Arginine Decarboxylase and Polyamines Required for Embryogenesis in the Wild Carrot

Abstract. *Embryogenic cultures of* Daucus carota *treated with 1 millimolar α-difluoromethylarginine, a specific inhibitor of arginine decarboxylase, exhibited nearly a 50 percent reduction in embryo formation compared with controls. Putrescine and spermidine concentrations in the treated cells were greatly reduced. Addition of putrescine, spermidine, or spermine to the culture medium restored embryogenesis in the treated cultures. Embryogenesis was not significantly affected by α-difluoromethylornithine, an inhibitor of ornithine decarboxylase. These results suggest that polyamines have a major function in plant embryo development and that the wild carrot synthesizes polyamines through the biosynthetic pathway involving arginine decarboxylase rather than ornithine decarboxylase.*

Polyamines are ubiquitous compounds in plants, animals, and bacteria. Mammalian cells synthesize the diamine putrescine, the precursor of the polyamines spermidine and spermine, from ornithine through ornithine decarboxylase (ODC) (E.C. 4.1.1.17). Changes in the concentration of polyamines and their biosynthetic enzymes accompany growth and differentiation in animal systems (*1, 2*). Many of the studies demonstrating polyamine functions have been made possible through the use of inhibitors of polyamine biosynthesis, such as α-difluoromethylornithine (DFMO), which selectively and irreversibly inhibits ODC (*3*). Inhibition of polyamine biosynthesis blocks differentiation or embryo development in invertebrates, amphibians, birds, and mammals, as well as differentiation in cultures of mammalian cells (*4, 5*). Embryo development in mice, rats, and rabbits and differentiation of cultured fibroblasts into adipocytes can be blocked by DFMO, and these effects can be reversed by the addition of polyamines (*4, 6*).

Polyamines stimulate DNA, RNA, and protein synthesis in plants as in animals, and elevated concentrations of polyamines are associated with rapidly proliferating cell cultures of tobacco and with plant parts undergoing rapid cell division, such as meristems of tomatoes and of potatoes (*7*). Active metabolism of polyamines has also been noted during the development and germination of *Phaseolus* and rice seeds (*8*). Unlike animal cells, plants synthesize putrescine through ODC and from arginine through an alternative pathway involving arginine decarboxylase (ADC) (E.C. 4.1.1.19). Although ODC activity has been associated with proliferative growth in plants and ADC is responsible for the increase in putrescine in oat leaves subjected to an osmotic stress (*7, 9*), it is not known which of the enzymes is involved in plant embryo development. Cohen *et al.* (*10*) recently used DFMO to inhibit ODC activity and fruit development in tomatoes; development was restored by applications of putrescine. Montague and co-workers (*11*) studied polyamine metabolism in embryogenic cultures of the carrot, a model system in which cells can easily be induced to differentiate into somatic embryos capable of forming a normal plant (*12*). Elevated levels of polyamines and ADC activity were closely correlated with the occurrence of embryogenesis in the cultures. We used embryogenic cultures of the wild carrot (*Daucus carota* L.) and the inhibitors of polyamine biosynthesis α-difluoromethylarginine (DFMA) and DFMO, which inhibit ADC and ODC, respectively (*3, 13*), to further investigate the role of polyamines in plant embryo differentiation.

A wild carrot cell line (obtained from D. Wetherell), derived from a petiole explant maintained as a suspension culture, served as the experimental tissue. Embryo development was initiated in the cultures by screening the culture to yield cell aggregates of a specific size (63 to 125 μm) and subculturing these cells in a medium lacking exogenous growth regulators (*12*). After 23 days of growth the embryos in the cultures were counted. At a concentration of 0.5 m*M* in the medium, DFMA did not significantly reduce embryogenesis, but higher concentrations of the inhibitor were capable of reducing and blocking embryo induction (Fig. 1A). Putrescine restored embryogenesis to control levels in cultures treated with 1.0 m*M* DFMA but not in cultures exposed to 5.0 m*M* DFMA. This high concentration of DFMA may have been toxic to the cells, because embryo-

