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Polyamine Biosynthesis and Effect of Dicyclohexylamine during the Cell Cycle of *Helianthus tuberosus* Tuber[1]

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ABSTRACT

Polyamine content and the activities of their main biosynthetic enzymes, ornithine decarboxylase (ODC, EC 4.1.1.17), arginine decarboxylase (ADC, EC 4.1.1.19), *S*-adenosylmethionine decarboxylase (SAMDC, EC 4.1.1.50), and arginase (EC 3.5.3.1.), were examined in crude extracts of *Helianthus tuberosus* tuber slices during the first synchronous cell cycle, induced by synthetic auxin, with or without the addition of 1 or 5 millimolar dicyclohexylamine (DCHA), an inhibitor of spermidine synthase. In the DCHA-treated slices a peak of accumulation of the drug was observed at 12 hours. Bound DCHA was also found. Free polyamine content generally increased, reaching a maximum at 12 to 18 hours in the S phase of the cycle; while spermidine content was decreased slightly with DCHA after 12 hours, putrescine almost doubled at 18 hours. Bound polyamines were also present. ODC and ADC showed a maximum activity at 15 and 18 to 21 hours, respectively, *i.e.* in the S phase; both activities increased slightly in the presence of 5 millimolar DCHA at or near the time of maximum activity. Arginase was initially very high and then rapidly decreased although a small peak of activity occurred at 15 hours. SAMDC, which had two peaks of activity, was initially inhibited by DCHA, and then stimulated, especially at 12 hours and in coincidence with the main peak, at 21 hours. Thus ODC, ADC, and SAMDC activities as well as polyamine titer increased before and during the S phase of the cell cycle and all declined during cell division. The slight inhibitory effect of DCHA was possibly due to its degradation in the tissue and to the fact that putrescine could substitute for the function(s) of spermidine.

Aliphatic polyamines, putrescine, spermidine, and spermine, present in bacteria and eukaryotic cells (23) are essential for cell growth.

In higher plants they represent a class of growth substances and are involved in cell division and differentiation and in other important physiological phenomena such as senescence (2, 22).

The mechanism of polyamine functions in the living cell is probably linked to their chemical physical interactions with nucleic acids, proteins, and phospholipids, and is due to their cationic nature (2, 23).

In plants polyamine biosynthesis, which is enhanced prior to cell division, results from the activity of several enzymes: ODC,[2] the only putrescine-forming enzyme in animals; ADC, and in some species citrulline decarboxylase for putrescine synthesis; SAMDC, spermidine synthase and spermine synthase for spermidine and spermine synthesis (22, 23).

An important contribution to the study of the physiology of polyamines has been given by the production and testing of several inhibitors of their biosynthetic enzymes (15) even though in plants the presence of at least two biosynthetic enzymes for putrescine synthesis complicates the problem considerably.

In particular, during the first cell cycle after dormancy break in *Helianthus tuberosus* tubers, a naturally synchronous model in which the cycle begins with all the cells in G_1 phase (20), several inhibitors of polyamine synthesis have been tested: α-difluoromethylornithine and canaline, ornithine analogs, partly reduce *in vitro* ODC activity; α-difluoromethylarginine and canavanine, arginine analogs, partly reduce *in vitro* ADC activity, while methylglyoxal bis (guanylhydrazone) totally inhibits SAMDC activity (4, 5). Also in long-term cultures of tuber explants all the above-mentioned drugs, except for the last one, strongly or totally inhibit cell division (2, 4).

In recent years a competitive inhibitor of spermidine synthase, DCHA, has been discovered (16) and shown to inhibit spermidine accumulation in bacteria (9), animal (13, 17) and plant cells (7, 26). Moreover DCHA inhibits growth in *Helianthus* tuber explants and carrot embryogenesis (5, 14).

In the present paper we studied the activity of the main polyamine biosynthetic enzymes, ODC, ADC, SAMDC, and arginase, in crude extracts, in relation to polyamine titer during the first synchronous division of *Helianthus* tuber cells (induced by synthetic auxin). In addition the effect of DCHA on polyamine enzymes and accumulation was examined.

