Session 1.

Chairman: DR L. G. COPPING

THE BIOSYNTHESIS OF AMINO ACIDS IN PLANTS

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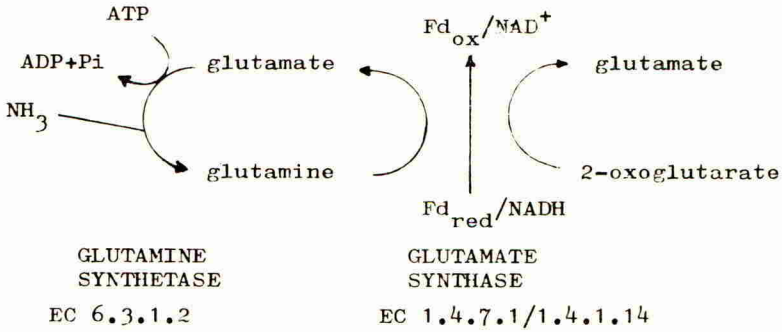
INTRODUCTION

In this brief review it would be impossible to cover in depth all aspects of the biosynthesis of amino acids in plants. I shall concentrate on outlining the biosynthetic pathways for all the protein amino acids, emphasising the metabolic regulation and intracellular localisation. Where possibly I shall highlight so far unexploited target enzymes or pathways for herbicidal action. For more comprehensive details of the biochemistry of amino acid synthesis in plants, the reader is recommended to consult Miflin (1980) or Lea *et al.* (1985). In addition to the 20 protein amino acids, higher plants synthesise many other amino and imino acids, sometimes in considerable quantities, and some of these have been shown to act as deterrents to herbivores and other pests. Interesting as they are, such compounds are outside the scope of this review. I have quoted only a minimum of references, for the sake of brevity, so I offer my apologies to the many authors not credited.

1) NITROGEN ASSIMILATION AND THE SYNTHESIS OF GLUTAMATE AND GLUTAMINE

The primary sources of nitrogen for higher plants are nitrate and ammonia in the soil, atmospheric N_2 gas (for plants with symbiotic N-fixing bacteria), and possibly atmospheric nitrogen oxides. Nitrates, N_2, and NOx are converted to ammonia prior to assimilation. It has now been firmly established that ammonia is assimilated into amino acids via the Glutamate Synthase Cycle, the concerted activities of glutamine synthetase (GS) and glutamate synthase (Fig.1). Many different isoforms of GS exist, in

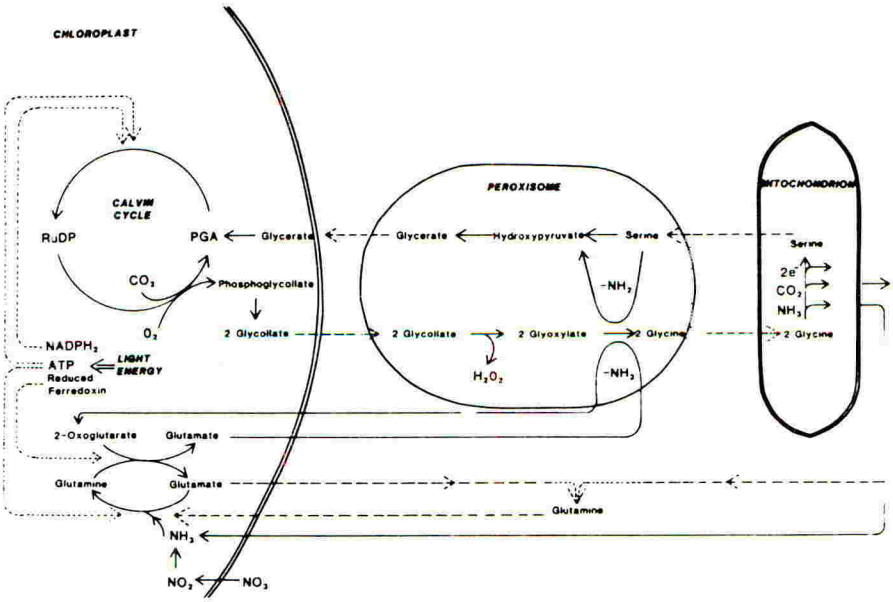
FIGURE 1 The Glutamate Synthase Cycle



different tissues and cellular compartments, and under different developmental regulation. For example, in *Phaseolus* there are four genes for GS, encoding sub-units for root nodule- and leaf-specific forms, plus a ubiquitous cytosolic isozyme present in all tissues. The reasons for this diversity are not clear, since the catalytic properties of the different GSs are very similar. Differential tissue-specific synthesis of these GS isozymes possibly relates to particular ammonia assimilation requirements. Two forms of glutamate synthase are present in higher plants, an NADH-specific enzyme and a ferredoxin-dependent enzyme. Both appear to be located in plastids, and to be present in all tissues, though the Fd-dependent enzyme is the major form in green, photosynthetic tissues. The Fd enzyme increases markedly during leaf development, or on greening of etiolated tissue. Although ferredoxin itself is not found in non-green tissues, an analogous electron-transfer protein has been found in such tissues.

Primary assimilation of ammonia for amino acid biosynthesis (and biosynthesis of all other nitrogen-containing compounds) is not the only role for the glutamate synthase cycle. There is considerable recycling of nitrogen within plants, such as the transport of nitrogen from roots to shoots, or from vegetative parts to developing seeds. A limited range of transport compounds are employed, depending on the species and nutritional status, including glutamine and asparagine, arginine, and the ureides. All except glutamine are broken down to release ammonia in the target, or sink, organ, such ammonia being reassimilated via GS. Another, quantitatively more significant, recycling occurs during the process of photorespiration (Fig.2). The oxygenase activity of RUBISCO produces phosphoglycolate, which must be returned to the Calvin cycle to avoid a disastrous loss of fixed carbon. The complex pathway used to achieve this is not wholly efficient,

FIGURE 2 The Photorespiratory Carbon and Nitrogen Cycle



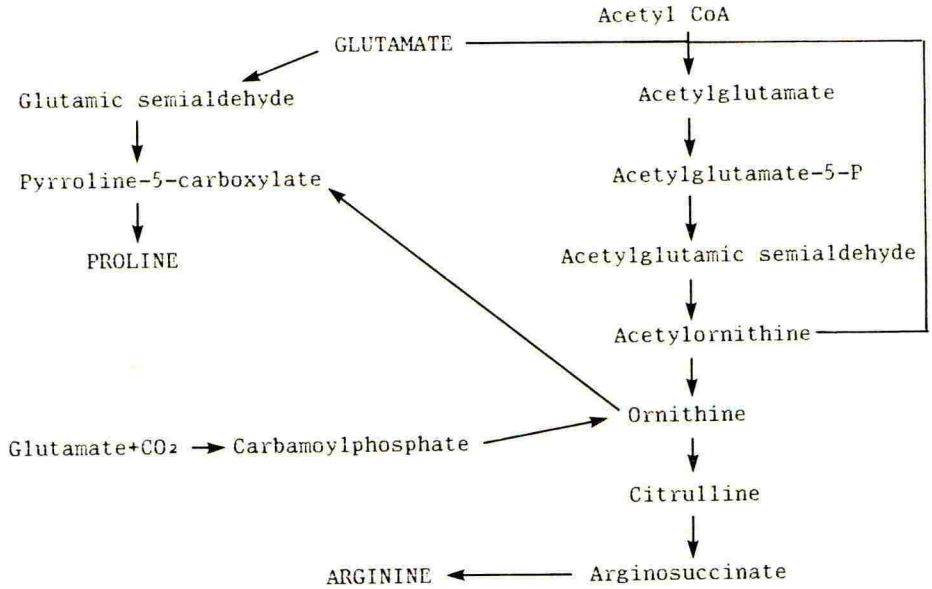
in that 25% of the carbon is lost by release of CO_2, accompanied by the stoichiometric release of ammonia. Studies with tracers, inhibitors, and specific mutants have established that such ammonia is efficiently reassimilated via the glutamate synthase cycle, at a rate that may be ten times greater than the primary assimilation of nitrogen (Wallsgrove *et al.* 1983). In barley, it has been found that the chloroplast-specific GS and the Fd-glutamate synthase are responsible, and are not required for any other purpose - mutants lacking either or both enzymes grow quite normally under conditions which suppress photorespiration (Kendall *et al.* 1987). Photorespiratory nitrogen cycling is not restricted to C3 plants such as barley, but has also been demonstrated in C4 plants such as maize, albeit at lower rates.

The very high rate of photorespiratory nitrogen cycling explains the success of GS inhibitors as herbicides (eg Glufosinate). A theoretical drawback to such inhibitors is the high concentration of both enzymes in leaf tissue (each may comprise up to 1% of total soluble proteins), and the fact that both activities are present in excess. Decreasing either activity by 50% has no effect on the plants ability to reassimilate ammonia from photorespiration. Otherwise GS and glutamate synthase are attractive potential targets for herbicide action. In the case of glutamate synthase, no comparable enzyme activity exists in animals.

2) AMINO ACIDS DERIVED FROM GLUTAMATE

All amino acids derive their nitrogen from glutamate (and/or glutamine), but we are here considering those that derive their carbon skeletons from glutamate, namely arginine and proline.

FIGURE 3 The Biosynthesis of Proline and Arginine



The biosynthetic pathways for these two amino acids are shown in Fig.3. The enzymes of proline synthesis, apart from P-5-C reductase, have not been characterised, though labelling studies have confirmed that [14C]-glutamate can be converted to proline. There is indirect evidence for feedback regulation of proline synthesis, but the enzyme involved has not been identified (Kueh *et al.* 1984). Plants can also convert ornithine to proline, but it is not clear whether this represents an alternative biosynthetic pathway or merely catabolism of ornithine. Despite the significance of proline in stress metabolism in plants, its biosynthesis is far from understood.

In contrast, all the enzymes of arginine biosynthesis have been identified, and the recycling of the acetyl group confirmed. Localisation studies indicate that the conversion of glutamate to citrulline takes place in the plastid, but the final steps are cytosolic (Taylor and Stewart 1981). Plants seem to contain a single carbamoylphosphate synthetase, involved in both arginine and pyrimidine biosynthesis (Kolloffel and Verkerk 1982), which is regulated by metabolites of both pathways. The bulk of this activity is found associated with chloroplasts (Taylor and Stewart 1981).

3) AMINO ACIDS DERIVED FROM ASPARTATE

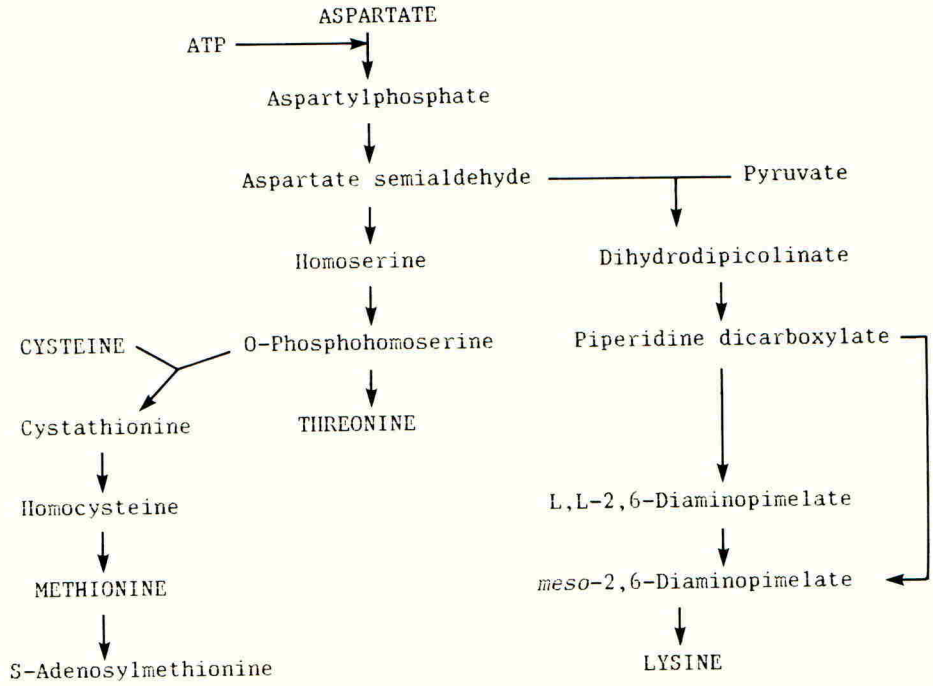
Aspartate itself is produced via glutamate:oxaloacetate aminotransferase. It forms the starting point for the synthesis of lysine, threonine, methionine, and isoleucine, as shown in Fig.4. The biosynthesis of isoleucine will be covered later, with the other branched-chain amino acids.

Isolated chloroplasts are capable of synthesising lysine and threonine (Mills *et al.* 1980), and most of the enzymes involved have been demonstrated to be restricted to plastids (Wallsgrove *et al.* 1983). Homocysteine is also synthesised in the chloroplast, but the final step in methionine synthesis takes place in the cytosol.

Whilst this pathway is broadly similar to that found in micro-organisms, there are aspects unique to plants. The activated form of homoserine used is phosphohomoserine, rather than O-acetyl or O-succinyl homoserine. There is also now some evidence for a single enzyme in plants that converts piperidine dicarboxylate directly to meso-diaminopimelate, rather than the three separate enzymes found in other organisms (Wenko et al. 1985).

The regulation of the aspartate pathway in plants is complex, and has yet to be fully elucidated. Isozymes of aspartate kinase exist, feedback regulated by either (lysine + S-adenosylmethionine) or threonine. In barley there are two genetically distinct lysine-sensitive forms (see Arruda *et al.* 1984). Mutations affecting the regulation of either isozyme can lead to an accumulation of threonine, as is found for similar mutants in maize and carrot. An ethionine-resistant line of tobacco, with elevated levels of lys-sensitive AK, accumulates lysine and methionine in addition to threonine (Gonzales *et al.* 1984). The precise metabolic role of the different AK isozymes has yet to be established.

FIGURE 4 The Biosynthesis of Lysine, Threonine and Methionine



Two plant homoserine dehydrogenases are known, either threonine sensitive or insensitive. The regulated form is found in the chloroplast, whilst the insensitive enzyme is cytosolic (Sainis *et al.* 1981), and its metabolic role is unknown. The elegant studies of Bryan and co-workers have established some complex conformational changes in maize homoserine dehydrogenase, mediated by substrate and pH, which alter both catalytic and regulatory properties (see Krishnaswamy and Bryan 1986). Feedback inhibition of homoserine kinase has been found in some plants but not in others.

Threonine synthase is regulated by S-adenosylmethionine (SAM), which is effectively the end-product of the methionine branch of the pathway. Low concentrations of SAM activate the enzyme, which is inactive in its absence. In both barley and *Lemna*, exogenous methionine reduces the activity of threonine synthase and cystathionine synthase, a rare example of metabolite repression in plants (see Rognes *et al.* 1986). Cystathionine synthase activity increases several fold under conditions of methionine starvation. No feedback regulator of cystathionine synthase has yet been identified.

Lysine synthesis is regulated by feedback inhibition of dihydrodipicolinate synthase by lysine. Mutants with a lysine-insensitive enzyme overproduce lysine (Negrutiu *et al.* 1984).

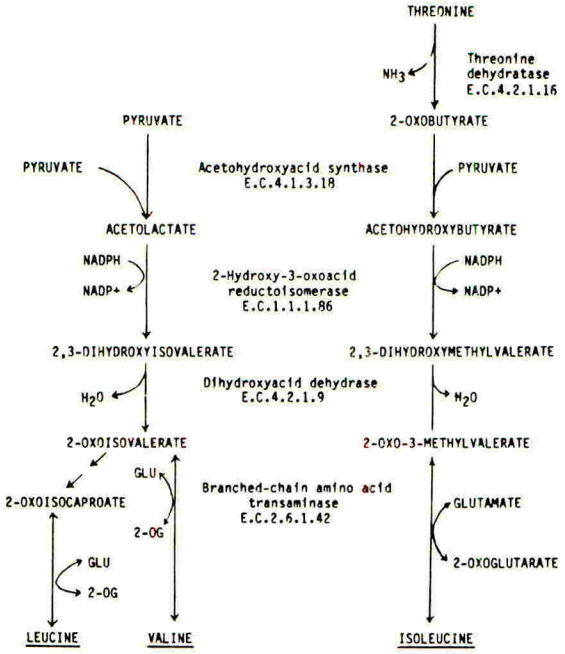
Aspartate kinase is a tantalisingly attractive target for herbicide action. It is present at low concentration (in all the species and tissues so far assayed), and a block here would starve the plant of four amino acids. However, the plant enzyme has never been successfully purified (see Relton *et al.* 1988 for the highest degree of purification so far reported), so detailed physico-chemical characterisation has been impossible. Preliminary studies suggest little homology with bacterial AK.

4) AMINO ACIDS DERIVED FROM PYRUVATE

Alanine is produced by transamination of pyruvate, the aminotransferase involved apparently not found in the plastid, surprisingly. The branched-chain amino acids leucine, isoleucine, and valine are synthesised via a common pathway from pyruvate (leu, val) or pyruvate plus threonine (ile) (Fig.5). This pathway has been extensively studied since the discovery of herbicides acting on acetohydroxyacid synthase (AHAS), the first common enzyme.

Overall, the pathway is very similar to that found in bacteria and fungi. In plants, isoleucine biosynthesis is regulated by feedback inhibition of threonine dehydratase, such inhibition being partly relieved by valine. Leucine and valine acting cooperatively inhibit AHAS, whilst leucine also inhibits isopropylmalate synthase, the first enzyme unique to its synthesis. No evidence could be found for induction/repression of AHAS in barley, or of IPMS in maize, but there has been a recent report of

FIGURE 5 The Biosynthesis of Branched-Chain Amino Acids



valine repression of AHAS in *Nicotiana plumbaginifolia* (Nielsen and Forlani, unpublished). This has yet to be confirmed, or demonstrated in any other species, but does raise the intriguing possibility that plants vary in their regulation of this (and other?) pathways. More subtle regulatory controls may also operate. AHAS preferentially acts on 2-oxobutyrate, the presence of which almost completely blocks acetolactate synthesis (see Crout, this volume). The final transaminase also discriminates between its substrates, at least in barley, the order of preference being oxoisocaproate > oxomethylvalerate > oxovalerate (Wallsgrove, unpublished).

Threonine dehydratase, AHAS, and dihydroxyacid dehydratase have all been purified from plants, and AHAS in particular extensively studied. The other enzymes are less well studied, and the enzymes of leucine synthesis not at all. Auxotrophic mutants have been isolated lacking either threonine dehydratase or dihydroxyacid dehydratase (see Wallsgrove *et al.* 1986), suggesting that both exist as single isozymes. However, two apparent isoforms of threonine dehydratase have been reported in maize (Kirchner and Bryan 1985). The existence of AHAS isozymes has not yet been satisfactorily resolved, but two distinct isozymes of BCAA transaminase have been found in both barley and *Phaseolus*.

It is assumed that the whole pathway is contained within the plastids, since isolated chloroplasts will synthesise isoleucine from threonine (Mills *et al.* 1980) and leucine and valine from pyruvate (Schulze-Siebert *et al.* 1984). With the exception of AHAS, demonstrated in both leaf and root plastids (Miflin 1974), other enzymes of the pathway have not been localised.

Given the effectiveness of AHAS inhibitors as herbicides, it is obvious that inhibitors of other steps in this pathway could be equally valuable. Considerable effort is undoubtedly going into the design of such inhibitors.

5) BIOSYNTHESIS OF AROMATIC AMINO ACIDS

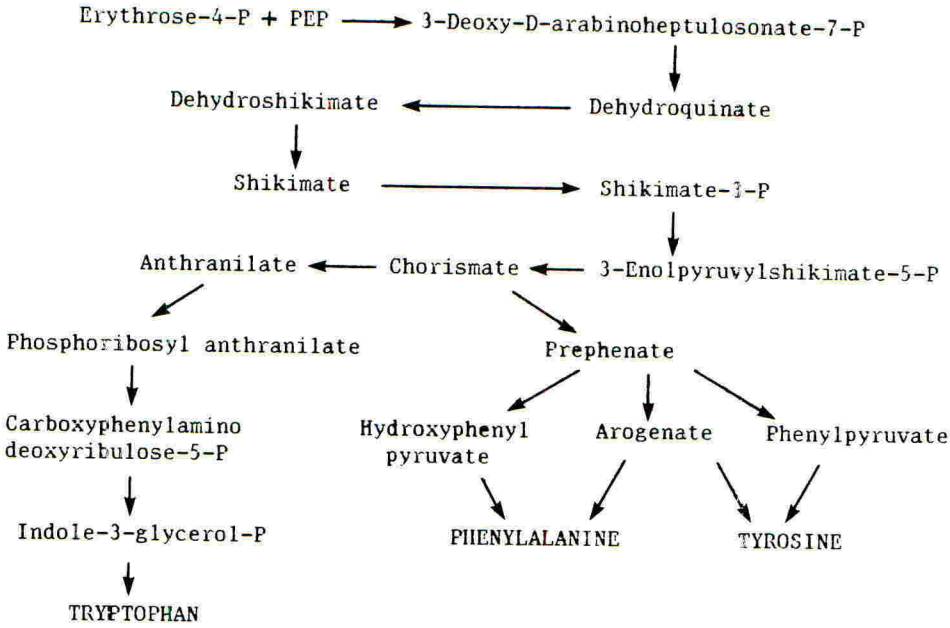
The biosynthetic route(s) to tyrosine, phenylalanine, and tryptophan are shown in Fig.6. These pathways also provide carbon skeletons for several groups of plant secondary products, and it is not always clear whether a particular enzyme or isozyme is involved in protein amino acid synthesis or secondary product synthesis. Such confusion also accounts for some of the observed differences betwen plant species.

