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Control by Ethylene of Arginine Decarboxylase Activity in Pea Seedlings and Its Implication for Hormonal Regulation of Plant Growth[1]

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ABSTRACT

Activity of arginine decarboxylase in etiolated pea seedlings appears 24 hours after seed imbibition, reaches its highest level on the 4th day, and levels off until the 7th day. This activity was found in the apical and subapical tissue of the roots and shoots where intensive DNA synthesis occurs. Exposure of the seedlings to ethylene greatly reduced the specific activity of this enzyme. The inhibition was observed within 30 min of the hormone application, and maximal effect—90% inhibition—after 18 hours. Ethylene at physiological concentrations affected the enzyme activity; 50% inhibitory rate was recorded at 0.12 microliters per liter ethylene and maximal response at 1.2 microliters per liter. Ethylene provoked a 5-fold increase in the K_m^{app} of arginine decarboxylase for its substrate and reduced the V_{max}^{app} by 10-fold. However, the enzyme recovered from the inhibition and regained control activity 7 hours after transferral of the seedlings to ethylene-free atmosphere. Reducing the endogenous level of ethylene in the tissue by hypobaric pressure, or by exposure to light, as well as interfering with ethylene action by treatment with silver thiosulfate or 2,5-norbornadiene, caused a gradual increase in the specific activity of arginine decarboxylase in the apical tissue of the etiolated seedlings. On the basis of these findings, the possible control of arginine decarboxylase activity by endogenous ethylene, and its implication for the hormone effect on plant growth, are discussed.

Considerable evidence links polyamines to the control of cell division and growth. Increased polyamine synthesis in rapidly dividing cells has been reported in microorganisms (10, 29) and in plant tumors (27). Inhibition of polyamine synthesis retarded growth (7), blockage of polyamine biosynthesis with specific inhibitors retarded tomato ovary growth, and supplying the polyamine beyond the block restored normal growth (11). The fact that inhibition of polyamine biosynthesis blocks the cells in the G_1 stage of the cell cycle (22) supports the view that polyamine biosynthesis is required for nucleic acid synthesis and for the completion of the mitotic cycle. Interactions between polyamines and nucleic acid are well known; spermine has been reported to be concentrated in the nuclei (20) and to play an important role in the configuration of DNA (19).

Association between polyamines, plant hormones, and growth arises from the study of Bagni (5), who first reported that growth of dormant *Helianthus tuberosus* was initiated when either auxin or one of the polyamines was added to the medium and that growth was accompanied by an increase in polyamine titer. There are now recorded instances of increased polyamine biosynthesis and titer in plants following application of each of the growth-promoting plant hormones: auxin (6), GA_3 (13), and cytokinin (28). On the other hand, retardation of growth by ABA has been shown to be accompanied by decreased polyamine biosynthesis (28). Consequently, the role of 'second messenger' mediating the effect of plant hormones has been proposed for polyamines (14).

Ethylene has been shown to inhibit cell division, DNA synthesis, and growth in meristems of roots, shoots, and axillary buds of etiolated pea seedlings (1). In the shoot apex, cell division was almost completely annulled by ethylene and only 60% inhibition was observed in the root apex (1). The gas inhibits cell division by blocking a stage before prophase (1). In the subapical regions of the seedling, the hormone inhibited cell expansion and DNA synthesis (2). In the various parts of the seedling, a qualitative relationship was found between the inhibition of DNA synthesis, cell division, and growth caused by ethylene (1).

In view of the above, a study was undertaken to pursue a possible involvement of polyamines in the effects of ethylene on plant growth. In this communication, we show that ethylene provokes a pronounced and reversible inhibition of ADC[2] activity and present evidence that indicates possible control of ADC activity by endogenous ethylene.

