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# Studies on the use of toxic precursor analogs of opines to select transformed plant cells

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**Summary.** S-(2-aminoethyl-)L-cysteine and L-canavanine were less toxic for octopine-type crown gall tissues that contained lysopine dehydrogenase than for other crown gall or habituated tissues. These analogs are substrates for lysopine dehydrogenase in vitro and in vivo. Thus toxic analogs of amino acid precursors of opines may be useful in selecting for cells that contain an opine dehydrogenase.

**Key words:** Selection – Amino acid analogs – Opines – Lysopine dehydrogenase – Crown gall – *Agrobacterium*

## Introduction

Crown gall tumours result from the transfer and integration into plant nuclear DNA of a 10–20 kilobase segment derived from a Ti-plasmid of a virulent *Agrobacterium tumefaciens* strain (see Kahl and Schell 1982 for reviews on the crown gall system). This transferred DNA codes for enzymes that catalyze the biosynthesis of new substances, generally called "opines", that are not present in uninfected plant cells. For example, lysopine dehydrogenase, which is usually present in octopine-type tumors, catalyzes the reductive condensation of pyruvate and L-arginine, L-lysine, L-histidine, or L-ornithine to yield octopine, lysopine, histopine, or octopinic acid, respectively (Fig. 1). Nopaline dehydrogenase catalyzes similar types of reactions in nopaline-type tumors, resulting in reductive condensation of alpha-ketoglutarate and arginine or ornithine to yield nopaline or nopalinic acid.

This paper presents our initial results on the use of toxic analogs of the amino acid precursors of the octopine family of opines to select transformed cells. These studies were based on the idea that a toxic amino acid might be metabolized in vivo to a less toxic or non-toxic opine analog by lysopine dehydrogenase. Thus, transformed cells that contain lysopine dehydro-

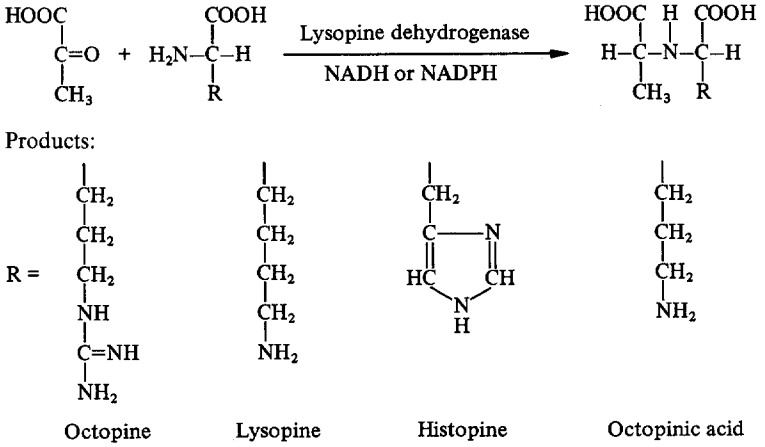



**Fig. 1.** Biosynthesis of octopine-type opines

genase might detoxify the amino acid analog and grow in the presence of amounts of the analog that are inhibitory for cells that do not contain this enzyme.

After this work was completed, Van Slogteren et al. (1982) reported that homoarginine was less toxic for transformed cells than for normal cells. Their work will be discussed in relation to the results presented here.

## Materials and methods

*Analogs tested*

The following analogs, for which the corresponding normal amino acids are given in parenthesis, were purchased from Sigma Chemical Company, St. Louis, MO: S-(2-aminoethyl-)-L-cysteine-HCl, "AEC", (L-lysine); L-canavanine-$SO_4$, (L-arginine); L-homoarginine-HCl, (L-arginine); and L-2,4-diamino-n-butyric acid-2HCl, (L-ornithine). 2-Thiazolyl-alanine, a histidine analog, was synthesized in the laboratory (Jones et al. 1950).

