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# Polyamine Metabolism in Embryogenic Cells of *Daucus carota*

## II. CHANGES IN ARGININE DECARBOXYLASE ACTIVITY

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### ABSTRACT

**Embryogenic cultured cells of *Daucus carota* have been shown to synthesize putrescine from exogenously supplied [$^{14}$C]arginine at twice the rate of control nonembryogenic cells. In the present paper, the activity of arginine decarboxylase (arginine carboxy-lyase, EC 4.1.1.19), an important enzyme in the synthesis of putrescine, was assayed and also found to be elevated by as much as 2-fold in embryogenic cells. This difference between embryogenic and nonembryogenic cells was observed as early as 6 hours after the induction of embryogenesis and appeared not to result from the presence of a diffusible inhibitor or activator. It seemed to be dependent upon concomitant RNA and protein synthesis, as judged using 6-methylpurine and cycloheximide. After cycloheximide addition to the culture medium, arginine decarboxylase activity declined with a half-time of about 30 minutes in both embryogenic and nonembryogenic cells. It is suggested that elevated arginine decarboxylase activity is involved in the mechanism leading to elevated putrescine levels in these cells and hence may play a role in the embryogenic process.**

In a previous paper (8), results were presented which showed substantial differences between embryogenic and nonembryogenic carrot cells with regard to their metabolism of polyamines. The levels of putrescine and spermidine rose in embryogenic cells compared with the control, whereas the level of spermine fell. Also, the rate of synthesis of putrescine appeared to be about 2-fold higher in embryogenic cells, as judged by the incorporation of radioactivity after a pulse of [$^{14}$C]arginine. These differences occurred within 24 hr after transfer of the cells to embryogenic medium.

Because polyamines may therefore be involved in the process of carrot embryogenesis, it seemed important to examine the basis for this alteration in polyamine metabolism. To this end, the activity of arginine decarboxylase (arginine carboxy-lyase EC 4.1.1.19) was determined. ADC[1] has been implicated in the regulation of polyamine biosynthesis in other plant and bacterial systems (4, 11-15). Its mammalian counterpart, ornithine decarboxylase, varies in response to many kinds of developmental events and environmental stimuli (7, 9, 10, 16, 17). In the present paper, time course changes for ADC activity in embryogenic carrot cells are reported, together with the effects of inhibitors of protein and RNA synthesis.

### MATERIALS AND METHODS

**Cell Culture and Enzyme Extraction.** Cells were cultured and embryogenesis was induced as before (8). Cycloheximide and 6-methylpurine were filter-sterilized prior to addition to the culture media.

For enzyme extraction, carrot cells (200-600 mg fresh weight) were harvested by filtration, weighed, and placed in a glass, hand-held homogenizer. The cells were ground (25 strokes) on ice with 2 ml of cold 100 mM HEPES (pH 7.0) containing 5 mM DTT, and the extracts were centrifuged at 12,000*g* for 30 min. The supernatant (hereafter called crude extract) was usually collected and frozen at −80 C for later assay. No change in activity with time was noted under these conditions of storage.

**Assay of ADC and SAMDC Activities.** The ADC enzymic reaction was started by adding 0.15 ml of crude extract to 0.01 ml of DL-[1-$^{14}$C]arginine monhydrate (Amersham/Searle, 0.5 $\mu$Ci, 20 mCi/mmol) and 0.01 ml of 5 mM pyridoxal-5′-phosphate. In some cases, these volumes were halved. The test tubes containing all components of the assay were capped with special rubber stoppers fitted with plastic center wells (Kontes Glass Co., K-882310 and K-882320) containing 0.1 ml of 2 N KOH on a Whatman No. 1 paper wick. The reaction was allowed to proceed for 60 min at 30 C in a shaking water bath (100 oscillations/min) and was terminated by the injection of 0.5 ml of 1 N $H_2SO_4$ into the reaction solution. After an additional 60 min of shaking, the center wells were removed, and the paper wicks were placed in scintillation vials with 3 ml of $H_2O$ and 10 ml of Packard Insta-gel scintillation fluid. Counting efficiency was about 80%. Blank values were obtained either by using boiled crude extract or by terminating the reaction with acid immediately after extract addition. The reaction was linear throughout the 60-min incubation period and was linearly proportional to the amount of crude extract added.

