid-Insoluble Material

To determine the amount of radioactivity that was being incorporated into proteins or covalently bound to other macromolecules, the PCA-insoluble fraction of the cells was analyzed at the same time as the soluble fraction. Dansylated polyamines were separated on TLC and radioactivity was counted after elution of bands. The data for 0 to 4 h and 4 to 24 h were taken from two separate experiments. Values are means ± se of three replicates. Different lowercase letters indicate that the values are significantly different \((P \leq 0.05)\) from each other at a given time. FW, Fresh weight.

### Table 1. Relative amounts of radiolabeled polyamines collected up to 24 h in transgenic (N14) and nontransgenic (NT) carrot cells when \([U-14C]Orn\) or \([U-14C]Arg\) were used as precursors

<table>
<thead>
<tr>
<th>Time</th>
<th>Putrescine</th>
<th>Spermidine</th>
<th>Spermine</th>
<th>Total Label in Polyamines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NT</td>
<td>N14</td>
<td>NT</td>
<td>N14</td>
</tr>
<tr>
<td>h</td>
<td>%</td>
<td>dpm/g fresh wt</td>
<td>%</td>
<td>dpm/g fresh wt</td>
</tr>
<tr>
<td>1</td>
<td>29</td>
<td>91</td>
<td>27</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>46</td>
<td>91</td>
<td>29</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>89</td>
<td>16</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>76</td>
<td>94</td>
<td>17</td>
<td>4</td>
</tr>
<tr>
<td>8</td>
<td>70</td>
<td>90</td>
<td>20</td>
<td>7</td>
</tr>
<tr>
<td>24</td>
<td>64</td>
<td>65</td>
<td>27</td>
<td>9</td>
</tr>
<tr>
<td>Arg</td>
<td>1</td>
<td>90</td>
<td>90</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>90</td>
<td>80</td>
<td>7</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>82</td>
<td>81</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>82</td>
<td>82</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>8</td>
<td>72</td>
<td>72</td>
<td>23</td>
<td>16</td>
</tr>
<tr>
<td>24</td>
<td>54</td>
<td>37</td>
<td>35</td>
<td>50</td>
</tr>
</tbody>
</table>

### Figure 4. The incorporation of \([U-14C]Arg\) into labeled putrescine (A and B), spermidine (C and D), and spermine (E and F) in nontransgenic (NT) and transgenic (ODC-N14) carrot cells during a 24-h incubation period. Dansylated polyamines were separated on TLC and radioactivity was counted after elution of bands. The data for 0 to 4 h and 4 to 24 h were taken from two separate experiments. Values are means ± se of three replicates. Different lowercase letters indicate that the values are significantly different \((P \leq 0.05)\) from each other at a given time. FW, Fresh weight.

### Figure 5. The amounts of label from \([U-14C]Orn\) (A) and \([U-14C]Arg\) (B) present in the aqueous fraction (remaining after the removal of dansyl-polyamines by toluene) in transgenic (NT) and nontransgenic (NT) carrot cells during a 4-h incubation period. Values are means ± se of three replicates. Different lowercase letters indicate that the values are significantly different \((P \leq 0.05)\) from each other at a given time. FW, Fresh weight.
Metabolism of [1,4-14C]Putrescine

The experimental design for this part of the study was similar to that employed for Arg and Orn metabolism. The rates of 14CO2 production, amount of label present in the aqueous fraction, incorporation of label into acid-insoluble material, and the conversion of putrescine into spermidine and spermine were compared between the two cell lines over a period of 24 h. The rates of 14CO2 produced from [1,4-14C]putrescine in the transgenic cells were significantly higher than those in the nontransgenic cells for the periods of 0 to 4 h and 4 to 8 h (Fig. 7A). This difference, however, was not observed for the period of 8 to 24 h.

The amount of label appearing in the PCA-insoluble fraction that was derived from [1,4-14C]putrescine was generally similar in the two cell lines (Fig. 7B). The total amount of label in this fraction did not change with time, indicating a continuous turnover of this fraction. Figure 7C shows the amount of radioactivity present in the total dansyl-polyamine fraction that partitioned into toluene. The transgenic cells generally had significantly less label than the nontransgenic cells at all times tested. The amount of label in this fraction generally decreased with time.

