SESSION 7A

BIO-RATIONAL DESIGN OF NEW HERBICIDES AND PGRs

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INVITED PAPERS 7A-1 to 7A-4

PREDICTING AND OPTIMISING THE TRANSLOCATION OF FOLIAGE-APPLIED HERBICIDES - A PLANT PHYSIOLOGIST'S PERSPECTIVE

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ABSTRACT

The mechanisms controlling the entry of foliage-applied herbicides into plant cells, and the factors affecting their short- and long-distance translocation in the whole plant are reviewed. The recent attempts to model the systemicity in phloem tissues of organic compounds, based primarily on their lipophilicity and dissociation constants, are critically examined. The need for a better understanding of the physiological and phytotoxic factors affecting cellular uptake, compartmentation and transport processes is emphasised.

INTRODUCTION

Within the last decade, the results from different areas of research have culminated in a better understanding of how the physicochemical properties of herbicide molecules and of co-formulated spray additives, such as surfactants, influence the mode of action of herbicides in plants. "Mode of action" is used in its fullest sense, encompassing herbicide absorption, transport (both inter- and intra-cellular), herbicide metabolism and the biochemical activity at the target site. Taken together, these findings have enabled a much more mechanistic approach to be made in the design, improvement and usage of herbicides than has been possible in the past.

In this review, I intend to discuss these recent innovative ideas and apply them to the complex and intriguing problem of optimising the translocation of foliage-applied herbicides. This comprises uptake into cells immediately underlying the cuticle and short-distance transport to adjacent cells, as well as long-distance transport to remote sites involving the vascular tissues. Emphasis will be placed on physiological and phytotoxic effects, that is, the interaction of the plant with the herbicide. Other papers by Pillmoor and by Brown (this volume) will discuss the more 'down-stream' aspects of herbicide mode of action, in particular, the rational approach to discovery of biochemical activity and metabolic detoxification. However, there will inevitably be some degree of 'common ground' with the above-mentioned papers, and, indeed, it would be unwise to try and understand herbicide action simply by looking at effects either at the cell, or at the whole plant level.

HERBICIDE TRANSPORT

General comments

Transport of herbicides can occur in two separate 'compartments' which are well characterised -- the non-living apoplast and the symplast, the living part of the plant. In general, water solubility of the active ingredient (AI) is an important factor which largely determines the amount of AI partitioning into the apoplast. With reservations, lipid solubility is an important factor for uptake into the symplast, since this 'compartment' is delimited by the (predominantly) lipoidal plant membrane, the plasmalemma (PM). Both the symplast and apoplast may be regarded as inter-linked networks, the former due to the presence of plasmodesmata, the latter due to a water continuum. In turn, these two systems may be regarded as further integrated since their respective vascular tissues, the phloem (symplast) and the xylem (apoplast), are usually physically close together, and transfer of AI between them often occurs. The importance of this is discussed in more detail below.

In many of the studies dealing with herbicide transport, it is has been impossible to determine precisely how the AI is taken up and subsequently translocated because whole plants or tissue explants have been used. Such techniques, while producing an 'overall' assessment of how, for example, AI lipophilicity affects translocation, do not allow a mechanistic analysis to be made. Subsequently, the results from these studies cannot be used in a predictive way simply because it is not possible to say which process (uptake, transport, adsorption, etc.) is being most affected. In order to do this, isolated cells or microsomal vesicles need to be

used. However, it should be stressed that although these cellular or sub-cellular systems provide the necessary 'resolution' to determine the characteristics of the cellular uptake mechanisms involved, they do not give any indication of their relative importance or involvement at the whole plant level.