SAMDC was assayed in a similar way. The cells were extracted as above except that the pH of the buffer was 7.5. The enzymic reaction was started by adding 0.075 ml of crude extract to 0.01 ml of [$^{14}$C]carboxyl-S-adenosylmethionine (ICN, 0.1 $\mu$Ci, 47.5 mCi/mmol) and 0.01 ml of 5 mM pyridoxal-5′-phosphate. Radioactive $^{14}CO_2$ was collected in the same way as above after a 60-min incubation. The reaction was linear throughout the incubation period and was linearly proportional to the amount of crude extract added.

**Determination of Protein.** Protein in the extracts was determined by the method of Lowry *et al.* (5) after precipitation with trichloroacetic acid and solubilization in 1 N NaOH to avoid interference from HEPES.

### RESULTS

**Time Course of ADC Activity.** After the completion of preliminary experiments in which conditions for the assay of ADC activity were established, the time course of activity in embryogenic and control cells was determined. Ornithine decarboxylase activity was also detected in carrot cells under similar conditions of assay but because its activity was very low compared with ADC activity and since it paralleled changes in ADC activity quite closely, no further investigations were carried out. The results of

[1] Abbreviations: ADC: arginine decarboxylase; SAMDC: S-adenosylmethionine decarboxylase.

the ADC time course are presented in Figure 1. In this experiment, ADC activity rose rapidly after transfer of the cells to fresh 0B5 (embryogenic) or 0.1B5 (nonembryogenic) medium and remained at a high level throughout the 24-hr time course. Even as early as 6 hr, embryogenic cells showed higher ADC activity than did nonembryogenic cells. At 12 hr, embryogenic cell extracts possessed almost twice the level of ADC activity as nonembryogenic cell extracts.

Figure 1 also presents the amount of protein used in each reaction mixture. Because a constant volume of buffer was employed to extract the cells, this plot also reflects the amount of protein extracted from each cell culture. Note that the protein content increased quite linearly over this 24-hr period for both embryogenic and nonembryogenic cells. No consistent difference in protein content was found between the two culture conditions, demonstrating that the elevation in the specific activity of ADC in embryogenic cells was not an artifact of changes in extracted protein.

In another experiment (Fig. 2), the elevated ADC activity in embryogenic cells was observed for as long as 3 days after transfer to 0B5 medium. The elevation in ADC activity varied from approximately 1.3- to more than 2-fold depending upon the experiment. The enzyme activity paralleled changes in putrescine levels quite well (see ref. 8) but the correlation was not exact. It appears that elevated ADC activity may be at least partially responsible for altered polyamine metabolism and especially for increased putrescine levels in embryogenic carrot cells.

**Time Course of SAMDC Activity.** The biosynthesis of spermidine and spermine is dependent upon the decarboxylation of S-adenosylmethionine via SAMDC (1). In order to determine what role SAMDC might play in the changes in polyamine levels found in embryogenic carrot cells, its activity was assayed over a 48-hr time course (Fig. 3). Note that SAMDC activity was initially very low but rose rapidly in both embryogenic and nonembryogenic cells, very much like the ADC activity in Figures 1 and 2. Unlike ADC, there was no clear difference between embryogenic and nonembryogenic cell cultures until about 24 hr and then the nonembryogenic cells had an activity slightly higher than the embryogenic cells. This higher activity in the nonembryogenic cells may be related to their generally higher levels of spermine (see ref. 8). Because the difference in ADC activity between

