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Modulation of Arginine Decarboxylase Activity in Cucumber (*Cucumis sativus*) Cotyledons in Short-term Organ Culture

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Abstract. Among the various amines administered to excised *Cucumis sativus* cotyledons in short-term organ culture, agmatine (AGM) inhibited arginine decarboxylase (ADC) activity to around 50%, and putrescine was the most potent entity in this regard. Homoarginine (HARG) dramatically stimulated (3- to 4-fold) the enzyme activity. Both AGM inhibition and HARG stimulation of ADC were transient, the maximum response being elicited at 12 h of culture. Mixing experiments ruled out involvement of a macromolecular effector in the observed modulation of ADC. HARG-stimulated ADC activity was completely abolished by cycloheximide, whereas AGM-mediated inhibition was unaffected. Half-life of the enzyme did not alter on treatment with either HARG or AGM. The observed alterations in ADC activity are accompanied by change in K_m of the enzyme. HARG-stimulated ADC activity is additive to that induced by benzyladenine (BA) whereas in presence of KCl, HARG failed to enhance ADC activity, thus demonstrating the overriding influence of K^+ on amine metabolism.

The molecular and regulatory aspects of ornithine decarboxylase (ODC), catalyzing the rate-limiting step in putrescine and hence polyamine biogenesis in mammalian systems, have been the subject of extensive investigations in recent years (Bachrach 1980, Canellakis et al. 1979, McCann 1980, Russell 1980). Among the various modulators that influence ODC activity are several amino acids and amides (in particular asparagine—Canellakis et al. 1979), their analogues (Chideckel and Edwards 1983, Viceps-Madore et al. 1982), cAMP (Wright and Boyle 1982), and the polyamines themselves, the last exerting a negative control *in vivo* (Fong et al. 1976, Heller et al. 1976, Kay and Lindsay 1973). With the higher plant systems, similar studies on the regulatory aspects of polyamine elaboration are relatively meager, despite the recent recognition that these ubiquitous polycationic molecules play a vital role in such diverse control processes as growth and differentiation (Bagni et al. 1981, Feirer et al.

1984, Galston 1983), various conditions of stress (Altman et al. 1982, Flores and Galston 1982, Smith and Sinclair 1967, Young and Galston 1983), senescence (Altman 1982), and membrane stability (Altman et al. 1977). Another feature that distinguishes the higher plants from mammalian systems in terms of polyamine elaboration is that arginine (rather than ornithine), through agmatine, is the principal precursor of putrescine; therefore arginine decarboxylase (ADC) catalyzes the first and rate-limiting step in the biogenic pathway (Smith 1975), notwithstanding the fact that ODC activity has been recently encountered in some specialized plant tissues (Altman et al. 1983).

Earlier investigations in this laboratory have led to the purification of ADC from *Lathyrus sativus* seedlings (Ramakrishna and Adiga 1975) to homogeneity and elucidation of some of its physicochemical and molecular properties. More recent studies using excised cucumber cotyledons in organ culture as a model system have revealed that ADC in plants can be modulated by growth regulatory factors like cytokinins in a fashion analogous to ODC in mammalian systems (Suresh et al. 1978). This paper deals with the *in vivo* modulatory influence of effectors such as various amines and amino acids on ADC activity in cucumber cotyledons in short-term organ culture. The results described herein reveal, for the first time, that these small-molecular-weight compounds, particularly those related to the biosynthetic pathway of polyamines and substrate analogues, do exert significant modulatory influence on ADC in this higher plant system in a fashion reminiscent of that observed with ODC in mammalian and microbial systems.

Materials and Methods

Catalase (beef liver) was purchased from Sigma Chemical Company, St. Louis, MO, USA. Radioactive [U-^{14}C]arginine (specific activity 246 mCi/mmole) was obtained from Bhabha Atomic Research Centre, Bombay, India. [U-^{14}C]Arginine was purified on Dowex-50 column, and its radiochemical purity was established by paper chromatography. Agmatine (AGM) (Sigma) was purified according to Srivenugopal and Adiga (1981). N-carbamyl putrescine was synthesized as described by Srivenugopal and Adiga (1983). The sources of cucumber seeds and other chemicals employed have been referred to earlier (Ramakrishna and Adiga 1975, Suresh et al. 1978).