MATERIALS AND METHODS

Plant Material and Tuber Activation. *Helianthus tuberosus* L. cv OB1 (Jerusalem artichoke) was grown and vegetatively propagated in the Botanical Garden of Bologna University. The tubers were harvested at the beginning of dormancy in November and stored in moist sand at 4°C. The present work was done by using tubers at the end of January during deep dormancy. They were sterilized by immersion for 30 min in an 8% solution of commercial NaOCl and then rinsed three times in sterile H_2O. Slices of homogeneous medullary parenchyma (9 mm diameter, 1 mm thick) were aseptically excised transversely to the axis of the dormant tuber; to activate the tissue, *i.e.* to induce the first synchronous cell cycle, about 10 g of slices were put into 100 ml Erlenmeyer flasks containing 30 ml of Bonner and Addicott (10) liquid medium to which 4% sucrose plus 10 μM 2,4-D had been added; the pH was adjusted to 5.5 with 1 N NaOH; in some experiments 1, 5, or 10 mM DCHA (Sigma) was also added to the medium. The activation of the slices was performed on a rotatory shaker (75 rpm) in the dark at 24°C.

In one experiment [6-^{3}H]thymidine (2.22 MBq; 851 GBq/mmol, Amersham, UK) was added to the activation medium. Following nucleic acid determination, as described by Serafini-

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[2] Abbreviations: ODC, ornithine decarboxylase; ADC, arginine decarboxylase: SAMDC, *S*-adenosylmethionine decarboxylase; DCHA, dicyclohexylamine.

Fracassini *et al.* (20), label incorporation in DNA was measured using a scintillation counter Beckman LS-1500.

Polyamine Determination. About 1 g fresh weight of tuber slices were homogenized in 3 ml cold 5% (w/v) TCA. Extracts were centrifuged for 10 min at 1500*g*; the supernatant was set aside (TCA-soluble fraction); the TCA-washed pellet (TCA-insoluble fraction) was resuspended in 2 ml of TCA; two replicates of 1 ml each were hydrolyzed with 1 ml of 12 N HCl for 24 h at 110°C in flame-sealed ampoules according to Slocum and Galston (21). Following centrifugation at low speed to remove charred debris, the hydrolysates were taken to dryness under vacuum in a 60°C bath and resuspended in 200 μl TCA. Replicates of 0.1 ml of both supernatant and hydrolysate were processed following the dansylation method as described by Smith and Best (24); dansylated polyamines and DCHA were separated and determined using TLC precoated plates of silicagel 60 with concentrating zone. Ethylacetate:cyclohexane 2:3 (v/v) or chloroform:triethylamine 10:2 (v/v) was used as the solvent. Spots were scraped from the plates and suspended in 2 ml of pure anhydrous acetone on a Vortex mixer, and centrifuged; fluorescence was measured using a Jasco FP-550 spectrofluorimeter (excitation 360 nm, emission 505.5 nm) and the results compared with dansylated standards. Standard DCHA was dansylated using the same method as for standard polyamines, as described by Biondi *et al.* (7).

Enzyme Extraction and Assay. All enzyme procedures were carried out in an ice bath unless otherwise indicated. Tuber slices (10 g) were homogenized in an Omni Mixer at 180 rpm in 100 mM Tris-HCl buffer (pH 8.3), containing 50 μM EDTA, and 25 μM pyridoxal phosphate; the presence of 2.5 mM DTT did not affect the ADC and SAMDC activity but inhibited (by 70%) that of ODC; buffer pH and molarity were previously optimized (25); the homogenate was centrifuged at 26,000*g* for 30 min at 0°C and the supernatant was used for the enzyme assay. ODC, ADC, and SAMDC activities were determined by incubating 0.5 ml aliquots of the supernatant with, respectively, 14.8 KBq in 40 μl of L-[1-^{14}C]ornithine (1.93 GBq/mmol), DL-[1-^{14}C]arginine (12.43 GBq/mmol), or *S*-adenosyl-L-[carboxyl-^{14}C]methionine (2.22 GBq/mmol), and measuring the rate of decarboxylation, *i.e.* $^{14}CO_2$ evolution from these substrates. The enzyme assay was carried out as previously described (25).