Perhaps one way of overcoming these problems is to model this movement between the various 'compartments' within the plant, both at the cell and at the tissue level. Such an approach has been attempted (Bridges & Farrington, 1974; McCall, 1989) but without the level of sophistication or complexity needed to be able to predict the behaviour of agrochemicals in the plant. Recent research (Balke, pers. comm.) has been more successful and the distribution of several sulfonylurea herbicides between various cell compartments (cell wall, cytoplasm, vacuole, etc.) has been accurately modelled using pharmacokinetic principles. Obvious benefits from this type of research are: i) a more accurate assessment of the amounts of AI available for translocation (*i.e.* not metabolically altered or sequestered in any way); ii) better estimates of cell membrane permeabilities to herbicides; and iii) the potential to predict inter-cellular movement better.

Regulation of the amount of AI available for transport

Although it may seem obvious, the amount of AI transported is dependent, perhaps throughout the entire treatment period, on the amount that is available for transport. Many so-called systemic herbicides, in fact, are characterised by being relatively *immobile* and a much larger quantity remains in the treatment area compared with that which is translocated (reviewed by Coupland, 1989). Even for compounds which are normally regarded as highly systemic, such as glyphosate, there are examples where a large proportion of the applied compound remains in the treated leaves (*e.g.* Whitwell *et al.*, 1980). In most of the references cited by Coupland (1989), the reasons why so much AI remains non-translocated are not known. Nevertheless, values of $\geq$ 80% "immobile" are likely to be the result of extensive conversion to non-mobile metabolites, compartmentation of AI or metabolites in the vacuole, binding to plant polymers, such as lignin, or a combination of these (Crisp & Larson, 1983; Cole *et al.*, 1987; Coupland, 1991). Whatever mechanisms are involved, relatively small reductions in herbicide detoxification would significantly increase the amount of AI available for transport. This could be achieved either by chemical means, or through genetic manipulation. In weed species, possibilities exist for the specific inhibition of enzymes, in particular mixed-function oxidases, involved in herbicide detoxification (Cole *et al.*, 1987; Kemp *et al.*, 1988). In crops, safeners are designed to prevent damage, principally by enhancing herbicide degradative metabolism (Hatzios, 1989). The potential for genetic modification of plants resulting in altered herbicide metabolism has attracted considerable attention over the past few years. Transformation of crops using various genes which code for herbicide-detoxifying enzymes has been described (Rubin, 1991). However, several environmental questions on the effects of such 'resistant' crops in the ecosystem need to be answered before there is successful commercialisation of transgenic crop plants.

Short-distance movement

Mechanisms

Movement from the inner side of the cuticle to the epidermal cell wall will occur by diffusion. Once through the cuticle there are four alternatives for subsequent transport. First, the AI may remain in aqueous solution and, disregarding any adsorption onto cellular constituents, move with the transpiration stream. Second, the AI may partition into the lipid PM and from there move into the cell. Third, the AI may be transported directly into the cell by naturally-occurring permeases, proteins which are resident in the PM and which facilitate the absorption of both nutrient ions and organic solutes into the cell. Alternatively, cotransporters may be involved, with uptake of AI being linked to H^+-pumps. The fourth mechanism is endocytosis.

Uptake into cells - diffusion: Where uptake is related linearly to the extra-cellular concentration, the primary mechanism for uptake is non-facilitated diffusion. This is how the majority of herbicides, so far investigated, enter into the plant cell: *e.g.* aminotriazole (Lichtner, 1983); diclofop-methyl (Tritter *et al.*, 1987); bentazon (Sterling *et al.*, 1990); and various sulfonylureas (Ooka & Balke, 1990). In this last study, the relative effects of log K_{ow}, the octanol/water partition coefficient, and the dissociation coefficient (pKa) were determined, and accurate measurements of membrane permeabilities to the various compounds were also made.