Germination and growth conditions have been described (Suresh et al. 1978). Cell-free extracts of the cotyledons for the enzyme assays were prepared as described earlier (Suresh et al. 1978) except that Tris-HCl buffers (20 mM) were employed. ADC assay was conducted as described earlier by Suresh et al. (1978). Catalase was also included in the assay mixture.

Definition of Enzyme Activity Unit

One activity unit is defined as 1 nmole of CO_2 liberated under standard assay conditions. Specific activity is defined as the number of activity units per milligram of protein. Total activity is the product of the specific activity units and total protein content expressed in milligrams.

Table 1. Inhibition of ADC by AGM in cucumber cotyledons in organ culture.[a]

Addition (mM)	Fresh weight gain (in mg)	Activity units/mg protein	Total activity units
Nil (control)	16.3 ± 3.7[b]	9.4	157
Agmatine (10)	21.3 ± 2.4	3.4 (63.9)[c]	54 (65.5)[c]

[a] Cultured for 12 h.
[b] Mean ± SD (n = 3).
[c] Values in parentheses represent percent of inhibition. Cucumber cotyledons were cultured with 10 mM AGM for 12 h and ADC activity was assayed.

Protein estimation was carried out according to Lowry et al. (1951), using bovine serum albumin as standard.

Results

Inhibition of ADC Activity by Agmatine

ADC assay was linear up to 2.0 mg protein included in the assay and 2 h. To investigate whether AGM has any influence on ADC activity, cucumber cotyledons were cultured with AGM (10 mM) for 12 h and the enzyme activity was measured subsequently. The specific activity of ADC in AGM-treated cotyledons was inhibited by 64% compared to that in the control cotyledons (Table 1). Morphologically, AGM-treated and the control cotyledons were indistinguishable even after 72 h of culture; neither a decrease in total protein content nor a change in the fresh weight of the AGM-treated cotyledons was observed. Total enzyme activity exhibited alterations in parallel with the specific activity in all the experiments described herein, thus indicating that the fluctuations recorded in both the activities reflect the true picture of enzyme content, uninfluenced by possible changes in the total reserve protein stores of the cotyledons during the culture period.

Inclusion of as low as 1 mM AGM in the culture medium significantly decreased ADC activity to 81%. Maximum inhibition (50%) in a 12-h culture was attained at 10 mM; beyond this amine concentration, no further inhibition could be observed (Fig. 1).

The specific activity of ADC decreased progressively with time on culturing with AGM. The guanidoamine exerted marginal, yet consistent, inhibitory influence as early as 3 h. Maximum inhibition (~50%) was recorded at 12 h, and this decreased ADC activity was sustained at this level for nearly 24 h. After this period, the enzyme activity gradually returned to the control values during the next 48 h (Fig. 2).

Effect of Di- and Polyamines

Under the optimal culture conditions for ADC inhibition by AGM (*viz.*, 12 h and 10 mM), a number of other amines were tested for their ability to influence