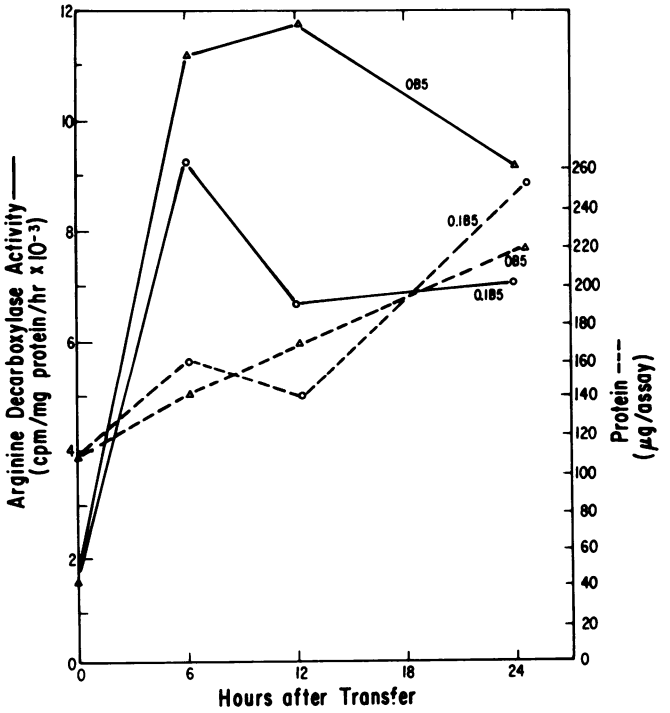


FIG. 1. Early time course of ADC activity and protein content. Carrot cells were transferred either to embryogenic (0B5) or nonembryogenic (0.1B5) medium and harvested at the indicated times.

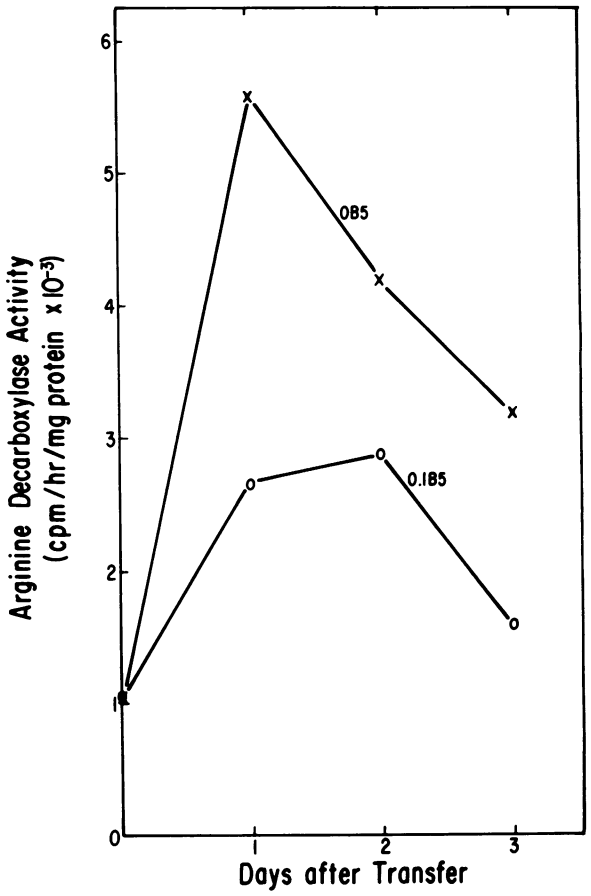


FIG. 2. Later time course of ADC activity. See legend of Figure 1.

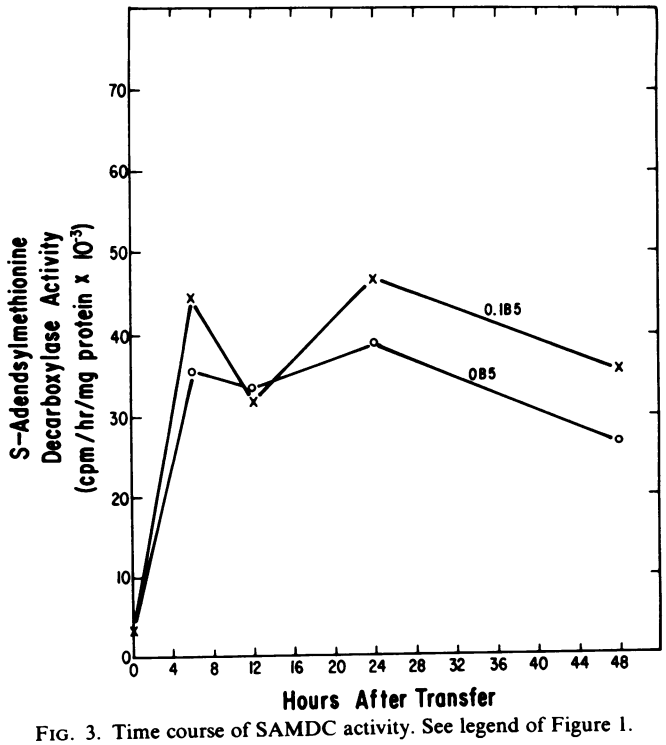


FIG. 3. Time course of SAMDC activity. See legend of Figure 1.

embryogenic and nonembryogenic cells occurred earlier and was more marked than the difference in SAMDC activity, the remainder of this study is devoted to ADC.