*Other chemicals*

Pyruvic acid, alpha-ketoglutaric acid, L-arginine-HCl, reduced beta-nicotinamide adenine dinucleotide (NADH), Orange G, phenanthrene quinone, and iodoplatinate spray reagent were obtained from Sigma. Sodium cyanoborohydride (90% practical grade) was purchased from FLUKA, Buchs, Switzerland.

*Tissue lines*

The following tissue lines were used: PSCG-B6, PSCG-15955, PSCG-C58, and HSSS, isolated from *Helianthus annuus* (sunflower) cv. 'Russian Mammoth' (Kemp 1982); 15955/1 and 15955/01, from *Nicotiana tabacum* (tobacco) cv. 'Xanthi' (Gelvin et al. 1982); E228 and Bo542, from *N. tabacum* cv. 'Samsun' (Sacristan and Melchers 1977); Braun's teratoma, from *N. tabacum* cv. 'Havana' (Braun and Wood 1976); A66, isolated from *N. tabacum* cv. 'White Burley' (Firmin and Fenwick 1978); and W-A6, W-B634, W-C58, and W-T37, isolated by J. Tourneur, CNRA, Versailles, from tumors incited on *N. tabacum* cv. 'Wisconsin 38' by octopine-type *A. tumefaciens* strains A6 or B634, or nopaline-type strains C58 or T37, respectively.

*Relative toxicities of amino acid analogs*

Aqueous solutions of the analogs were adjusted to pH 5.6–5.8 and sterilized by filtration (0.45 micron Millipore filter). Serial dilutions of each analog were added to cytokinin- and auxin-free agar medium (Linsmaier and Skoog 1965) that had been autoclaved and allowed to cool to about 60 °C. For initial screening of the analogs, three 100-mg pieces of 4–6-week-old tissue were planted on analog-containing medium in 55 mm Petri dishes. For the toxicity studies with the 15955/1 and 15955/01 tumor lines, three 50-mg pieces of 4–6-week-old tissue were planted on medium in 90 mm dishes. In all cases, duplicate or triplicate dishes were used for each tissue and each concentration of an analog. The tissues were weighed after 5–7 weeks of growth at 25 °C in the dark. Growth was expressed as a percentage of the fresh weight increase of the same tissue on medium that did not contain any analog or amino acid. Experiments were repeated after initial results delimited the range of concentrations to use for a given analog. Experiments with the 15955/1 and 15955/01 lines were repeated at least twice with the appropriate concentration ranges.

*Assays for opine dehydrogenases*

Lysopine dehydrogenase and nopaline dehydrogenase were assayed using modifications of previous methods (Birnberg et al. 1977; Goldman 1977; Hack and Kemp 1977; Lejeune 1967; Otten and Schilperoort 1978). Thus, for each mg of tissue in a 1.5-ml Eppendorf tube 3 µl of a buffered (0.15 M potassium phosphate, pH 6.9) reaction mixture was added which contained 20 mM L-arginine, 50 mM pyruvate, and 13.5 mM NADH for the lysopine dehydrogenase assay or 40 mM L-arginine, 40 mM alpha-ketoglutarate, and 5.5 mM NADH for the nopaline dehydrogenase assay. The tissue was carefully macerated in the reaction mixture with a glass rod and incubated at 25 °C for 1 h. The samples were then clarified by centrifugation and 2 µl of each supernate was spotted on Whatman 3MM paper. Up to 30 µl of sample was used to verify a negative assay result. The Whatman 3MM paper with the samples, as well as 1–10 µg of octopine or nopaline as an authentic standard and Orange G as a migration marker, was carefully wetted with electrophoresis buffer (formic acid/acetic acid/water; 3/6/91, v/v/v) and electrophoresed at 50–75 V/cm (Gilson High Voltage Electrophoresis Apparatus, Model D) for 10–20 min. The electrophoretograms were dried in a current of hot air and stained for octopine or nopaline with either the phenanthrene quinone reagent (Yamada and Itano 1966) or the Sakaguchi reagent (Easley 1965); the latter reagent was several-fold less sensitive than the former, but was more specific for guanidinyl compounds. Experiments showed that under our conditions the tissue extraction buffers used by other workers (Hack and Kemp 1977; Otten and Schilperoort 1978) did not improve assay results and in fact, retarded electrophoretic migration when larger sample volumes were spotted. Electrophoretic mobilities were measured from the origin (0) relative to Orange G (1.0).