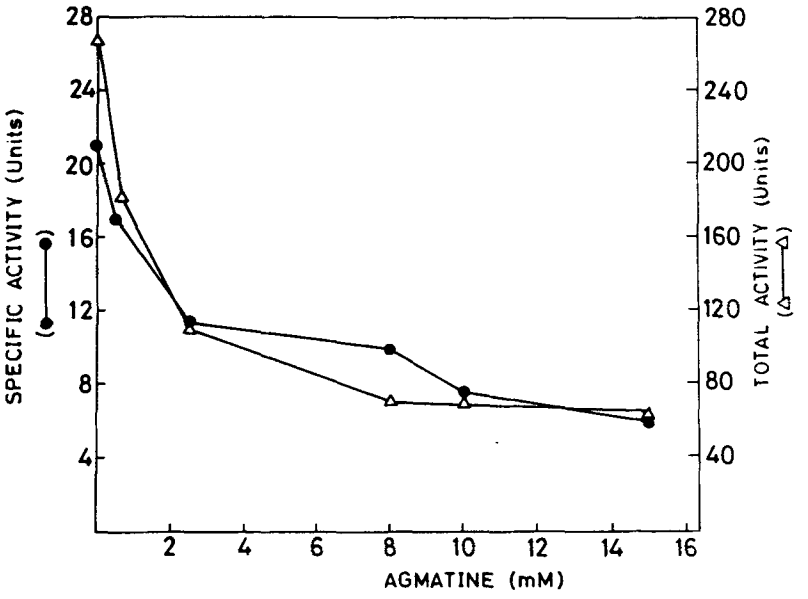

Fig. 1. Concentration-dependent inhibition of ADC by AGM in cucumber cotyledons in organ culture. AGM at indicated concentrations was included in the culture medium and incubated for 12 h.

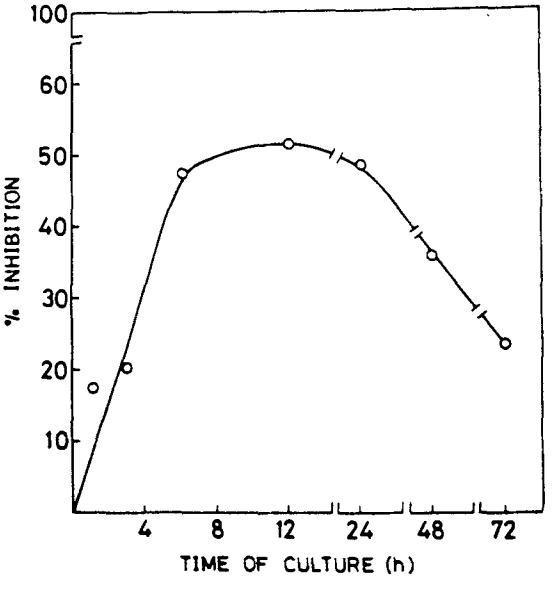

Fig. 2. Time course of *in vitro* inhibition of ADC by AGM in cucumber cotyledons in organ culture.

the enzyme activity (Fig. 3). Putrescine was the most potent amine (65% inhibition) in this regard , and NCP appeared to be least effective. Other amines, spermidine, spermine, cadaverine and 1,3-diaminopropane were also inhibitory to the extents of 48%, 55%, 60%, and 58%, respectively.

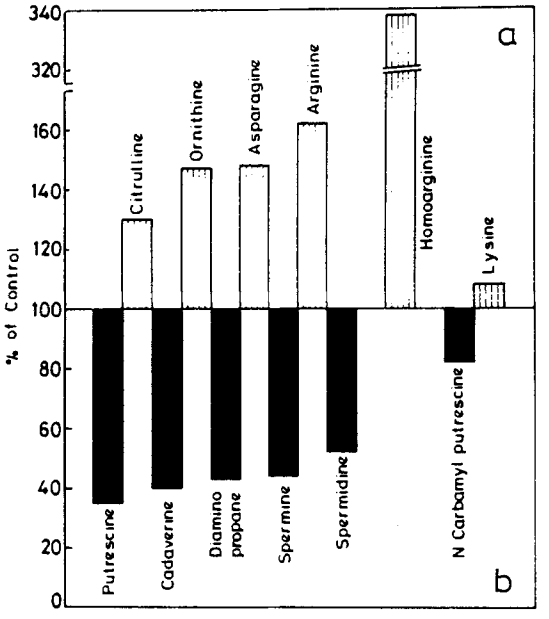

Fig. 3. Effect of amino acids (a) and amines (b) on ADC activity of *Cucumis sativus* cotyledons.

