Plant Physiol. (1978) 62, 430–433

Polyamine Metabolism in Embryogenic Cells of *Daucus carota*

I. CHANGES IN INTRACELLULAR CONTENT AND RATES OF SYNTHESIS

Received for publication December 5, 1977 and in revised form May 4, 1978

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ABSTRACT

Changes in the metabolism of polyamines, which seem to be involved in transcription and translation in animal systems, have been studied in cultured cells of *Daucus carota* (carrot) undergoing embryogenesis. Putrescine levels were elevated by as much as 2-fold over the control within 24 hours after transfer of the cells to embryogenic medium. Spermidine levels were elevated also but spermine levels appeared to be lower in embryogenic cells. Embryogenic cells incorporated [^{14}C]arginine into putrescine at two times the rate of control cells. These changes suggest that polyamines may be involved in cellular differentiation during embryogenesis.

The metabolism of the polyamines, putrescine, spermidine, and spermine, has been the subject of considerable investigation, especially in animal systems (2). Elevated levels of at least one of these compounds have been noted in developing rat fetuses (15), in sea urchin embryos (11), following partial hepatectomy (14), and in transformed cells (3). In addition, there is evidence that polyamines are involved in the regulation of transcription (9, 13) and translation (8, 10). Relatively little work has been carried out in developing plant systems with regard to changes in polyamine metabolism.

Unlike cultured animal cells, many cultured plant cells are able to regenerate into whole plants when provided with appropriate hormonal and nutritional conditions. In this laboratory, suspension cultures of carrot, *Daucus carota*, undergoing embryogenesis, have been employed as a model for the study of biochemical events involved in plant cell differentiation. As part of this study, the metabolism of polyamines was investigated in an effort to determine whether they play a role in the initiation of carrot cell embryogenesis. Earlier work (unpublished) suggested that respiratory quotients in embryogenic cell cultures were higher than in nonembryogenic ones and that this could indicate increased decarboxylation reactions such as those leading to polyamine biosynthesis. This paper reports on: (*a*) the intracellular levels of polyamines; (*b*) the rates of synthesis of polyamines from exogenous, labeled precursor; and (*c*) the rates of turnover of labeled polyamines in embryogenic and in nonembryogenic (control) cells.

MATERIALS AND METHODS

Maintenance of *D. carota* Cell Cultures and Induction of Embryogenesis. Carrot cells (obtained from D. Dougall, W. A. Jones, Cell Science Center) in liquid suspension were grown on B5 medium as described by Gamborg *et al.* (7). Cultures were routinely maintained on B5 medium containing 0.1 mg/liter 2,4-D (designated 0.1B5 medium) for 3.5 days prior to the initiation of embryogenesis. By 3.5 days, the cells had reached a density of about 40 mg fresh weight per ml. These cells were harvested by gentle centrifugation (500*g* for 5 min), washed in 0B5 medium (B5 medium containing no 2,4-D), and transferred to 80 ml of either 0B5 (embryogenic) or 0.1B5 (nonembryogenic) medium at about 2.5 mg fresh weight per ml. After 14 days, approximately 1.4×10^4 embryos were produced per 80 ml of 0B5 culture medium.

Extraction and Assay of Polyamines. Approximately 200 to 600 mg of fresh cells were harvested by filtration, washed, and homogenized in 1.5 volumes of 0.1 N HCl (5, 16). After addition of an equal volume of 10% (w/v) trichloroacetic acid, the homogenates were heated at 80 C for 10 min. The cooled mixture was then centrifuged at 20,000*g* for 20 min and the supernatant retained for further extraction. After noting the volume of supernatant, a 1-ml aliquot was withdrawn for the dansylation procedures (3, 6) used to quantitate the polyamines.

A 0.2-ml volume of the supernatant obtained after deproteination corresponding to approximately 7 mg dry cell weight was sufficient for densylation. After making this volume of supernatant basic (pH greater than 9) with 18.5 mg of anhydrous Na_2CO_3, 0.4 ml of dansyl chloride in acetone (30 mg/ml) was added. The stoppered tubes were incubated overnight at 27 C. The excess dansyl chloride was destroyed by the addition of 0.1 ml of a proline solution (100 mg/ml in H_2O) and the dansylated products were extracted into 0.5 ml of benzene. Aliquots of 10 to 15 μl of the benzene extract were supplied to activated (45 min at 100 C) silica gel TLC plates. The plates were developed for 5 hr in ethyl acetate-cyclohexane (2:3, v/v) and immediately sprayed with 10% (v/v) triethanolamine in isopropyl alcohol. The polyamines were quantified with a Turner model III fluorometer equipped with a Camag T Scanner Mark III (activation at 365 mm) and peak areas (>512 nm) from the extracts were compared to those obtained from dansylation of standards. It was necessary to include at least two standard spots on each plate along with the extract samples because there was a variation of fluorescence both between plates and upon a single plate.