As expected, putrescine was by far the most abundant of the three polyamines at 4 h in both the cell lines followed by spermidine and spermine (Table II). Between 4 and 24 h, the relative proportion of labeled putrescine declined from more than 70% in both cell lines to below 30%. By 24 h, the proportion of label in spermidine in both cell lines was substantially higher than that in putrescine. It increased from about 20% at 4 h to about 60% by 24 h. The amount of label in spermine also increased with time but never exceeded 11% of the total label in polyamines.

DISCUSSION

In the present study we compared the metabolism of putrescine and its precursors Arg and Orn in a transgenic cell line constitutively expressing a mouse ODC cDNA and the control nontransgenic cell line. We showed earlier that the transgenic cells contain significantly higher concentrations of free putrescine with little change in free spermidine and spermine (Bastola and Minocha, 1995). This was correlated with high levels of mouse ODC-specific enzyme

Table II. Relative amounts of radiolabeled polyamines collected up to 24 h in transgenic (N14) and nontransgenic (NT) carrot cells when [1,4-14C]putrescine was used as a precursor

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Putrescine</th>
<th>Spermidine</th>
<th>Spermine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NT</td>
<td>N14</td>
<td>NT</td>
</tr>
<tr>
<td>4</td>
<td>71</td>
<td>77</td>
<td>25</td>
</tr>
<tr>
<td>8</td>
<td>53</td>
<td>61</td>
<td>40</td>
</tr>
<tr>
<td>24</td>
<td>28</td>
<td>29</td>
<td>61</td>
</tr>
</tbody>
</table>

Figure 6. The incorporation of [U-14C]Orn (A) and [U-14C]Arg (B) into acid-insoluble material in nontransgenic (NT) and transgenic (ODC-N14) carrots cells during a 4-h incubation period. Values are means ± se of three replicates. Different lowercase letters indicate that the values are significantly different (P ≤ 0.05) from each other at a given time. FW, Fresh weight.

Figure 7. The amount of 14CO2 released (A), 14C present in the acid-insoluble fraction (B), and 14C present in the dansyl-polyamine fraction from [1,4-14C]putrescine (C) in nontransgenic (NT) and transgenic (ODC-N14) carrot cells during a 4-h incubation period. Values are means ± se of three replicates. Different lowercase letters indicate that the values are significantly different (P ≤ 0.05) from each other at a given time. FW, Fresh weight.
activity in the transgenic cells. The results presented here provide a direct measurement of in vivo enzyme activity of ADC and ODC in the transgenic as well as nontransgenic cells. To our knowledge this type of information has not been available before for these enzymes. As expected, $[^{14}\text{C}]\text{Orn}$ was decarboxylated at a severalfold higher rate in transgenic cells than in the nontransgenic cells. This observation further shows that exogenously supplied Orn was rapidly absorbed and became accessible as a substrate to the mouse ODC in transgenic cells. The fact that the nontransgenic cells showed very little decarboxylation of Orn is consistent with the low levels of extractable ODC activity reported earlier for carrot cells (Montague et al., 1978; Robie and Minocha, 1989; for review, see Minocha and Minocha, 1995).

Except for the 1st h of incubation, the amount of $^{14}\text{CO}_2$ produced from $[^{14}\text{C}]\text{Arg}$ was similar in the two cell lines, indicating that there was no general reduction in cellular ADC activity in the transgenic cells. This is consistent with the ADC activity measurements in transgenic carrot cells (Bastola and Minocha, 1995). Similar results have been reported earlier by Hamill et al. (1990) in Nicotiana rustica transgenic roots expressing a yeast ODC gene, and by DeScenzo and Minocha (1993) in Nicotiana tabacum cv Xanthi expressing the mouse ODC.

The relatively little change in putrescine biosynthesis via the ADC pathway in cells that are producing large quantities of putrescine via ODC is rather unexpected. It is commonly known that there is a strong homeostatic regulation of the polyamine biosynthetic pathway in plants as well as animals, and this is achieved by feedback regulation of the three decarboxylases, i.e., ADC, ODC, and SAMDC (for reviews, see Pegg, 1986; Kumar et al., 1997; Walden et al., 1997). Data presented here indicate no such feedback inhibition of ADC activity by putrescine in carrot cells.