The steps leading to chorismate synthesis are common to all three amino acids. Deoxyarabinoheptulosonate phosphate synthase (DAHPS) has been reported to occur as multiple isozymes in various plant tissues (see Rubin and Jensen 1985). These differ in requirement for divalent metal ions, pH optimum, kinetics, and other properties, and the precise role of feedback regulation on this enzyme is unclear. Other enzymes of this part of the pathway have been isolated as separate proteins, rather than the pentafunctional complex found in *Euglena gracilis* (Patel and Giles 1979).

Recently considerable attention has focussed on enolpyruvylshikimate phosphate (EPSP) synthase, as this is the target of the herbicidal action of glyphosate. Cell lines resistant to glyphosate have ben found to have elevated levels of EPSP synthase (eg. Nafziger *et al.* 1984).

Conversion of chorismate to anthranilate is the first step in the pathway unique to tryptophan synthesis. Two isozymes of anthranilate synthase have been resolved in both potato and tobacco. Control over this part of the pathway is by feedback regulation of the enzyme by tryptophan. The other enzymes of this branch have all been found in plant tissues.

FIGURE 6 The Biosynthesis of the Aromatic Amino Acids



Chorismate mutase, the first step in phenylalanine and tyrosine synthesis, also exists in multiple forms. One class of isozyme is sensitive to regulation by the end products (inhibited by phe/tyr, activated by trp), and the other is not. It had been thought that prephenate was converted to either phenylpyruvate (phe) or hydroxyphenylpyruvate (tyr). Recent evidence has indicated the existence of an alternative biosynthetic route, via arogenate, in both algae (Byng *et al.* 1981) and higher plants (Bonner and Jensen 1985). It appears that there may well be significant species differences in the existence and significance of the alternative pathways, and more work is required to clarify the situation.

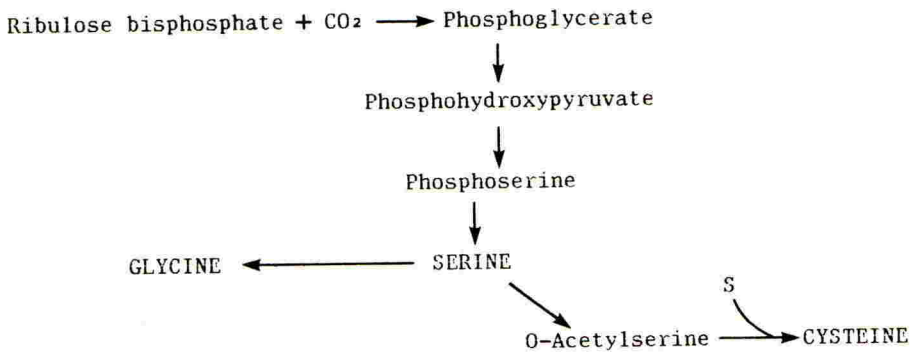
Synthesis of aromatic amino acids by isolated chloroplasts has been demonstrated (Schulze-Siebert *et al.* 1984), and enzymes of the pathway located in plastids (eg. Mousdale and Coggins 1985). However, there is also evidence for at least a partial biosynthetic pathway OUTSIDE the plastid, and this may be related to secondary product biosynthesis.

Again, the potency of glyphosate encourages us to believe that inhibitors of other steps in aromatic amino acid biosynthesis may be effective herbicides. No single enzyme stands out as an obvious target.

6) BIOSYNTHESIS OF SERINE, GLYCINE, AND CYSTEINE

Glycine and serine are synthesised as part of the photorespiratory pathway (Fig.2) in green leaves, in peroxisomes and mitochondria. However, there are good reasons for assuming that such synthesis is not significant in terms of net production for protein synthesis or other biosynthetic reactions. Growth of plants in high CO_2 (when photorespiration is suppressed) does not produce a deficiency in gly/ser, and in both *Arabidopsis* and barley mutants have been isolated that are unable to make serine in the mitochondria (Somerville and Ogren 1981, Lea *et al.* 1984), yet do not require exogenous glycine or serine. In addition, blocking the carbon recycling of the photorespiratory pathway by mutation or with enzyme inhibitors rapidly decreases photosynthesis, and ultimately kills the leaf, making it unlikely that any significant fraction of photorespiratory glycine or serine could be diverted from the cycle *in vivo*.

FIGURE 7 The Biosynthesis of Serine, Glycine, and Cysteine



Photorespiratory glycine and serine metabolism is an attractive target for potential herbicides, as are other steps in the cycle. The potential drawback noted for nitrogen cycle inhibitors also applies, though.

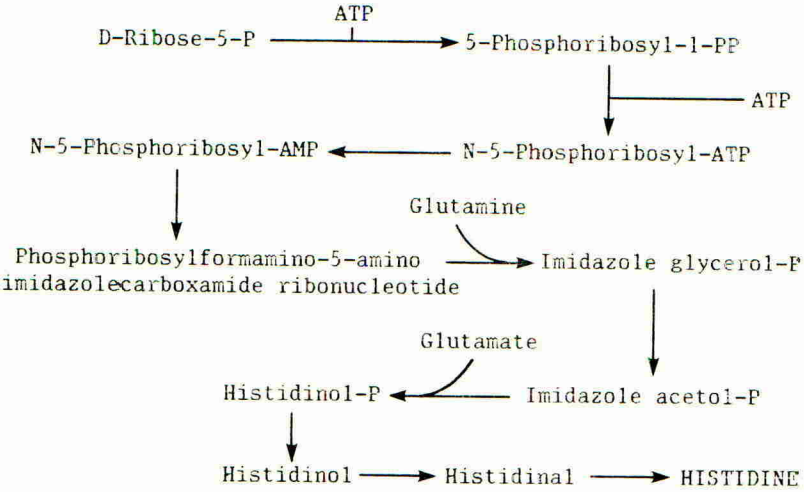
A more likely pathway for the biosynthesis of these amino acids is from 3-phosphoglycerate, as shown in Fig.7. The enzymes involved in serine synthesis have all been found in spinach chloroplasts (Larsson and Albertson 1979). Serine hydroxymethytransferase has also been found in plastids, distinct from the mitochondrial enzyme involved in photorespiratory metabolism. Cysteine is synthesised via O-acetylserine, and at least part of the total cysteine synthase is found in plastids.

7) BIOSYNTHESIS OF HISTIDINE

The biosynthesis of histidine in plants is assumed to occur via the pathway shown in Fig.8, as elucidated for micro-organisms. There is however little direct evidence, few of the enzymes having been isolated from plant sources. Histidine auxotrophs have been isolated but not fully characterised, though indirect evidence was found for deficiencies in imidazole glycerol phosphate dehydratase or histidinol phosphate

aminotransferase (see Negrutiu *et al.* 1985). Where in the plant cell histidine is synthesised is totally unknown.

FIGURE 8 The Biosynthesis of Histidine



CONCLUSIONS

Even at the basic level of enzyme biochemistry, there is a great deal still to be learned about amino acid biosynthesis in plants. From what we do know, the central role of the plastids is already obvious. In many pathways, the full details of *in vivo* regulation are still unclear, though in most cases the primary role of feedback regulation by end products is certain, rather than induction/repression of enzyme synthesis as found in bacteria. Developmental regulation and differences between tissues have hardly begun to be explored.

The striking effectiveness of amino acid biosynthesis inhibitors as herbicides suggests that there is immense potential for other novel classes of inhibitors to be developed. So far, detailed work on the enzymes has followed, not preceded, the discovery of effective compounds - there must surely be scope for this pattern to be reversed, by rational design based on an understanding of enzyme structure and mechanism. Even ignoring commercial agrochemical development, the basic biochemistry and molecular biology of amino acid biosynthesis are important and challenging areas for plant scientists.

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NATURAL AMINO ACIDS AS ENZYME INHIBITORS

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According to a recent review (Hunt 1985), over 700 natural amino acids have been reported in the literature. For most of these compounds, the only information available consists of the structure and the organism of origin. However, in a limited but increasing number of cases, a biological effect has been identified. The identification of this function can either be due to:-

a) The screening of natural products for a particular property (eg: pepstatin (17) and Asp - proteases; Katunuma et al, 1983.

b) The identification of the active principle in a biological extract of known activity
(eg: hypoglycine (19) in Ackee fruit; Bell, 1980)

c) The discovery of a new function for an old compound
(eg: Methoxy vinyl-glycine (1) and transaminase inhibition; Rando, 1974).

It is far beyond the aim of this review to document every natural amino acid with enzyme inhibitory properties. Instead, a few examples will be selected which, hopefully will illustrate how diverse and sophisticated natural product chemistry can be; how it can lead the discovery of new principles or even mechanisms of enzyme inhibition and last, but not least, how it can be exploited to transform a biochemical tool into a commercial compound for medical or agricultural use -

The review will deal with the following three sections:

I Pyridoxal-5'-phosphate dependant enzymes

II Inhibitors of enzymes acting on the γ-carboxyl group of glutamate/glutamine

III Inhibitors of proteases/peptidases

Note All chemical compounds mentioned in the text are numbered and their structure is given at the end.

I PYRIDOXAL - DEPENDANT ENZYMES

The biological effects of propargylglycine (2) and cycloserine (4) on bacterial growth, of aminoethoxyvinyl glycine on ethylene biosynthesis in plants have been known for some years. The report (Rando, 1974) that methoxyvinyl glycine (1), previously characterised by Roche Scientists as a "methionine antimetabolite", produces a time-dependant, irreversible inhibition of Ala-transaminase brought the concept of mechanism - based irreversible enzyme inhibitors to public attention. An explosion of publications by several groups described similar or different concepts of suicide inhibitors of pyridoxal-enzymes (for review see Jung and Danzin 1989). Selected examples of inhibitors of amino acid decarboxylases, transaminases, racemases, , -lyases will be given, with properties ranging from herbicidal to antibiotic and human pharmacology applications.

II ENZYMES ACTING ON γ-COOH OF GLUTAMATE

Inhibitors of several enzymes of the γ-glutamyl cycle are regrouped in table 1.

TABLE 1

Some compounds interfering with γ-carboxyl activation in Glu

Name	Source	Bio. Effect	Ent. Inhib.	Ref
Methioninesulfo ximine (5)	Bleached flour	Convulsant	G.S.	Meister, 1978
Phosphinothricine (6)	Strept.	Herbicidal Antibiotic	G.S.	Fraser & Ridley, 1984
Acivicin (7)	Strept.	Antitumoral	G.T.	T5O et al, 1980
Tabtoxinine (8)	Pseudom	Herbicidal	G.S.	Langston-Unkeferer, et al, 1987
Anticapsin (9)		Antifungal	Glc.S	Milewski, et al, 1986
A 19009 (10)		Antifungal	Glc.S	Badet, et al, 1988
Anthglutin (11)	Penicillin	Antitumoral	G.T.	Kinoshta, et al, 1981

Note: G.S.: Glutamine synthetase; G.T.:Glutamic acid transpeptidase; Glc.S: glucosamine synthesis

III INHIBITORS OF PROTEASES/PEPTIDASES:

This very important class of enzymes is subdivided in four groups based on the specific mode of action. Nature has devised inhibitors with different chemical structures taking advantage of the specific mode of amide bond activation. Table II summarises the state of the art, by giving some important enzymes for which inhibitors based on the natural concepts are presently evaluated clinically.

TABLE II

Proteases and their (small molecular weight) inhibitors

Class of protease	Inhibitor type	Important enzymes	Ref
Serine-prot	pept. aldehyde (12)	- elastase - serum factors - kallikreins	Stein & Stimpler 1987
Cysteine-prot	pept. ald. expoxyde (13) diazoketone	cathepsin B	Barrett, et al, 1982
Metalloprotease	thiol deriv (14) bestatin (15) amastatin 16)	Angiot. Conv. Enz. Aminopept. B	Ondetti & Cushman, 1982 Wilkes & Prescott 1985
Asp-protease	statine (17) arphamenine (18)	pepsin renine HIV-protease	Salituro, et al 1987

Other concepts either devised from the natural chemistry (fluorinated ketones from aldehydes) or from an educated guess of what the transition state might look like (phosphonamides or phosphinic acids) have also been described in the literature (Gelb et al, 1985).

CONCLUSION

Examples of natural enzyme inhibitors with a pharmacological potential are of course also to be found in families of compounds other than amino acids (eg sterols, sugars, nucleosides ..). This brief review was meant to give a fore-taste of the diversity and usefulness of natural products as leads into novel chemistry. Secondary metabolites in bacteria represent probably an endless source of novel chemicals (suffice to leaf through an issue of Journal of Antibiotics). It has been estimated that only a few percent of plant species have been thoroughly investigated (see for instance Chinese and Indian pharmacopeias or the folk medicines in S.America/Africa). Clearly, natural product chemistry has not finished to surprise the Scientific Community.

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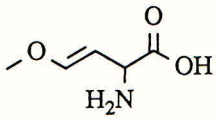

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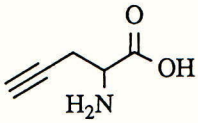

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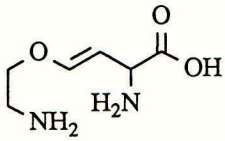

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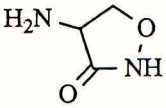

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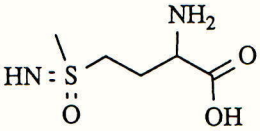

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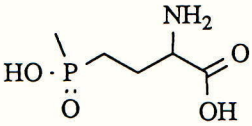

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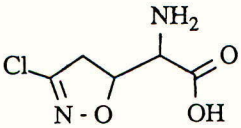

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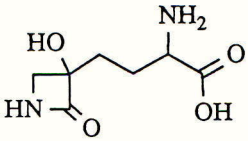

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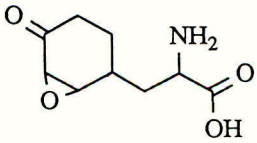

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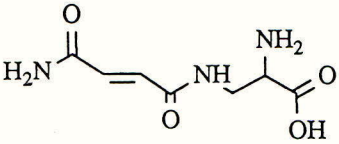

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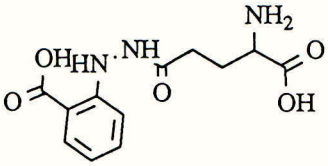

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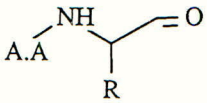

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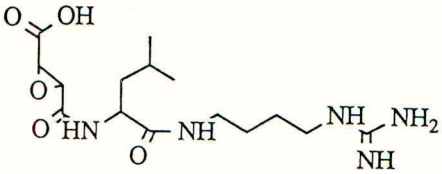

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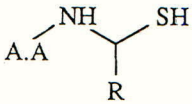

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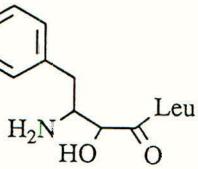

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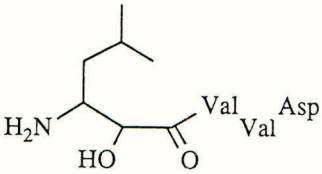

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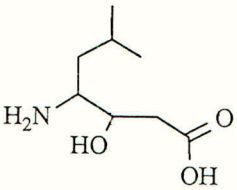

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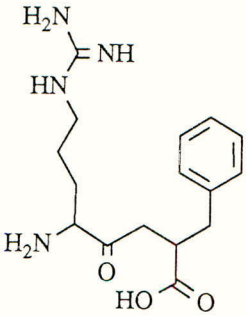

18

AMINO ACID BIOSYNTHESIS - AN ALADDIN'S CAVE OF NEW PESTICIDE TARGETS?

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ABSTRACT

The inhibition of amino acid biosynthesis is well established as a target for herbicidally active compounds. To date inhibition of only five enzymes from the 75 or so involved in the biosynthesis of the twenty common amino acids has been shown to cause herbicidal effects. It is undoubtedly true that some of the, as yet, unexploited enzymes will have greater potential as targets for pesticidal compounds than others. The approaches that might be used to identify the best targets and to discover novel pesticidally active compounds are reviewed.

INTRODUCTION

The inhibition of amino acid biosynthesis as an excellent target for herbicidally active compounds needs little introduction. Three enzymes have been well established as the primary sites of action of commercial herbicides: acetohydroxyacid synthase [EC 4.1.3.18] (eg Ray, 1984; Shaner et al., 1984), 5-enolpyruvylshikimate 3-phosphate synthase [EC 2.5.1.19] (Steinrücken & Amrhein, 1980) and glutamine synthetase [EC 6.3.1.2] (Bayer et al., 1972). Imidazoleglycerol phosphate dehydratase [EC 4.2.1.19] is suspected, although not unequivocally proven, to be the site of action of aminotriazole (Hilton et al., 1965). Most recently, inhibition of acetohydroxyacid reductoisomerase [EC 1.1.1.86] has been shown to cause herbicidal effects, although no compound with commercial levels of activity has yet been reported for this enzyme (Schulz et al., 1988).

The purpose of this paper is to consider the general directions in which research might be profitably concentrated in the future. To this end some of the outstanding questions that need resolving will be highlighted and the approaches and techniques that can be employed in the search for both new targets and novel pesticides will be reviewed.

THE POSSIBLE BIOLOGICAL TARGETS

As all the commercial examples of inhibitors of amino acid biosynthesis are herbicides, it is natural that most attention is directed towards this area and this review will be no exception. However, we should perhaps not completely ignore the possibility of discovering novel fungicides or insecticides that act by this means. In the fungicide area it is difficult to believe that a fungal pathogen cannot obtain sufficient supplies of most nutrients, including amino acids, from its host once penetration has been completed and an intimate association with the plant established. However, during the initial stages of infection, it is entirely possible that the fungus may need to synthesise its own amino acids. Indeed, some fungicidal activity with a

number of sulphonanilide inhibitors of acetohydroxyacid synthase has been noted at Chesterford Park. Studies of the pathogenicity of fungal mutants that are auxotrophic for a specific amino acid when cultured in vitro would be an ideal way to define the potential of this area, although it may be difficult to unequivocally distinguish between loss of pathogenicity due to spontaneous events (for example due to extended in vitro cultivation) and loss due to a mutation in a particular amino acid biosynthesis pathway. Should it be possible to prove that the biosynthesis of amino acids by fungi is important for pathogenicity, then an interesting area to consider would be fungal lysine biosynthesis. It is well recognised that most fungi synthesise this amino acid via the aminoadipate pathway, in contrast to the diaminopimelate pathway employed by plants, thereby giving the potential for built-in crop safety. In the insecticide area, much the same problems as detailed above are present. However, one interesting possibility is proline biosynthesis. This amino acid has been implicated as a major energy source during flight (Beenakkers et al., 1985). As rapid resynthesis is required during rest periods, inhibition of this process could lead to loss of mobility.

ARE ALL AMINO ACID BIOSYNTHETIC PATHWAYS EQUALLY GOOD TARGETS?