Effect of Amino Acids

The various amino acids added to the culture medium at 10 mM concentration during a 12-h incubation period produced different effects (Fig. 3a). Lysine increased ADC activity only marginally. Although ornithine (146%), citrulline (148%), arginine (162%), and asparagine (148%) stimulated ADC activity compared to the control, the most dramatic rise in ADC was brought about by L-homoarginine (HARG). Maximum stimulation of the enzyme activity (3- to 4-fold) by homoarginine was registered at around 12 h of culture. Interestingly, no parallel increase in fresh weight of the treated cotyledons was noticed.

Time Course of Enhancement of ADC Activity by HARG

A twofold increase in ADC activity could be elicited by 8 h after adding 10 mM HARG to the culture medium. Maximum of three- to fourfold stimulation of the enzyme activity was measured by 12 h; thereafter the enzyme activity tended to decline significantly. But even at 24 h considerably elevated ADC activity was encountered when compared to corresponding control values. Enhancement in total activity also followed a similar time course (Fig. 4).

Effect of HARG Withdrawal

To establish whether the continued presence of HARG is required in the culture medium for sustained stimulation of ADC activity, cotyledons were exposed to HARG for specified periods and subsequently transferred to the amino acid–

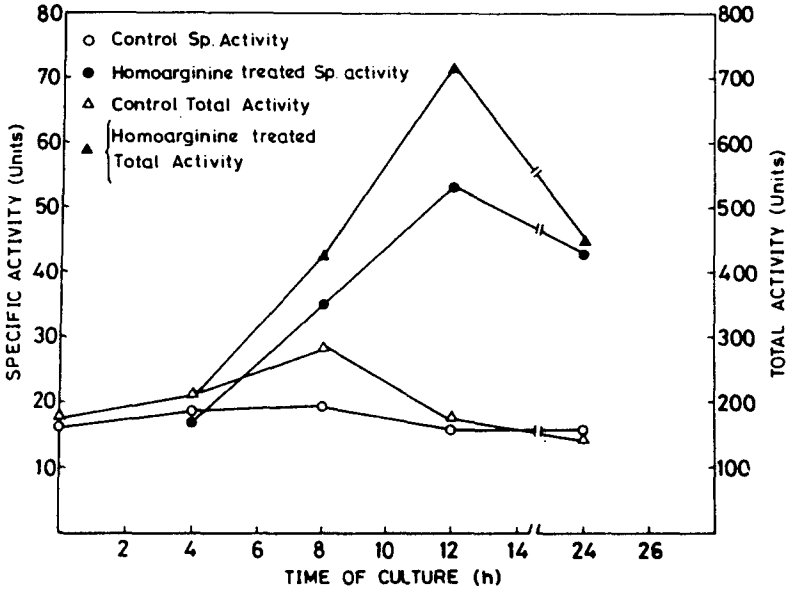

Fig. 4. Time course of ADC induction by 10 mM HARG.

free medium. After 4, 8, and 12 h in the presence of homoarginine, the washed cotyledons were incubated with the fresh medium for 8 h and 4 h in case of the first two sets (i.e., a total period of 12 h) and 12 h more in case of third set (i.e., 24 h total). In those sets of cotyledons that received HARG for 4 and 8 h harvested at 12 h, ADC activity was higher by 2.6- and 3.4-fold respectively over the corresponding controls. But the third set exhibited relatively lower ADC levels (increase of 2.6-fold) despite the fact that incubation with HARG was prolonged. This value of ADC activity was comparable to that assayed in 24-h homoarginine-treated sample (Fig. 5a,b). These observations clearly show that prolonged exposure to HARG is not a prerequisite for eliciting the maximum response.

Effect of Mixing of Crude Enzyme Extracts of Untreated Cotyledons with Those from AGM- and HARG-treated Cotyledons on ADC Activity

Upon mixing equal volumes of crude dialyzed enzyme preparation from the control cotyledons with those from AGM- or HARG-treated cotyledons, the resultant specific activities were mere mathematical means of the individual experimental values (Table 2). The numbers in parenthesis are the expected values if no macromolecular factor is involved in the observed modulation of enzyme activity.