Based upon the amount of label in the aqueous fraction, it appears that both labeled Orn as well as labeled Arg were taken up at similar rates in the two cell lines. Moreover, these rates changed only slightly with time. Thus, the observed differences in the rates of decarboxylation of Orn and Arg, as well as their incorporation into putrescine in the two cell lines, probably reflect the activities of the respective enzymes. A potential problem with this argument, however, is that different sizes of endogenous pools of these substrates would directly affect the amount of $^{14}\text{CO}_2$ released at short intervals. Cellular Orn and Arg pools in the transgenic cells during normal growth conditions are not presently known. However, it is conceivable that cellular Orn in the transgenic cells may be lower than that in the nontransgenic cells because of its increased utilization by mouse ODC (Bastola and Minocha, 1995). This could, therefore, result in an overestimation of the rates of decarboxylation of $[^{14}\text{C}]\text{Orn}$ as well as its conversion into putrescine.

The metabolic fate of Orn involves (a) its conversion into putrescine by ODC and subsequently into other polyamines, (b) its conversion into citrulline and its deriatives, including Arg by Orn carbamoyltransferase, and (c) its metabolism into glutamate-$\gamma$-semialdehyde and its derivatives, including Pro and Glu by Orn transaminase (Slocum et al., 1984; Davis, 1986, and refs. therein). Labeled Orn can be incorporated into acid-insoluble material either as Arg or after catabolic conversion into Pro and Glu. A part of this fraction may include cell-wall-bound material. A large fraction of Orn can also be stored unchanged in the vacuole from where it is only slowly metabolized. The results presented here show that relatively little of the radiolabeled Orn supplied from outside is converted into Arg in the nontransgenic cells, since it does not appear as putrescine in any significant amounts.

The data presented here provide further an insight into changes in the metabolic flux of putrescine into spermidine and spermine. It has been reported earlier by us and others that in plants (Hamill et al., 1990; DeScenzo and Minocha, 1993; Bastola and Minocha, 1995) as well as in animals (Halmekytö et al., 1995; Kauppinen and Alhonen, 1995; Heljasvaara et al., 1997), increased putrescine biosynthesis by transgenic overexpression of ODC has relatively little effect on the cellular pools of spermidine and spermine. No studies have been reported on the changes in metabolic turnover of these two polyamines in the transgenic cells. Paulus and Davis (1981) found that whereas $[^{14}\text{C}]\text{Orn}$ was quickly converted into putrescine in Neurospora crassa, the latter remained sequestered for several hours. Its conversion into spermidine and spermine and its catabolism were relatively slow. It is also obvious from the published literature that the cellular contents of spermidine and spermine in plants are much more tightly regulated than those of putrescine (Hiatt and Malmberg, 1988; Minocha et al., 1995; Minocha and Minocha, 1995). Our results point to something of a similar situation in that there was only a slow appearance of labeled spermidine and spermine either from $[^{14}\text{C}]\text{Orn}$ or $[^{14}\text{C}]\text{Arg}$. The exogenously supplied $[^{14}\text{C}]\text{putrescine}$ was also converted into spermidine and spermine at a slow rate. What most of the previous studies do not reveal is the extent to which spermidine and spermine turnover might be affected in relation to changes in putrescine levels.

In spite of the overall low rates of conversion of putrescine into spermidine and spermine, it is apparent from the data presented here that the rates of spermidine and spermine biosynthesis were significantly higher in the transgenic cells as compared with the nontransgenic cells when putrescine was produced from $[\text{U}-^{14}\text{C}]\text{Orn}$ (Fig. 3) but not from $[\text{U}-^{14}\text{C}]\text{Arg}$ (Fig. 4). If anything, the observed amounts of labeled spermidine and spermine in the transgenic cells are underestimates of the actual rates of conversion of putrescine into spermidine because of the fact that the pools of nonlabeled spermidine and spermine in the nontransgenic cells are much higher (as much as 5- to 10-fold) than in the transgenic cells (Bastola and Minocha, 1995). Since the amount of radioactivity present in spermidine and spermine was always small relative to the amount present in putrescine (whether synthesized from $[^{14}\text{C}]\text{Orn}$, $[^{14}\text{C}]\text{Arg}$, or provided exogenously as $[^{14}\text{C}]\text{putrescine}$), it can be argued that (a) the overall rates of biosynthesis of spermidine and spermine in both cell lines were indeed low either due to the limitation of decarboxylated SAM or the enzymes spermidine and/or spermine synthase; or (b) newly syn-
thesized spermidine was rapidly turned over, so as not to affect its overall cellular levels. It is the latter possibility that will cause an overall stimulation of polyamine metabolism that may competitively affect the availability of SAM for ethylene biosynthesis. Our preliminary investigation on the metabolism of [U-14C]Met does indeed show significantly lower rates of its conversion into ethylene in the transgenic cells as compared with the nontransgenic cells (S.E. Andersen and S.C. Minocha, unpublished data).