Is it safe to assume that inhibition of the biosynthesis of any of the twenty 'common' amino acids will inevitably result in a herbicidal effect? In principle, this is probably true as a ready supply of all the amino acids must be required to maintain the growth and health of a plant. Nevertheless it is also probably true that the absence of some amino acids will have different consequences for the plant than the absence of others. For example, specific effects might be expected if the biosynthesis of amino acids that are themselves precursors for other pathways was to be inhibited. Two obvious possibilities are tryptophan and α-ketoisovalerate where inhibition of the biosynthesis of these two metabolites could lead to reduced levels of auxin and pantothenate respectively. Equally, inhibition of branched chain amino acid biosynthesis shows different symptomology to that evident following the inhibition of aromatic amino acid biosynthesis, possibly reflecting differences in the physiological consequences following starvation of these two groups of amino acids. However, the accumulation of potentially toxic intermediates (see later) could also influence the observed symptomology. Despite having completely established that the inhibition of acetohydroxyacid synthase is the primary site of action of a number of different groups of compounds, we still have no clear explanation of how the inhibition of this enzyme causes plants to die. In particular, an explanation of the remarkably close association between the inhibition of acetohydroxyacid synthase and the subsequent blockage of the cell division cycle is still required (Rost & Reynolds, 1985). There is also a similar absence of a clear explanation of why plants die following the application of glyphosate. Answers to these questions could help in two areas: firstly, is there something unique about these two pathways and, secondly, is it necessary to inhibit the biosynthesis of all the products of the pathways to achieve such a herbicidal effect or can a similar level of activity be achieved by specifically inhibiting the biosynthesis of only one of the amino acids produced by the pathway?

The above then raises the question of how we can identify new potential targets within amino acid biosynthesis. There is a tremendous

temptation to concentrate exclusively on the ten, so-called essential amino acids. This is at least partly justifiable as it is undoubtedly true that targeting on an enzyme not present in mammals must improve, although in no-way guarantee, the chances of avoiding mammalian toxicity. However, we do need to be careful of the definition of an essential amino acid (Bender, 1985). For example, arginine and histidine are now classified as essential amino acids, despite the fact that mammals can synthesise at least part of their daily requirement. Equally, just because a non-essential amino acid can be synthesised by mammals, there may be no necessity for this de novo synthesis to occur when a balanced diet is available. This argues that the biosynthesis of the non-essential amino acids should not be ignored as a potential target. It should also be borne in mind that one of the three commercial targets in amino acid biosynthesis, glutamine synthetase, is universally distributed, thereby emphasising that acceptable levels of safety can be achieved with compounds whose biochemical target is also present in mammals.

Returning to how new targets might be identified, studies with plant auxotrophs can provide a rich hunting ground for new ideas. Although plant mutants are much rarer than those for bacteria (King, 1986), a number of mutants in amino acid biosynthesis have now been reported. For example, proline biosynthesis (Gavazzi et al., 1975); tryptophan biosynthesis (Last & Fink, 1988); histidine biosynthesis (Shimamoto & King, 1983); isoleucine biosynthesis (Sidorov et al., 1981). These reports indicate that there is no absolute impediment to obtaining plant amino acid auxotrophs and give encouragement to the possibility of isolating further mutants in the future. However, specialist isolation strategies may be required to enable such mutants to be detected. For example, the successful isolation of tryptophan mutants of Arabidopsis thaliana (Last & Fink, 1988) was greatly dependent on the inclusion of 5-methyl anthranilate (5-MA) to give the tryptophan mutants a growth advantage over the wild types due to the ability of the latter to convert 5-MA to the toxic compound 5-methyltryptophan. Where whole plant auxotrophs can be obtained, the physiological consequences of withdrawing the nutrient supplement after the plant is established can then be investigated. This could give valuable information about the symptomology and speed of kill that might be expected if the biosynthesis was to be blocked through application of a herbicide.

ARE ALL ENZYMES IN A PARTICULAR PATHWAY EQUALLY GOOD TARGETS?

Once a particular amino acid biosynthetic pathway has been chosen for further study, the question of whether all the enzymes in the pathway are equally good targets must be addressed. If mutants are available and the lesion characterised, then the answer is obvious. In other cases the choice of which enzyme to work on can be more difficult. Study of the flux control of a pathway can sometimes identify a particular enzyme as a rate-limiting step in the pathway where even a relatively small reduction in the effective enzyme concentration, for example through inhibition by our potential pesticide, can lead to a dramatic change in the overall flux through the whole pathway. However, it is now generally accepted that in many cases there is no single "control" step and that the flux through a pathway is influenced by all elements of the system (Kacser and Burns, 1973). Feedback-regulated steps in a pathway have an added

advantage in that the allosteric nature of these enzymes provides additional binding sites that could be exploited for inhibitor design. However, in circumstances where different isozymes with different feedback regulators are present some caution is required as any uninhibited isozyme could provide sufficient flux through the pathway to overcome the inhibition. For example, differential sensitivity of isozymes of acetolactate synthase to metsulphuron-methyl have been noted in the bacterium Salmonella typhimurium (LaRossa & Schloss, 1984). However, the potential of feed-back regulated enzymes in multiple end-product pathways as herbicide targets can be demonstrated by the inhibitory effect noted when plants are exogenously supplied with either an excess of one or more of the end products or amino acid analogues (Bryan, 1980).

In some cases the choice is complicated even further by the lack of information about the exact biosynthetic route employed by plants. For example, the biosynthesis of phenylalanine and tyrosine from prephenate has been reported to occur by two routes: either via phenylpyruvate or via arogenate (Jung et al., 1986). Although lysine biosynthesis is generally thought to occur by the classical route employed by Escherichia coli, the enzyme meso-diaminopimelate dehydrogenase has been reported to be present in soybeans (Wenko et al., 1985), thereby giving the possibility of by-passing five of the enzymic steps of the 'classical' route. Only a few of the enzymes of histidine biosynthesis have been reported in plants and we can only assume that the route of synthesis is the same as in bacteria. Proline can potentially be synthesised by two routes, either from glutamate or ornithine, but it is not clear whether both routes are present in all plants. Answers to these questions are required in order to help us make rational choices about new targets for potential herbicides. Where multiple pathways are present it is important to determine whether crops and weeds differ in the routes used as any differences could be exploitable in terms of building in crop selectivity.

A further question to address when selecting new enzyme targets is whether the inhibition of the enzyme could lead to the accumulation of a toxic intermediate, as this could potentiate the herbicidal effect of the compound. The accumulation of a α-ketobutyrate following the inhibition of acetohydroxyacid synthase by the sulphonylureas has been elegantly demonstrated to contribute to the toxicity of these compounds to bacteria (LaRossa et al., 1987). However, whether the same situation applies in plants is more doubtful as similar symptomology is apparently evident for compounds acting at acetolactate reductoisomerase, another enzyme of the pathway (Schulz et al., 1988). These authors showed that large amounts of acetolactate and its decarboxylated product, acetoin, accumulated under the influence of an inhibitor of acetolactate reductoisomerase but did not, however, report whether α-ketobutyrate also accumulated. Protection against inhibitors of acetohydroxyacid synthase is not evident when plants are provided with isoleucine alone (Ray, 1984; Shaner & Reider, 1986). In this case isoleucine would have been expected to inhibit threonine dehydratase [EC 4.2.1.16] and hence reduce the levels of α-ketobutyrate accumulating. For inhibitors of glutamine synthetase a number of factors has now been implicated in causing treated plants to die and it is no longer considered that accumulation of toxic levels of

ammonia can, alone, fully explain all the observed effects (Sauer et al., 1986).

POSSIBLE APPROACHES TO THE DESIGN AND DISCOVERY OF NOVEL INHIBITORS

In this section the approaches that might be employed to discover a lead inhibitor of a chosen target enzyme will be considered. The subsequent work that is required to maximize the biochemical potency and translate the biochemical effect into a herbicidal effect will not be considered but, of course, should not be underestimated.

Probably the most common starting point for a new synthesis programme is the random discovery, or a literature report, of a new type of inhibitor of either the target enzyme or a closely related (in mechanistic terms) enzyme. Leads for the rational design of novel inhibitors can come from information about the characteristics of the substrates and products, the specificity of the enzyme with respect to substrate analogues, the mechanism of the reaction and the possible transition states involved. There must be great potential for 3-dimensional structural information about a target enzyme to aid in the rational design process in the future. Indeed, X-ray crystallographic studies have been successfully used in the pharmaceutical industry, for example in the design of new inhibitors for dihydrofolate reductase (e.g. Goodford, 1984), but there are, as yet, no examples available in the agrochemical area. Progress in this field is rather painstaking at the moment. Cloning and overexpression of the enzymes of interest can help provide the large amounts of pure enzyme required for this type of study. However, even when crystals of the enzyme are obtained, by no means a simple or guaranteed process in itself, the acquisition and interpretation of the X-ray data is a long term and expensive business. Nevertheless, this type of information will be a cornerstone of the design process in the future.

There is often considerable debate about the 'ideal' type of inhibitor for an enzyme. As the literature contains herbicidally active examples of most types of inhibitors, it would be imprudent to rule out any possibility. However, some points to consider are discussed below. Transition state analogues are likely to yield the most potent and highly specific inhibitors whose theoretical dissociation constant can be as low as 10^{-20}M (Schloss, 1988). However, transition state analogues may have only narrow potential for analogue synthesis. Suicide-type and irreversible inhibitors would not be the best type of inhibitor for enzymes that are destroyed and synthesised at a high rate as this could provide an effective, in-built detoxification mechanism. Competitive inhibitors and substrate analogues would not be the ideal choice when considering an enzyme whose substrate can be present in high concentrations.

In the final analysis, the choice of which enzyme to work on will be greatly determined by the perceived potential for the synthesis of inhibitors. The acquisition of the necessary biochemical information about the enzymes of interest should continue to be a priority in order to provide the inspirational basis for the synthesis of the future.

So far the emphasis has been on a rational design approach to the discovery of inhibitors. However, having spent time and effort identifying a good candidate enzyme and working-up an assay, it is attractive to consider positively trying to identify new inhibitors through a biochemical screening approach. Biochemical screening is widely used by the pharmaceutical industry but is perhaps less common within the agrochemical industry, probably due to the ready accessibility of the true *in vivo* targets in the latter. However, many pesticide companies are now adopting some limited biochemical screening and it is possible that this will increase in the future. Biochemical screening of natural products is also a possibility, although this is perhaps less readily attractive than screening synthetic chemicals due to the effort required to identify the active component from the source material before being able to determine the quality of the lead. As well as screening single enzymes, the possibility of using whole amino acid biosynthetic pathways should also be borne in mind in view of the substantial advantages to be gained from testing several enzymes at once. If the *in vitro* test can closely mimic the *in vivo* situation in terms of enzyme levels and relative flux rates, this has a further advantage in biasing the screen towards detecting those inhibitors that are likely to cause the maximum perturbation of the flux through the pathway in the whole organism.

CONCLUSIONS

Novel, pesticidally active compounds, acting at both the established enzyme targets and at the, as yet, unexploited enzymes of amino acid biosynthesis, will undoubtedly be discovered in the future. The study of amino acid auxotrophs is suggested to be a powerful means of unequivocally establishing which of the many enzymes involved in amino acid biosynthesis have the greatest potential as new targets. This type of hard evidence can give real incentive to attempts to discover novel inhibitors of these selected enzymes, especially in those areas where the exact, or major, route of amino acid biosynthesis has not yet been fully established. However, the perceived potential for the design and synthesis of novel inhibitors is still a major factor that determines which particular enzymes are actually investigated in depth. The future will depend greatly on our ability successfully to apply a multidisciplinary approach to gaining the necessary information about the enzymes of interest in order to initiate and support the synthesis effort.

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SCREENING TECHNIQUES FOR AMINO ACID BIOSYNTHESIS INHIBITORS

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ABSTRACT

Amino acid biosynthesis inhibition has become increasingly a focus for herbicide research, not least because of the action of glyphosate, phosphinothricin and the acetolactate synthesis inhibitors. An important question with novel herbicides is how to find out their mode of action. In experimental studies with new compounds it is important to understand how the compound inhibits plant growth. A number of systems have been used to examine how compounds work. The power of these systems relies upon the ability to rapidly screen compounds for inhibition of amino acid biosynthesis and to identify further the site of activity. Additional studies can then elucidate more precisely the specific site of chemical inhibition. In this short review attempts are made to focus on the methods used, their advantages and disadvantages and the evidence for successful use of such systems.

INTRODUCTION

Information concerning the precise biochemistry of growth inhibition by compounds used as herbicides is not extensive. Even more fragmentry is the information on newly discovered chemicals. But knowledge of a mode of action is important as it can help design of more effective, more specific and safe compounds. As is shown by the acetolactate synthesis inhibitors it can also provide evidence leading to methods for the engineering of crop plants to be resistant to the herbicidal compounds.

The particular problem is the first step of recognition of the pathway of biosynthesis which is inhibited, once this is discovered, then clear routes exist for further study. This paper will address some of the key issues in carrying out investigations of herbicide mode of action.

SYSTEMS USED TO STUDY AMINO ACID BIOSYNTHESIS INHIBITION

Table 1 lists a sample of some published examples of systems used for the study of amino acid biosynthesis inhibition. A range of tissues in cell cultures have been used in conjunction with a variety of amino acid biosynthesis inhibitors. Clearly there are large number of assay systems available.

The common parts of all these cell culture assays are the growth in a defined medium followed by standardisation of the biomass concentration. If the addition of inhibitor causes the death, or inhibition, of the cells and this is reversed by the addition of the amino acid or mixture, then the first important information on mode of action is available.

TABLE 1

SOME EXAMPLES OF THE USE OF CELL CULTURE TO STUDY MODE OF ACTION OF INHIBITORS

System	Inhibitor(s)	Authors
Tomato cell culture	*Alternaria alternata* toxin	Fuson and Pratt (1988)
Carrot (*Daucus carota*) culture	Glyphosate	Kilmer, Widholm and Slife (1981)
Duckweed (*Lemna gibba*)	"	Jaworski (1972)
Rhizobium japonicum	"	"
Escherichia coli	"	Gresshoff (1979)
Chlamydomonas reinhardi	"	"
Carrot (*Daucus carota*) culture	"	Haderlie, Widholm and Slife (1977)
Tobacco (*Nicotiana tabacum*) cell culture	"	Haderlie, Widholm and Slife (1977)
Soybean *(Glycine max)* cell culture	"	"
Lemna gibba	HOE 704	Schulz, Sponemann, Kocker and Wengenmayer (1988)
Corn (*Zea mays*) cell culture	Tabtoxinine-B-Lactam	Bush, Durbin and Langston-Unkefer (1987)
Soybean (*Glycine max*) cell culture	Chlorsulfuron	Scheel and Cassida (1985)
Escherichia coli	Sulfometuron methyl	"
Salmonella typhimurium		"
Corn (*Zea mays*) cell culture	AC243997 (Imidazolinone)	Anderson and Hibberd (1985)

WHOLE PLANT VERSUS CELL ASSAY

Analysis of mode of action can be done in a number of systems. For some types of inhibition the whole plant provides the best solution (for example, photosynthesis inhibitors). For anti-metabolites plant cell culture offers a rapid diagnostic method which enables large numbers of experiments to be carried out with homogenous material (Table 2).

TABLE 2

WHOLE PLANT VERSUS CELL ASSAY

	Advantages	Disadvantages
Whole plant	Reflects 'real' situation. Site-specific effects are detectable with differentiated tissue. Symptomology can give clues	Slow. Difficult to ensure uniform exposure. Non-homogenous. Difficult to ensure uptake of defined medium addition.
Cell assay	More rapid assays Homogeneous Simple to add compounds Amenable to high throughput Direct quantitive measurement Uses much less compound (10 - 100x)	Tissue undifferentiated may therefore not give specific sensitivity Not suitable for all modes of action. e.g. photosynthesis inhibition Different quantitative responses may be seen in comparison with whole plants

WHICH TISSUE?

Three types of cell culture have been used. Monocot, and dicot plant cell culture, bacterial culture and unicellular algae. The use of *Lemna gibba* as a plant analogue has also proved useful. The choice of system would depend on the particular case (Table 3). Bacterial cells can be very useful because they are simple and very quick to grow, but they do not always have the same pathways as plant cells and mechanism of uptake of compounds may be different. Inhibitors of plant cells do not always inhibit bacterial cultures. For example Fusilade (fluazifop-P-butyl) a grass selective herbicide does not inhibit bacterial cell culture but does inhibit plant cell culture. For most amino acid biosynthesis inhibitors bacterial systems will give an indication of mode of action which can be followed up by plant cell culture analysis. Table 4 shows the type of reversals obtained with a number of known amino acid biosynthesis inhibitors in bacteria, in tissue culture cells and in *Lemna gibba*.

TABLE 3

	days to grow culture	assay length in days	Sensitivity to known herbicidal amino acid inhibitors			
			glyphosate	Als inhibitors	hadacidin	tabtoxinine
Bacterial cell culture	0.5	1	+	+	+	+
Plant cell culture	7	7	+	+	+	+
Duck weed (*L.gibba)*	7	10	+	+	+	+

While bacterial systems can give good clues on herbicide mode of action, it is clear that plant tissue culture, in some cases, gives different results. These data may be important in determining secondary mode of action or in revealing different isoenzyme patterns. Two examples are given below:

Glyphosate

Early results with glyphosate implicated the aromatic amino acid pathway (Gresshoff, 1979; Haderlie *et al.*, 1981) in both bacteria and plant cells. Later further work on plant cells (Killmer *et al*, 1981) showed reversal of glyphosate inhibition in plant cells by aspartate, and α-ketoglutarate suggesting a second mode of action where glyphosate acts to deplete respiratory substrate and aromatic amino acids help to avoid a drain on the respiratory substrate by preventing the accumulation of shikimate. This case will be discussed in more detail below.

ALS inhibitors

In *E.coli* sulphonylurea inhibition is reversed by isoleucine but not leucine, in plants reversal is seen with a combination of valine and leucine. In *E.coli* sulphonylurea inhibitors appear to act by causing inhibition of isozyme 3 specialised for the biosynthesis of isoleucine (Scheel and Casida, 1985). Scheel and Casida (1985) concluded that there are some similarities but there are also many differences in the action of sulphonylureas in bacteria and plants.

TABLE 4

REVERSAL OF SPECIFIC AMINO ACID BIOSYNTHESIS INHIBITORS

	phosphinothricin	hadacidin	glyphosate	chlorsulfuron	HOE 704
Plant cell culture	glu + gln (his)	asp	aromatic amino acids & KG, malate succinate	val + ile	-
Bacterial	glu	asp	Phe+tyr+trp	ile (aromatic amino acids)	val ile leu
L.gibba			Phe (Phe+tyr+trp)		val, ile leu

PRACTICAL ASPECTS OF USING A PLANT CELL CULTURE SCREEN FOR AMINO ACID ANTIMETABOLITES

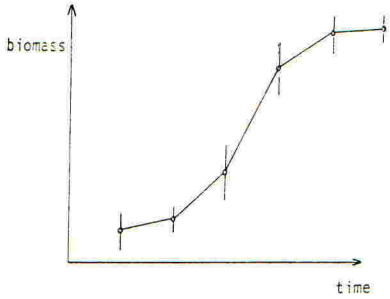
The value of plant cell suspension culture is that quantitative effects can be measured fairly accurately, however it can offer only limited information of a qualitative nature, such as symptomology provides from a whole plant screen.

The first principle to consider is that one is dealing with a virtually unicellular organism which will show logarithmic biomass growth in shaken liquid culture. In this respect many rules that apply to the growth of microbial culture are useful. It is best to choose a cell suspension that is friable, while the cells will in clumps analogous to pellets, these are ideally relatively small and consistently distributed throughout the whole suspension.

Firstly a system to measure biomass should be determined, this might be cell volume (after centrifugation) or optical density. As one of the main advantages of using plant cell culture is the potential to run a high throughput screen, this part of the process should be simple and, if possible, automated. Equipment such as a microtitre-plate absorbance reader can be used.

To demonstrate logarithmic growth over a convenient time course in a confined vessel, standardisation of the inoculum is essential. It should be of a size and physiological state such as to demonstrate a steep gradient growth curve, ie. from lag through log to stationary, over say, 7 days, figure 1. Optimisation of the growth curve has to be studied individually for each variety of cell suspension used as different varieties of the same species have been shown to respond very differently. This is best considered in conjunction with medium optimisation where, for example, levels of hormones can significantly affect growth rates.

figure 1



The process of addition of test chemicals requires some thought. It is imperative that the cell suspension remains sterile throughout the process which means that contamination from the test chemical must be avoided. The options here are to sterilise either by filter or autoclave, the latter will present problems if compounds are likely to be thermo-unstable. Another possibility is the use of non-phytotoxic antibiotics.

Any inhibition of growth mediated by the presence of herbicidal compounds will be demonstrated by a signficant reduction of biomass by the end point of the test. It is also possible to study the dynamics of growth inhibition by sequential assay throughout the course of the test.

There are always inconsistencies between activity of certain chemicals on *in vivo* and *in vitro* tests, some possible causes, eg. relating to photosynthesis have already been mentioned. However in other cases the appropriate metabolic active site may be present in cell suspension but activity is not observed. This may be because the compound is not taken up into the plant cell; this can be facilitated by the addition of surfactants, but again preliminary testing is advisable as many such reagents can be phytotoxic at quite low rates.