Arginase activity was determined as the release of $^{14}CO_2$, due to urease activity, from the [^{14}C]urea stoichiometrically produced by arginase from L-[guanidino-^{14}C]arginine (2.07 GBq/mmol), as previously described by Torrigiani *et al.* (25). The arginase assay was carried out in the same experimental conditions as for ADC (buffer pH and molarity) using an equimolar amount of substrate as for ADC assay. In these experiments, the substrate concentration was not modified by adding cold substrate in order to maintain the physiological conditions as unaltered as possible; it is known in fact that in activated slices of *Helianthus* tuber arginine has a concentration of the order of 10 mM and ornithine of 100 μM (18). Under these conditions the addition of the labeled precursor (16 μM) in the enzyme assay did not affect the endogenous pool of arginine and only to a small extent that of ornithine.

All the radioactive compounds were supplied by Amersham, UK.

Replications. Experiments were done twice at 6, 12, 15, 21, and 24 h; the first was performed during deep dormancy (January) and the second during the natural release of dormancy (March). All data presented are referred to the first experiment and are the mean of three (polyamine and DCHA determination) to four-six (enzyme assay) replicates. In the figures, the lack of an error bar indicates that the SD was too small to show.

RESULTS

Polyamine and DCHA Content. As previously described (1, 19), tuber tissue reacts differently according to the different stages of dormancy; that is, its response to activation diminishes with the progression of dormancy. In fact, the experiment performed in March gave a similar pattern but a lower yield in polyamines as well as enzyme activity with respect to that performed in January.

During the first cell cycle in the 5 mM DCHA-treated tuber slices, a peak of accumulation of the drug was observed at 12 h (Fig. 1) while DCHA content in the 1 mM-treated tissue was rather constant after 6 h. The uptake of DCHA appeared concentration-dependent. DCHA bound to the TCA-precipitable molecules was also found, both in the 1 mM- and 5 mM-treated tissue, and ranged from 2 to 7% to 10 to 23% of the free one, respectively. When expressed as mM concentration, considering that the fresh tuber tissue contains 90% water, data showed that DCHA uptake occurred against a concentration gradient at 12 h and then followed the concentration gradient (Table I).

Free polyamine content generally increased, reaching a maximum at 12 to 18 h, corresponding to the first half of the S phase (Fig. 2). In particular, a sharp peak of putrescine was observed at 18 h while a spermidine peak occurred at 12 h.

Whereas 1 mM DCHA did not affect free polyamine content (data not shown), 5 mM did (Fig. 2). Unexpectedly, at 6 h (during G_1 phase) both spermidine and spermine content increased in the presence of DCHA; subsequently, however, spermidine content remained lower than in the control. Putrescine levels increased markedly (doubled) at 18 h in the DCHA-treated tissue

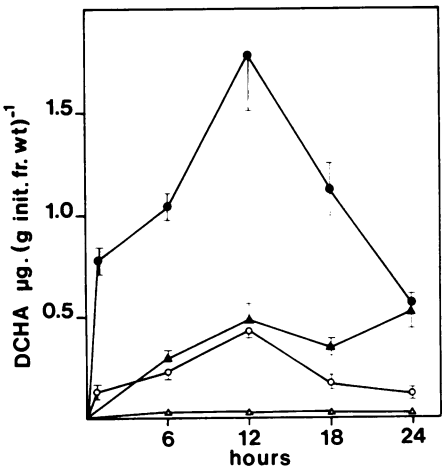

FIG. 1. DCHA content during the cell cycle in *Helianthus* tuber slices activated in a medium containing 1 mM (triangles) or 5 mM (circles) DCHA. (○, △), bound DCHA; (●, ▲), free DCHA. Bars represent ±SD.

Table I. *DCHA Concentration in Tuber Slices and in the Culture Medium (to Which 5 mM DCHA Was Added) at Different Times of Activation during the First Cell Cycle of Helianthus*

Time of Activation	DCHA	
	Slices	Medium
h	*mM*	
3	ND[a]	1.49 ± 0.18
6	1.34 ± 0.11	1.40 ± 0.15
9	ND	1.17 ± 0.18
12	2.44 ± 0.27	1.08 ± 0.12
15	ND	1.15 ± 0.09
18	1.42 ± 0.21	1.29 ± 0.14
24	0.74 ± 0.07	1.20 ± 0.16

[a] Not determined.