*Chemical syntheses of putative enzymatic products*

AEC or L-canavanine-$SO_4$ (1 mmole) and pyruvate (1 or 3 mmole, respectively) were dissolved in 2–4 ml of water, the pH adjusted to 6–7, and sodium cyanoborohydride (3 mmole) added. After standing for 48 h at 25 °C, samples from the reaction mixtures were spotted on Whatman 3MM paper and electrophoresed as described above. Dried electrophoretograms were stained with ninhydrin or phenanthrene quinone for the canavanine reaction or with ninhydrin or iodoplatinate for the AEC reaction.

*Amino acid analogs as lysopine dehydrogenase substrates*

*In vitro.* An amino acid analog (20 mM) was used instead of L-arginine in the lysopine dehydrogenase assay to test for its ability to serve as a substrate for the enzyme. The analog and any enzymatic product formed were initially identified by ninhydrin staining of the electrophoretogram. Iodoplatinate, which reacts with thioethers (Easley 1965), was used (16 ml of Sigma reagent in 100 ml of acetone, used as a dip) to more specifically identify the enzymatic product formed from AEC. Canavanine and its enzymatically-formed reaction product reacted with ninhydrin and weakly with phenanthrene quinone. In some experiments, lysine was used, in addition to an analog, as a competitive substrate for lysopine dehydrogenase.

*In vivo.* A crown gall tumor tissue that had been grown on medium that contained an analog was macerated in a 1.5-ml

Eppendorf tube with a glass rod. After centrifugation, 2–10 µl of supernate was spotted on Whatman 3MM paper, electrophoresed as described above, and examined for the presence of a compound that had the same electrophoretic migration and staining properties as the opine analog that was formed in vitro, and the chemically-synthesized product.

## Results

### *Relative toxicities of various analogs to crown gall and habituated tissues*

Among the 14 habituated and crown gall tumour tissues that were on medium with varying levels of different analogs, there was an approximate correspondence between the presence of lysopine dehydrogenase activity and a relative decrease in the toxicity of certain analogs. For example, the maximum concentration of canavanine that still permitted growth was more than two-fold higher for a sunflower crown gall tumor line (PSCG-B6) that contained lysopine dehydrogenase than for an habituated sunflower line (HSSS) that did not contain this enzyme. The differences with AEC were even greater: the maximum tolerated concentration of AEC in the medium was at least several-fold greater for tissues that contained lysopine dehydrogenase than for tissues that did not. The presence of lysopine dehydrogenase in a tissue did not appear to correlate with a reduction in toxicity by homoarginine, but further work with this analog was pursued because it is an in vitro (Otten 1979) and an in vivo (Petit and Morel 1966) substrate for the enzyme. The relative differences in growth of tissues on medium that contained L-2,4-diamino-n-butyric acid or 2-thiazolyl-alanine were small and inconsistent from experiment to experiment; therefore, work with these analogs was abandoned. There was no correlation between the presence of nopaline dehydrogenase in a tissue and its ability to tolerate higher concentrations of any of the analogs tested.