One of the major advantages of a plant cell suspension assay is the relatively low levels of compound needed to carry out a test, perhaps one tenth of that needed for a whole plant test. Again the optimum level to apply for any one particular test should be considered separately for each cell type as there can be considerable differences in sensitivity.

If a test is designed to look for reversal of inhibition by addition of appropriate metabolites then appropriate controls are essential. For example if the effect of inhibition reversal by an amino acid is being studied the control for the reversal test should be the cells plus the amino acid as this can often promote the plant cell growth:

test	control
1. cells + herbicide	cells
2. cells + herbicide + amino acid	cells + amino acid

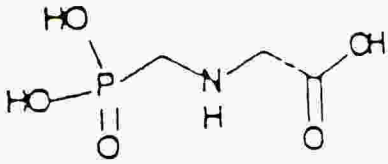
Throughout the process of assaying plant cells the steps involving handling and transfer of a cell suspension are always likely to provide a source of experimental error. It is critical to ensure that the suspension remains as homogenous as possible, thereby avoiding variation caused by sampling from a cell-settling suspension. The recommendation is to keep the number of transfer steps in the process to a minimum. Similar caution should be observed if biomass assay is by optical density measurement. It is most practicable to use a system where the light source travels through the base of the vessel rather than the sides. However, even if a uniform "settling time" is used in the operation, account still needs to be made of the "tea leaves in the tea cup" phenomenon where the depth of cells will vary on the bottom of a vessel. This margin of error in an OD measurement must be taken into account and it will be advisable to make multiple readings, either by carrying out the test in replicate or, at least, taking successive readings of the same vessel after renewed disturbance.

Another advantage that plant cell culture offers over whole plant assays is in affording a higher throughput, this can be further exploited with the use of automated systems. However, there is a note of caution to sound here; plant cell culture is very sensitive to many external conditions, especially rough handling. This eliminates the possibility for using many liquid-handling gadgets as the gentler of these are often incompatible with the requirement to maintain a non-settling suspension.

If the aim of a plant tissue culture screen is to identify chemicals which are herbicidal by inhibiting plant amino acid biosynthesis the hit rate can be expected to be low; in this case a high throughput is important. Consideration will need to be given to the most efficient cascade. Since most herbicidal compounds will not arrest plant cell culture growth by inhibiting amino acid biosynthesis it is probably reasonable to screen compounds simply for phytotoxicity first. Positives can be subsequently rescreened to confirm the initial result and also for relief of inhibition by the addition of one or more amino acids. Unless there is reason to guess which amino acid(s) are likely to mediate relief of inhibition then it is probably most efficient to test amino acids as mixtures, and subsequently follow positives by reducing the number of amino acids in the mixture until the reversal can be attributed to one or more particular amino acid.

However, while this sounds neat, very often the situation does not resolve so easily. A typical example is the work of Killmer *et al.* on the investigation of the mode of action of glyphosate. Working with carrot cells they showed that 2.5mM aspartate would relieve growth inhibition, although this contrasted with a previous, parallel, experiment with duckweed where there was no reversal with aspartate. Nevertheless Killmer and his colleagues went on to investigate further the possibility that glyphosate was involved in the inhibition of the asparagine pathway in carrot cell culture, perhaps by acting as an aspartate analogue.

glyphosate



The first results were not encouraging, far from promoting cell growth Killmer saw high levels of asparagine, 6.6mM, inhibiting cell growth. Regulation of the aspartate amino acid family is fairly complex and other components of the pathway were added in varying concentrations and combinations, *ie.* lysine, methionine, threonine and homoserine, (0.1-0.3 mM); but again no convincing reversal of glyphosate was seen.

This lack of reversal by asparagine and aspartate lead Killmer and co-workers to consider that aspartate reversal of glyphosate inhibition might be occurring through deamination or transamination of aspartate, resulting in a donation of carbon skeletons to the tricarboxylic acid (TCA) cycle via oxaloacetate. They went on to show that this was indeed what could be happening by demonstrating that TCA cycle intermediates such as α-ketoglutarate, succinate and malate would effectively relieve glyphosate inhibition. Further experiments revealed more reversal agents: pyruvate, phospho-enolpyruvate, glycerate, glucose-1-phosphate, glucose-6-phosphate and fructose-6-phosphate.

At first sight this long list of reversal agents for the phytotoxic activity of glyphosate is at odds with what is believed to be the major mode of action of the herbicide in whole plants, namely the inhibition of 5-enolpyruvylshikimic acid-3-P synthase, (EPSP synthase). The evidence points to glyphosate inhibition in plant cell culture being due to insufficient respiratory substrate. In whole plants the effect of EPSP synthase inhibition is manifested in the accumulation of shikimate as a consequence of poor regulation of the shikimic acid pathway. However, this has the knock-on effect of depleting respiratory substrate in the form of phospo-enolpyruvate and erythrose-4-phosphate, hence the reversal of glyphosate by an assortment of respiratory substrates.

In the case of glyphosate the patterns of reversal observed have lead to insights into the complexities of plant biochemistry which might otherwise have been missed. However, for the biologist using plant cell culture as a model to study the mode of action of herbicides a note of caution is sounded, care should be exercised in the interpretation of results and it would be as well to prove the hypothesis suggested by the initial results.

Results in any tissue culture system must be carefully analysed and the method used fully validated. Often reports of inhibition are not repeatable and claims of inhibition of protein synthesis may omit the fact that dead cells do not synthesise proteins (Gressel, 1984). It is also essential to ensure that the chemical used is pure and that the inhibition and reversal are not measured on a very active contaminant.

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Session 2.

Chairman: DR J. DALZIEL

Use of Mutagenesis to Probe the Structure and Function of AATase

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L-Aspartate aminotransferase (L-AATase) is a pyridoxal phosphate (PLP) dependent enzyme involved in amino acid metabolism. More than 60 transaminases have been identified, differing in substrate specificity, and capable of transforming over 100 amino acids including ω-amino acids and D-amino acids (Cooper and Meister, 1985; John and Fowler, 1985). L-AATase is the best characterized of these (see reviews by Braunstein, 1973, and Jansonius and Vincent, 1987). The three-dimensional structures of several vertebrate isozymes and various derivatives are available (Ford et al., 1980; Borisov et al., 1980; Arnone et al., 1985; Harutyunyan et al., 1982; Torchinsky et al., 1982) and the information from these sources has been molded into a detailed mechanism of action for this enzyme (Kirsch et al., 1984; Jansonius and Vincent, 1987).

The L-AATase reaction involves a double displacement (ping pong Bi Bi; Snell, 1945; Metzler and Snell, 1952; Velick and Varva, 1962; Kiick and Cook, 1983) in which the enzyme shuttles between the pyridoxal phosphate form of the cofactor and pyridoxamine phosphate (PMP) form of the cofactor (Figure 1). Various coenzyme-substrate intermediates, formed and transformed during the course of the reaction, have been identified by characteristic absorption and circular dichroism spectra (Braunstein, 1973; Metzler, 1979), and kinetic analysis (Fasella and Hammes, 1967). Formation of these intermediates is accompanied by conformational changes (Birchmeier et al., 1973; Gehring and Christen, 1978). Crystallographic studies of the enzyme in the presence of substrate analogs and inhibitors such as succinate (Arnone, 1985), 2-methylaspartate (Arnone, 1985; Jansonius and Vincent, 1987), and pyridoxyl amino acid derivatives (Eichele et al., 1979) have been

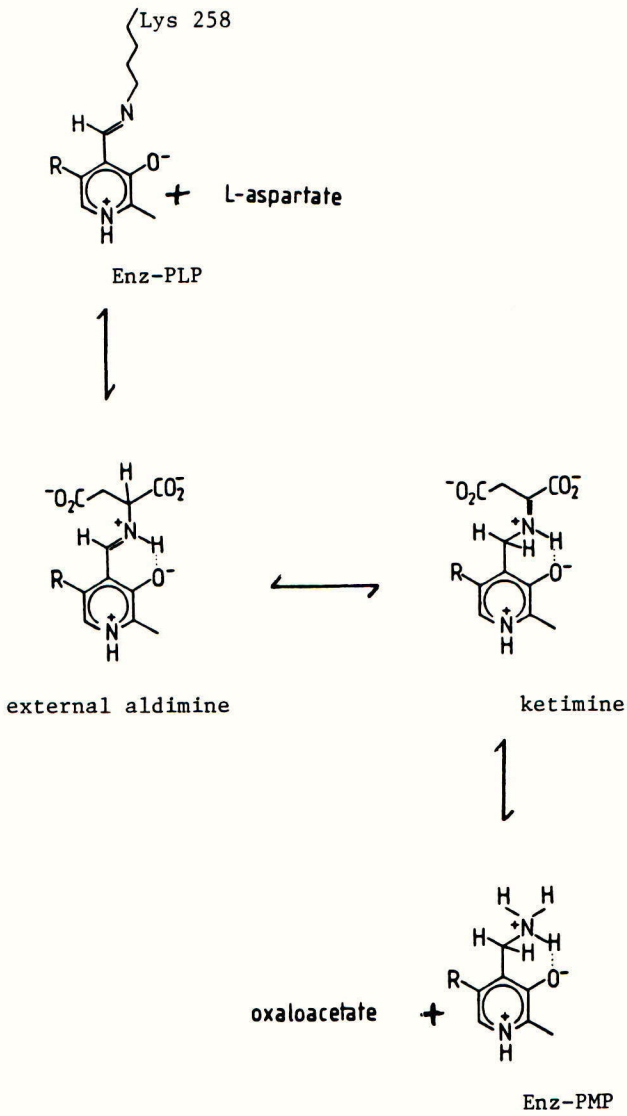

Figure 1. Reaction of aspartate aminotransferase with aspartic acid.
R = $CH_2OPO_3^{2-}$

crucial in elucidating the nature of the intermediates of the reaction and in mapping some of the conformational changes that occur at different stages of the reaction. This discussion will focus on these conformational changes and try to identify some of the factors which contribute to these structural movements.

The enzyme being studied is the L-AATase from E. coli which has been cloned, sequenced and expressed (Malcolm and Kirsch, 1985). Site-directed mutagenesis has been used to probe precisely the roles of individual amino acid residues in the function of this protein. A combination of kinetic and structural studies is being used to answer questions about the roles of these individual amino acid residues in promoting and/or stabilizing these conformational changes.

A diagram of the structure of the enzyme is shown in Figure 2. The enzyme consists of two subunits, each with two domains. The large domain of one subunit contains the lysine which forms the Schiff's base with PLP in the wild type enzyme, and the specificity site for the distal-carboxyl group of a substrate molecule. The small domain interacts with the α-carboxyl group of the substrate. Two arginine residues, Arg386 on the small domain and Arg292 on the large domain of the adjacent subunit, interact with the two substrate carboxylate groups. The two domains are connected by a long helix.

The conformational change which accompanies the binding of specific substrates and inhibitors has been described as a rigid body rotation of the small domain with respect to the large domain, bringing the two domains into closer apposition (Arnone et al., 1985). Domain closure is thought to be required for efficient catalysis (Kirsch et al., 1984); however, the precise nature of this contribution to the mechanism is

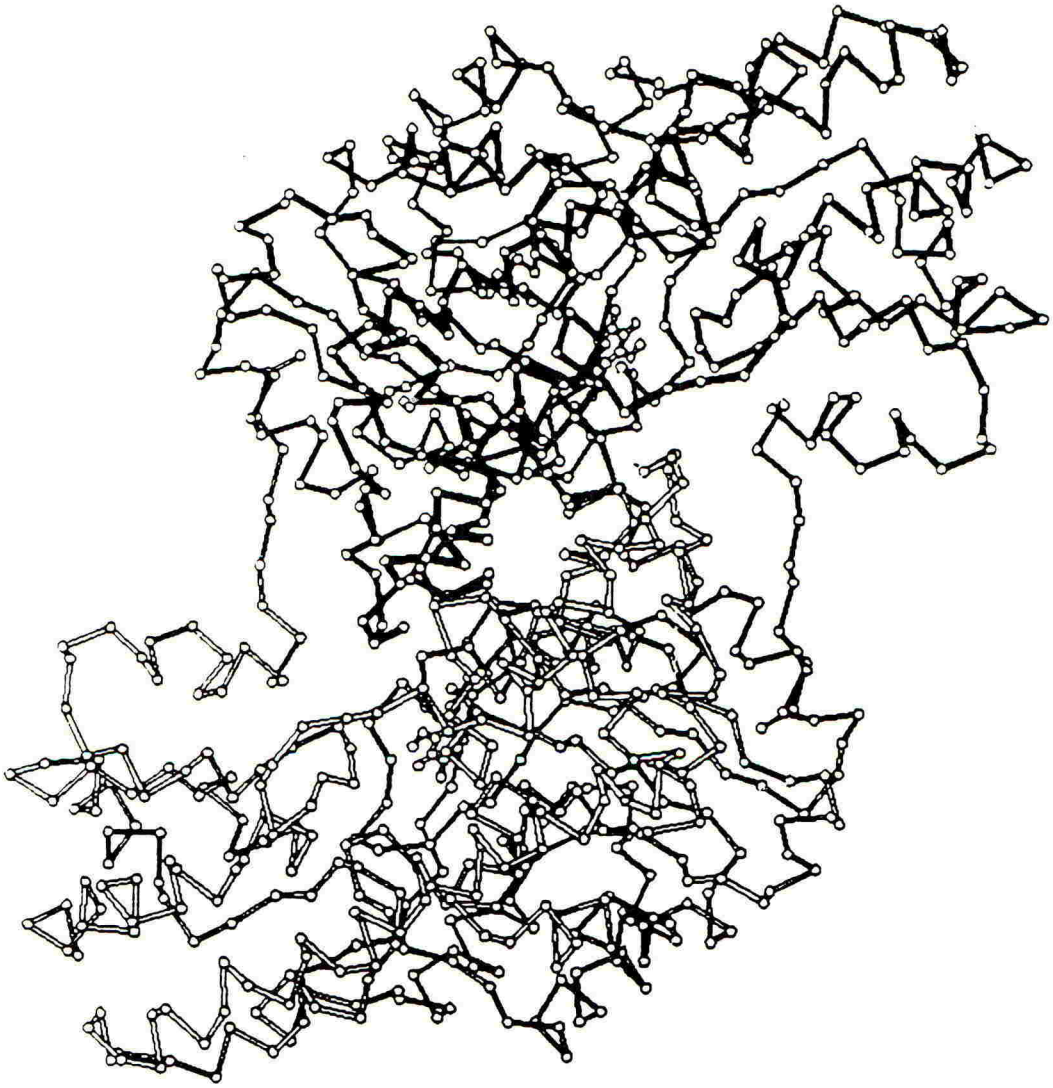

Figure 2. An illustration of the alpha-carbon backbone of a dimer of L-AATase

uncertain, and the roles of individual amino acid residues in promoting such a conformational change are unknown. The overall effect of the conformational changes is a tightening of the protein structure around the substrate.

For the purposes of this discussion the structure of the wild type enzyme with PLP bound will be used as the standard open form to which all conformational changes are referred. This structure contains a bound sulfate ion in the active site and may therefore be considered to have an inhibitor bound. Comparison to the mitochondrial enzyme (Jansonius and Vincent, 1987) shows that the open form of the E. coli enzyme is less open than the mitochondrial enzyme, and may reflect the presence of the bound sulfate ion. The structure of the PLP-enzyme crystallized in the presence of succinate (a competitive inhibitor) shows the closed form of this structure, in which the small domain has rotated toward the active site around an axis perpendicular to the long helix.

This same conformational change has been observed in a mutant designed to probe the role of Lysine 258 (K258A) in the mechanism of this enzyme. The tertiary structure of this mutant (Smith et al., 1989) is practically identical to that of the wild type PLP-enzyme with the exception of the lysine 258 side chain (absent), the precise position of the PMP ring (*vide infra*), and the precise position of the sulfate ion. The important observation with regard to conformational change is that the large and small domains are in the same open conformation as those in the wild type PLP-enzyme. This would indicate that the conformational change which occurs during catalysis is not dependent on the chemical form of the cofactor (Schiff's base to lysine 258 or free

PMP), or the tilt of the pyridoxal ring. This ring is tipped away from the protein in the K258A-PMP form by approximately 7° relative to the wild type PLP-form of the enzyme.

This mutant forms a stable external aldimine in the presence of oxaloacetate. The structure of the protein in this complex is in the same closed form as it is in the wild type-succinate complex. The small domain has rotated toward the active site in the same way as it has in the structure of the inhibited wild type PLP-enzyme. It is therefore tempting to draw an analogy between these two structures and to assume that the external aldimine in the wild type enzyme will be very similar to the external aldimine in the K258A mutant structure. This assumption is not entirely unwarranted since an external aldimine of the mitochondrial enzyme is in the same closed conformation as the maleate inhibited enzyme (Jansonius and Vincent, 1987).

Which parts of the enzyme are involved in this substrate/inhibitor induced conformational change? One area which has been studied is the helix which connects the large and small domains. This helix has been likened to a stiff spring which supports the movements of the domains when substrate binds (Fasella and Hammes, 1967). Analysis of the mitochondrial enzyme has indicated the presence of a kink at Glycine 325 in the center of this helix, which may act as a hinge for the conformational change. The _E. coli_ enzyme contains an aspartate residue at this position (Kondo _et al._, 1984). If the conformational change which occurs is dependent on a hinge, mutations at positions around this residue should either facilitate or hinder any movement. Several mutants have been studied (M326V, M326I, and M326L) to determine whether changes in flexibility in this region have any effect on the structure

of the relatively distant active site. Kinetic measurements with aspartate as substrate give values of k_{cat} and K_M which are not different from those for the wild type enzyme (Table I). Preliminary structural data for the complex between succinate and M326I in the PLP form indicates that the same closed form exists in this complex as in the succinate-wild type complex. The implications of these results is that the flexibility of the helix is not dictated by the interactions of a single amino acid with its neighbors, but is instead spread over a larger region of the helix and/or a larger region of the whole protein. This would indicate that the analogy to a spring is more accurate than the analogy to a hinge. It also indicates that the forces which drive the equilibrium from the open to the closed conformation are considerably stronger than possible steric interactions at the interface between the helix and the rest of the protein.

A second set of interactions which may be instrumental in forcing the conformational change leading to the closed form of the enzyme is that which includes the electrostatic interactions of the substrate (inhibitor) carboxylate group with the arginine side chains in the specificity pocket (R292) and in the α-carboxylate site (R386). The importance of these two interactions is demonstrated by the low activity of L-AATase towards monocarboxylic acids such as alanine, which is significantly increased in the presence of a second carboxylic acid such as formate (Morino and Okamoto, 1972; Danishefsky, unpublished results). The implication of these results is that the second carboxylic acid is required for the conformational change to occur, and that the absence of such a conformational change significantly slows down the enzyme-catalyzed reaction.

The roles of these arginine residues have been probed with site-specific mutations at sites R292 and R386. The specificity of the wild type enzyme for L-aspartate and L-glutamate is, in part, dictated by a favorable salt bridge between the side-chain carboxylate and the guanidinium group of arginine 292. The preference of the enzyme for negatively charged substrates over neutral or positively charged substrates is reflected in values of k_{cat}/K_M which differ by a factor of 10^5 or more (Cronin et al., 1987; Cronin and Kirsch, 1988). This difference might be the consequence of altered substrate binding, a retardation of the rate-determining step, or an altered mechanism. The structure of the R292D mutant has been studied in some detail. This mutant was expected to show specificity toward basic amino acids, arginine and lysine, over acidic amino acids, aspartate and glutamate. This prediction was qualitatively observed, as the mutant enzyme transaminates arginine 16 times more readily than it does aspartate and is five orders of magnitude less efficient than wild type toward aspartate (Table II; Cronin and Kirsch, 1988). However, the apparent increase in specificity of the mutant toward arginine is far lower than would be expected based solely on a simple charge reversal model. The mutant is essentially a nonspecific transaminase with reduced but relatively uniform activity for positively charged and neutral substrates.