At present, the fate of putrescine produced by ODC in transgenic cells remains unclear. Mengoli et al. (1989) showed that exogenously supplied putrescine was concentrated in the vacuole of carrot cells. It is not known if the same happens to the mouse ODC-derived putrescine in the transgenic cells. It is quite apparent that increased putrescine production is correlated with its increased catabolism as measured by $^{14}$CO$_2$ evolution from [14C]putrescine as well as increased incorporation of its label into PCA-insoluble material. The chemical nature of the label from putrescine incorporated into the acid-insoluble fraction is difficult to interpret because of the binding of putrescine to the cell wall material, which is also present in this fraction (Mengoli et al., 1989). In solanaceous plants, putrescine is incorporated into many secondary compounds, such as tropane alkaloids and pyrrolidine (Hamill et al., 1990; Altman and Levin, 1993). Likewise, polyamines can be conjugated to various phenolic and other secondary metabolites (Evans and Malmberg, 1989). Although in some tissues the conjugated polyamines constitute a major proportion of the total polyamines, carrot cell cultures have polyamines mostly in the soluble form; only less than 10% are conjugated (Minocha and Minocha, 1995). Rastogi and Davies (1989) showed that in fruit pericarp discs of tomato, putrescine is metabolized into spermidine, GABA, Glu, and a polar fraction eluting with sugars and organic acids. Similarly, in Pinus radiata cotyledons, Kumar and Thorne (1989) showed the metabolism of putrescine into GABA, aspartate, and glutamate; with GABA accounting for as much as 24% of the total label. Using the dansylation procedure with [14C]GABA, we found that GABA remained in the aqueous fraction after partitioning with toluene (S.E. Andersen and S.C. Minocha, unpublished data).

In contrast to the possibility of overestimation of decarboxylation of [14C]Orn in the transgenic cells (due to low cellular Orn pools), the observed catabolism of [14C]putrescine must be an underestimation because of the fact that cellular pools of nonradioactive putrescine are severalfold higher in the transgenic than in the nontransgenic cells. Another possibility that is currently being investigated in our laboratory to follow the fate of excessive putrescine in transgenic cells is its secretion into the surrounding medium. No information is currently available on the secretion of polyamines by plant cells.

In conclusion, the results presented here show that (a) the higher amount of putrescine in the transgenic cells is due to its increased biosynthesis through the mouse ODC pathway; (b) the presence of elevated cellular amounts of putrescine due to the mouse ODC has no significant effect on the metabolism of Arg into putrescine by the native ADC pathway, and (c) although cellular concentrations of spermidine and spermine are not affected by the overproduction of putrescine, their biosynthetic rates are, nevertheless, increased. Furthermore, as suggested by Minocha and Minocha (1995), increased putrescine production leads to an overall stimulation of the metabolic pathway, which could adversely affect the biosynthesis of ethylene through increased utilization of SAM. In this respect, studies are underway to analyze the effects of increased polyamine metabolism on the biosynthesis of ethylene in several transgenic cell lines of carrot.

ACKNOWLEDGMENTS

The authors would like to thank Stephanie Long and Daniel Coughlin for technical help and Dr. Curtis Givan, Dr. Rakesh Minocha, and Dr. Thomas Davis for helpful suggestions for improvement of the manuscript.

Received May 2, 1997; accepted September 18, 1997.

LITERATURE CITED


physiological polyamine homeostasis in their tissues. Biochem J 323: 457–462