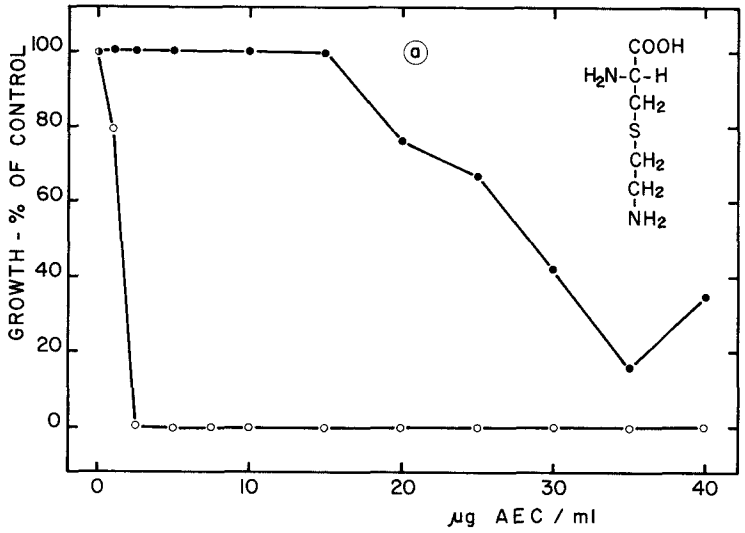
Crown gall tumor lines 15955/1 and 15955/01 were used for subsequent studies: these tissues are derived from single-cell clones obtained from tumors incited on *N. tabacum* cv. 'Xanthi' by *A. tumefaciens* strain 15955 (Gelvin et al. 1982). Both lines grow on medium that lacks cytokinins and auxins and both contain T-DNA. However, the T-DNA in line 15955/1 lacks an internal segment (Thomashow et al. 1980) and its corresponding mRNA transcript (Gelvin et al. 1982) that are present in line 15955/01. The former line also does not contain octopine, whereas the latter line does (Gelvin et al. 1982). We confirmed that lysopine dehydrogenase activity was present in line 15955/01 but not in line 15955/1. Thus, these tissues seemed to be ideal for testing whether or not toxic analogs of amino acid precursors of opines could be used to select lysopine dehydrogenase-containing cells.

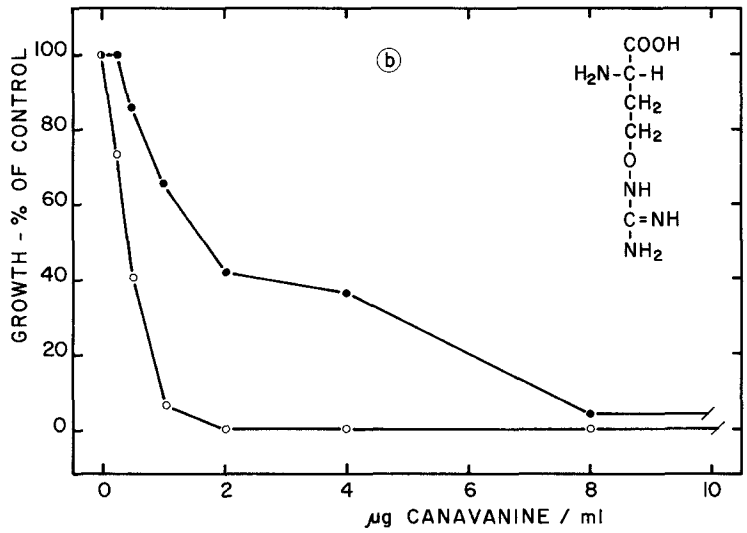
Figure 2 shows the toxicity curves obtained when lines 15955/1 and 15955/01 were grown on medium that contained varying concentrations of AEC, canavanine, or homoarginine. As was observed during the initial toxicity screenings with various habituated and crown gall tumor lines, AEC was by far the best of the analogs tested to select the tumor tissue that contained lysopine dehydrogenase. Thus, the maximum tolerated concentration of AEC in the medium was more than 20 times greater for the 15955/01 tumor line than for the 15955/1 line. The maximum tolerated concentration of canavanine was several-fold higher for the 15955/01 tissue than for 15955/1. Homoarginine affected the growth of the 2 tumor lines about equally, indicating that this is not a good analog for selection of cells containing lysopine dehydrogenase.