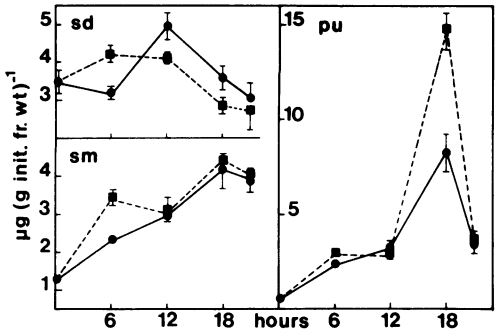

FIG. 2. Free polyamine content during the cell cycle in *Helianthus* tuber slices activated in a medium with (■) or without (●) 5 mM DCHA; pu, putrescine; sd, spermidine; sm, spermine. Bars represent ±SD.

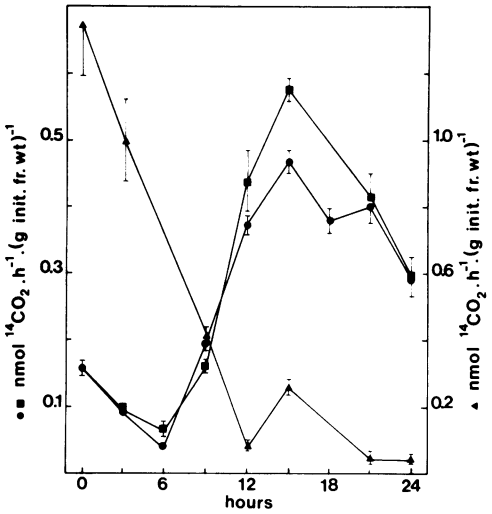

FIG. 3. ODC activity during the cell cycle in tuber slices activated in a medium with (■) or without (●) 5 mM DCHA; arginase activity (▲) from slices activated without DCHA. Bars represent ±SD.

while spermine remained unchanged (Fig. 2).

To verify if the polyamine pool was sufficient to ensure normal DNA synthesis in the DCHA-treated tissue, the following experiment was carried out: [^{3}H]thymidine was added to a growth medium containing 10 mM DCHA; nucleotide incorporation and nucleic acid content were examined in slices activated for 21 h, in the S phase, when [^{3}H]thymidine incorporation in nuclei is highest (20); DCHA did not affect DNA synthesis (control: 384 dpm/µg DNA; DCHA-treated; 394 dpm/µg DNA), and slightly affected DNA (control: 67 µg/g initial fresh weight; DCHA-treated: 57 µg/g initial fresh weight) and RNA (control: 230 µg/g initial fresh weight; DCHA-treated: 189 µg/g initial fresh weight) content.

Polyamine Biosynthetic Enzymes. After an initial decline, ODC showed a peak of activity at 15 h during the S phase (Fig. 3); DCHA induced a slight increase (about 20%) in ODC activity in correspondence with this peak. ADC activity also showed, after an initial decrease, a maximum around 18 to 21 h again in the S phase (Fig. 4); a slight enhancement of the enzyme activity by DCHA was observed in this case too between 12 and 15 h.

The activity of arginase, which cleaves arginine yielding ornithine and urea, was investigated (Fig. 3) to evaluate if it affects the determinations of the activity of the above-mentioned enzymes. At the onset of the cycle, arginase activity was high (10-fold that of ADC); thereafter, it decreased rapidly to one-fifteenth at 12 h; a small peak of activity occurred at 15 h in correspondence with maximum ODC activity.