Effect of Cycloheximide on ADC Activity

To test whether altered ADC activity during either AGM-dependent inhibition or HARG-mediated increase was dependent on *de novo* protein synthesis,

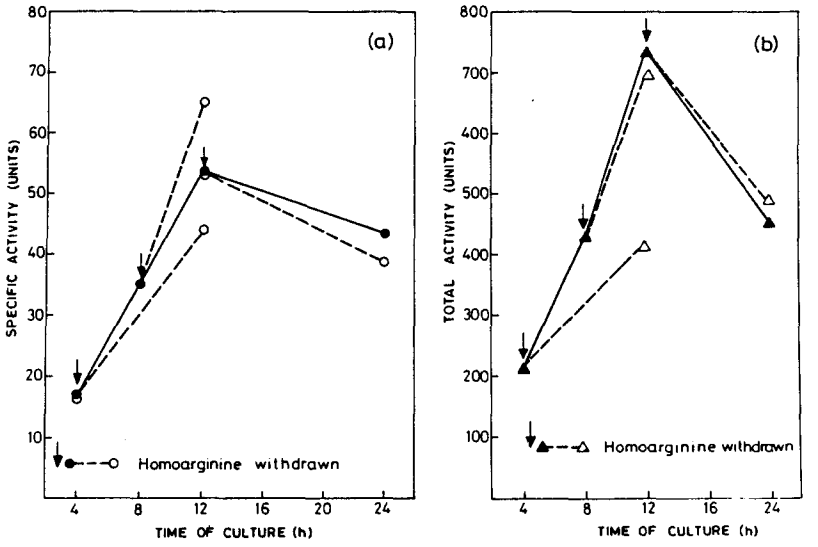

Fig. 5. Effect of HARG withdrawal on ADC activity of *Cucumis sativus* cotyledons. Cucumber cotyledons were incubated with 10 mM HARG for different lengths of time. At indicated time points cotyledons were washed with culture buffer (minus HARG) and transferred to HARG-free culture medium, and incubation was continued till the tissue was processed for crude enzyme extracts. (a) ADC specific activity. (b) ADC total activity. Normal time course is indicated by solid line.

Table 2. Effect of mixing of crude extracts of untreated cotyledons with those from AGM- and HARG-treated cotyledons on the activity of ADC.

Sl. No.	Treatment	ADC activity units/mg protein
1	Nil (control)	12.1
2	AGM (10 mM)	5.6
3	HARG (10 mM)	37.9
4	1 + 2	9.3 (8.8)[a]
5	1 + 3	23.7 (25.5)[a]

Cotyledons were cultured with AGM or HARG for 12 h, and the crude enzyme extracts were prepared separately. Equal volumes of control and treated extracts were mixed prior to enzyme assay.

[a] Values in parentheses are expected specific activities.

cycloheximide at 25 μg/ml concentration was included in the culture medium at the beginning of incubation with either of the effectors. Under these conditions the basal activity in the control cotyledons remained unaltered, whereas the stimulatory effect of HARG was completely abolished (Table 3). On the other hand, AGM exerted its inhibitory influence even in the presence of cycloheximide. Morphologically, the cycloheximide-treated cotyledons were similar to the control plant tissue when examined at 12 h of incubation.

Table 3. Effect of cycloheximide on ADC activity of cucumber cotyledons.

Treatment	ADC activity units/mg protein	
	− Cycloheximide	+ Cycloheximide
Nil (control)	12.6	10
AGM (10 mM)	7.5	6.0
HARG (10 mM)	48.7	11.0

Cycloheximide at 25 μg/ml concentration was added along with AGM or HARG or alone at the beginning of 12-h culture.