At carefully-chosen levels, the corresponding normal amino acids diminished the toxicities of the analogs. Thus, 50 µg of L-lysine per ml of medium completely eliminated toxicity by 10 µg of AEC/ml for the 15955/1 tissue, while not affecting its lack of toxicity for the 15955/01 tissue. Lysine itself had no toxicity for these tissues at concentrations less than or equal to 50 µg/ml. However, 100 µg of L-lysine/ml inhibited growth of the 15955/1 and 15955/01 tissues by 97% and 45%, respectively. L-Arginine counteracted the toxicity of L-canavanine. Whereas 8 µg of canavanine/ml of medium inhibited growth of the 15955/1 and 15955/01 tissues by 100% and 97%, respectively, the addition of 150 µg of arginine/ml to medium that contained the same level of canavanine resulted in growth inhibitions of 97% and 51%, respectively.

### *Amino acid analogs as lysopine dehydrogenase substrates*

*In vitro.* As shown in Fig. 3, AEC appears to be a substrate for lysopine dehydrogenase from crown gall tumor tissues. Thus, the 15955/01, but not the 15955/1 line, contained an enzyme that metabolized AEC in vitro to a new product that had the properties expected for $N^2$-(D-1-carboxyethyl-)-S-(2-aminoethyl-)-L-cysteine (or "carboxyethyl-AEC"): this product was formed only if pyruvic acid, NADH, AEC, and the appropriate ground tissue were simultaneously present in the reaction mixture. Omission of any of the components or boiling the tissue for 3 min before adding it to the reaction mixture led to loss of formation of the product. The electrophoretic mobility of the new product (–0.53) relative to that of AEC (–1.15) was as would be expected for carboxyethyl-AEC based on the electrophoretic mobility of the structurally-similar lysopine (–0.62) relative to lysine (–1.18). Further, both the new product and AEC gave white spots on a rose back-ground when electrophoretograms were



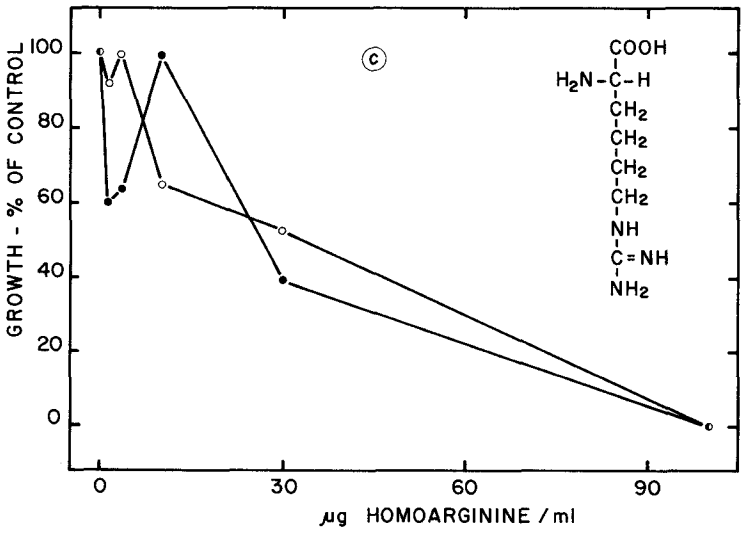




**Fig. 2a–c.** Growth of the 15955/1 (*open circles*) and 15955/01 (*closed circles*) tissue lines after 6 weeks on medium containing AEC **(a)** L-canavanine **(b)** or L-homoarginine **(c)** relative to the average weights of 7.5 g and 5.4 g, respectively, for the 15955/1 and 15955/01 tissues on analog-free medium