One possible explanation for the reduced activity of the R292D mutant is the inability of facile domain closure to occur. If domain closure is required for efficient catalysis, then the favorable interactions of arginine 292 (and arginine 386) with the carboxylate group(s) of the substrate may be the driving force. Two lines of

evidence indicate that this explanation is, at least in part, correct. The cytosolic enzyme from pig contains a cysteine residue (390) which exhibits increased reactivity toward sulfhydryl reagents in the presence of specific substrates (aspartate; Christen and Gehring, 1982). This enhanced reactivity has been interpreted as a diagnostic for the conformational change, which alters solvent accessibility or chemical environment of this residue. This increase in reactivity is not observed in the presence of nonspecific substrates such as alanine. These observations can be interpreted to mean that catalysis of nonspecific substrates involves a mechanism which does not include domain closure, or the requirement for domain closure is the same for nonspecific and specific substrates, but the favorable energetic interactions required to effect the conformational change are absent in nonspecific substrates. Therefore, the enzyme may have to rely on thermal energy to occasionally lead to domain closure and subsequent catalysis in the presence of nonspecific substrates. Further support for the idea that conformational change only accompanies binding of specific substrates (inhibitors) comes from structural studies of the mitochondrial enzyme containing the stable reduced PLP-amino acid analog of alanine or iodo-tyrosine (Kirsch et al., 1984; Eichele, 1979). In both cases the enzyme is in the open form as would be expected based on a lack of complementarity between substrate side chain and specificity site residues.

Finally, the interactions of the α-carboxylate of substrates with arginine 386 has been studied. Two mutants, R386F and R386Y, have been made and evaluated kinetically and structurally (preliminary). Both mutant enzymes are approximately five orders of magnitude less active in

the presence of aspartate than the wild type enzyme. The tyrosine mutant R386Y is slightly more active than the phenylalanine mutant (Table II), presumably because the phenolic hydroxyl group can at least form a hydrogen bond to the substrate carboxylate group. It is interesting to note that the reduction in activity almost parallels the reduction in activity of the R292D mutant relative to the wild type enzyme. The implication of this finding is that the electrostatic interaction at either end of the substrate is almost equally important and the cause of the reduced activity may be similar. The structure of the R386F mutant has been solved and appears as the open form (with sulfate bound). Further studies are required to determine whether the closed form can be induced in this mutant.

To approach the design of a mutant in a rational way, careful model building experiments must be performed which take into account all potential polar and non-bonded contacts of the newly introduced amino acid. When altered specificity is desired, in an effort to use an existing protein to catalyze the transformation of a substrate which the naturally occurring enzyme does not accept, interactions of the new substrates with the entire active site of the protein must be examined. Careful consideration of such effects might allow potential changes in mechanism or nonspecific binding modes to be predicted. If the enzyme undergoes a conformational change during catalysis, the possible effect of the proposed mutation on the catalytic machinery must also be considered.

TABLE I. Kinetic Constants of AATase Mutants

Enzyme	K_m (mM)	k_{cat} (sec^{-1})
Wild type	.95	111
M326I	.85	105
M326V	.95	101
M326L	.90	94

TABLE II. Substrate Specificities of *E. coli* Wild type and Mutant AATase

K_{cat}/K_M, $M^{-1}S^{-1}$

Substrate	Wild type	R292D	R386F	R386Y
L-aspartate	18500	0.0695	0.5	0.8
L-arginine	0.0276	0.429		

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NEW TECHNIQUES FOR TRANSFERRING AND SPECIFICALLY EXPRESSING TRAITS IN CROP PLANTS

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ABSTRACT

Transformation, that is the transfer of foreign DNA into a plant by non-sexual means and its stable integration into the germ line, has been shown to occur for many crop species. However, examples of convincing transformation of monocotyledons, particularly from the major crops, wheat, maize and rice, are comparatively rare. Control of the expression of introduced genes has been studied and is becoming relatively well understood in a number of specific systems. A recent advance has been the demonstration that it is possible to control the expression of genes in the host plant by the introduction of DNA in the 'anti-sense' configuration. This opens up the possibilities for the directed control of biochemical pathways including those of amino acid biosynthesis.

INTRODUCTION

Any discussion of plant transformation requires some careful definitions and measurement of achievements. Claims of successful transformation of a range of crop plants far outweigh actual achievements. In the author's opinion, successful transformation of CROP plants can only be claimed when the seed (or commercial propagative form) that will eventually be used to plant the crop has been shown to contain foreign DNA introduced by means other than sexual crossing. Furthermore, for seed crops, it is necessary to show that this DNA is stably integrated and can be passed on to subsequent generations. For the rest of the discussion I will limit my comments to crops propagated by seed.

Transformation as a process can be broken down into three components; the preparation of the recipient tissue, the isolation and transfer of the foreign DNA into that tissue, and the regeneration of fully fertile transformed plants. Only when all three components are fully capable of successful manipulation is transformation achieved.

RECIPIENT SYSTEMS

Protoplasts provide the easiest target cells for the delivery of DNA. Virtually all species from which protoplasts can be isolated and cultured can be transformed, in the sense that foreign DNA can be introduced and integrated into the DNA of that species. The problem lies in the regeneration of fully fertile plants from protoplasts. Whilst this is easily achieved for many Solanaceous species, it has proved much more difficult for Cruciferous, Leguminous and Graminaceous crops (for further details see Fraley et al. 1986). The latter group of crops have been the focus of a large amount of effort, particularly because other routes to transformation of the major important cereal crops - wheat, maize and rice - have not proved successful. This effort has brought success in that protoplasts from, first, rice (Abdullah et al. 1986, Toriyama et al.

1986, Yamada et al. 1986, Kyozuka et al. 1987) and later maize (Prioli & Söndahl 1989, Shillito et al. 1989) have been regenerated into fertile plants. Subsequently transformed, fertile rice plants have been obtained (Shimamoto et al. 1989) and maize protoplasts have also been transformed and regenerated, but unfortunately into non-fertile plants (Rhodes et al. 1988).

Cells from a large number of different plant explants, including portions of roots, leaves and stems, are capable of being induced to regenerate to fertile plants and have been utilised as targets for DNA delivery. These have been particularly useful for dicotyledonous plants in conjunction with Agrobacterium as a vector. Major crops that have been successfully transformed this way include oil-seed rape (Fry et al. 1987), soybeans (Hinchee et al. 1988) and cotton (Umbeck et al. 1987, see also Gasser & Fraley 1989, for other species).

Reproductive tissues such as immature infloresences, whole gametes (usually pollen cells) and young zygotes have also been used as targets for transformation. These have the definite advantage of regeneration and the transformation of gametes should ensure direct entry into the germ-line. However, despite many claims (e.g. see Picard et al. 1988 for wheat, Luo & Wu 1989 for rice and McCabe et al. 1988a for maize) the major crops have not yet been convincingly (i.e. frequently and repeatedly) shown to be transformed by DNA transfer to gametes. Zygotic tissues have been successfully used as targets for physical transfer of DNA and subsequent regeneration of transformed brassica (Neuhaus et al. 1987) and soybean (McCabe et al. 1988b) plants. However, in both cases the embryonic tissue was subsequently cultured and multiple plants derived from single original embryos. A report of transformation via delivery of DNA to the immature infloresence of rye (de la Pena et al. 1987) has appeared but has proved difficult to repeat in many laboratories.

DNA TRANSFER

DNA may be transferred via biological or physical means. Of those crops so far transformed by far the most have been transformed using Agrobacterium tumefaciens or Agrobacterium rhizogenes. The major disadvantage of this biological system is that the natural host range of these organisms is restricted to dicots. However, there is some hope for the application of this biological approach to monocots since Agrobacterium will attach to monocot cells and has been shown to transfer DNA successfully into maize (Grimsley et al. 1987) and wheat (Woolston et al. 1988) plants, albeit without any evidence to date of integration of that DNA into the host.

The need for alternative delivery systems has stimulated the ingenuity of scientists into using a wide range of physical techniques. Where protoplasts are the targets, fusion of the protoplasts or electroporation have proved to be successful methods. The real problem occurs in transferring DNA across cell walls into complex explants or reproductive tissues. Approaches include directed microinjection into individual cells (Neuhaus et al. 1987), macroinjection of whole infloresences (de la Pena et al. 1987), and the use of microprojectiles coated with DNA (Sanford et al. 1987, Klein et al. 1987, Sanford 1988). The latter has been used to produce large numbers of transformed soybean plants (McCabe et al. 1988b). Although, there are several examples of

transformed cereal tissues being produced from biolistic bombardment only one claim of a fertile transformed monocot plant is known to date (McCabe et al. 1988a).

GENE EXPRESSION

Specific improvement of crop plants requires the controlled expression of transferred genes and the targeting of the gene product to the correct biochemical environment. A large amount of research on plant promoters has been carried out and examples of sequences that control the expression of genes under a whole range of internal (e.g. position and time) and external (e.g. temperature, amount and spectral quality of light, oxygen tension, presence of certain chemicals, physical pressure and osmotic pressure) factors. In many cases the DNA sequences concerned have been isolated and used to drive the expression of other genes. One particularly favoured reporter gene has been the GUS gene (Jefferson et al. 1987) which has the advantage of producing a coloured product and thus large numbers of impressive graphical descriptions of controlled gene expression.

Targeting of gene products to organelles has also been studied. Of particular relevance to a symposium on amino acid metabolism has been the delivery of genes involved in amino acid metabolism into the chloroplasts. Thus della-Cioppa et al. (1987) have shown that altered versions of EPSP synthase can be successfully targeted into chloroplasts.

Transfer and control of gene expression need not always imply the addition of gene function to crop plants. In particular, it is possible to envisage that the control of gene expression might be used to manipulate amino acid biosynthesis in plant cells to change the balance of end products produced by specifically turning off certain genes. These possibilities exist by virtue of the demonstrations that transformation of plants by DNA expressing the anti-sense version of a gene leads to the diminution or loss of expression of the target host gene (see Rothstein & Lagramini 1989). This technology has lead to the control of expression of several enzymes including polygalacturonase (Sheehy et al. 1988, Smith et al. 1988) and the small subunit of Rubisco (Rodermel et al. 1988). This has the effect of generating specific mutations in a pathway similar in nature to those selected in bacteria to enhance the fermentative production of amino acids. Furthermore, the exploitation of some of the control sequences mentioned above in front of the anti-sense construction would lead to the possibility of inducing conditional mutations at will. There is the promise of even better technology for turning off gene function by interfering with the messenger RNA produced from specific genes. This arises because it has been shown that RNA sequences can act catalytically to cleave other specific RNA sequences (Cech 1987, Haseloff & Gerlach 1988). If such 'ribozymes' can be tailored to control gene expression in vivo they could be more effective than the stoichiometric control given by anti-sense RNA.

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DISCOVERY, TRANSFER TO CROPS, EXPRESSION AND BIOLOGICAL SIGNIFICANCE OF A *BIALAPHOS RESISTANCE GENE

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ABSTRACT

Glufosinate and bialaphos are non-selective herbicides which act by inhibiting the plant glutamine synthetase. A gene which confers resistance to bialaphos (bar) was isolated from Streptomyces hygroscopicus, an organism which produces bialaphos and was shown to encode a phosphinothricin acetyl transferase. Using Agrobacterium-mediated Ti plasmid transformation methodology, the bar gene has been introduced and expressed into several plant species. Transgenic tobacco, tomato, potato, alfalfa, oilseed rape and sugarbeet plants showed a complete resistance towards the commercial glufosinate and bialaphos.
The followed strategy presented a successful approach to obtain herbicide-resistant plants by introducing a pathway for detoxification of the herbicide. Subsequent field tests demonstrated complete resistance of engineered plants to field dose applications and confirmed that glufosinate can be applied as a selective post emergence herbicide on engineered crops.
The bar gene also revealed a convenient selectable and scoreable marker gene in transgenic plants.

INTRODUCTION

In modern agriculture, herbicides are indispensable as they allow economic weed control and increase the efficiency of crop production.
A number of new herbicides combine high effectivity with non-toxicity to animals and rapid degradation in the soil. However, they often lack selectivity, limiting their use to pre-emergence applications.
The engineering of plants to become resistant to broad spectrum herbicides that are highly effective, safe for animals and rapidly biodegraded, would allow the selective use of these chemicals and their application for post emergence applications in more effective and flexible weed control programs. Success with resistance engineered towards a number of commercially used herbicides has been reported (for review, see Botterman and Leemans, 1988). An approach to engineering crop resistance is the introduction in plants of a pathway that degrades and/or detoxifies the herbicide. Plants were engineered resistant to the non-selective herbicides glufosinate or phosphinothricin (PPT) and bialaphos by transferring and expressing a herbicide inactivating enzyme in transgenic plants. The gene which confers resistance to the herbicide was isolated from Streptomyces hygroscopicus, the organism which produces the tripeptide bialaphos as a secondary metabolite.

* The accepted common name for this compound is bilanafos

HERBICIDES

Bialaphos (Kondo et al., 1973) and phosphinothricin (PPT) (Bayer et al., 1972) are potent new herbicides. Bialaphos is a tripeptide antibiotic produced by Streptomyces hygroscopicus or Streptomyces viridochromogenes. It consists of PPT and two L-alanine residues. Upon removal of these residues by peptidases, PPT is an inhibitor of glutamine synthetase (GS). This enzyme plays a central role in the assimilation of ammonia and in the regulation of nitrogen metabolism in plants. It is the only enzyme in plants that can detoxify ammonia released by nitrate reduction, amino acid degradation and photorespiration. Inhibition of GS by PPT causes rapid accumulation of ammonia which leads to death of the plant cell (Tachibana et al., 1986). Glufosinate is chemically synthesized ('Basta' 200 g a.i./l), while bialaphos is produced by fermentation of Streptomyces hygroscopicus ('Herbiace', 330 g a.i./l).

BIALAPHOS RESISTANCE GENE

Streptomyces strains which produce different antibiotics have evolved mechanisms to avoid the toxicity of their own products. Similar as observed with other clusters of antibiotic production genes, a gene coding for antibiotic resistance was physically linked to the biosynthesis pathway for bialaphos (Murakami et al., 1986). It has been shown that the bialaphos is synthesized from three carbon precursors in a series of at least thirteen conversions. Many of these genes have been defined by blocked mutants. One of these intermediate steps contained an acetyl-coenzyme A - dependent activity which can modify either demethylphosphinothricin, an intermediate in the pathway, or PPT itself. This gene which confers resistance to bialaphos (bar) has been isolated from S. hygroscopicus and characterized (Thompson et al., 1987). It has been shown that the bar gene is at the same time an antibiotic-resistance and a biosynthetic resistance gene since it plays a role in both self-defense and bialaphos biosynthesis (Kumada et al., 1988). The gene was shown to encode a polypeptide of 22kDal with acetyl transferase activity (phosphinothricin acetyl transferase - PAT), which converts PPT into a non-herbicidal acetylated form, by transferring the acetyl group from acetyl-coenzyme A on the free amino group of PPT. Analysis of the substrate specificity of the acetyl transferase by determining the Km and kcat value for PPT and chemically related compounds, showed that the enzyme has a very specific substrate requirement and preferentially acetylates PPT. Optimal activity of the enzyme was found at 35°C and neutral pH. These reaction conditions looked at first hand adequate for use as a herbicide resistance trait in transgenic plants.

EXPRESSION IN TRANSGENIC PLANTS

A possible strategy to engineer herbicide resistance in plants is based on expression of an enzyme that detoxifies the herbicide. A chimeric gene construct with the bar gene under control of the CaMV 35S promoter was transferred in tobacco, tomato and potato using Agrobacterium mediated Ti plasmid transformation methodology (De Block et al., 1987).
Transformed calli were selected on 2.5 to 25 mg/l and were resistant up to 1g/l PPT, while no growth was observed with control plants. The bar gene proved to be a convenient dominant selectable marker for plant transformation. Transgenic tobacco and potato plants expressing the bar gene are resistant to applications of glufosinate under greenhouse conditions. An active PAT enzyme was produced in the plant cells.

The growth of transgenic plants was indistinguishable from non-transformed control plants. Glufosinate at 400 g a.i./ha killed control plants in ten days. Transgenic plants were resistant to herbicide treatments of 4000 g a.i./ha. Additional applications of the herbicide did not affect growth of the plants. Transgenic plants expressing various levels of PAT were fully resistant to the herbicide. Treated resistant plants did not show any increase in NH_4^+ content and showed a complete protection of the plant glutamine synthetase from the action of the herbicide. Treated plants also flowered normal and produced normal amounts of seed. These plants proved also resistant to applications of bialaphos. Similar results obtained with transgenic sugarbeet, oilseed rape and alfalfa proved that a complete resistance towards high doses of glufosinate was obtained independent of the plant species used. The trait was also shown to be inherited as a dominant Mendelian trait. Beyond its practical use, the bar gene also revealed a convenient selectable and scoreable marker gene in plant transformation and expression technology. The enzymatic activity can be detected chromatographically and a spectrophotometric acetyl transferase assay allows quantification by measuring enzyme kinetics (De Block, 1988; Denecke et al., 1989; De Almeida et al., 1989).

FIELD TRIALS

Although the results with transgenic plants in the greenhouse look very promising, different important questions have still to be addressed. Observations in the laboratory and the greenhouse, which are needed to monitor the expression of the introduced gene and the behavior of the genetically modified crop have to be followed by open field trials to prove statistically significant performance and yield data. They answer two recurring questions: 1) how stable is the expression of the introduced gene under the highly variable conditions in the field, opposed to the tightly controlled conditions in the greenhouse, and 2) do plants suffer any undesired effects as a result of the genetic modification ?
Moreover, these field trials with engineered crops have to teach us more about the qualitative and quantitative characteristics of engineered crops relative to competitive existing products, and potential risks coupled to the cultivation of genetically engineered plants. The following risks are to be considered: 1) the offspring of the genetically engineered plant may become a pest; 2) the newly introduced information is transmitted to a related wild species; 3) the transgenic crop may have an ecological impact. The most significant risk with genetically modified plants is the transfer of newly-acquired genes to wild and weedy relatives.
Several precautions are taken during the present small-scale field trials to eliminate the risk of spread of the recombinant genes: absence of relatives in test areas, prevention of pollen transfer (by early harvesting, buffer zones or deflowering) daily plant monitoring, access restricted to authorized personnel, destruction of the removed plant and seed material, control of volunteer weeds and follow-up of the field in subsequent sessions. In a first trial in 1987, two transgenic tobacco and four potato lines were evaluated under field conditions (De Greef et al., 1989). Transgenic plants of the tobacco dwarf variety SR1 as well as untransformed control plants were planted in the field according to a factorial design to compare different genotypes and four levels of herbicide application. Untransformed plants were used as control instead of plants obtained from the same regeneration protocol in order to evaluate the agronomic performance of transgenic crops.

In the weeks following the herbicide applications, no visible effects or damages were observed on the resistant plants. The tobacco trial was assessed by measuring the length of the largest leaf before and after herbicide application. This method is a standard method for evaluating tobacco growth data. The analysis of variance demonstrated that there was no significant difference between the treatments. Since flowers have to be removed from the plants according to the field test protocol, only a subset of plants were qualitatively analysed for flowering and were bagged for selfing. The flowering and seed set were not influenced by the herbicide treatments and confirmed previous greenhouse tests. This first field test proved the complete resistance to field dose applications of glufosinate in both lines tested, even although the expression of the resistance gene in these lines varied by two orders of magnitude.
Transgenic lines obtained from three commercial potato cultivars were chosen for field tests: Bintje, Berolina and Desiree. In the weeks following the herbicide treatment, there was no difference between unsprayed controls and transformed plants sprayed with the herbicide, even at the highest dose. At harvest, the number of surviving plants per plot and the tuber weight were recorded. In general, there was no difference between the unsprayed controls and the sprayed transformed lines in percentage of surviving plants. The production of tubers varied strongly depending on the source of planting material; but these differences were observed in the control material as well as in the transgenic lines, and are most likely a consequence of the in vitro propagation conditions of the planting material. For the statistical analysis, the fresh tuber weight per surviving plant was determined for each plot. The analysis of variance indicated no significant difference between the control and any of the sprayed herbicide resistant lines.
The growth of the transgenic tobacco and potato lines was indistinguishable in all treatments from the non-transformed non-treated control lines over the whole crop cycle and they showed the same agronomic performance (De Greef et al., 1989).
In 1988, field trials have been performed with transgenic commercial lines of tobacco cultivar pBD6. The results showed that the presence of the bar gene conferred total resistance to the herbicide. There were no phenotypically differences observed between the transgenic lines and the untransformed control lines. Moreover, weed control with the commercial preparation of glufosinate allowed to perform optimal weed control during the whole growth season.
In 1989 field trials are planned with herbicide resistant tobacco, tomato, oilseed rape, sugarbeet and alfalfa.

CONCLUSION

As opposed to other strategies in which a nonsensitive mutant enzyme is produced or in which the target enzyme is overproduced, we introduced a detoxification pathway for the herbicide. The strategy followed here to engineer plants resistant against a broad spectrum herbicides clearly illustrate the advantage for using bacterial detoxifying enzymes.
This system is independent from the plant species, highly effective and has no effect on crop performance.
The herbicide-resistance genes can also be used as dominant selectable markers for both basic research and plant breeding. For basic research, the resistance genes will serve as analogues of the widely used antibiotic resistance genes in selecting transformants. For plant breeding, the resistance genes can be physically linked to other genes conferring agronomically useful traits which are not easy to follow during transformation and regeneration procedures.