**Effect of Cell Density and Fresh Medium on ADC Activity.** One interesting result shown in Figure 1 is the large rise in ADC activity which occurred in the cells whether they were transferred to embryogenic or to nonembryogenic medium. In some cases (data not shown), this rise in activity occurred at least as soon as 3 hr after transfer. In order to determine whether the initial rise in activity was due to fresh medium, lowered cell density, or both, the experiment presented in Table I was conducted. Carrot cells (3.5 days old in 0.1B5, 42 mg fresh weight/ml) were transferred to fresh 0.1B5 medium either at 42 mg/ml or 3 mg/ml for 5 hr. Similarly, they were also transferred to depleted (the original) 0.1B5 medium either at 42 mg/ml or 3 mg/ml for 5 hr. At that time, the cells were extracted and assayed for ADC activity. It appears from the results that simple manipulation of the cells (treatment 3) caused a 2.2-fold rise in activity. This rise was enhanced somewhat by a lowered cell density (3.5-fold, treatment 4). In fresh medium, the activity rose about 13-fold in 5 hr, whether the cells were at low (treatment 2) or at high density (treatment 1). It appears that fresh medium is the primary cause of the rapid rise in ADC activity observed in carrot cells under these experimental conditions. This finding is in good agreement with results obtained for mammalian cells, where a shift from nongrowing to growing conditions results in a substantial increase in ornithine decarboxylase activity (16, 17).

**Effect of Removal of Small Molecules and Mixing Extracts.** At this point, all data on arginine decarboxylase activity were derived from experiments using crude, undialyzed extracts. It was of interest to determine whether the removal of small molecules, through dialysis or passage of the extract through a Sephadex G-25 column, would affect the activity. Table II presents the results of three experiments. In experiment 1, extracts from both embryogenic and nonembryogenic carrot cells (24 hr after transfer) were either assayed directly or dialyzed overnight against 125 volumes of extraction buffer. In experiment 2, carrot cells were grown in the same way, extracted, and a portion of the crude extract was passed through a Sephadex G-25 column, using the extraction buffer as eluant.

Activity in crude extracts from cells grown in 0B5 medium was elevated over that from 0.1B5-grown cells (about 1.4-fold for both experiments). Similarly, an elevation of the 0B5 to 0.1B5 ratio was apparent in the dialyzed (1.25-fold) and Sephadex-treated (1.5-fold) preparations. Some increase in total activity was observed in each case (about 20% after dialysis and about 50% after passage through Sephadex). These results indicate that: (*a*) the elevation of ADC activity in embryogenic carrot cells occurred independently of the presence or absence of small mol wt compounds; and (*b*) the removal of small molecules from the extracts caused some increase in ADC activity. This second effect is especially pronounced after passage through Sephadex, perhaps because this is a milder treatment than overnight dialysis.

Table I. Effect of fresh medium and cell density on arginine decarboxylase activity

Carrot cells (3.5 days) were transferred from 0.1B5 medium (42 mg/ml) either to fresh or to depleted medium at 42 or 3 mg fresh weight per ml. ADC activity was assayed at 5 hr.

| Treatment | Arginine decarboxylase activity |
|---|---|
| | cpm/mg protein·hr |
| 0 hr | 579 |
| 1. Fresh medium, 42 mg/ml | 7714 |
| 2. Fresh medium, 3 mg/ml | 7899 |
| 3. Depleted medium, 42 mg/ml | 1249 |
| 4. Depleted medium, 3 mg/ml | 2000 |

Table II. Effect of dialysis and Sephadex G-25 on arginine decarboxylase activity

In experiment 1, carrot cells, which had grown either in embryogenic (0B5) or non-embryogenic (0.1B5) medium for 24 hrs, were harvested and extracted. Crude extracts were either assayed directly or dialyzed overnight against 125 volumes of extraction buffer. In a separate experiment 2, the same protocol was followed except that a portion of the crude extracts was passed through a Sephadex G-25 column. In a separate experiment 3, the cells were grown for 18 hr before extraction.