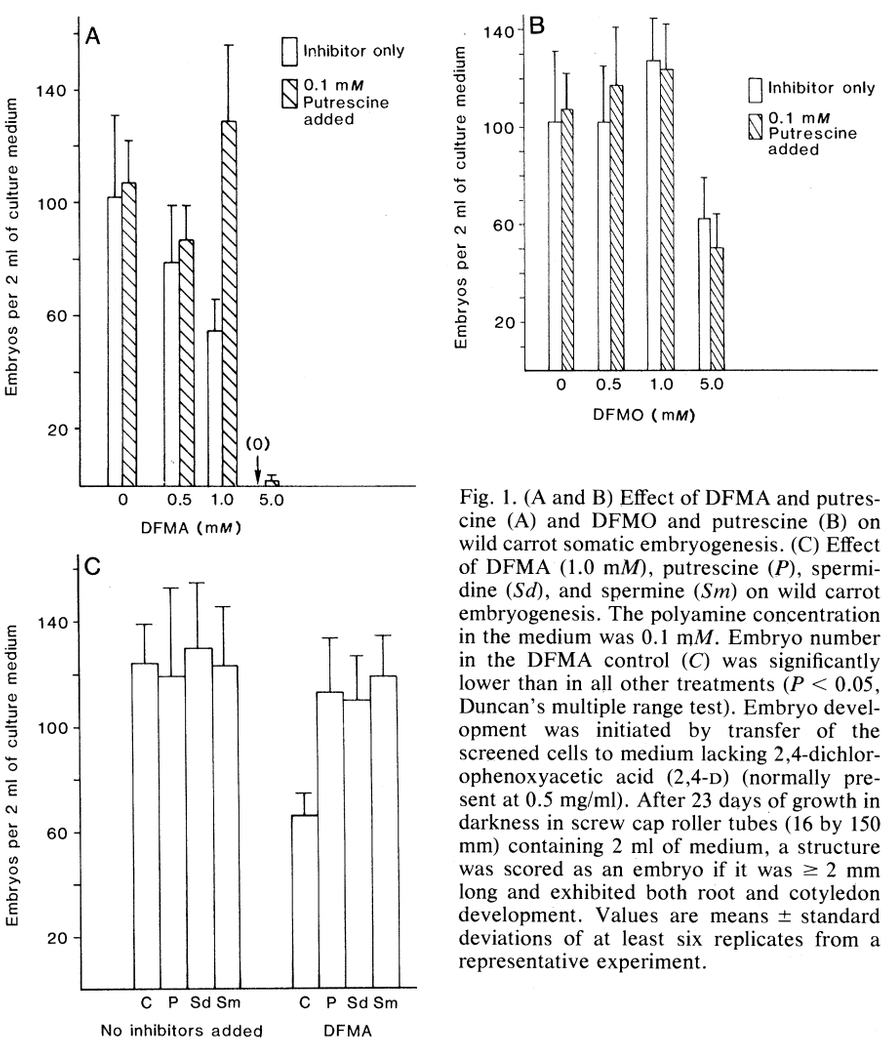

Fig. 1. (A and B) Effect of DFMA and putrescine (A) and DFMO and putrescine (B) on wild carrot somatic embryogenesis. (C) Effect of DFMA (1.0 m*M*), putrescine (*P*), spermidine (*Sd*), and spermine (*Sm*) on wild carrot embryogenesis. The polyamine concentration in the medium was 0.1 m*M*. Embryo number in the DFMA control (*C*) was significantly lower than in all other treatments ($P < 0.05$, Duncan's multiple range test). Embryo development was initiated by transfer of the screened cells to medium lacking 2,4-dichlorophenoxyacetic acid (2,4-D) (normally present at 0.5 mg/ml). After 23 days of growth in darkness in screw cap roller tubes (16 by 150 mm) containing 2 ml of medium, a structure was scored as an embryo if it was ≥ 2 mm long and exhibited both root and cotyledon development. Values are means ± standard deviations of at least six replicates from a representative experiment.