They are introduced in plants via transformation using selection for resistance to the herbicide. Subsequently, in a plant breeding program the trait can be transferred to various cultivars through standard genetic crosses by following the easily assayable herbicide resistance phenotype associated with the linked marker gene. Moreover, the spraying of the seedlings can be used to follow segregation and offers a great advantage for selection of progeny grown in soil, this in contrast with many other marker genes.

ACKNOWLEDGEMENTS

We thank our collaborators at PGS, who participated in most of this research: M. De Block, W. De Greef, K. D'Halluin, D. Rahier and C. Thompson at Institut Pasteur Paris.

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TRANSFER OF *IN VITRO* SELECTED IMIDAZOLINONE RESISTANCE TO COMMERCIAL MAIZE HYBRIDS

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ABSTRACT

In vitro selection has been used to develop a maize inbred line resistant to imidazolinones. Resistance is due to a single gene coding for a resistant form of acetohydroxyacid synthase, the enzyme affected by imidazolinones. The *in vitro* selected line is not suitable for use in producing commercial maize hybrids due to agronomic deficiencies. Therefore, the single gene is being transferred to over 100 commercial maize inbred lines by backcrossing. Backcrossing and subsequent hybrid testing require seven years. This delay in releasing resistant commercial hybrids is an important consideration when projecting the commercialization of biotechnology modified cultivars. These considerations are relevant whether *in vitro* selection or transformation is used to add a new trait to a cultivar.

In vitro selection and transformation are providing novel variation for crop improvement. Researchers are seeing the results of biotechnology in the form of herbicide resistance, insect resistance, and disease resistance. These are at various stages of development but whether from *in vitro* selection or through recombinant DNA technology they are a source of improved traits that will be used to improve existing or new cultivars.

In nearly every instance, these improved traits, or more specifically the genes that control them, will be carried in crop strains that cannot be marketed directly. The ability to form callus that can be used for *in vitro* selection varies among genotypes. Certain genotypes respond better than others so that in some species, certain varieties or inbred lines are favored for producing callus or suspension cultures needed for selection. The process of cell culture itself can lead to unpredicted genetic changes known as somaclonal variation. *In vitro* selection usually prolongs the length of time in culture and leads to deleterious changes in a crop strain even though selection for the desired trait was successful. Thus for many crops, researchers are limited to certain strains for *in vitro* selection and can not expect to regenerate plants from selected cultures that show only the changes in the selected trait.

In a similar vein, many transformation systems require cell cultures such as callus or protoplasts as target tissues. The culturing and subsequent selection of putative transformants may lead to undesired genetic changes. Also, as for *in vitro* selection, cell culture systems needed for transformation favor certain genotypes.

These may or may not be genotypes that can be used directly for commercial production. Particle gun technology may allow gene introduction into elite genotypes, but this technology is not yet fully developed. Thus for most crops, transformation and in vitro systems which allow directed changes in any cultivar or inbred line without deleterious side effects do not yet exist. Therefore, researchers see successes from biotechnology in the introduction of novel traits into crop species, but are not in a position to introduce these changes into any crop variety and move them directly to the market place. Usually the gene or genes controlling the trait must be transferred to elite varieties or inbred lines. This will require time for the breeding process and for retesting of the converted cultivar. These considerations should be kept in mind when projecting the commercialization of biotechnology modified cultivars.

As an example of the steps involved in transferring an in vitro selected trait into breeding lines, the following is a review of a collaborative project between the American Cyanamid Company and Pioneer Hi-Bred International, Inc. to develop imidazolinone resistant maize hybrids.

The imidazolinone family of herbicides has been developed by the American Cyanamid Company. Some of the imidazolinones such as imazaquin and imazethapyr are for soybean weed control. They are effective herbicides with a broad spectrum of weed control, versatility of application, and all evidence indicates they are environmentaly safe chemicals. However, these imidazolinones are toxic to maize. Having a source of resistance in maize would add the useful imidazolinones to the array of herbicides available to maize farmers. In conjunction with American Cyanamid, Molecular Genetics, Inc. of Minneapolis used in vitro selection to develop maize resistant to imidazolinones.

Herbicide resistance is a good target for in vitro selection as the selective agent can be included in the media. Callus derived from the F_1 cross A188/B73 was grown on media containing an imidazolinone herbicide. Most callus died as expected but a cell line was identified which survived and grew in the presence of imidazolinones. Plants were regenerated from callus and grown to maturity in the greenhouse. Subsequent progeny were resistant to field applications of imidazolinones. Imidazolinones' mode of action is to interfere with the AHAS (acetohydroxyacid synthase or acetolactate synthase) enzyme and thus reduce synthesis of valine, leucine, and isoleucine. Biochemical analysis of the resistant maize line showed that it can synthesize these amino acids in the presence of imidazolinones. Genetic analysis indicates inheritance is controlled by a single partially dominate gene. Thus the evidence supports a hypothesis that the resistant maize inbred has an altered form of AHAS that is resistant to imidazolinones. The inheritance is described as partial dominance in that with appropriate rates of certain imidazolinones, all three genotypes can be differentiated. Heterozygous plants are very resistant but with certain imidazolinones and at high rates they can be affected, whereas the homozygous resistant plants seem unaffected.

Resistance is dramatic. Using imidazolinones directly on maize at lethal use rates for susceptible genotypes, the resistant inbred thrives. This strongly expressed single gene inheritance lends itself readily to gene transfer through traditional breeding techniques. Following is a description of the process Pioneer is using to commercialize imidazolinone resistant hybrids and why such steps are necessary.

The original resistant inbred line derived from callus can not be used directly for producing commercial hybrids for two reasons. One is that this inbred is poor agronomically due to the realities of maize tissue culture and in vitro selection so that is not an acceptable hybrid parent. Even if it was, the resistance must be present in more than one inbred line for a seed company like Pioneer to offer resistance in several hybrids. Pioneer has a very large number of inbred lines that are used to make commercial hybrids and many more that may become parents of commercial hybrids. The situation would be similar for any commercial seed company. Thus, even with more favorable transformation and cell culture techniques, it is unlikely that these would be used to introduce a gene into all the inbreds Pioneer is currently testing. This same reasoning would apply to any other crop with a large number of different hybrids or varieties being sold.

Backcrossing is the breeding method used to introduce the imidazolinone resistance gene into over 100 inbred lines of maize. For those unfamiliar with breeding, backcrossing is a well known method for transferring single genes from one variety to another creating a nearly isogenic version of the original variety or inbred line.

The resistant donor line is crossed to a susceptible elite line. The F_1 generation is all heterozygous and resistant and contains 50% of its genes from each parent. This generation is then crossed back to the elite (recurrent) parent. This introduces an entire haploid genome from the recurrent parent. The gamete from the F_1 plant on the average will contain half of its chromosomes from the donor and half from the recurrent parent due to independent assortment at meiosis. Thus, after the first backcross, the offspring are 75% recurrent parent and 25% donor parent. Half of these individuals will be heterozygous for resistance and half will be homozygous susceptible. They are planted and sprayed to identify the heterozygous resistant plants. These are again crossed back to the recurrent parent giving a generation that is 87.5% recurrent parent and 12.5% donor parent. This process is repeated until approximately 99% of the recurrent parent has been recovered. The inbreds are then selfed to derive homozygous resistant seed.

This procedure is straight forward if you have a good selection system for differentiating between heterozygous resistant plants and homozygous susceptible plants. One significant consideration is that by selecting the resistant plant, that entire donor chromosome is being selected. Hopefully a crossover will transfer that allele to the recurrent parent chromosome and by selecting those plants most similar in phenotype to the recurrent parent, breeders are selecting those that have the allele recombined into the recurrent parent

chromosome. If not, the derived inbred will not likely be close enough in phenotype to the recurrent parent to use and additional backcrosses will be necessary. This is one of the drawbacks of backcrossing. If the resistance gene is linked tightly to an undesirable gene in the donor parent, it can be difficult to obtain progeny that have had the desired recombination.

Another limitation to backcrossing is that the time involved to reach a 99% recovery of recurrent parent requires seven seasons. While an inbred is being converted, it is already being used to produce imidazolinone susceptible commercial hybrids. Meanwhile an aggressive breeding program will have developed many new inbreds so that when backcrossing is complete, the converted inbred has been replaced in the production of new hybrids. This is a real concern for Pioneer because its breeders are introducing many new inbreds each year and backcrossing can not keep up with them. This problem is reduced by using winter nurseries to make backcrosses. Pioneer can obtain two seasons per year using a cornbelt nursery in the summer and a winter nursery or can use a winter nursery such as Hawaii and obtain three seasons per year. This is probably necessary to make the backcrossing a viable process in a commercial seed company with a productive breeding program. This would reduce the number of years required for backcrossing to a minimum of two and probably a maximum of three.

The next stage in the process is hybrid testing. Once an inbred in converted, it must be tested in hybrid combinations to evaluate not only resistance but hybrid performance for yield, lodging resistance and all other agronomic traits. Because of linkage to deleterious genes you can not assume a converted inbred can be substituted for the original susceptible inbred in hybrid production. The inbred must be tested in comparison to the original. If performance of the converted inbred is equal or better than the original inbred, then an imidazolinone resistant hybrid can be introduced. This testing can take a period of years depending on the philosophy of the company and past experience with converted inbreds. Additionally, time must be allowed for increasing inbred seed for commercial hybrid production.

With all of these considerations, converting an existing hybrid to resistance could take at least seven years. Most maize hybrids would be considered old at that point. However, if researchers begin conversion of inbreds before they have become parents of commercial hybrids then a few years can be gained. This is why so many inbred lines are in the conversion program. Pioneer does not expect to make commercial hybrids with all of those inbreds but by starting with a large number of inbreds that have not yet reached commercial status, the chance of having included those inbreds that will be parents of commercial hybrids in the near future is increased. It is a matter of making predictions based on the use of these inbreds in experimental hybrids by Pioneer's maize breeders.

On the positive side, if several inbreds are converted and testing demonstrates that they are good conversions, then those inbreds can be used by breeders in their pedigree breeding programs where two inbreds are crossed to make a segregating F_2 population. If both parents are converted then their breeding population is fixed for resistance and

the gene will be present in any new inbred derived from that population. It remains to be seen if that becomes reality. Backcrossing of single genes has not played a major role in maize breeding and researchers have had few examples of a single gene being introduced into so many inbreds. The Ht gene for northern leaf blight (Exserohilum turcicum) resistance has been backcrossed into certain inbreds that are most susceptible to northern leaf blight but not generally into all inbred lines. Some companies convert their inbreds to the wx phenotype but then those inbred will not likely be used in a non-waxy breeding program.

There are several considerations before this project becomes a commercial success. The level of imidazolinone resistance must be adequate for commercial use. All evidence to date indicates that it is, but thorough testing must be completed before the hybrids are sold.

The resistant AHAS gene could have a negative effect on agronomic traits. One inbred line has been converted to resistance by backcrossing and this line appears isogenic to the original, susceptible recurrent parent. In 1988, the two lines were compared in extensive field tests in Kansas, Missouri, Iowa, and Illinois. The resistant line was equal to the susceptible line for grain yield, harvest moisture, plant height, lodging resistance, and flowering date. The results indicate that the resistant AHAS gene has no negative effect on these important agronomic traits. This is encouraging but subsequent converted lines must still be field tested because of potential linkage drag of undesirable genes from the donor parent.

The utility of imidazolinone resistant maize hybrids will be determined by farmers. The availability of resistant hybrids offers the farmer the option of using imidazolinone herbicides which do offer some advantages over currently available maize herbicides. These hybrids can still be treated with standard maize herbicides and the farmer is not forced into using certain herbicides. Adding resistance via biotechnology has simply provided another management option.

In summary, in vitro selection and transformation offer exciting possibilities for introducing traits into crops that might not be possible through traditional breeding. This author believes it is certain that the public will see such modifications reach the market in the near future. However, because of the considerations discussed, transformed or in vitro selected cultivars will probably have to pass through the hands of breeders again before they can be introduced for sale. This is a clear example of why biotechnology for crop improvement must go hand in hand with crop breeding.

Session 3.

Chairman: DR B. J. MIFLIN

SYNTHESIS OF ANALOGUES OF 5-FLUORO-4-AMINOPENTANOIC ACID

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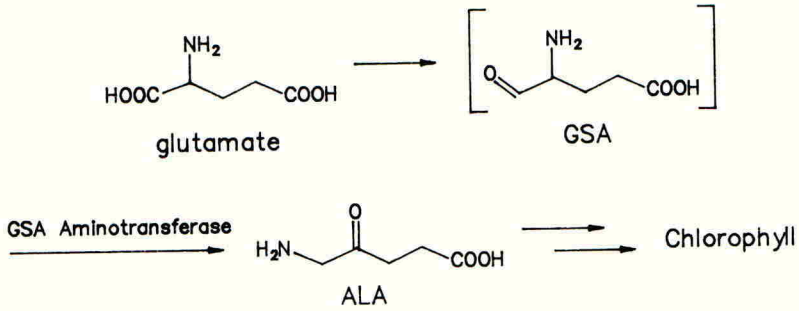
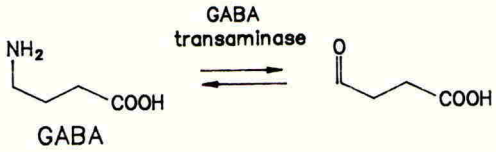
The rate-limiting step in the biosynthesis of chlorophyll is the conversion of glutamate to 5-aminolevulinic acid (ALA). This conversion (Scheme I) appears to involve a unique tRNA activation of the C-1 carboxylate followed by a reduction to produce the unstable intermediate, glutamate-1-semi-aldehyde (GSA).[1] GSA is then transaminated by GSA aminotransferase to provide ALA. This transamination of GSA to ALA is



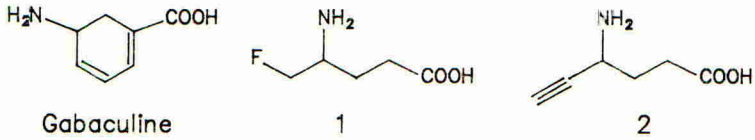
SCHEME I

quite similar to the catabolism of 4-aminobutyric acid (GABA) by GABA transaminase (GABA-T), an enzyme which has been extensively studied due to the importance of GABA as a neurotransmitter.



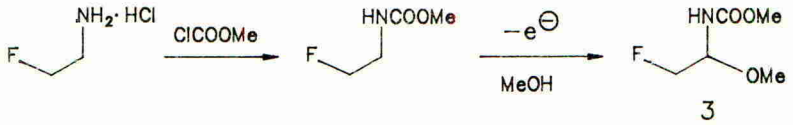
SCHEME II

Researchers at Merrell-Dow have designed numerous irreversible ("suicide") inhibitors of GABA-T as potential pharmaceuticals.[2] Following reports of inhibition of chlorophyll biosynthesis by another known GABA-T inhibitor, gabaculine, the Merrell-Dow compounds were screened for herbicidal activity.[3] Substantial activity was observed for two of these, 5-fluoro-4-aminopentanoic acid (1) and 4-aminohex-1-ynoic acid (2), and is attributed to inhibition of GSA aminotransferase activity.



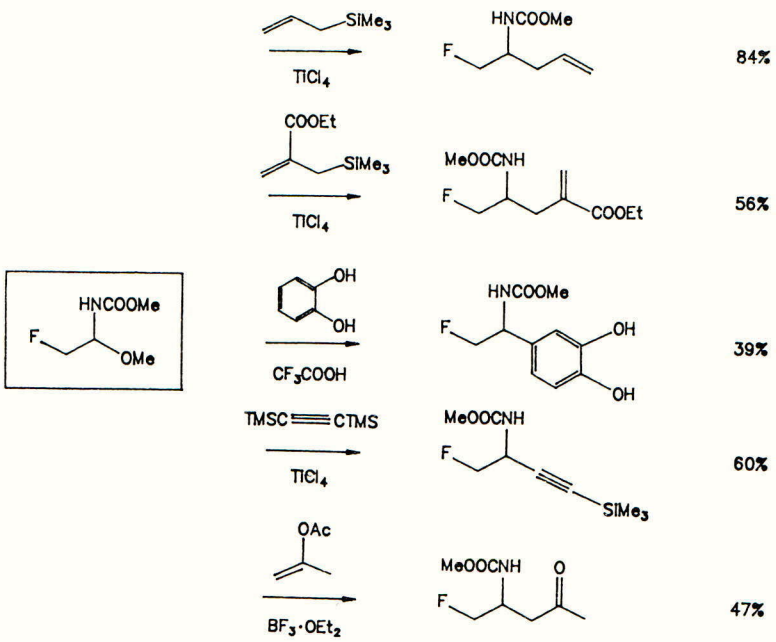
The mechanism proposed for GSA aminotransferase inhibition is directly analogous to that of other suicide inhibitors of pyridoxal-linked enzymes. The symptomology observed for the herbicidal compounds is consistent with the expected inhibition of chlorophyll synthesis: treated plants turn a characteristic yellow color associated with the absence of chlorophyll. Both 1 and 2 demonstrated broad spectrum activity with no useful selectivities at effective rates of about 2 kg/ha.

A number of new compounds based on these structures have been synthesized by straightforward transformations of glutamic acid. However, such chemistry is of limited utility for making substantial changes in the basic skeleton. Efforts to do this led us to the development of a new synthon which should be applicable to the synthesis of irreversible inhibitors for various pyridoxal-linked enzymes. 2-Fluoro-1-methoxy-(methoxycarbonylamino)ethane (3) was designed as a versatile intermediate to allow easy incorporation of the 2-fluoroethylamine subunit which serves as a mechanism-based inhibitor 'handle'.[4]



SCHEME III

Preparation of 3 is accomplished in two steps from 2-fluoroethylamine, including an electrochemical oxidation. An overall yield of 50-60% is obtained after fractional distillation. This provides material which is approximately 90% pure and is suitable for further reactions. Treatment of 3 with any of several Lewis acids generates an iminium which is efficiently alkylated by allylsilanes, enol ethers, and other carbon nucleophiles. The following examples represent the variety of substrates and catalysts employed successfully. While no significant biological activity was observed for any of these products, the reactions illustrate the versatility of the new reagent.



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IN VITRO RECONSTITUTION OF THE DIAMINPIMELATE PATHWAY

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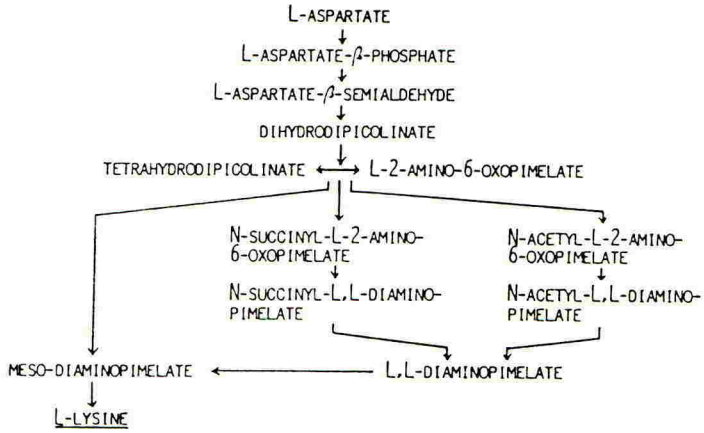
ABSTRACT

Bacteria and plants synthesize lysine via the diaminopimelate pathway. Using aspartate kinase from *Pseudomonas fluorescens*, aspartate semialdehyde dehydrogenase, dihydrodipicolinate synthase, dihydrodipicolinate reductase and diaminopimelate decarboxylase from *Escherichia coli*, as well as diaminopimelate dehydrogenase from *Corynebacterium glutamicum*, we have reconstituted this pathway *in vitro* as a model of the plant pathway. The formation of lysine from ^{14}C-labelled aspartate can be conveniently followed in this system, which has potential use in the screening for inhibitors of the lysine biosynthetic pathway.

INTRODUCTION

There are two distinct pathways for lysine biosynthesis. Some lower fungi, euglenids, yeasts and higher fungi utilize the -aminoadipate pathway, while bacteria, some other lower fungi and green plants synthesize lysine from aspartate via the diaminopimelate pathway (for overview see Herrmann and Somerville 1983). The following scheme of the latter pathway indicates three different routes from tetrahydrodipicolinate to *meso*-diaminopimelate, the immediate precursor of lysine:



There is some evidence that higher plants utilize diaminopimelate

dehydrogenase to form meso-diaminopimelate directly from tetrahydrodipicolinate, but admittedly diaminopimelate epimerase activity has also been found in plants indicating the (co)existence of the other pathway. Substantial homologies recently found for the primary structures of some enzymes of amino acid biosynthesis in plants and bacteria, as well as the comparable susceptibility of these enzymes to certain inhibitors with herbicidal activity (Kishore and Shah 1988) indicate that the more easily accessible bacterial enzymes may be useful in the development and study of inhibitors of the corresponding enzymes from higher plants. Reconstitution of an entire biosynthetic pathway, as reported here, should allow its use in the identification of the target site(s) of putative inhibitors and, furthermore, make it feasible to follow the metabolism of antimetabolites (alternate substrates) along the pathway.