MATERIALS AND METHODS

Plant Material. Seeds of *Pisum sativum* (var 'Kelvedon Wonder') were soaked for 6 h in tap water, planted in pots containing moist vermiculite, and grown in the darkness at 23°C and 80% RH. Several pots of seedlings were placed in a 10-L desiccator and treated with 2,5-norbornadiene (24) or ethylene at various concentrations using a flow system at a rate of 100 ml/min. A comparable lot of plants was retained in air for each experiment and regarded as controls. All manipulations were carried out under dim green light that was tested and found to have no effect on elongation, otherwise the seedlings were kept in total darkness. Pots of seedlings were also grown in the dark under hypobaric conditions by placing them in a 10-L desiccator which was evacuated continuously at approximately 250 ml/min with a vented exhaust oil-seal pump. The pressure within the desiccator was maintained at 120 mm Hg by continuously admitting pure O_2 to the desiccator through a Matheson No. 449 regulator. The

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[2] Abbreviations: ADC, arginine decarboxylase; ODC, ornithine decarboxylase; STS, silver thiosulfate.

incoming O_2 was saturated at the reduced pressure by passing it through water. In some cases, the seedlings were exposed for 4 or 6 h to light with an intensity of 80 μE m^{-2} s^{-1} provided by cool white tubes. Before harvesting, seedlings were transferred to 3°C. The plumular hook, along with the plumule, where intensive cell division occurs (1), were excised and are referred to as the apical region; the region below that, where only cell expansion occurs (2), was excised and is referred to as the subapical region. In some cases, the apical and subapical regions of the primary root were harvested for determination of ADC activity.

Enzyme Extraction. One gram of chilled sections was ground in a chilled mechanized ground glass grinder with 3 volumes of grinding medium composed of 160 mM Tris-HCl buffer (pH 8.5), 50 μM pyridoxal phosphate, and 2 mM DTT. The homogenate was centrifuged for 20 min at 27,000*g* at 4°C, and the supernatant fraction was used for enzyme assay and protein estimation.

Enzyme Assay. ADC activity was determined by measuring $^{14}CO_2$ release from L-[U-^{14}C]arginine. A 50-μl aliquot of crude enzyme (0.2–0.4 mg protein) was added to a reaction mixture consisting of 1.2 μmol L-arginine containing 0.2 μCi L-[U-^{14}C] arginine (32.1 mCi/mmol) (New England Nuclear), 20 μmol Tris-HCl (pH 8.5), 6.5 nmol pyridoxal phosphate, and 0.26 μmol of DTT in a total volume of 150 μl. Each reaction was carried out in triplicate in test tubes capped with vaccine caps fitted with plastic center wells containing a paper wick soaked in 250 μl soluene 350 (Packard, IL). Incubation was carried out in a shaking water bath for 30 min at 45°C, and terminated by injecting 200 μl 6 N H_2SO_4. The tubes were further shaken for 60 min to liberate $^{14}CO_2$ trapped in the incubation mixture. The center wells containing the paper wicks soaked in the trapping solution were transferred to plastic scintillation vials containing 4.5 ml of Aqualuma plus scintillation liquid (Lumac, B.V., Holland) and counted in a Kontron liquid scintillation counter. Blank values were obtained by using boiled or acid-treated extracts. These two nonenzymic controls were always included in the experiments and their values were subtracted from those obtained from the enzymic reactions.

Protein concentration in the crude enzyme was determined by the method of Bradford (8), using BSA as a standard. ODC was extracted and assayed following the procedure described by Dai *et al.* (13). Ethylene production was determined as described elsewhere (3).

The data presented are from single experiments which are representative of a group of three to five experiments each involving triplicate sets of experimental treatments and controls.

RESULTS

Characterization of the Enzyme. Activity of ADC could be demonstrated in crude extracts from different tissues of etiolated pea seedlings. The optimal temperature for the enzyme activity was found to be 45°C. The enzyme exhibited a single pH optimum at pH 8.5, as measured at 45°C in the reaction mixture. Activity was linear with time for at least 40 min and with concentration of protein up to 2 mg/ml. Activity was increased with the addition of pyridoxal phosphate up to 50 μM. No apparent requirement for divalent metals was recorded. The enzyme exhibited typical Michaelis-Menten kinetics and a Lineweaver-Burk plot gave an apparent K_m value of 0.15 mM for L-arginine and V_{max}^{app} of 830 nmol CO_2/mg protein·h (Fig. 1).