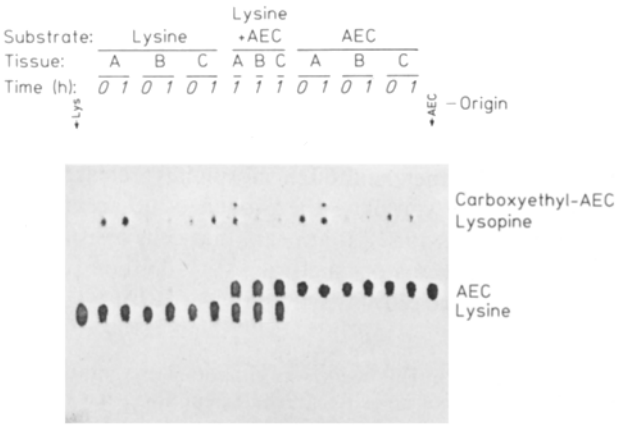



**Fig. 3.** Ninhydrin-stained electrophoretogram of lysopine dehydrogenase assays using 20 mM lysine and/or AEC as substrates in the presence of 15955/01 (*A*), 15955/1 (*B*), or PSCG-B6 (*C*) tissue extracts. The light ninhydrin spots on this electrophoretogram from the carboxyethyl-AEC formed in the presence of the PSCG-B6 tissue were difficult to photographically reproduce; other experiments confirmed the formation of lysopine and carboxyethyl-AEC in the presence of this tissue, although at lower rates than for the 15955/01 tissue. A small amount of ninhydrin-positive compound, which co-migrated with lysopine, was present in the 15955/1 tissue. However, this compound did not increase in amount during the assay and was degraded by acid hydrolysis (5 N HCl, 100 °C for 6 h); lysopine is stable under these conditions

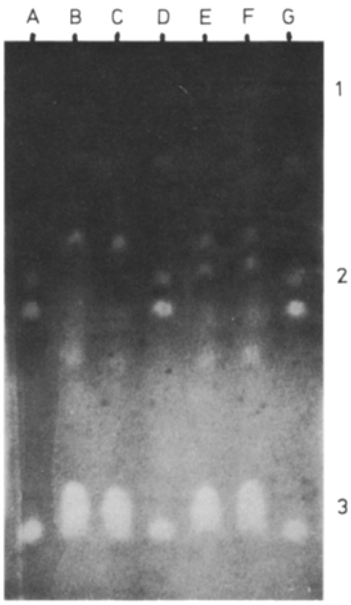



**Fig. 4.** Identification of carboxyethyl-AEC (*2*) on iodoplatinate-stained electrophoretograms of lysopine dehydrogenase assays 1 h after incubation of AEC (*3*) with ground 15955/1 (*B, C*) or 15955/01 (*E, F*) tissues, using the crude reaction mixture of chemically-synthesized carboxyethyl-AEC as a standard (*A, D, G*). Migration is from the origin (*1*) towards the cathode

stained with iodoplatinate (Fig. 4), which reacts with such thioethers (Easley 1965).

Canavanine was also an in vitro substrate for lysopine dehydrogenase. The product had the same electrophoretic mobility (–0.53) and staining properties as canavanooctopine, which was previously identified as an in vivo product in canavanine-containing tumors (Petit et al. 1968).

An enzyme in the 15955/01 line, but not in the 15955/1 line, metabolized lysine to lysopine in vitro. Synthesis of lysopine competed with synthesis of an opine analog from an amino acid analog. Thus, no detectable canavanooctopine was formed when 40 or 80 mM lysine was present in the reaction mixture in addition to 20 mM canavanine, and there was a parallel increase in the amount of lysopine synthesized. However, AEC seemed to be a better substrate for lysopine dehydrogenase than canavanine: addition of lysine to the reaction mixture (up to 80 mM) in addition to 20 mM AEC only slightly decreased the amount of the putative carboxyethyl-AEC that was formed; the amount of lysopine synthesized was also not increased by much.