| Extract | Arginine decarboxylase activity |
|---|---|
| | cpm/mg protein.hr |
| Experiment 1 (dialysis) | |
| 0B5 crude | 12,714 |
| 0.1B5 crude | 9,321 |
| 0B5 dialyzed | 14,948 |
| 0.1B5 dialyzed | 11,944 |
| Experiment 2 (Sephadex) | |
| 0B5 crude | 9,289 |
| 0.1B5 crude | 6,601 |
| 0B5 Sephadex | 14,363 |
| 0.1B5 Sephadex | 9,774 |
| Experiment 3 (mixing) | |
| 0B5 crude | 13,189 |
| 0.1B5 crude | 6,588 |
| 0B5 + 0.1B5 (mixed) | 9,101 |
| Predicted for mixture | 9,130 |

Also presented in Table II are the results of a mixing experiment (experiment 3). Crude extracts were prepared from embryogenic and nonembryogenic carrot cells and assayed for ADC activity. As usual, the embryogenic cells showed higher activity, and in this case, the activity was elevated by about 2-fold over the nonembryogenic control. ADC activity was also assayed in a mixture of embryogenic and nonembryogenic extracts. Here, the activity observed was simply additive. This result indicates that no diffusible molecule was responsible for the elevated activity in embryogenic cells.

**Effect of Cycloheximide and 6-Methylpurine on ADC Activity.** To determine whether concurrent protein synthesis was required for enhanced ADC activity in the embryogenic carrot cells, cycloheximide (*cf.* 2) was supplied 18 hr after transfer to either 0B5 or 0.1B5 medium. The results of time course determinations of ADC activity are presented in Figure 4. At the time of addition of cycloheximide (10 μg/ml), ADC activity from embryogenic cells was more than 2-fold higher than the control. Both the ratio of ADC activity in embryogenic compared with control cells and the time of maximum ADC activity after transfer varied from experiment to experiment.

When cycloheximide was added at 18 hr, it caused a rapid decrease in ADC activity in both embryogenic and nonembryogenic cells. If it is assumed that cycloheximide caused immediate, total inhibition of protein synthesis, the half-life of ADC activity was about 30 min, both in embryogenic and in control cells. Essentially the same results were obtained with dialyzed and nondialyzed extracts. This finding suggests that the elevation of ADC activity in embryogenic compared with control cells does not result from a decreased rate of degradation of the enzyme. Rather, the rates of degradation in both embryogenic and control cells appear to be the same.

In a similar experiment (Fig. 5), 5 mM 6-methylpurine, an inhibitor of RNA synthesis (6), was supplied to the carrot cells 24 hr after transfer to embryogenic or nonembryogenic medium. At this point, embryogenic cells, as usual, showed higher ADC activity than the control cells. After about 3 hr, 6-methylpurine decidedly reduced ADC activity both in embryogenic and in control cells, completely abolishing the difference between cells in 0B5 and 0.1B5 media by 24 hr after application.