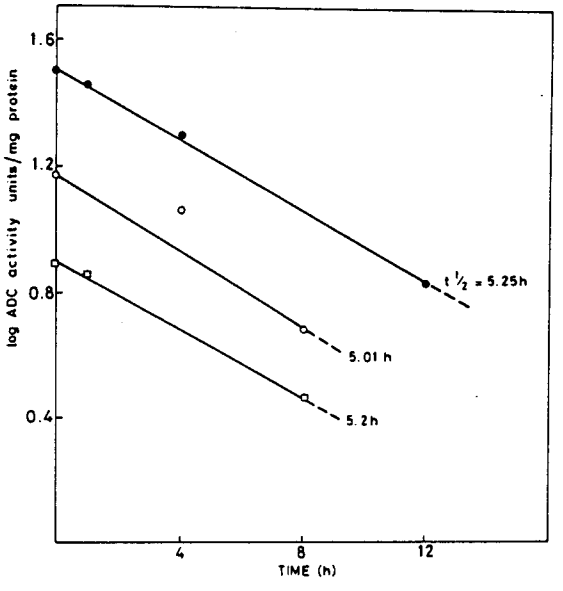

Fig. 6. Half-life of ADC of *Cucumis sativus* cotyledons. Cotyledons were incubated with HARG or AGM for 12 h and then transferred to a medium containing cycloheximide (25 μg/ml) and either of the effectors. At different time points cotyledons were harvested and the rate of disappearance of ADC activity was followed. Half-life was computed by least-squares method. ○——○, Control; ●——●, HARG; □——□, AGM.

Rate of Disappearance of ADC Activity in Control and HARG- and AGM-treated Cotyledons

Cotyledons pretreated with HARG or AGM (10 mM each) for 12 h were thoroughly washed and subsequently incubated with cycloheximide (25 μg/ml) for different periods of time. The enzyme activity decayed with the half-life ($t_{1/2}$) of about 5 h in control and HARG- and AGM-treated cotyledons, indicating that the rate of degradation of the enzyme does not account for the observed alternations in enzyme activity (Fig. 6).

Effect of Treatment with HARG and AGM In Vivo *on the Kinetic Parameters of ADC*

When HARG enhanced ADC activity threefold, it also lowered the K_m for arginine to a similar extent (Table 4). AGM treatment resulted in a twofold

Table 4. Kinetic parameters of ADC prepared from control and AGM- and HARG-treated cucumber cotyledons

Treatment	K_m (mM)	V_{max}[a]
Nil (control)	0.83	3.5
AGM (10 mM)	1.45	2.1
HARG (10 mM)	0.31	4.8

[a] V_{max} expressed as nmole of CO_2 liberated/h/mg protein.

Table 5. Effect of BA and potassium on HARG-induced ADC activity of cucumber cotyledons.

Treatment	ADC activity (units/mg protein)
Nil (control)	10.2
HARG (10 mM)	15.0
BA (30 μM)	19.0
BA (30 μM) + HARG (10 mM)	23.0
Potassium (30 mM)	7.1
Potassium (30 mM) + HARG (10 mM)	7.5

Cotyledons were incubated with BA or K^+ for 60 h, followed by 12-h culture with HARG + potassium or HARG + benzyladenine mixture. To one set, HARG was added only after 60 h of culture during which period cotyledons were not exposed to either BA or K^+ and ADC activity was assayed in the cotyledons after 12 h (i.e., total of 72 h) of culture.

increase of ADC K_m with respect to arginine. V_{max} of the enzyme did not change significantly under these conditions.

Effect of HARG on Benzyladenine- and Potassium-Modulated ADC Activity

Cotyledons were cultured with benzyladenine (BA) or KCl for 60 h in order to elicit maximum response in terms of ADC activity. At the end of 60 h, cotyledons were washed and incubated with either HARG plus BA or HARG plus K^+. HARG (10 mM) alone was also added to one set which was not exposed to either BA or K^+; similarly, one set each of BA-treated cotyledons and K^+-treated cotyledons were not exposed to HARG. At the end of a total culture period of 72 h, cotyledons were processed and ADC activity was measured. As is clear from Table 5, the enhanced response to BA and HARG together was additive in terms of ADC activity. On the other hand, HARG in the presence of potassium failed to bring about an increment in ADC activity since the ADC activity in potassium plus HARG plant tissue was comparable to that in presence of K^+ alone. This is in agreement with the earlier finding of Suresh et al. (1978) that potassium exerts an "overriding" influence over other modulators of ADC activity.