Substrate Specificity and Diamines Effect. To ensure the specificity of the decarboxylation, specific inhibitors, substrate, or reaction end-products were added to the reaction mixture and their effect on the rate of decarboxylation was recorded. The results are presented in Table I. Addition of DL-α-difluoromethyl arginine, a compound known to be an irreversible specific inhibitor of ADC (17), caused a 65% inhibition at 50 μM, and at 0.5 mM the compound almost completely abolished the decarboxylation. However, addition of 1 mM DL-α-difluoromethyl ornithine, which is known to be a specific inhibitor of ODC activity (16), had no effect. This implies that the decarboxylation observed in this reaction is solely due to the ADC activity. Addition of 10 mM unlabeled L-arginine resulted in a 50% reduction of $^{14}CO_2$ release due to a dilution of the label, whereas addition of L-ornithine had no effect (Table I). This indicated that arginine is the substrate for the decarboxylation reaction, adding more support to the specificity of the reaction. The inclusion of 1 mM agmatine, an end-product of ADC activity, in the reaction mixture, resulted in about 50% inhibition of the decarboxylation, whereas putrescine, an end-product of ODC activity, had no effect, providing more support for the activity of ADC in this

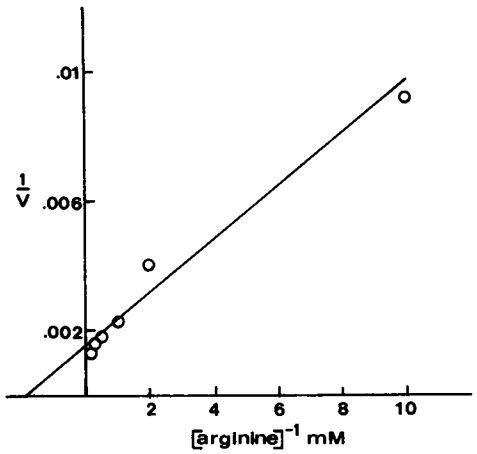

FIG. 1. Double-reciprocal plot of ADC activity *versus* arginine concentration. The apical portion of 4-d-old etiolated pea seedlings was excised and homogenized. ADC activity was determined as described in "Materials and Methods."

Table I. *Effect of Specific Inhibitors, Putative Substrates, and Amines on ADC Activity*

The effectors were added to a crude extract prepared from the apical region of 4-d-old etiolated pea seedlings and preincubated for 5 min at 45°C and then aliquots were added to the reaction mixture containing the substrate. The enzyme assay was conducted as described in "Materials and Methods" (100% = 380 nmol CO_2 mg protein·h).

Effector Added		$^{14}CO_2$ Released
	mM	*% control*
DL-α-DFMA	0.005	85
	0.05	35
	0.5	5
DL-α-DFMO	0.5	100
	1	98
L-Arginine	1	85
	10	50
L-Ornithine	1	100
	10	96
Agmatine	0.1	90
	1	53
Putrescine	0.1	98
	1	92

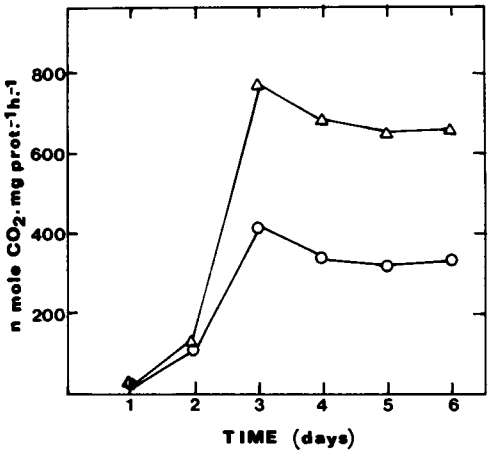

FIG. 2. ADC activity in the aerial part of etiolated pea seedlings during the early stages of the plant development. Activity was determined in the apical (O) and subapical (Δ) zones, as described in "Materials and Methods."

Table II. *Distribution of ADC Activity in Different Parts of the Etiolated Pea Plant*

ADC activity and ethylene production were determined in 7-d-old etiolated pea seedlings. Rates of ethylene production were determined in the different parts of the shoot, as described in "Materials and Methods."

Plant Tissue	ADC Activity	Ethylene Production
	nmol CO_2/mg protein·h	*nl/g·h*
Apical bud	350	0.43
Subapex	780	0.09
Root apex	77	
Root subapex	235	

Table III. *Effect of Ethylene on ADC Activity in Different Parts of the Pea Seedling*

ADC activity was determined on 7-d-old control or ethylene-treated (24 h) etiolated pea seedlings as described in "Materials and Methods."