Table 1. Effect of DFMA on wild carrot polyamine concentrations. Polyamines were determined in 5 percent perchloric acid extracts of tissue from 6-day-old cultures. We previously determined that polyamine concentrations in our wild carrot cultures are elevated on day 6. Embryogenesis was initiated as described in the legend to Fig. 1, except that 250-ml Erlenmeyer flasks containing 50 ml of medium were used to yield an adequate amount of tissue for analysis. Benzoylated derivatives of the polyamines were separated and measured by high-performance liquid chromatography (*9, 21*). Values are means ± standard deviations of quadruplicate determinations and are expressed as nanomoles per gram (fresh weight). N.D., none detected.

Treatment	Putrescine	Spermidine	Spermine
Control	890 ± 121	506 ± 46	66 ± 25
DFMA (1.0 m*M*)	N.D.	39 ± 16	260 ± 38
DFMA (1.0 m*M*) + putrescine (0.1 m*M*)	185 ± 18	463 ± 143	115 ± 18
DFMO (1.0 m*M*)	645 ± 69	415 ± 17	66 ± 10

genesis was not restored in these cultures even when higher concentrations of putrescine were added (> 0.1 m*M*). DFMO did not significantly inhibit development except at 5.0 m*M*, which was judged to be toxic (Fig. 1B). These findings suggest that ODC does not have a major role in wild carrot embryogenesis.

Putrescine, spermidine, or spermine were equally effective in restoring embryogenesis in DFMA-treated cultures (Fig. 1C). Metabolic interconversions of the polyamines back to putrescine are possible and may account for these results, although spermidine can serve as a polyamine source in ODC-deficient hamster cells and spermidine levels may be more closely correlated to growth in bacteria than is putrescine (*14*). Studies of oat leaf senescence have suggested that spermidine and its biosynthetic enzyme spermidine synthase may be physiologically important (*15*). We recently observed that dicyclohexylammonium sulfate and methylglyoxal bis(guanylhydrazone), which reduce spermidine synthesis by inhibiting spermidine synthase and *S*-adenosylmethionine decarboxylase, respectively (*14, 16*), can also reduce wild carrot embryogenesis.

Because DFMA is an effective inhibitor of ADC in certain bacterial species (*13*), we determined its effects on wild carrot ADC activity and cellular polyamine concentrations. The effect of DFMA on wild carrot ADC was tested in an in vitro enzyme assay in which ADC activity was determined by measuring the release of $^{14}CO_2$ from [1-^{14}C]arginine (*7, 17*). DFMA inhibited in vitro ADC activity more than 88 percent when present at concentrations higher than 0.005 m*M* (Fig. 2). The inhibitor had a strong effect on free polyamine concentrations in the cultured tissues, decreasing putrescine below our limit of detection and causing a more than tenfold reduction in spermidine (Table 1). The spermine titer rose significantly in the treated cultures, possibly because of increased activity of enzymes leading to spermine biosynthesis (*4*). Addition of putrescine to DFMA-treated cultures at concentrations that restored embryogenesis increased putrescine and restored the spermidine level to that of untreated cells; this is more evidence that spermidine may be physiologically important. These results indicate that DFMA can be used to inhibit ADC in wild carrot tissues and in other plants as well, as DFMA was recently shown to prevent the increases in ADC activity and polyamine concentrations in stressed oat leaves (*9*). Although DMFO can inhibit wild carrot ODC, it did not dramatically reduce putrescine or spermidine (Table 1). Since ADC activity was ten times higher than ODC activity in our cultures, it appears that DFMO did not reduce embryogenesis (except when present at toxic levels) because of the relatively minor contributions of ODC to putrescine biosynthesis.