In order to determine radioactivity present in the polyamines after exposure to [^{14}C]arginine, 2.5 ml of the supernatant obtained after deproteination were extracted four times with 2 volumes of ether to remove the trichloroacetic acid. Then, the extract was made basic by the addition of 0.05 ml of 10 N NaOH per ml and 0.2 g of a salt mixture (62.5 g anhydrous Na_2SO_4 + 9 g Na_3PO_4) per ml. To separate polyamines from other compounds, an equal volume of water-saturated 1-butanol was shaken with the aqueous extract for 30 min and then the phases were allowed to separate. The butanol phase was removed and its volume noted. The polyamines in the organic phase were converted to the hydrochlorides by the addition of 0.3 ml of 6 N HCl per ml of butanol followed by evaporation to dryness with N_2 gas. After reconstitution with 0.5 ml of 0.1 N HCl, a portion of each extract was spotted on cellulose thin layer plates along with polyamine standards. The plates were developed with isopropyl alcohol-concentrated HCl-H_2O (8:3:2, v/v/v) for 20 hr. They were then dried, neutralized with ammonia vapors, and the regions corresponding to the polyamines (determined using ninhydrin) were cut out, placed in scintillation vials, and counted.

Supply of Radioactive Arginine. Radioactive arginine (L-[u-^{14}C]arginine, 295 mCi/mmol) was filtered-sterilized before being supplied to the cultures. In each pulse experiment, the cells were washed thoroughly with 1 mM [^{12}C]arginine and the washes monitored for radioactivity in order to ensure complete removal of the radioactive precursor. Appropriate details of each experiment are presented in the figure legends.

Protein Determination. Protein in the extracts was determined by the method of Lowry *et al.* (12) after the trichloroacetic acid-precipitated material had been dissolved in 0.3 N NaOH.

RESULTS

Determination of Polyamine Levels. Initially it was necessary to determine the nature and levels of polyamines found in cultured carrot cells under embryogenic and nonembryogenic conditions. The predominant polyamines in these cells were found to be putrescine, spermidine, and spermine, which are also the polyamines usually present in eukaryotic cells (2). Time course changes in their intracellular levels are presented in Figure 1, both for embryogenic (0B5-grown) and for nonembryogenic (0.1B5-grown) cells. From the results, it can be seen that spermidine was present in greatest abundance, followed by spermine (about half the spermidine level), and putrescine (about one-tenth the spermidine level). Putrescine levels rose and then fell during the first 20 hr after transfer to embryogenic and nonembryogenic media. Spermidine levels rose slightly during the time course, whereas spermine levels remained quite constant for the first 6 hr and then rose considerably in nonembryogenic cells. The level of putrescine showed the opposite trend, that is, it was higher in embryogenic cells at the end of the time course. These results indicate that embryogenic and nonembryogenic carrot cells differ with regard to polyamine metabolism. Furthermore, these differences seem most apparent after 20 hr of growth in fresh medium.

Synthesis of Polyamines from [^{14}C]Arginine. In order to investigate the metabolism of polyamines further in embryogenic carrot cells, the synthesis of polyamines from exogenously supplied [^{14}C]arginine was studied. Arginine has been shown to be a precursor of putrescine in other plant systems (17). Figure 2 presents the results of an experiment where a small amount of [^{14}C]arginine (3.4 nmol) was supplied to nonembryogenic carrot cells (200 mg fresh weight). Radioactivity was followed as it appeared in putrescine, spermidine, and spermine. It seems that putrescine was the first polyamine to become labeled, and it also quickly began to lose radioactivity after 4 hr, presumably due to depletion of the exogenous labeled arginine from the medium. Spermidine seemed to be the next polyamine labeled and spermine was clearly the last to become radioactive, increasing in radioactivity at a rate much slower than either putrescine or spermidine.