Plant Tissue	ADC Activity	
	Control	Ethylene (50 μl/L)
	nmol CO_2/mg protein·h	
Apical bud	390	23
Subapex	710	78
Root apex	83	51

decarboxylation reaction.

Distribution of ADC Activity in Pea Seedlings. The relative distribution of ADC activity in the apical and subapical regions during the early stages of development of the plant was studied over a 7-d growth period. The data are depicted in Figure 2 and Table II. Activity was barely detectable in the embryo on the 1st d after imbibition, but was steeply enhanced subsequently reaching a maximal value on the 3rd d after imbibition (Fig. 2). A higher specific activity was found in the subapical region, as compared with the apical tissue: 780 and 400 nmol CO_2/mg protein·h, respectively. ADC activity in both regions declined slightly towards the 4th d after imbibition, leveling off thereafter. The distribution of ADC specific activity in the various parts of the pea seedling, as was recorded on the 7th d after imbibition, is given in Table II. The highest specific activity was recorded in the subapical region, and was more than 2-fold higher than in the apex. ADC specific activity was generally lower in the root system, compared with the aerial part, and was 3-fold higher in the root subapex than in the root apex; 235 and 77 nmol CO_2/

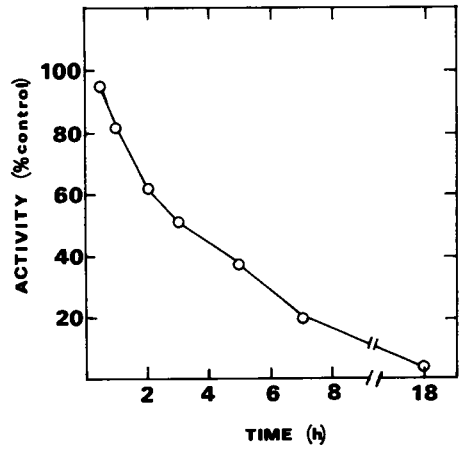

FIG. 3. Time course for the effect of ethylene on ADC activity. Etiolated pea seedlings—6 d old—were ventilated with ethylene (50 μl/L). ADC activity was determined in the apical zone as described in "Materials and Methods." (100% = 360 nmol CO_2/mg protein·h).

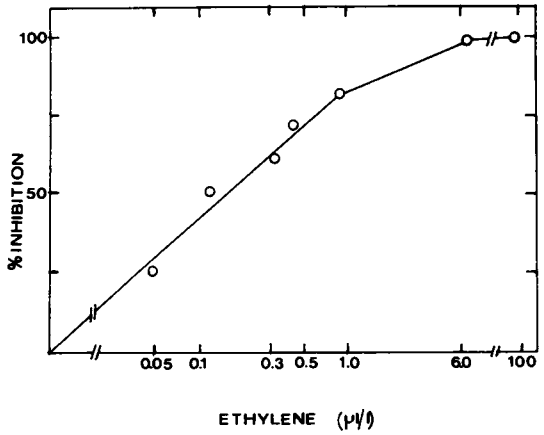

FIG. 4. Effect of various concentrations of ethylene on ADC activity. Activity was determined in the apical portion of 6-d-old pea seedlings treated for 18 h with the indicated concentrations of ethylene. Determination of ADC activity is described in "Materials and Methods." Control activity = 360 nmol CO_2/mg protein·h.

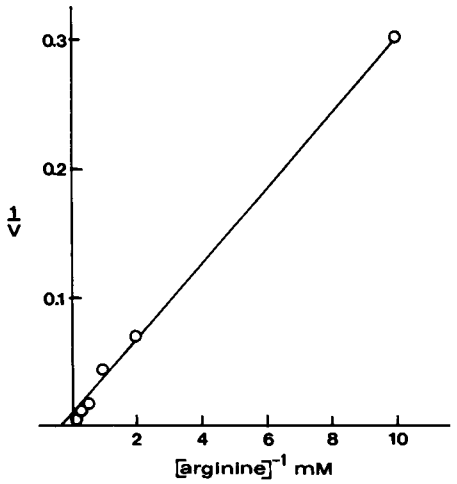

FIG. 5. Double reciprocal plot of ADC activity *versus* arginine concentrations in ethylene-treated plants. The seedlings were ventilated for 18 h with ethylene (50 μl/L). The apical portion of 4-d-old seedlings was excised and ADC activity determined as described in "Materials and Methods."