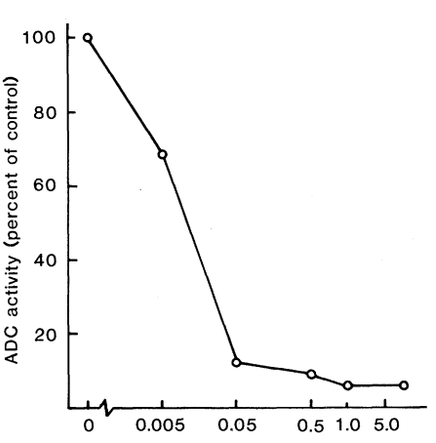

Fig. 2. Effect of DFMA on in vitro ADC activity. Various amounts of DFMA were added to centrifuged tissue extracts. Samples were incubated with the inhibitor for 15 minutes at 30°C before the [^{14}C]arginine substrate was added (*17*). Tissue from unscreened 4-day-old wild carrot stock cultures (0.5 mg per milliliter of 2,4-D) served as the source of ADC. Values are means of triplicate determinations. Similar results were obtained in tests of the effects of DFMO on ODC activity. DFMA was found to have no effect on ODC, and DFMO did not significantly affect ADC activity

Our findings show that DFMA can effectively inhibit embryogenesis in suspension cultures of wild carrot cells. This inhibition of development by DFMA, which acts by decreasing ADC activity and lowering putrescine and spermidine concentrations in the cells, demonstrates that putrescine or spermidine are among the factors essential for embryogenesis. The importance of polyamines in development was also recently demonstrated in tobacco plants having altered polyamine metabolism (*16*). Mutant plants regenerated from a cell line resistant to methylglyoxal bis(guanylhydrazone) developed flowers with anthers in place of ovules. Baker *et al.* (*18*) have recently showed that the activity of ornithine carbamoyltransferase (E.C. 2.1.3.3), an enzyme leading to the biosynthesis of arginine, increased shortly after the induction of embryogenesis in wild carrot cultures. This did not occur in cultures that had lost their embryogenic capacity, providing additional evidence that arginine, ADC, and the polyamines are involved in wild carrot embryogenesis.

Of the two alternative pathways leading to polyamine biosynthesis in plants, it appears that the route involving ADC rather than ODC is utilized in wild carrot embryo development. ODC has been associated with proliferative growth in plants (*7*), but preliminary data from several trials in our laboratory suggest that inhibition of ADC with DFMA affected only embryo differentiation and not culture growth as measured by fresh weight.

Our observations are consistent with the effects of inhibitors of polyamine biosynthesis on embryo development in animals (*4, 5*). Inhibition of polyamine biosynthesis does not block developmental processes in all systems, however. DFMO affected only proliferation and not differentiation of human leukemic cells and murine erythroid precursor cells (*19*). Differentiation of embryonal carcinoma cells in vitro was induced by DFMO, suggesting that polyamines may have widely diverse functions in different cell types (*20*). It appears, however, that polyamines and their biosynthetic enzymes are essential for differentiation in a number of species and cell types.

RUSSELL P. FEIRER*
GAIL MIGNON
JOHN D. LITVAY

Forestry Division,
Institute of Paper Chemistry,
Appleton, Wisconsin 54912

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17. We extracted ADC from approximately 100 mg of cells in 1 ml of cold extraction medium containing 10 m*M* Hepes, 1 m*M* dithiothreitol, and 1 m*M* EDTA. The assay was performed at *p*H 7.0, the *p*H at which wild carrot ADC exhibited optimum activity in our reaction system. Ten microliters of DL-[1-^{14}C]arginine (0.5 μCi, 12 mCi/mmole; Research Products International) was used as a substrate in the reaction.
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22. We thank Merrell Dow Research Institute for the generous gift of DFMA, D. Einspahr for his encouragement and support, and H. E. Flores, S. Wann, and others for reviewing the manuscript.

* To whom correspondence should be addressed.