RESULTS

We chose the diaminopimelate pathway of lysine biosynthesis for the present study because this pathway has been insufficiently characterized in plants. The six enzymes involved in the conversion of aspartate to lysine via the direct formation of tetrahydrodipicolinate from meso-diaminopimelate (see scheme) were purified, either to homogeneity or only partially (Table 1). As all three aspartate kinase isozymes of Escherichia coli were unsuitable for the reconstitution of the pathway in vitro (due either to their bifunctionality or to their feedback inhibition by lysine) the aspartate kinase of Pseudomonas fluorescens was chosen because it is a monofunctional enzyme sensitive to feedback inhibition only by the concerted action of both lysine and threonine. All other enzymes, with the exception of diaminopimelate dehydrogenase which does not occur in E. coli, were isolated from this organism and reconstituted in vitro in the approximate ratio of their activities previously described for stationary cells of Bacillus sphaericus (White 1983). The system containing the six enzymes, as well as aspartate, pyruvate, Mg-ATP, NADPH, NH_4^+ and pyridoxalphosphate as substrates and cosubstrates, respectively, produced lysine at a rate of approx. 6nmol h^{-1}. Incorporation of radioactivity from ^{14}C-aspartate into lysine was confirmed by automated amino acid analysis and by the reaction of the product with saccharopine dehydrogenase.

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TABLE 1

Sources for and purification factors of the enzymes used in the reconstituted system

ENZYME	SOURCE	PURIFICATION (-FOLD)
Aspartate Kinase	*Pseudomonas fluorescens*	101*
Aspartate Semialdehyde dehydrogenase	*Escherichia coli*	54
Dihydrodipicolinate Synthase	*Escherichia coli*	217
Dihydrodipicolinate Reductase	*Escherichia coli*	278
Meso-Diaminopimelate Dehydrogenase	*Corynebacterium glutamicum*	288
Meso-Diaminopimelate Decarboxylase	*Escherichia coli*	267

* 1% Homoserine Dehydrogenase Activity

Inhibition of purified acetolactate synthase from barley (*Hordeum vulgare L.*) by chlorsulfuron and imazaquin

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Acetolactate synthase (ALS), the first common enzyme in the biosynthesis of the branched-chain amino acids valine, leucine and isoleucine, has been extracted from etiolated barley shoots (*Hordeum vulgare L.*) and purified to near homogeneity. Purification was performed by hydrophobic interaction, gel filtration, anion-exchange and hydroxylapatite chromatography. Due to the use of FPLC- and HPLC-techniques only small amounts of pure acetolactate synthase could be purified. While loss of activity in the presence of 50 µM FAD and 20% glycerol was below 25% when stored at 70K, purification of the enzyme was hampered by an extreme lability during the first chromatographic steps. Stabilizing the enzyme by adding co-factors and substrates did not show any preservation. The same holds true for incubation in the presence of various protease inhibitors. Purified ALS used for inhibitor studies was stored at 70K with a loss of activity of less than 30% after 2 months. Feedback inhibition by valine, leucine and isoleucine and the Michaelis-Menten kinetics with respect to pyruvate (K_m = 5.5 mM) were not affected by the storage. Random tests between stored and freshly purified enzyme did not show a significant difference in inhibition by herbicides.

Both chlorsulfuron and imazaquin exhibit slow-binding inhibition of purified barley ALS as reported for chlorsulfuron assayed with pea extract (1) and imazapyr (an imidazolinone derivative) assayed with maize extract (2). The I_{50} value for inhibition by chlorsulfuron was determined as 33 nM in a 30 min fixed-time assay. The time-dependent, biphasic inhibition delivers an initial K_i of 67.5 µM (a 0 to 4 min assay was set as the initial inhibition), and a final steady state K_i of 3 nM. The corresponding data for imazaquin are 3.75 µM (I_{50}), and 10.1

and 0.55 µM (initial and steady state K_i). While chlorsulfuron was reported as being a competitive inhibitor of ALS II from Salmonella typhimurium with respect to pyruvate (3), the system barley ALS - chlorsulfuron always exhibited a mixed-type character of inhibition. In contrast, the uncompetitive mode of action of the imidazolinones could be confirmed both for the initial and the final inhibition.

Whether the binding of the inhibitors to ALS is reversible or not was determined by gel filtration on Superose (FPLC) using ^{14}C-chlorsulfuron and ^{14}C-imazaquin. The enzyme was incubated for 2 hours in the presence of 5 µM chlorsulfuron (20 µM imazaquin, respectively), which led to an almost 100% inhibition in the steady state. Separation of the enzyme from free herbicide by desalting and subsequent gel filtration of the enzyme-inhibitor complex showed that the activity can be restored completely. Surprisingly, the release of the radiolabeled inhibitors seems to be much faster than reported (2). A comparison of the elution profiles of the enzyme-inhibitor complex and inhibitors chromatographed alone leads to an estimated half-time of dissociation of less than 10 min.

Unlike enterobacteria, the question of isozymes in higher plants is still unclear. However, with respect to inhibition by herbicides there are different sensitive forms of ALS (4). Using gel filtration two enzymatically active species were eluted with apparent molecular weights of about 440,000 and 200,000, respectively (5). Splitting into two active peaks also occured during anion-exchange using HPLC. We are currently investigating whether active fractions obtained by chromatography represent true ALS isozymes or are induced by chromatographic conditions. It seems certain, however, that (in the case of etiolated barley shoots) there are no significant differences in inhibition of different ALS-fractions by herbicides. Differences in feedback inhibition and pH-dependency of activity might be due to different oligomeric states of the ALS. This is under further scrutiny.

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ACETOHYDROXYACID SYNTHASE-IMIDAZOLINONE INTERACTION

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ABSTRACT

Imidazolinone herbicides kill plants by inhibiting acetohydroxyacid synthase (AHAS). Although AHAS is an extremely labile enzyme, it can be stabilized by maintenance in the presence of substrate and co-factors. Flavin adenine dinucleotide (FAD), a co-factor, was found to cause aggregation of AHAS to a tetrameric form. The different aggregation states of the enzyme have differential sensitivities to inhibition by imidazolinones. This observation indicates that FAD is of structural as well as functional importance for the AHAS enzyme. Imidazolinone-resistant corn lines that have been selected *in vitro* contain an AHAS activity that is insensitive to imidazolinones. Studies of these mutant AHAS activities have shown that the sulfonylureas, another class of inhibitors, bind the enzyme differently than do the imidazolinones. Furthermore, the binding sites for the imidazolinones and sulfonylureas are separate from the feedback regulation sites of valine and leucine.

INTRODUCTION

Acetohydroxyacid synthase, the first enzyme unique to the biosynthesis of valine, leucine and isoleucine, is the sole site of action of imidazolinone herbicides (Shaner *et al.* 1984, Muhitch *et al.* 1987, Newhouse *et al.* 1989). Two other classes of herbicides (sulfonylurea and triazolo primidine) also inhibit the same enzyme (Chaleff and Mauvais 1984, LaRossa and Schloss 1984, Ray 1984, Schloss *et al.* 1988). That this enzyme is a target for three different classes of herbicides has stimulated much interest in understanding its biochemical and physiological properties.

Studies of eukaryotic AHAS have been plagued by its extreme lability (Magee and DeRobichon-Szulmaster 1968, Takenaka and Kuwana 1972, Muhitch *et al.* 1987). We have been successful in partially stabilizing plant AHAS. The results of these studies are presented here. FAD, one of the stabilizing factor, causes aggregation of AHAS to a tetrameric form. The significance of this finding has been discussed. A comparison of properties of AHAS from sensitive normal corn and AHAS from imidazolinone resistant corn lines is also presented.

STABILIZATION OF AHAS ACTIVITY

Rapid loss of AHAS activity has hampered the purification and characterization of plant AHAS. While optimizing the incubation temperature of the AHAS assay, we discovered factors that contributed to the stabilization of AHAS activity.

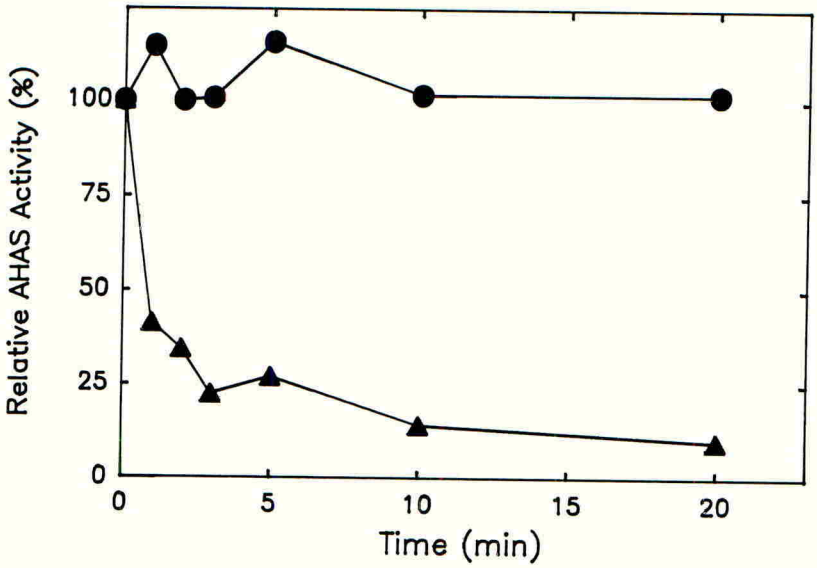


FIGURE 1. Effects on AHAS activity from BMS cells of incubation at 50°C for different periods. Incubation was in either assay buffer (50 mM potassium phosphate, pH 7.5, containing 100 mM pyruvate, 10 mM $MgCl_2$, 1 mM thiamine pyrophosphate, and 10 uM FAD) or desalting buffer (50 mM potassium phosphate, pH 7.5, containing 1 mM EDTA and 100 mM NaCl). AHAS activity was determined following incubation and desalting. Assay buffer (●); desalting buffer (▲).

AHAS from Black Mexican Sweet (BMS) corn cells has maximum *in vitro* activity between 46 and 50°C (Singh *et al.* 1988). At this temperature, protein precipitates were observed in the assay tubes. Further experimentation revealed a rapid loss of AHAS activity at 50°C in 50 mM phosphate buffer (pH 7.5) in the absence of substrate or co-factors (Fig. 1), whereas activity was fully preserved when incubated in the assay buffer containing substrates and co-factors. It is apparent that the substrates and co-factors in the assay buffer must be stabilizing the enzyme.

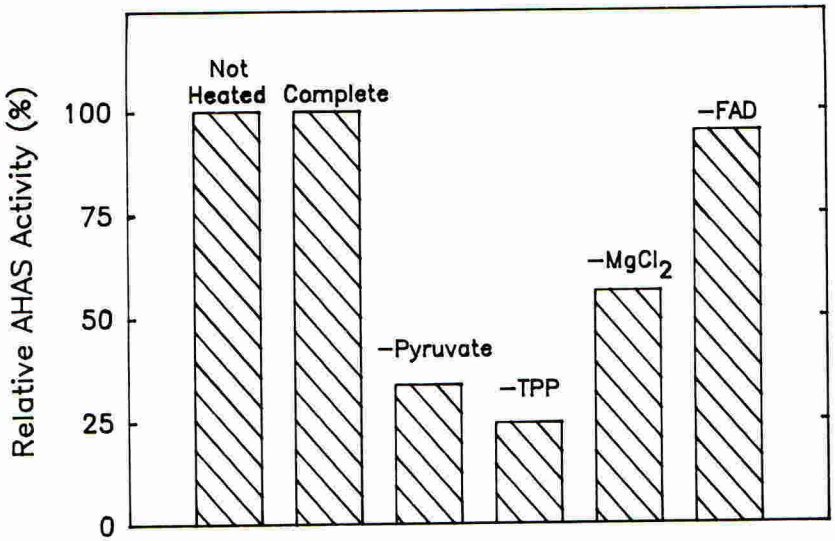


FIGURE 2. Effects on AHAS activity from BMS cells of incubation at 50°C for 20 min. The enzyme was incubated in the complete assay buffer (50 mM potassium phosphate, pH 7.5, containing 10 mM $MgCl_2$, 1 mM TPP, and 10 uM FAD) or in the same assay buffer from which one item was omitted at a time as indicated in the figure. AHAS activity was determined following incubation and desalting.

To examine this point further, the enzyme was incubated either in the complete assay buffer or in the assay buffer from which one factor was omitted at a time (Fig. 2). Omission of pyruvate and thiamine pyrophosphate (TPP) were most detrimental to AHAS activity. Omission of $MgCl_2$ caused the next greatest loss of activity. The stability of AHAS activity was unaffected by the omission of FAD. These results appear to contrast with an earlier report from our laboratory that FAD was essential for stabilization of AHAS activity (Muhitch *et al.* 1987). However, because FAD binds the enzyme tightly (Schloss et al. 1985) and the enzyme was extracted in the presence of 100 uM FAD in the present experiment, sufficient FAD to stabilize the enzyme might be bound to the enzyme. Therefore, inclusion of substrate and all of the co-factors is recommended for the stabilization of AHAS.

ROLE OF FAD IN THE AGGREGATION OF AHAS

Even though AHAS is not involved in an oxidation-reduction reaction, an unusual feature of the bacterial enzyme is its absolute requirement for FAD (Stormer and Umbarger 1964, Schloss *et al.* 1985). A possible explanation for this requirement for FAD by AHAS was proposed on the basis of the strong amino acid sequence homologies between the large subunits of the three *E. coli* AHAS isozymes and pyruvate oxidase (Grabau and Cronan 1986). Results of experiments with pyruvate

oxidase-AHAS hybrid protein have led to the suggestion that the AHAS enzymes are descended from pyruvate oxidase and the flavin requirement of the AHAS activity is a vestigial remnant, which may have been conserved to play a structural rather than a chemical function (Chang and Cronan, 1988).

AHAS from plants also require FAD for the stabilization and stimulation of enzyme activity (Muhitch *et al.* 1987, Singh *et al.* 1988). However, the mechanism by which FAD performs its role is not understood. We now have evidence that FAD causes aggregation of AHAS and may play a role in the quarternary structure and function of the plant enzyme.

Gel permeation chromatography of AHAS from BMS cells in the absence of FAD revealed two peaks of activity of about equal peak area (Fig. 3A). However, the amount of enzyme activity in the first peak (peak I) was about twice the amount of enzyme activity in the second peak (peak II) when chromatographed in the presence of FAD (Fig. 3B). Based on the deduced molecular weight of the mature protein from the AHAS gene sequence, AHAS in peak II is the dimeric form of the enzyme (MW = 150,000) whereas AHAS in peak I represents the tetrameric form of the enzyme (MW = 300,000).

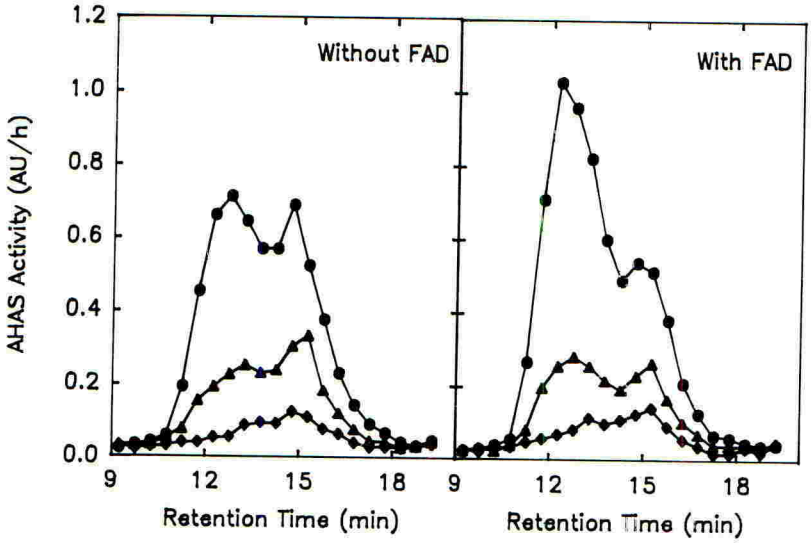


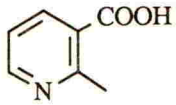
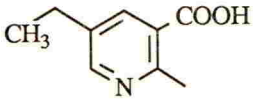
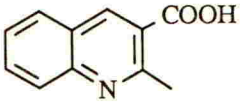
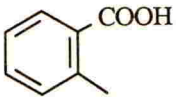
FIGURE 3. Gel permeation chromatography of AHAS on Waters Protein Pak SW300 column in the absence or presence of FAD. AHAS was extracted from BMS cells in 50 mM phosphate (pH 7.0), centrifuged to remove particulate material, and chromatographed on the column equilibrated with the extraction buffer or the extraction buffer containing 10 uM FAD. Control (●), valine + leucine (▲), imazapyr (◆).

The present results are the first demonstration of aggregation of plant AHAS to a higher molecular weight form in the presence of FAD. Similar results with bacterial AHAS have been previously observed (Chang and Cronan 1988, Eoyang and Silverman 1984, Grimminger and Umbarger 1979). This observation has some important implications: first, AHAS may be predominantly in the tetrameric form *in vivo* because FAD is present within the chloroplast. Second, the dual role of FAD in stabilizing AHAS activity and in converting the enzyme predominantly to the tetrameric form suggests that the tetrameric form of the enzyme is the more stable form of the enzyme. Finally, limited kinetic data suggest that the tetrameric form of the enzyme is more sensitive to inhibition by feedback inhibitors (valine and leucine) and imazapyr (Fig. 3). On the basis of these observations, a dual role is proposed for FAD in plant AHAS, a structural and a protective role.

IMIDAZOLINONE INHIBITION OF PLANT AHAS

An imidazolinone must have both an imidazolinone ring and the R-group to inhibit AHAS activity (Table 1). Neither the R-group nor the imidazolinone ring alone inhibits AHAS activity. The R-groups of imidazolinone herbicides currently on market are benzene (ASSERT) quinoline (SCEPTER) or pyridine (ARSENAL and PURSUIT). Among these herbicides, the benzene imidazolinone is the best inhibitor of AHAS activity, followed by the quinoline and then by the pyridine (Table 1).

TABLE 1. Inhibition of acetohydroxyacid synthase from corn seedlings by different imidazolinones.

COMMERCIAL NAME	COMMON NAME	R	K_i (uM)
ARSENAL ®	Imazapyr		12.0
PURSUIT ®	Imazethapyr		5.4
SCEPTER ®	Imazaquin		3.4
	Imazabenz		1.7

Inhibition of AHAS activity by imazapyr is uncompetitive (Shaner *et al.* 1984) with respect to both pyruvate and TPP. This result implies that binding of imazapyr could occur only to the AHAS-pyruvate-TPP complex.

Inhibition kinetics of AHAS suggest that imidazolinones are slow-tight binding inhibitors (Muhitch *et al.* 1987). The initial and final Ki for imazapyr in these studies were found to be 15 and 0.9 uM, respectively. The tight binding of imidazolinones with AHAS is also suggested by two other observations. First, pretreatment of tissue with imazapyr prior to extraction causes a loss in extractable AHAS activity. Second, AHAS activity is also lost when the enzyme is preincubated *in vitro* for an extended period of time with an imidazolinone prior to desalting and assaying. These results indicate that the inhibitor does not readily dissociate from the enzyme.

AHAS FROM IMIDAZOLINONE RESISTANT CORN LINES

Imidazolinone resistant mutants of corn were isolated by *in vitro* selection (Anderson *et al.* 1985). Plants were regenerated from two such mutant cell lines designated XA17 and XI12. These plants were selfed to obtain plants homozygous for the mutation conferring imidazolinone resistance. Homozygous resistant corn lines and a sensitive inbred line (B73) were used in the present study. The sensitivity of these lines to two imidazolinone herbicides (imazethapyr and imazaquin) and a sulfonylurea herbicide (sulfometuron methyl) at the whole plant level as well as at the enzyme level was determined.