Because of the lag period before 6-methylpurine took effect,

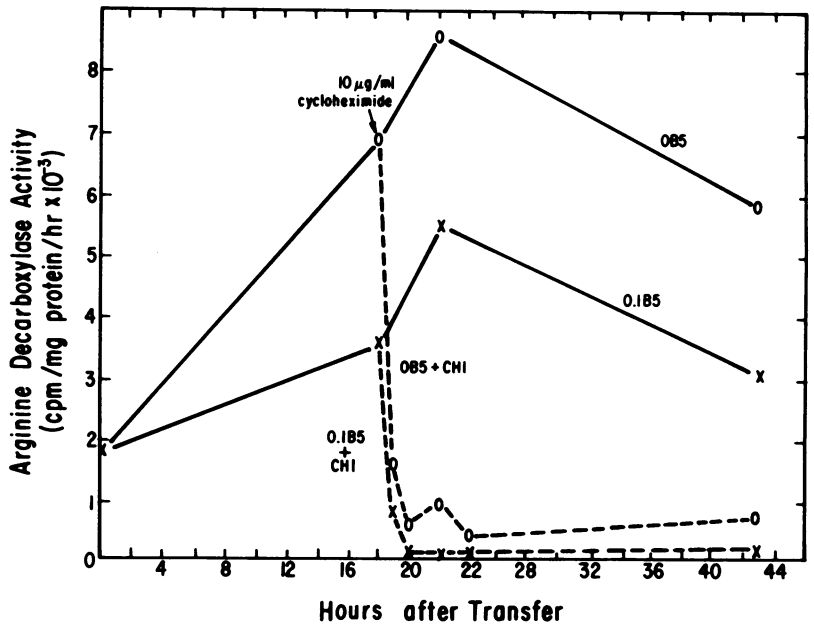


FIG. 4. Time course pattern of effect of cycloheximide on arginine decarboxylase activity. Carrot cells were transferred either to embryogenic (0B5) or to nonembryogenic (0.1B5) medium. At 18 hr after transfer, some of the cells were supplied with cycloheximide (10 µg/ml). ADC activity was assayed at the indicated times.

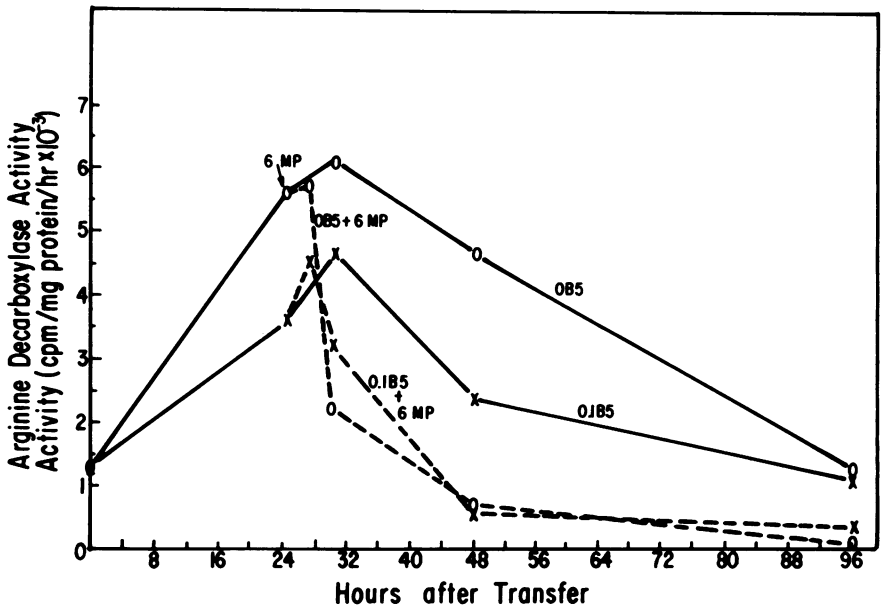


FIG. 5. Time course pattern of effect of 6-methylpurine on arginine decarboxylase activity. Carrot cells were transferred either to embryogenic (0B5) or to nonembryogenic (0.1B5) medium. At 24 hr after transfer, 6-methylpurine (6 MP) was supplied at a concentration of 5 mM to certain cultures. ADC activity was assayed at the indicated times.

half-life measurements under these conditions were not very meaningful. This experiment indicates that elevated ADC activity is dependent upon concomitant RNA synthesis, and the results indicate that the difference in ADC activity between embryogenic and nonembryogenic cells is dependent upon RNA synthesis.

## DISCUSSION

Previous results (8) obtained in this laboratory have shown elevated levels of putrescine in embryogenic compared with nonembryogenic carrot cells. A 2-fold elevation in the rate of synthesis of putrescine from exogenously supplied [$^{14}$C]arginine was reported. Results of the present study show a consistent elevation in ADC activity in embryogenic compared with nonembryogenic cells. This elevated activity occurred within 6 hr after transfer (Fig. 1) and continued in some experiments for as long as 72 hr (Fig. 2). Although the time course of elevated ADC activity did not correlate exactly with the previously observed time course changes in putrescine levels (Figs. 1 and 4; ref. 8), it is important to note that both the putrescine level and ADC activity were elevated by about 2-fold at 24 hr after transfer to embryogenic medium. The intracellular level of putrescine is a function of both its rate of synthesis and its rate of degradation. Complex compartmentation may play a role in this regulation. Therefore, it is not surprising that ADC activity and putrescine levels did not correlate exactly. It appears that elevated ADC activity may well be one factor in the regulation of putrescine levels in carrot cells.