SAMDC activity exhibited a biphasic pattern with a first peak

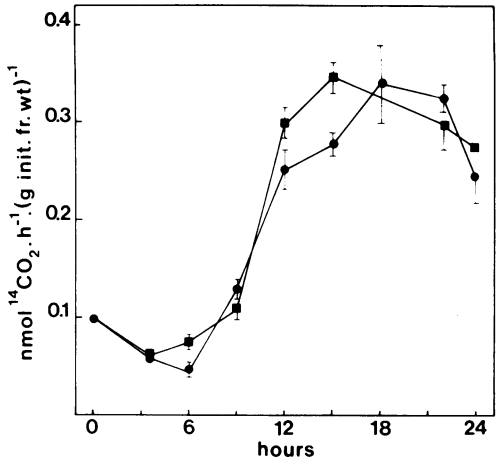

FIG. 4. ADC activity during the cell cycle in tuber slices activated in a medium with (■) or without (●) 5 mM DCHA. Bars represent ±SD.

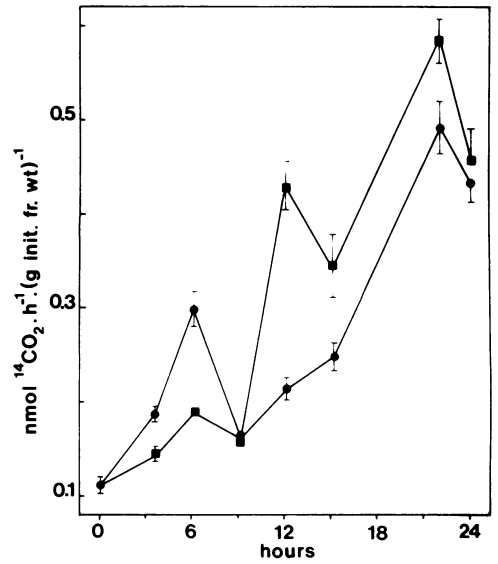

FIG. 5. SAMDC activity during the cell cycle in tuber slices activated in a medium with (■) or without (●) 5 mM DCHA. Bars represent ±SD.

at 6 h and a second one at 21 h which were differently affected by DCHA (Fig. 5). In fact, the inhibitor reduced the enzyme activity at 6 h (37%) and subsequently enhanced it, particularly at 12 h (100%).

DISCUSSION

Polyamine titer depends on the physiological state of the tuber tissue at the time of activation (19), as well as on the environmental conditions under which the plant is grown.

A rise in polyamine content and synthesis before and during nuclear DNA synthesis is a general feature in plants (11, 20) and animal systems (15). Moreover in ours, as in other systems (22), this rise is well correlated with a high activity of the biosynthetic enzymes.

A decline in spermidine content and a corresponding increase in putrescine induced by DCHA is a common feature in animal tumor cells (13, 17), bacteria (9), tobacco mosaic virus (28), pine cotyledons (7), and germinating seeds of *Acer* (26). Generally, in animal and bacterial cells, DCHA is effective at lower concentrations than in plant tissues (7, 26).

The putrescine accumulation induced by DCHA at 18 h may be explained in terms of its inhibited conversion to spermidine

and may be due to the competition of DCHA with putrescine in the spermidine synthase (aminopropyltransferase) reaction (17). At 21 h the putrescine titer returns to control values and this may be correlated with the sharp decrease of free DCHA (Fig. 1).

Polyamines bound to TCA-precipitable molecules (about 10% of the free ones) were found during the entire cell cycle both in the control and in the 5 mM DCHA-treated slices; DCHA induced an accumulation of bound putrescine which was not detectable in the control. The recovery seemed to be affected by different methods used (19); for this reason, further studies are in progress on this topic.

The effect of DCHA on DNA synthesis and accumulation may indicate that the S phase is not affected by the inhibitor. In cells infected by turnip yellow mosaic virus, the production of virus nucleic acids or particles is not inhibited by DCHA, as spermine replaces the depleted spermidine (28).

The rise in both ODC and ADC activity from 6 h onward (Figs. 3 and 4) can be correlated with the accumulation of putrescine. In our system the two enzymes operate *in vitro* at about the same rate and this again raises the question as to why two biosynthetic pathways for putrescine synthesis are activated in plants at the same time. During *Helianthus* tuber formation a correlation was found between ODC activity and cell division and between ADC activity and cell enlargement (3); a similar correlation was described also in other systems (22).