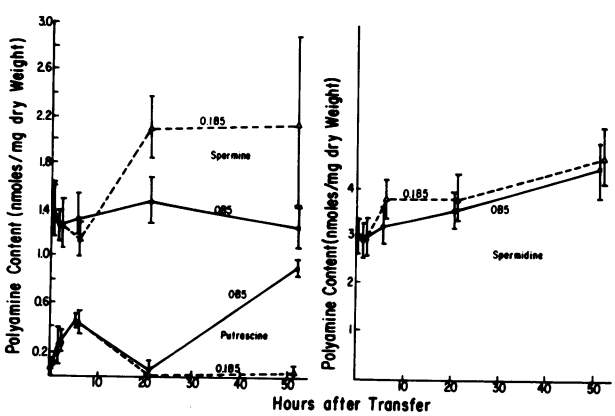

FIG. 1. Early time course changes in polyamine levels of cultured carrot cells. Carrot cells were transferred to embryogenic (OB5) or nonembryogenic (0.1B5) medium at 2.5 mg fresh weight per ml and harvested at the indicated times. Standard deviations of triplicate determinations are indicated by vertical bars.

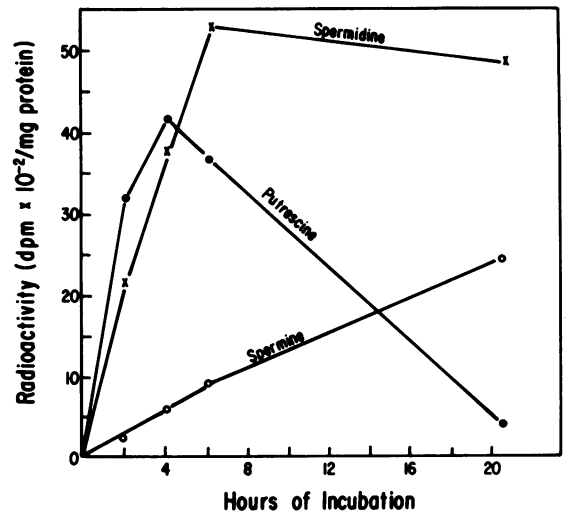

FIG. 2. Time course of incorporation of radioactivity from [^{14}C]arginine into polyamine from carrot cells. Carrot cells were supplied with 1 μCi of [^{14}C]arginine (295 μCi/μmol) per 280 mg fresh weight of cells. After the indicated times, the cells were harvested and extracted for polyamines.

These results tend to confirm the generally accepted conversion of arginine to putrescine to spermidine to spermine.

Turnover of Polyamines. Because embryogenic carrot cells showed alterations in absolute intracellular polyamine levels compared with the control and since exogenously supplied [^{14}C]arginine was an effective precursor of polyamines in carrot cells, an experiment was carried out to study the turnover of polyamines in embryogenic and control carrot cells after labeling with [^{14}C]-arginine. A pulse of [^{14}C]arginine was supplied after 24 hr of growth in either embryogenic or nonembryogenic medium. At this point in time, differences in polyamine metabolism would be observable, as indicated by the results of Figure 1. After collection by filtration and washing following the pulse of radioactivity, the cells were reinoculated into fresh 0B5 or 0.1B5 medium. Samples were collected at various times after transfer to ^{14}C-free medium. The results of determining total radioactivity as well as radioactivity present in the trichloroacetic acid-insoluble and trichloroacetic acid-soluble fractions are presented in Figure 3. Note that uptake of label was nearly the same in embryogenic and nonembryogenic cells. Although radioactivity in the trichloroacetic acid-soluble fraction declined somewhat, total radioactivity remained fairly constant over the time course.

The levels of putrescine, spermidine, and spermine determined in this experiment are presented in Figure 4. These results are quite similar, although not identical to those of Figure 1. Note that the putrescine level was considerably higher in embryogenic cells than in the control. Spermidine levels were also elevated, but spermine levels were lower in embryogenic cells than in nonembryogenic cells.

Specific radioactivities for putrescine, spermidine, and spermine are presented in Figure 5. The specific radioactivity of putrescine declined rapidly after completion of the pulse of radioactive arginine (with a half-time of approximately 6 hr). The specific radioactivity of spermidine rose briefly during this time course and then declined at a much slower rate than putrescine. The specific radioactivity of spermine increased throughout most of the time course, and it was the only case which showed a clear difference between embryogenic and nonembryogenic cells. At 48 and 72 hr, the specific radioactivity of spermine was higher under embryogenic conditions. Perhaps this difference in specific radioactivity reflects a different metabolic fate for spermine under the two growth conditions. It should also be noted that the specific radioactivity of putrescine was slightly lower under embryogenic conditions throughout most of the time course, perhaps reflecting more rapid turnover in embryogenic cells.