*Chemical syntheses of putative enzymatic products*

After 48 h, the reductive condensation of AEC and pyruvate by sodium cyanoborohydride yielded 2 products, presumably the carboxyethyl derivatives resulting from a reaction at each of the 2 amino groups of AEC. One of these products had the same electrophoretic mobility (–0.53) and staining properties as the putative carboxyethyl-AEC formed in vitro (Fig. 4). The other product had an electrophoretic mobility of –0.60. After a longer reaction time and/or with a higher concentration of pyruvate in the reaction mixture, another product with an electrophoretic mobility of –0.26 was formed; this presumably resulted from carboxyethylation of both amino groups. Reduction of the Schiff base resulting from canavanine (electrophoretic mobility, –1.10) and pyruvate yielded a single product that had an electrophoretic mobility (–0.53) and staining properties that were identical to those of the putative canavanooctopine formed in vitro.

*In vivo.* Carboxyethyl-AEC was identified by its electrophoretic mobility and staining in extracts of the 15955/01 tumor line that had been grown on medium that contained 40 µg of AEC/ml; no AEC could be detected in these tissues that contained carboxyethyl-AEC. The product was not present in the same tissue line that was grown on medium that did not contain AEC. The 15955/1 line, which grew only on medium that contained a much lower level of AEC (1.2 µg/ml), and the 15955/01 tissue that was grown on 1.2 µg of

AEC/ml contained AEC but no detectable product; the assay was limited by the overloading of the electrophoretogram when more than 10–20 µl of sample was spotted.

## Discussion

The results obtained are consistent with the idea that the toxicity of AEC or canavanine for plant cells was decreased by the intracellular presence of lysopine dehydrogenase, presumably because metabolism of an amino acid analog to an opine analog constituted a detoxification pathway.

It is reasonable to assume that metabolism of AEC or canavanine to an opine analog would result in detoxification: that lysine or arginine, respectively, competed out some of the toxicity of AEC or canavanine indicates that the toxicities of these analogs were probably due to the fact that they substitute for the normal amino acids in important metabolic pathways. At least part of the toxicity of each of these analogs appears to be due to its incorporation into cellular proteins (Bright et al. 1979; Rosenthal 1977). The opine analogs resulting from metabolism of AEC or canavanine by lysopine dehydrogenase would not likely be incorporated into proteins.

On the other hand, the results do not prove that lysopine dehydrogenase provided an important detoxification pathway. Further work will be necessary to determine whether the observed toxicity differences could not be due to other factors, such as differential uptake of the analogs from the medium, differential metabolism of the analogs by other biochemical pathways, or different intracellular concentrations of the corresponding normal amino acids. These and other mechanisms for detoxification of AEC or canavanine in plant cells have been observed (Bright et al. 1979; Cattoir et al. 1980; Chaleff and Carlson 1975; Rosenthal 1977).

Van Slogteren et al. (1982) reported that homoarginine could be used to select lysopine dehydrogenase-containing cells. Although homoarginine is a substrate for lysopine dehydrogenase (Otten 1979; Petit and Morel 1966), we observed little or no selectivity by homoarginine for lysopine dehydrogenase-containing tissues compared to other crown gall or habituated tissues. AEC appeared to be by far the most selective of the analogs tested, followed by canavanine. Perhaps the toxicity differences with homoarginine observed by Van Slogteren et al. for their particular plant materials were due to one of the other detoxification mechanisms mentioned above. Alternatively, perhaps homoarginine had lysopine dehydrogenase-related selectivity at the protoplast level that we did not observe at the tissue level.

Although further work is needed to determine if amino acid analogs of opine precursors can be used to select cells that contain an opine dehydrogenase, the results obtained offer promise that such a selection system will be possible. Such a selection might provide an alternative to the present requirement to select crown gall cells based on their growth substance autonomy, thus allowing the selection of cells that have the enzyme even if they do not have tumorous growth properties. Further, although hormones released by tumor cells can stimulate the growth of adjacent normal cells (Meins 1982), it appears that cells resistant to an amino acid analog such as AEC do not protect adjacent sensitive cells (White and Vasil 1979).

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