In contrast to the clear elevation of ADC activity in embry-

ogenic compared with control cells, another enzyme involved in polyamine synthesis, SAMDC, showed a different pattern (Fig. 3). Although there was a large initial rise in activity, no difference was found between embryogenic and control cells until 24 hr, when the activity in the control cells was slightly higher. This result indicates that the elevation in ADC activity is not a characteristic of all enzymes in embryogenic cells and also that the synthesis of spermidine and spermine may be regulated differently from the synthesis of putrescine. Spermine was found at generally lower levels in embryogenic compared with control cells (8).

The initial rapid rise in ADC activity upon transfer of the carrot cells seemed to be due simply to fresh medium (Figs. 1 and 2; Table I). A similar phenomenon occurs in cultured animal cells. For example, Yamasaki and Ichihara (17) found that fresh medium but not conditioned medium induced ornithine decarboxylase activity in confluent cultured mouse L cells. The activity was also induced by a mixture of amino acids. Prouty (10) found that ornithine decarboxylase activity was induced up to 100-fold over basal leves 4 hr after the addition of glutamine to cultured HeLa cells. Thus, cultured plant and animal cells seem to be somewhat similar with regard to the induction of these polyamine-synthesizing enzymes.

Elevated ADC activity in embryogenic carrot cells did not seem to result from the presence of a small dialyzable molecule because the enhanced activity was found even after dialysis or passage of the crude extract through a Sephadex G-25 column (Table II). As judged from a mixing experiment, no diffusible inhibitor or activator appears to play a role in establishing the difference in ADC activity between embryogenic and control cells. In this regard, a protein inhibitor of ornithine decarboxylase has been found in H-35 rat hepatoma cell cultures after the addition of putrescine (3). No such substance seemed to regulate ADC activity in cucumber cotyledons (14).

Cycloheximide rapidly inhibited ADC activity in both embryogenic and control carrot cells (Fig. 4). In both of these cultures, the half-life of ADC was about 30 min, indicating that the cause of elevated ADC activity in embryogenic cells was probably not simply a decreased rate of enzyme degradation. Suresh *et al.* (14) found that the half-life of ADC in cucumber cotyledons varied from 3 to 10.7 hr depending upon whether the cotyledons were treated with putrescine or KCl, respectively. The half-life of carrot ADC seems, therefore, to be shorter than that of cucumber ADC and, in fact, it approaches the very short half-lives of 9 to 112 min found for ornithine decarboxylases from mammalian cells (10, 16).

The RNA synthesis inhibitor, 6-methylpurine (Fig. 5), also caused a substantial decrease in ADC activity, abolishing the difference between embryogenic and control cells. This result indicates that RNA synthesis is essential for elevated ADC activity in embryogenic cells and that at least part of the regulation of ADC activity in this system probably resides at the level of transcription. In contrast, actinomycin D stimulated ornithine decarboxylase activity in cultured mouse L cells and this effect was much greater in nongrowing cells than in growing cells (16).

Several workers have demonstrated changes in plant ADC activities in response to various hormonal or nutritional treatments. For example, ADC activity was shown to increase in potassium-deficient barley (11) and in soybean seedlings deprived of their cotyledons and grown on ammonium instead of nitrate (4). ADC activity was altered in response to tumor induction in *Scorzonera hispanica* L. by *Agrobacterium tumefaciens* (13). Suresh *et al.* (14) found that benzyladenine and its ribotide enhanced ADC activity in cucumber cotyledons, whereas abscisic acid was inhibitory.

It does not appear that the elevated ADC activity in embryogenic cells is due simply to an increased rate of growth because growth rates (measured as fresh weight or protein increase) in control and embryogenic media are similar for at least 2 days (unpublished results). Moreover, ADC activity and growth rate are not necessarily positively correlated in plant systems (14). Based on the present data, it is not possible to determine whether increased ADC activity is a causal factor in the embryogensis of carrot cells. The early onset of elevated activity (6 hr) and the short half-life of the enzyme are noteworthy considerations. Taken together with the findings in the literature cited above, these results do indicate some role for polyamines, and specifically for ADC, in the embryogenic process.

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