Rates of Synthesis of Polyamines. In order to provide some estimation of the *in vivo* rates of synthesis of polyamines in embryogenic compared with nonembryogenic cells, [^{14}C]arginine was employed in a different way. In this experiment, carrot cells were allowed to grow either in embryogenic or in nonembryogenic medium for 24, 48, or 72 hr. At those times, the cells were supplied with [^{14}C]arginine for 1 hr and then extracted. The radioactivity present in the polyamines was determined. Although radioactivity in spermine was undetectable, there was substantial ^{14}C in putrescine and in spermidine. The results are shown in Table I. Note that putrescine from embryogenic cells became labeled at about twice the rate of putrescine from nonembryogenic cells at all three time points. There was no difference in the rate of labeling of spermidine from embryogenic and nonembryogenic cells. Although the data are not shown, the uptake of [^{14}C]arginine was comparable under the two growth conditions. These results indicate that putrescine may be synthesized at twice the rate in embryogenic as in control cells, thus accounting for its higher level.

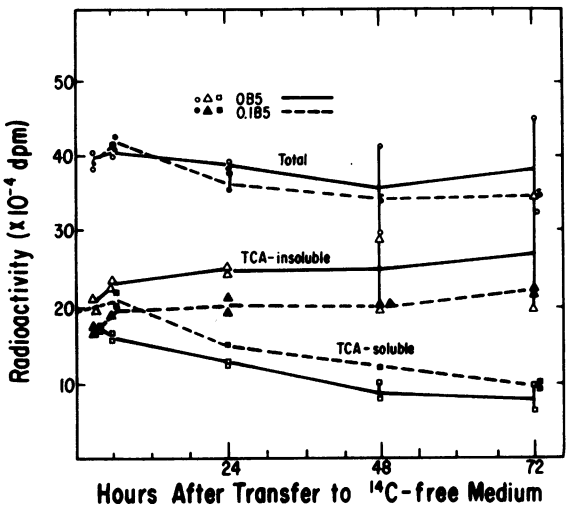

FIG. 3. Time course of incorporation of radioactivity from [^{14}C]arginine into various fractions extracted from carrot cells. Carrot cells were transferred into either embryogenic (OB5) or nonembryogenic medium (0.1B5) and allowed to grow for 24 hr. At that time, the cells were pulsed for 1 hr with [^{14}C]arginine (8 μCi/120 mg fresh weight; 3.4 nmol/μCi), washed and transferred to fresh, nonradioactive 0B5 or 0.1B5 medium. At the indicated times after transfer to ^{14}C-free medium, the cells were harvested and extracted.

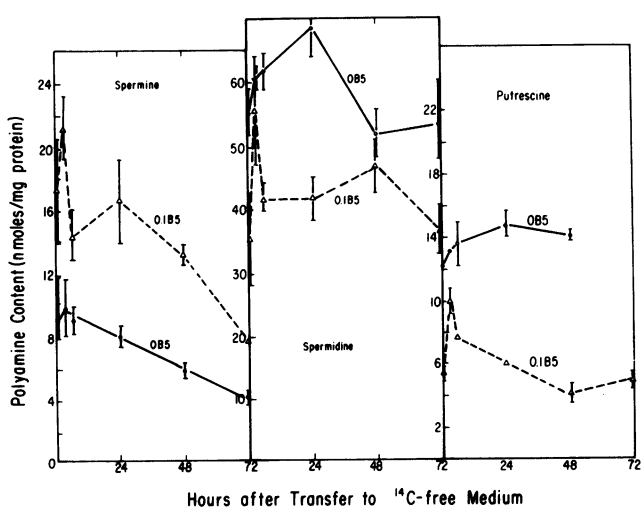

FIG. 4. Time course changes in levels of putrescine, spermidine, and spermine. See legend Figure 3. Vertical bars represent standard deviations of triplicate determinations.

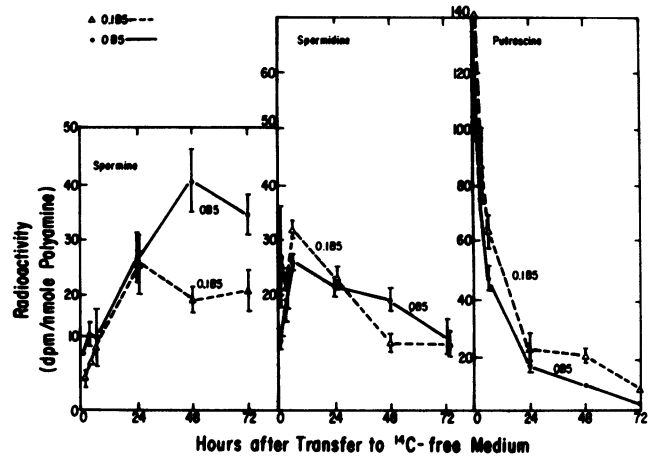

FIG. 5. Time course changes in specific activities of putrescine, spermidine, and spermine after a pulse of [^{14}C]arginine. See legend Figure 3. Vertical bars represent standard deviations.