Discussion

Prompted by earlier observations (Flores and Galston 1982, Murthy et al. 1971,

Suresh et al. 1978, Young and Galston 1983) that plant growth regulators and other environmental factors alter ADC activity and hence putrescine titers, attention was focused on the possible modulatory influence of various amines and amino acids on ADC activity in cucumber cotyledons in organ culture. A short-term culture system was preferred for this purpose to obviate generalized pleiotropic responses attendant on growth and differentiation. The data discussed above represent the first demonstration that in higher plants, ADC behaves like ODC in terms of the modulatory influence of low-molecular-weight effectors governing its activity *in vivo*.

The use of [U-^{14}C]arginine as the substrate for ADC assay raises the possibility that a part of $^{14}CO_2$ released may be contributed by ODC activity as a consequence of conversion of a portion of the labelled substrate to ^{14}C-ornithine through arginase-type of activity since arginase-urease type of activity has been demonstrated in *Cucurbita* seedlings (Lignowski and Splittstoesser 1971) and ODC activity is shown to be modulated during growth in some specialized plant tissues (Altman et al. 1983). However, it has been unequivocally established earlier (Suresh et al. 1978) that under the standard assay conditions employed here for ADC quantification, $^{14}CO_2$ liberated with [U-^{14}C]arginine as the substrate reflects true ADC activity and is uninfluenced by arginase-urease type of reaction. Furthermore we have been unable to measure ODC activity in crude extracts of cucumber cotyledons when [1-^{14}C]ornithine is used as the substrate.

It is clear from the data of Fig. 1 that the dose-dependent inhibition of ADC by AGM is rather transient, notwithstanding the continued presence of the guanidoamine in adequate amounts and prolonged periods in the culture medium. Another noteworthy feature is that the maximum inhibition in ADC activity elicited by this amine is limited to around 50% of the control value. It is conceivable that a variety of factors either singly or in combination may account for the observed short-lived and restricted inhibition; these may include limited uptake and hence inadequate intracellular amine accumulation, increased clearance due to catabolism through activated amine oxidase-mediated reactions to ineffective products, and/or accelerated depletion due to channeling through the polyamine biosynthetic pathway. However, the observation that putrescine and polyamines themselves are also more or less equipotent inhibitors of ADC *in vivo* (Fig. 3b) would argue against the last-mentioned alternative as the predominant mechanism. The possibility that the transient nature of agmatine inhibition of ADC is related to its instability in the medium appears remote since a considerable portion of added amine could still be recovered intact in the medium after 12 h of incubation. The effective concentrations of various amines required to inhibit *in vitro* the purified ADC from higher plants (e.g., *L. sativus*—Ramakrishna and Adiga 1975) are higher by an order of magnitude than those capable of eliciting a comparable degree of *in situ* inhibitions, when included in the culture system. This situation is analogous to observations with the HTC cell line in which low concentrations (10^{-5} M) of exogenous putrescine effectively inhibited ODC activity (McCann et al. 1977b). Furthermore, the finding that the addition of cycloheximide to AGM-inhibited cotyledons did not decrease the rate of ADC inactivation (as measured by $t_{1/2}$; Fig. 6) would favor the hypothesis that the inhibition in ADC