19 December 1983; accepted 24 January 1984

Coronary Arteries of Cardiac Patients Are Hyperreactive and Contain Stores of Amines: A Mechanism for Coronary Spasm

Abstract. *Coronary arteries from hearts of cardiac patients contain significantly higher concentrations of histamine than do those from noncardiac patients. The coronary vessels of cardiac patients are also hyperresponsive to histamine and serotonin. These differences between groups of patients suggest an explanation for coronary artery spasm in heart disease.*

Coronary artery spasm is now recognized as a clinical entity implicated in heart disease (*1*). A number of reports point to a sudden sustained contraction of a large surface artery feeding the heart muscle in the initiation of some cases of myocardial infarction, angina pectoris, and sudden death (*2*). Coronary spasm has been observed repeatedly during angiographic examination of the hearts of subsets of cardiac patients, but no satisfactory explanation of the vascular derangement that might induce sudden and protracted tone changes and the ensuing myocardial hypoxia and cardiac damage is yet available.

In the study reported here we found that coronary arteries obtained postmortem from patients with a history of coronary artery disease and pathological evidence of myocardial damage respond to biogenic amines with contractions that are significantly larger than those of vessels from patients with no history of cardiac disease. In addition, we found that the coronary vascular tissue from cardiac patients contains stores of these amines, and one of them, namely histamine, is substantially elevated above control values from the arteries of noncardiac patients. We studied the coronary arteries of ten patients whose cause of death was attributed to coronary heart disease. Sudden cardiac death (less than 1 hour) was considered to have occurred in two of these patients. In eight of the ten patients autopsy revealed old or recent infarct damage (scarring or necrosis). In a control group of 18 patients death was attributed to accident (two cases), suicide (one case), carcinomatosis (six cases), hemorrhagic pancreatitis (one case), Hodgkins lymphoma (one case), cerebral or brain stem hemorrhage (three cases), hepatic or renal failure (two cases), atypical pneumonia (one case), and idiopathic aplastic anemia (one case). In only one of the 18 control patients did the postmortem reveal evidence of an old (unreported) infarct.

For biochemical studies portions of the right, left, and circumflex coronary arteries were removed within 10 hours of death (average 6.3 ± 0.5 hours in 20 patients) and immediately placed in chilled (4°C) and previously oxygenated Krebs-Henseleit solution. The tissues were transported to the laboratory in vacuum bottles and carefully placed in fresh, oxygenated and chilled Krebs solution. They were trimmed of all adherent fat and connective tissue, then minced and homogenized in either 5 percent trichloroacetic acid (TCA) (for 5-hydroxytryptamine and histamine) or *n*-butanol (catecholamines) by means of a Kinematica Polytron (full speed for 30 seconds at 0°C). The samples were allowed to stand for at least 10 minutes and then were centrifuged at 10,000*g* for 15 to 20 minutes at 0°C, and the supernatants were stored at −20°C.

5-Hydroxytryptamine (serotonin) was analyzed by the method of Somerville and Hinterberger (*3*), which is based on the development of fluorescence with orthophthaldialdehyde. Total catecholamines were determined by the ethylenediamine condensation method essentially as described by Ogasahara *et al.* (*4*). Histamine was analyzed by a slightly modified version of the method described by Håkanson and Ronnberg (*5*, *6*).

All values were corrected for dilution during the extraction procedures and are expressed as nanograms of amine per gram of wet tissue. The chemical analyses were performed on numbered samples without knowledge of the patient's medical history. At least two coronary vessel segments were assayed from each patient (between 800 and 1000 mg each), and the results were pooled to obtain a single mean value for each patient unless indicated otherwise.

The coronary arteries contained surprisingly high concentrations of serotonin and histamine but a low concentration of catecholamines, the latter probably reflecting a paucity of sympathetic innervation (Table 1). No significant differences between the two groups of patients in the concentrations of serotonin or of noradrenaline plus adrenaline (catecholamines) were detected, but the concentration of histamine was nearly doubled in the arteries of cardiac patients (Table 1). If the data are described on the basis of individual vessel segments, rather than by patients, the level of histamine in 18 vessels from cardiac patients was clearly elevated above that of 27 vessels from noncardiac cases (Table 1). The concentration of serotonin was slightly diminished in the arteries of cardiac patients if values are expressed in terms of individual vessel segments. The values for noradrenaline did not differ between the vessels of the two patient groups.

The differences in the histamine content between cardiac and noncardiac patients was not attributable to postmortem times which averaged 6.4 ± 0.8