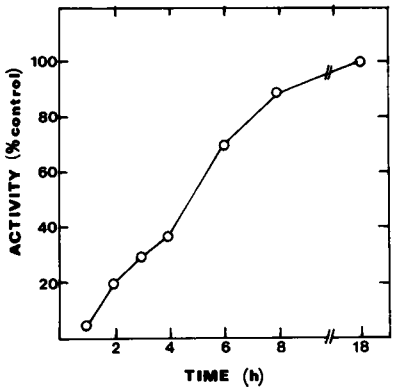

FIG. 6. Time course for the recovery of ADC activity from the inhibition by ethylene. Etiolated pea seedlings—6 d old—were treated with ethylene (50 μl/L) for 24 h, and then transferred to an ethylene-free atmosphere. Samples from the apical zone were excised at the indicated intervals and ADC activity was determined as described in "Materials and Methods." (100% = 390 nmol CO_2/mg protein·h).

Table IV. *Effect of Hypobaric Pressure, Exposure to Light, and Inhibitors of Ethylene Action on ADC Activity*

ADC activity was determined in crude extracts from the apical portion of 4-d-old etiolated pea seedlings. Light and hypobaric pressure treatments were conducted as described in "Materials and Methods." STS spray was applied to the plants in the dark 24 h prior to ADC determination. For 2,5-norbornadiene treatments, plants were enclosed for 12 h in 10-L desiccators containing various concentrations of the compound (100% for A, B = 295 nmol/mg protein·h, C = 305 nmol/mg protein·h).

Treatment	Specific Activity
	% control
A	
760 mm Hg	100
120 mm Hg—24 h	124
120 mm Hg—48 h	158
120 mm Hg—48 h + 1 μl/L C_2H_4	90
B	
Dark	100
Light—3 h	117
Light—6 h	140
C	
Control	100
STS—1 mM	96
STS—2 mM	112
STS—3 mM	142
2,5-Norbornadiene—200 μl/L	100
2,5-Norbornadiene—500 μl/L	110
2,5-Norbornadiene—700 μl/L	130

mg protein·h, respectively.

Effect of Ethylene on ADC Activity. When 6-d-old pea seedlings were ventilated with an air-stream containing 50 μl/L ethylene, ADC specific activity was markedly reduced in the various parts of the seedling examined; however, ODC activity was not affected (not shown). Inhibition of ADC activity by the gas was more pronounced in the aerial part than in the roots; 89 to 94% compared with 43% (Table III). The changes in ADC specific activity observed in the ethylene-treated tissue paralleled those in total activity, since no change in total protein content was observed upon ethylene treatment.

A time course study for the effect of ethylene on ADC specific activity in the apical region of pea seedlings revealed (Fig. 3) that the inhibition of ADC activity could be observed 60 min after exposing the intact plant to the gas. Almost 20% inhibition was observed within 1 h after the treatment, and 50% inhibition was recorded after 3 h. Exposure to ethylene for 18 h caused 95% inhibition of ADC specific activity.

ADC activity has the same sensitivity to ethylene as numerous other processes in pea seedlings; a threshold response with about 0.05 μl/L, half maximal at 0.12 μl/L, and a complete effect with a few μl/L ethylene (Fig. 4). Increasing the concentration from 6 μl/L to 100 μl did not have any additional inhibitory effect.

In an effort to understand the nature of the effect of ethylene on ADC activity, we studied the kinetics of arginine decarboxylation with increasing substrate concentration in extracts from the apical tissue of ethylene-treated plants. Lineweaver-Burk plot of the data revealed that the treatment caused a decrease in the affinity of the enzyme for arginine. A 5-fold increase in K_m was observed in the enzyme extracted from plants treated for 18 h with 50 μl/L ethylene; the apparent K_m value recorded for enzyme from ethylene-treated plants was 2.45 mM (Fig. 5) and 0.52 mM for enzyme extracted from controls (Fig. 1). In addition, the apparent V_{max} decreased by about 10-fold by the ethylene treatment; thus, the inhibitory effect does not fit either a classical competitive or noncompetitive pattern, since both K_m and V_{max} values were altered by the hormone treatment.