Table I. Incorporation of Radioactivity from ^{14}C-Arginine into Putrescine and Spermidine

Carrot cells were transferred to embryogenic (0B5) or nonembryogenic (0.1B5) medium (2.5mg fresh weight/ml). At 24, 48, or 72 hrs after transfer, the cells were pulsed for 1 hr with ^{14}C-arginine (1 μCi, 295 μCi/μmole). Immediately after completion of the pulse, the cells were harvested and extracted for polyamines. Values are the means of triplicate determinations with standard deviations.

Time after transfer (hr)	Culture	Polyamine	
		Putrescine	Spermidine
		(cpm/mg protein)	
24	0B5	969±39	536±77
	0.1B5	464±61	572±58
48	0B5	1217±30	349±10
	0.1B5	528±13	392±37
72	0B5	1051±19	284±34
	0.1B5	426±12	234±27

DISCUSSION

Although considerable research has been directed toward the study of polyamines in developing animal systems (2), little work has been carried out in plants. Because plants have the unique ability to regenerate from cells grown in culture, they provide a special opportunity to study biochemical events during differentiation. Results presented here indicate that polyamine metabolism differs significantly in embryogenic compared with nonembryogenic cultured carrot cells. Putrescine levels were elevated in embryogenic cells within 24 hr after transfer to 0B5 medium. Spermidine levels were elevated also, but spermine levels appeared to be lower in embryogenic cells (see Figs. 1 and 4). It is important to note that the results shown in Figure 1 emphasize polyamine levels early in the time course whereas those in Figure 4 are concerned with levels of polyamine 24 hr after transfer. Radioactive arginine, which served as a precursor for polyamines (Fig. 2) was used to determine rates of synthesis and turnover in embryogenic as compared with control cells. Although the rate of putrescine synthesis appeared to be 2-fold higher in embryogenic cells (Table I), little difference in its specific radioactivity after a pulse of [^{14}C]arginine could be demonstrated. No difference in the specific radioactivity of spermidine was found, but spermine had a higher specific radioactivity in embryogenic cells (Fig. 5). These findings may reflect a complex cellular compartmentation or interconversion of the polyamines.

While the significance of these changes is somewhat difficult to assess, some comparative data can be drawn from the literature. Bagni *et al.* (4) have studied the polyamine content of tissues

derived from *Scorzonera hispanica*. Their results for normal tissue were quite similar to those presented here (Fig. 1) in that they found the level of spermidine to be about 2-fold higher than the level of spermine, which was about 2-fold higher than the putrescine level. In addition, the absolute levels of polyamines (nmol/mg dry weight) are quite similar in carrot and in *S. hispanica* tissue. Bagni *et al.* (4) also compared polyamine levels of crown gall, habituated, and normal tissue from *S. hispanica*. They showed that the levels of putrescine and spermidine were higher in crown gall tissue than in habituated tissue, where the levels were in turn higher than in normal tissue. In fact, putrescine and spermidine were 100 times and two times higher, respectively, in crown gall than in normal tissue. Spermine was about half the level in crown gall compared with normal tissue.

Audisio *et al.* (1) showed that habituated tissue of *Nicotiana glauca* also possessed elevated levels of putrescine when compared with normal tobacco tissue. The putrescine content was nearly 17-fold higher and the spermidine content about 5-fold higher than in the normal tissue. Spermine was found only in trace amounts.

These results are quite similar to those reported in the present study for embryogenic carrot cells compared with the control. In all three cases, carrot, tobacco, and *S. hispanica*, a change in morphology was accompanied by changes in polyamine levels and the largest changes occurred in putrescine content specifically. In all three systems, it is clear that alterations in the level of one polyamine are not necessarily accompanied by similar or proportional changes in the levels of other polyamines. For example, putrescine may increase while spermine decreases. Perhaps some sort of common regulatory mechanism is operating in all three cell types, such as a general increase in the rate of protein synthesis. Still, caution is certainly required with such an interpretation of the data. In crown gall and habituated callus, growth is unorganized, whereas in embryogenic carrot cells, growth presumably leads to differentiation and consequently to increased organization. Nonetheless, a change in the metabolism of polyamines is clearly demonstrable as the cells undergo an alteration in their differentiative state. This result suggests that polyamines may play an important role in the growth and development of higher plant cells.

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