SAMDC, which catalyzes the synthesis of decarboxylated *S*-adenosylmethionine, which in turn furnishes the aminopropyl groups for spermidine synthase (aminopropyltransferase), shows two main peaks of activity in the course of the cell cycle, differently affected by DCHA. Possibly an alteration, different in G_1 phase with respect to S phase, occurs in the level of aminopropyl groups by DCHA treatment, as already suggested (13). The main peak of SAMDC (21 h; Fig. 5) followed the maximum accumulation of putrescine, while in other plants the enzyme was not putrescine stimulated (28). If spermidine and spermine are required for DNA synthesis (15, 23) the first peak of SAMDC activity could be related to organellar DNA synthesis, occurring in *Helianthus* in early G_1 phase (12, 20) and the second peak to nuclear DNA synthesis. During S phase, in *Helianthus*, a SAMDC activity was detectable mainly in the soluble fraction of the cytoplasm (25), as in other plants (28).

Concerning DCHA, it has been recently reported (6) that the anhydrous M_r of the Sigma compound is inconsistent with dicyclohexylammonium sulphate, but consistent with it being bis-cyclohexylammonium sulphate and thus the effect of DCHA, described in literature and in this paper, is due to cyclohexylamine but at twice the molarity quoted.

Arginase, purified and studied in *Helianthus* tuber throughout the period of dormancy (27), produces ornithine which is only in part transformed into putrescine via ODC, as demonstrated by the stoichiometric ratios between the two enzyme activities (Fig. 3); ornithine is in fact an intermediate also for proline and glutamate synthesis; moreover, the rise in ODC activity occurred when arginase activity decreased. The decrease in arginase activity is probably related to the decrease of its substrate, arginine, the main nitrogen reserve substance in *Helianthus* (18), which is rapidly metabolized to other compounds, such as polyamines, when the cells are induced to divide (20).

ODC, ADC, and arginase activities as well as free polyamines have been found to occur in *Helianthus* mitochondria and in chloroplasts from *Pinus* cotyledons (25); the compartmentation of these enzymes might be of some importance in explaining the effect of the inhibitors.

Recently the specificity of enzyme assays using crude plant extracts has been questioned (8). At present, we can only say that in *Helianthus* tubers ODC activity is inhibited in *in vitro* assays by difluoromethylornithine and canaline (both ornithine analogs) (4); moreover, ODC activity is enhanced by the addition of ornithine to the enzyme assay but not of a mixture of four amino acids (D D'Orazi, personal communication).

ADC activity determination does not exhibit problems of aspecificity (8). In *in vitro* assays difluoromethylarginine and canavanine (arginine analogs) effectively inhibited ADC activity in *Helianthus* slices (4, 5). However, ADC activity was probably overestimated in a small extent due to the release of $^{14}CO_2$ via ODC from the ornithine derived from arginine during the assay.

EDTA (1 mM) was used in the assay to minimize the arginase activity: arginase activity was reduced by 60%, ADC by 40%, and ODC by 20%; by adding 10 mM ornithine in the assay, ADC activity decreased by 16%; 10 mM arginine increased ODC by 15%. These data confirm that ADC determination are partly affected by arginase activity.

The SAMDC assay seems to be rather specific; in *Helianthus* it is inhibited *in vitro* by about 50% by 10 μM and almost completely by 1 mM methylglyoxal bis (guanylhydrazone) (5).

In conclusion ODC, ADC, and SAMDC activities as well as polyamine titer increase before and during the S phase and all decline during cell division. In *Helianthus*, DCHA, a very active growth inhibitor in *in vitro* cultures (5), acts, during the S phase of the first cell cycle, in causing a partial depletion of spermidine and a consistent enhancement of putrescine coincident with its maximum accumulation. It also affects indirectly ODC and SAMDC activity. The slight inhibitory effect of DCHA may be due in part to its degradation in the tissue (Fig. 1); moreover, putrescine might replace spermidine, at least in the short-term experiments. Perhaps, as shown in *Chlorella* (11), the effect of the inhibitor could become evident in the second cell cycle when polyamine depletion may be such that polyamine levels will no longer support cell division.

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