XA17 has a higher level of resistance to imidazolinones than does XI12 (Table 2). In addition, XA17 is cross-tolerant of sulfonylurea herbicides. Homozygous resistant XA17 mutant plants were not killed by application of a concentration of herbicide more than one thousand fold higher than the concentration that was lethal to B73 (Table 2). AHAS activity extracted from XA17 plants was similarly insensitive to these herbicides (Fig. 4).

TABLE 2. Increase in resistance to three AHAS-inhibiting herbicides provided by two imidazolinone-resistance mutants.

	Imidazolinone-resistance mutant	
Herbicide	XA17	XI12
	(Increased level of resistance)	
Imazethapyr	>1000X	1000X
mazaquin	>1000X	60X
Sulfometuron methyl	>1000X	<2X

Plants homozygous for the XI12 mutation are resistant to high rates of imazethapyr and to intermediate rates of imazaquin herbicides, but is sensitive to sulfometuron methyl (Table 2). The level of resistance of the XI12 plants to imazethapyr is similar to the level of resistance of XA17 plants. However, XI12 was less resistant than was XA17 to imazaquin, and the response of XI12 to sulfometuron methyl does not differ from the response of the sensitive B73 genotype. AHAS activity from XI12 was insensitive to imazethapyr and had an imazethapyr response curve similar to that of AHAS activity from XA17 (Fig. 4). The enzyme activity from XI12 was inhibited to a small degree at high concentrations of imazaquin, but the inhibition curve was displaced by an order of magnitude relative to the curve obtained for the B73 AHAS. AHAS activity from XI12 was inhibited by sulfometuron methyl.

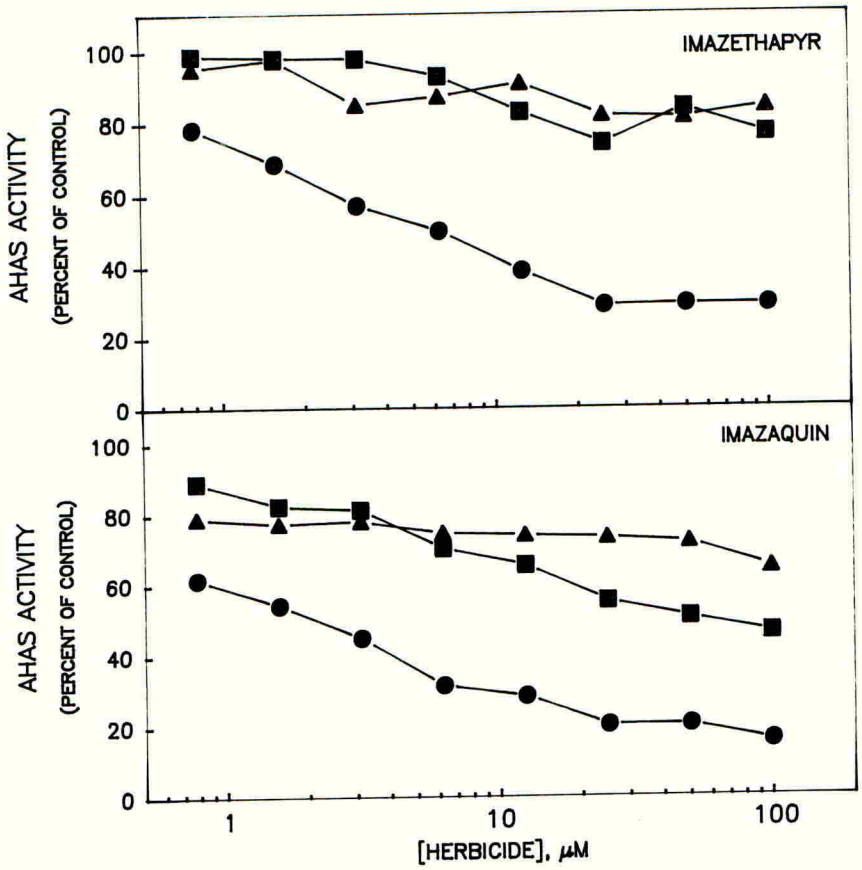


FIGURE 4. Effects of different herbicides on the AHAS activity from wild type corn (B73) and various imidazolinone resistant mutants. B73 (●), XA17 (▲), and XI12 (■).

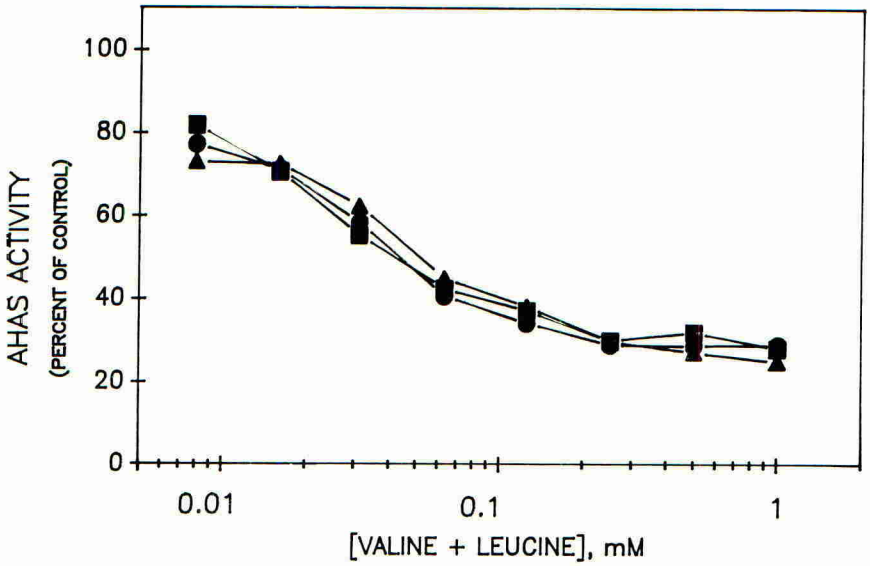


FIGURE 5. Inhibition of AHAS activity from imidazolinone sensitive and imidazolinone resistant corn lines by valine + leucine. B73 (●), XA17 (▲), and XI12 (■).

We have previously reported a form of AHAS from BMS cells that is insensitive to inhibition by the branched chain amino acids but highly sensitive to inhibition by imazapyr (Singh *et al.* 1988). In the present experiments, the enzyme activities from these imidazolinone resistant mutant lines were similar in their response to inhibition by valine + leucine (Fig. 5). This inhibition is identical to the kinetics of inhibition of AHAS activity from B73. These results indicate that the herbicide binding site is distinct from the feedback inhibitor binding site.

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MECHANISM OF ACTION OF 1,2,4-TRIAZOLO[1,5-a]PYRIMIDINE SULFONAMIDE HERBICIDES

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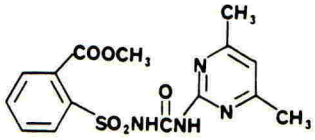
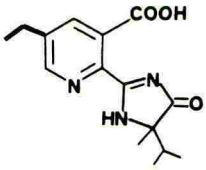
INTRODUCTION

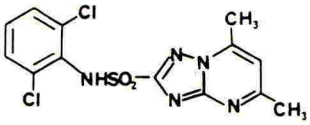
Acetolactate synthase (ALS, EC 4.1.3.18) is the first enzyme in the biosynthetic pathway to valine, leucine and isoleucine. It is the primary target site for at least three structurally diverse classes of herbicides, the imidazolinones (IM), sulfonylureas (SU) and triazolo-[1,5-a]pyrimidine sulfonamides (TP, Figure 1., Subramanian & Gerwick 1989). The substituted TP are a new class of herbicides under development at Dow Chemical Company whereas IM and SU are proprietary of DuPont (Levitt 1978) and American Cyanamid (Los 1987) respectively. Presented below are some recent studies on the mechanism of action of TP.



A. SULFONYLUREA



B. IMIDAZOLINONE



C. TRIAZOLOPYRIMIDINE

Figure 1: Herbicides known to inhibit acetolactate synthase, A. sulfonylurea (sulfometuron) B. imidazolinone (imazethapyr) and C. A representative triazolopyrimidine.

RESULTS AND DISCUSSION

Several analogs of TP completely blocked the growth of *Bacillus subtilis*, soybean suspension cultures as well as *Arabidopsis thaliana* (Subramanian & Gerwick 1989). The growth inhibition in all of the above systems was nullified by the addition of valine, leucine and isoleucine to the growth media (Subramanian & Gerwick 1989). The recovery of soybean suspension cultures from the effects of TP, upon addition of branched chain amino acids is shown in Figure 2. The requirement for

all the three amino acids to neutralize the effects of TP suggested that the target enzyme is ALS. This enzyme was isolated from a number of sources including barley and Arabidopsis seedlings, tobacco, soybean and cotton suspension cultures and found to be inhibited by TP. The I-50 (concentration of the compound required for 50% inhibition) for the representative TP shown in Figure 1 was in the range 32-46 nM for the enzyme from different sources.

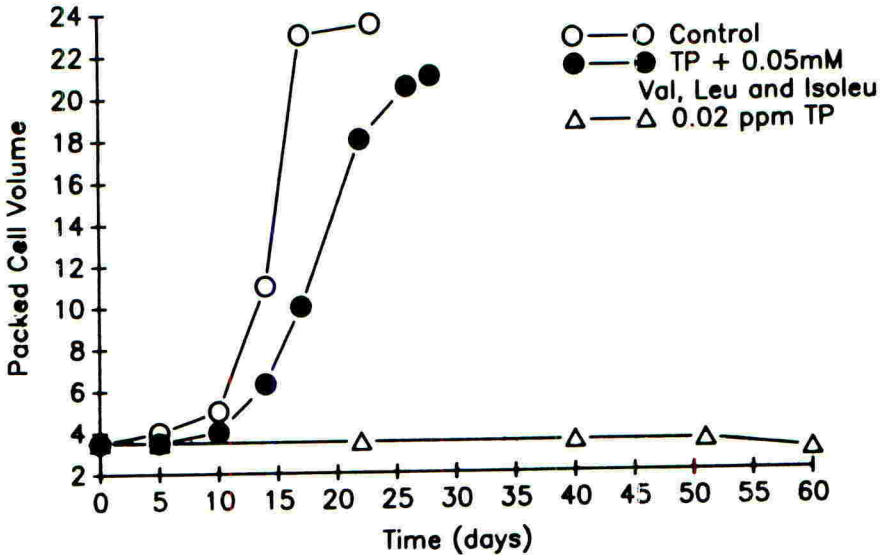


Figure 2: Effect of TP on soybean suspension cultures. Cell growth was measured as packed cell volume/50 ml under gravity after 15 min (ppm = parts per million by volume).

ALS from most plant sources was found to be very unstable (Subramanian & Gerwick 1989). Only the maize enzyme has been amenable to extensive purification (Muhitch et al. 1987). A rapid purification scheme for ALS from barley has been devised by a combination of Mono-Q h.p.l.c. and phenylagarose chromatography. The final enzyme preparation was not homogenous but 235-fold pure (recovery was >60%, Subramanian & Gerwick 1989) and suitable for kinetic studies. The enzyme displayed typical Michaelis-Menten kinetics in the presence of varying pyruvate (Km = 2.44mM) and thiamine pyrophosphate (TPP, Km = 5.77uM).

Radiolabelled TP bound to barley ALS was quantitatively recovered upon separation in a Sephadex G-25 column, indicating non-covalent interaction. Steady state kinetic analysis of TP inhibition of ALS in the presence varying pyruvate showed a linear mixed type inhibition (Ki= 59nM and Ki'= 69nM, Subramanian & Gerwick 1989). The inhibition pattern was similar with TPP as the variable substrate (Ki = 37.34nM and Ki' = 54nM, Figure 3). SU has been reported to be a competitive inhibitor of ALS from Salmonella typhimurium (with respect to pyruvate, Schloss 1984)

and an uncompetitive inhibitor of the enzyme from Methanococcus (Xing & Whitman 1987). IM is an uncompetitive inhibitor of maize ALS (Shaner et al. 1984). The kinetics of inhibition of ALS by TP, IM and perhaps SU is suggestive of a binding site on ALS distinct from that of substrate or cofactor.

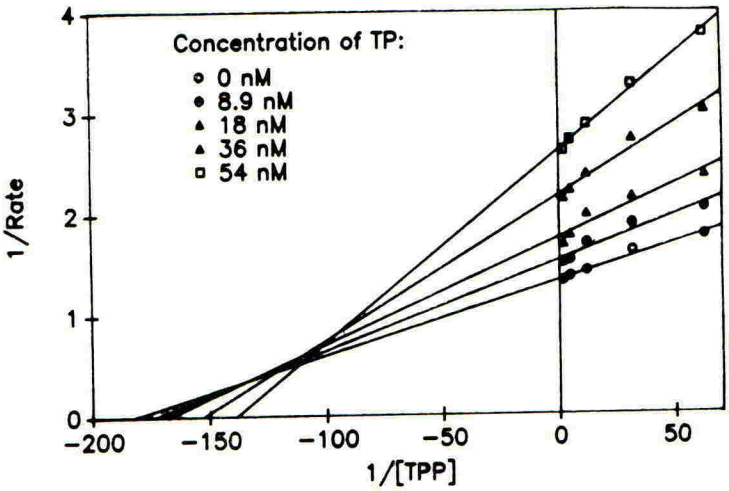


Figure 3: Kinetics of inhibiton of barley ALS by TP; varied substrate is thiamine pyrophosphate (TPP).

The similarity in the biochemistry of the three structural classes of ALS inhibitors is interesting as well as intriguing. One of the critical questions is whether these inhibitors compete for the same locus on the enzyme. This question has been addressed by using a number of mutants of tobacco and soybean cultures, 10-300 fold resistant to TP. Of the fifteen mutants tested, all were cross resistant to growth on chlorsulfuron (an SU), all but one to imazaquin (an IM). Both the compounds were used at 10-20 times the lethal concentration. Inhibition analyses of ALS from one of the tobacco mutants (KS43) is shown in Figure 4. Compared to that of wild type, the enzyme from KS43 was 350-fold less sensitive to inhibition by TP and 150-fold less sensitive to imazethapyr (an IM). These preliminary results suggest that there is substantial overlap in the binding site of the three classes of ALS inhibitors. Cross resistance between SU and IM has already been documented by others (Comai & Stalker 1986). Also, IM and TP have been shown to quantitatively displace a radiolabelled SU herbicide from ALS, indicating competitive binding (Schloss et al. 1988). Mutants resistant only to IM or SU have also been observed (Shaner et al. & McDevitt et al. 1988), indicating the existence of domains of specificity among these inhibitors.

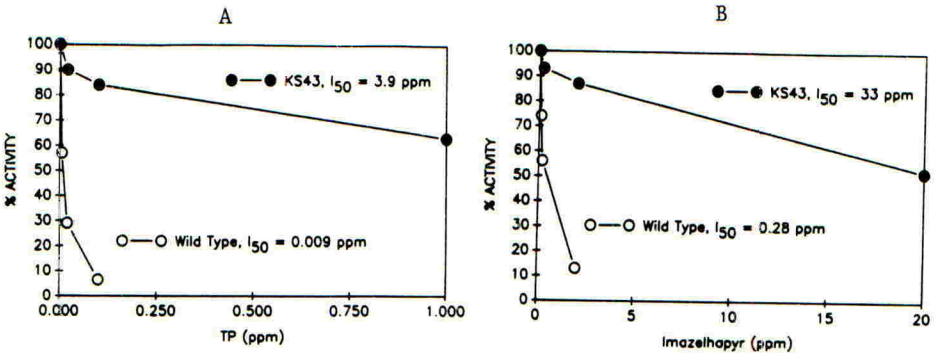


Figure 4: Inhibition of ALS from wild type and KS43 tobacco cultures by TP (A) and imazethapyr (B).

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ASPARTATE KINASE FROM AMINO ACID ANALOG-RESISTANT TOBACCO CELL CULTURES

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ABSTRACT

Purification of tobacco aspartate kinase to homogeneity has been impeded by its extreme instability at later stages of purification. Ion exchange and size seperations on FPLC have resulted in only a 25-fold enrichment of specific activity. However, SDS-PAGE analysis of fractions from FPLC purification followed by photoaffinity labelling with azido-ATP(^{32}P) has revealed a single labelled band that closely correlated with enzyme activity. Attempts to raise antibodies and to microsequence this protein using nitrocellulose blots will be discussed.

INTRODUCTION

A tobacco cell line, which overproduced methionine by 100-fold, was obtained by selection for resistance to the growth inhibitory effects of the methionine analog ethionine (Gonzales et al. 1984). Subsequent analysis revealed that methionine overproduction, and thus resistance, was most likely due to a 16-fold increase in the activity of one of two isoforms of aspartate kinase, the first enzyme in the branch pathway leading to the synthesis of methionine, threonine, isoleucine and lysine. Purification of this enzyme, with the aim of raising antibodies, is necessary for the elucidation of the mechanism of increased activity in the resistant cell line.

This enzyme is usually present in plants in at least two isoforms, exhibiting feedback regulation by either lysine or threonine (Davies and Miflin 1977). Purification to homogeneity of the tobacco enzyme has been impeded by its extreme instability at later stages of purification. Ion exchange and size separations using FPLC have resulted in only a 25-fold enrichment of specific activity (Gonzales et al., unpublished data). A greater enrichment was achieved for the carrot enzyme (Relton et al. 1988); however, purification to homogeneity was not obtained.

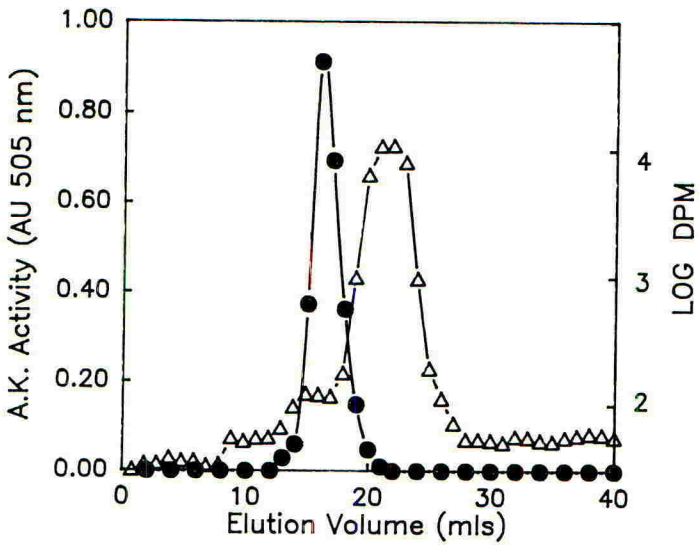
Azido-ATP(^{32}P) labelling

A crude protein extract, prepared from ethionine-resistant tobacco cells three days after subculture (Gonzales et al. 1984), was fractionated on DEAE Fast Flow, Pharmacia. The fractions containing aspartate kinase activity were precipitated with 50% (w/V) ammonium sulfate and desalted on Sephadex G25. Protein samples in 200 µl reaction buffer (Bryan et al. 1970), minus the hydroxylamine, were placed in the wells of a ceramic agglutination plate and exposed for 3 minutes to UV light at 254 nm in the presence of 5 µCi azido-ATP(^{32}P).

FPLC purification and SDS-PAGE

Figure 1 shows the elution profiles of aspartate kinase activity

and ^{32}P label on Superose 6. Western blots from SDS-PAGE gels of the fractions containing aspartate kinase activity exhibited numerous bands with Coomassie Blue staining (Figure 2A); however an autoradiogram of the blot showed only a single labelled band of about 42 kD (Figure 2B). The small amount of radioactivity detected in the fractions containing aspartate kinase activity was, apparently, associated with a single protein. The co-purification of the labelled 42kD band with aspartate kinase activity was subsequently confirmed by SDS-PAGE analysis of fractions from each of our enzyme purification steps.



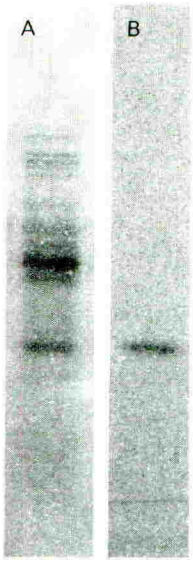


Figure 1. Elution of profiles of aspartate kinase activity (-) and protein bound azido-ATP(^{32}P) (-) on Superose 6.
Figure 2. SDS-PAGE analyais of partially purified aspartate kinase activity following photoaffinity labelling with azido-ATP(^{32}P). (A) Coomassie Blue stained western blot of Superose 6 fractions containing aspartate kinase activity. (B). Autoradiogram of western blot from A.

Initial attempts to miscrosequence the 42 kD band excised from western blots indicated that the protein was N-blocked; we are now attempting to circumvent this problem by microsequencing internal peptides separated by HPLC. A number of protocols are being used to raise antibodies against the 42 kD subunit in rabbits and rats.

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