activity may not be primarily due to enhanced rate of enzyme catabolism. Results presented in Table 3 also indicate that agmatine inhibition of ADC activity does not require new protein synthesis. Similar observations have been made (Clark and Fuller 1975, McCann et al. 1977ab) in different mammalian cell cultures wherein putrescine modulated ODC levels. The apparent increase in K_{mArg} of ADC observed (Table 4) would imply additional mechanisms of enzyme inactivation such as involvement of a macromolecular inhibitor in influencing the substrate-active site interaction (Fong et al. 1976). It is pertinent to recall that when higher concentrations of putrescine are added to animal cell cultures, an ODC-antizyme induced *de novo* comes into prominence with consequential more pronounced inhibition of ODC (McCann 1980). Whether such a mechanism is also operative in this plant system is unexplored at present and awaits further study. If in fact such an inducible ADC antizyme is operative in the plant system, its accumulation in free state in cucumber cotyledon system when AGM inhibited ADC activity appears remote in view of the outcome of the mixing experiments reported in Table 2.

It is noteworthy that among various amino acids that enhanced ADC activity in this plant culture system, HARG is the most potent entity (Fig. 3a). Again, as in the case of AGM-mediated ADC inhibition, HARG-enhanced ADC activity is also transient (Fig. 4) and the continued presence of the guanido amino acid in the culture medium is not essential for the maintenance of the inductive response (Fig. 5a,b).

Since cycloheximide could completely annul the stimulatory influence of HARG, it would appear that this enhanced ADC activity is primarily ascribable to augmented synthesis of the enzyme protein itself. However, this does not at present rule out additional mechanisms like posttranslational modifications of the enzyme protein (cf. ODC—Canellakis et al. 1979, Kuehn and Atmar 1982, Mitchell 1983, Russell 1982); lowered K_m value of induced ADC activity for its substrate is in fact in line with the latter possibility. Of interest is our earlier observation that HARG is a potent competitive inhibitor of the purified ADC from *L. sativus* seedlings (Ramakrishna and Adiga 1975). Furthermore, in mammalian systems, ODC and SAMDC activities assayed in cell-free extracts are dramatically enhanced when the cells are exposed to their corresponding competitive inhibitors (α-hydrazinoornithine [Harik et al. 1974] or α-methylornithine [McCann *et al.* 1977] for ODC and methylglyoxal bis [guanylhydrazone] for S-adenosyl-L-methionine decarboxylase [Pegg 1979], respectively) *in vivo*. This paradoxical phenomenon of inhibition of polyamine elaboration *in vivo* and enhanced enzyme activity *in vitro* has been traced to the considerable stabilization of the enzyme protein. However, in the present study, the data of Fig. 6 clearly show that HARG-enhanced ADC activity in cucumber cotyledons is not a reflection of altered half-life and that alternative mechanisms may be operative. Since cycloheximide could effectively block the increased ADC activity under these conditions, enzyme synthesis is presumably enhanced by HARG. This response conceivably is the reflection of an adaptive mechanism generated by the plant system to offset the depleted putrescine and polyamine levels *in vivo* precipitated by homoarginine functioning as a competitive inhibitor of ADC (Ramakrishna and Adiga 1975). A similar explanation has been offered when α-methylornithine inhibited ODC

activity in HTC cells (McCann et al. 1977a). Whatever the mechanism, the present study has shown that HARG-augmented ADC activity in the cucumber cotyledon system perhaps represents the most potent stimulation of ADC observed hitherto in any plant system in such a short time.

That multiple factors can interact to modulate the plant ADC activity *in vivo* is also evident from the data of the Table 5, which clearly show that HARG-enhanced ADC activity is additive to that consequent on cytokinin-stimulated growth and differentiation. However, interestingly HARG fails to stimulate ADC activity *in vivo* when high concentrations of K^+ strongly suppress the ADC activity. The overriding influence of K^+ on ADC activity has been noted earlier (Suresh et al. 1978), when the cytokinin stimulated growth and differentiation synergistically with K^+, but ADC activity remained blocked. It would therefore appear that strong control exercised by K^+ in this plant system is a major factor to be reckoned with in understanding the molecular mechanisms governing polyamine synthesis in higher plants.

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