Effect of Mixing Crude Extracts and *in Vitro* Ethylene Treatment. On mixing equal volumes of the control crude extract with extract from ethylene-treated plant tissue for 30 min at room temperature prior to the enzyme assay, the resultant specific activity of ADC was merely the mathematical means of the individual values. Incubation of the crude extract for 30 min at room temperature with various concentrations of ethylene up to 1000 μl/L prior to the enzyme assay and during the assay, did not affect ADC specific activity.

Recovery from Ethylene-Induced Inhibition. When pea seedlings exposed to 50 μl/L ethylene for 24 h were transferred to an ethylene-free atmosphere, ADC resumed activity. This recovery from inhibition could be observed within 2 h after removal of the plants from the ethylene atmosphere (Fig. 6). The enzyme regained 50% of its original activity within 5 h, and 90% after 8 h. Complete recovery from the ethylene-induced inhibition was observed 18 h after transferral of the plants to an ethylene-free atmosphere.

Effect of Reducing Endogenous Ethylene Levels on ADC Activity. When plants are exposed to hypobaric conditions, the outward diffusion of the endogenous ethylene is enhanced. Consequently, the endogenous concentration of ethylene at equilibrium is diminished in direct proportion to the reduction in atmospheric pressure (1). To avoid O_2 depletion and desiccation, the plants are continuously ventilated with pure water-saturated O_2 at 120 mm Hg, which was found to be the lowest pressure under which pea seedlings were able to grow at a normal rate (1). When 4-d-old plants were transferred for 24 h to hypobaric conditions, the activity of ADC increased in the extract from the apical tissue by 24%, compared with that of plants grown continuously at atmospheric pressure (Table IV, A). Prolonging the duration of growth under hypobaric conditions to 48 h resulted in a 58% increase in ADC activity. This increase implies that ADC activity is suppressed by the endogenous ethylene present in the apical tissue.

When 1 μl/L ethylene was added to the O_2 introduced into the chamber containing pea seedlings held at 120 mm Hg, the concentration of ethylene was reduced in direct proportion to the reduction in the atmospheric pressure, thus forming an atmosphere of about 0.15 μl/L ethylene in the chamber held at

the reduced pressure. When pea seedlings were grown under these conditions, the increase in ADC activity caused by the hypobaric conditions was reversed by the ethylene, resulting in 90% activity of the control (Table IV).

Exposure to light has been shown to inhibit ethylene production (9, 15, 23) in etiolated pea seedlings. Accordingly, when dark-grown pea seedlings were exposed for 3 h to light, ADC activity was increased by 17% compared with that of plants kept continuously in the dark. Exposure to light for an additional 3 h resulted in a 40% increase in ADC activity (Table IV, B). Similar results were reported by Dai and Galston (12), where red light illumination which inhibits ethylene production in etiolated pea seedlings (9) produced an increase in ADC activity in the bud. These results indicate that inhibition of ethylene production caused an increase in ADC activity.

Effect of Inhibitors of Ethylene Action. Spraying etiolated pea seedlings with STS, a compound known to inhibit ethylene action (30), resulted in an increase in ADC activity in the apical zone of the plants. This effect was found to be concentration-dependent. Spraying with 1 mM STS 24 h prior to ADC determination did not show any effect; however, increasing STS concentration to 2 and 3 mM resulted in a 12 and 42% increase in ADC activity, respectively (Table IV, C). Higher concentrations of STS were harmful to the etiolated seedlings.

When pea seedlings were enclosed in desiccators containing 2,5-norbornadiene, a compound known to interfere with ethylene binding to its site of action (24), an increase in ADC activity was observed. This effect was more pronounced when the compound concentration was increased up to 700 μl/L. Treatment for 24 h with 200 μl/L 2,5-norbornadiene had no effect, but increasing the concentration to 500 and 700 μl/L resulted in a 10 and 30% increase in ADC activity, respectively. Any further increase in norbornadiene concentration was harmful to the etiolated seedlings. These results indicate that inhibition of ethylene action can cause an increase in ADC activity.

DISCUSSION

Activity of arginine decarboxylase was demonstrated in crude extracts from various tissues of etiolated seedlings (*P. sativum* var Kelvedon Wonder). The results of the studies with specific inhibitors, putative substrates, and end-products provide evidence for the specificity of the reaction and for the operation of the arginine→agmatine→putrescine pathway (25) in this plant. The relatively high pH (8.5) and temperature (45°C) exhibited by the enzyme extracted from these seedlings is in line with properties of other plant arginine decarboxylases (21). The enzyme appears to require pyridoxal phosphate as a cofactor, but no metal requirement was observed. It exhibited higher affinity for arginine than that reported for *Lathyrus sativum*, *E. coli* and barley (21), but lower than that reported for oat (26).

The finding that activity of ADC is hardly detectable during the first 24 h of seed imbibition, and the rapid increase in specific activity observed thereafter, is in line with observations reported for other plants (21, 28). This serves to emphasize the obligatory relationship between enhanced ADC activity which gives rise to an increased rate of polyamine formation, accelerated rate of cell division, and subsequent growth.

The change in the specific activity caused by ethylene treatment always runs parallel to that in total activity, indicating that the modulation in the enzyme activity was not influenced by possible fluctuation in the total reserve protein contents of the plant during the treatment period. It could also be shown that the change in ADC activity due to the hormonal treatment is not accompanied by the appearance of a free inhibitor, as revealed by the mixing experiments. This observation was pertinent in view of the observation that a specific protein inhibitor for ornithine decarboxylase is induced by polyamines in plant tissue (18).

Several reports have implicated plant growth regulators as modulators of polyamine biosynthesis. In all these cases, polyamine biosynthesis was enhanced by growth promoters (6, 13, 18, 21), and suppressed by growth inhibitors (28).

Ethylene has been known to suppress growth in etiolated pea seedlings and numerous other plants (1, 9). The hormone has been shown to exert this effect by inhibiting cell division in the meristems of the root and shoot apecies (1) and slowing down cell expansion in the subapical regions of these tissues (1). Ethylene was shown to interfere with cell division by blocking a stage before prophase (1). In root and shoot apices of etiolated pea seedlings, a quantitative relationship was found (1) between inhibition of DNA synthesis, cell division, and growth caused by ethylene. Consequently, it was concluded that ethylene inhibits plant growth predominantly via inhibition of DNA synthesis. Since polyamines have been implicated to be required for DNA synthesis (14, 29), it is proposed that ethylene inhibits DNA synthesis by blocking polyamine formation via inhibition of ADC activity along with inhibition of *S*-adenosylmethionine decarboxylase activity (4). In fact, ADC has a sensitivity to ethylene similar to that found for cell division and DNA synthesis, and quantitative relationship can be observed between the magnitude of ADC inhibition and that of cell division and DNA synthesis. Active DNA synthesis occurs in the subapical region of pea seedlings in spite of a lack of cell division in this tissue (1, 2). Ethylene inhibited DNA synthesis and ADC activity in both tissues, indicating that inhibition of these processes is not a consequence of inhibition of cell division caused by the hormone, but rather can be responsible for it.

The fact that ethylene at physiological concentrations (9) inhibits ADC activity (Fig. 4) raised the possibility that this hormone at the endogenous level controls ADC activity within the tissue. The first indication that could support this notion was the observation that higher specific activity of ADC was found in the subapical region, where a lower rate of ethylene production is recorded (Table II) and only half of that specific activity was found in the apical region, where the rate of ethylene production is 4-fold higher.

In addition, inhibition of ethylene production by illumination or reduction of the endogenous ethylene concentration in the tissue by hypobaric pressure, as well as inhibition of ethylene action by STS or 2,5-norbornadiene, resulted in a substantial increase in ADC activity. Furthermore, ethylene inhibits ADC activity within a relatively short time, and the enzyme recovers from the inhibition shortly after removal from an ethylene atmosphere.

All the above support the notion that endogenous ethylene controls ADC activity, and provide presumptive evidence that fluctuations in the rate of ethylene production, like under stress conditions (3), could produce the consequent fluctuations in ADC activity (14).

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