Uptake - 'Ion-trapping': With compounds which dissociate into ionised moieties, and for most phloem-translocated herbicides weak acids predominate, the pH outside and inside of the cell is most important. Lipophilicity decreases dramatically with an increase in ionic charge. In general, the partition coefficient of the

charged molecule is lower by a factor of 10^4 compared with that of the undissociated molecule (Leo *et al.*, 1971). Thus the undissociated molecule will be able to diffuse into the cell more readily than the dissociated moiety can diffuse back out. Accumulation in this way is known as the 'ion-trap' mechanism and has been reported for several different classes of herbicide including: phenoxyacids (Darmstadt *et al.*, 1983; Minocha & Nissen, 1985); pyridine carboxylic acids (Swanson & Baur, 1969; Devine *et al.*, 1985); maleic hydrazide (Grimm *et al.*, 1985) and sulfonylureas (Devine *et al.*, 1987; Ooka & Balke, 1990). As the apoplast is normally ≈ pH5, and that of the cytoplasm ≈ pH7.5, it should be possible for molecules to be specifically designed so that penetration of the PM, and retention in the cell are both maximised. Indeed, it has been demonstrated that carboxylic acid analogues of certain compounds are more phloem-mobile than the parent compounds (Fig. 1). However, this is not to say that any simple acid derivative will enhance phloem systemicity since anions which are highly lipophilic will just as easily be able to diffuse out of the sieve element (SE) (*e.g.* flamprop).

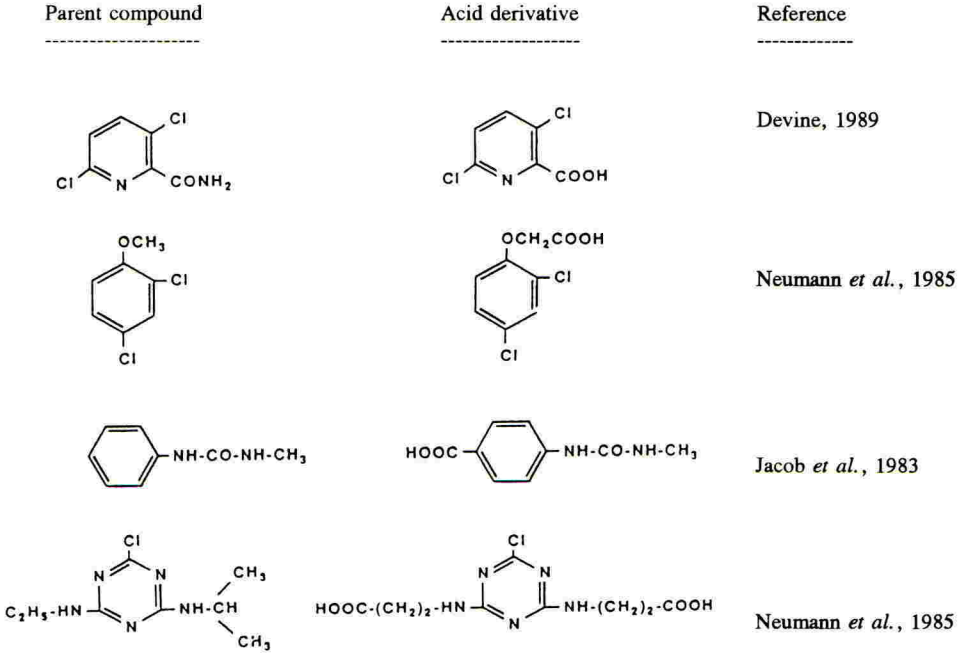

FIGURE. 1 Conferring phloem systemicity by acid derivatisation.

Uptake - permeases: Solute uptake can occur via active transport systems, either using a carrier protein (permease) which facilitates diffusion, or by a transport protein which becomes energised by proton binding. In the latter case, the AI and H^+ ions are co-transported, the free energy gradient of the protons being harnessed to the 'uphill' transport of the AI. The proton energy gradient is established through the action of H^+-translocating ATPases in the PM. Such systems are well-characterised for naturally-occurring metabolites, in particular amino acids (Bush & Langston-Unkefer, 1988) and sugars (Bush, 1989). There are only a few examples where there is evidence of carrier-mediated transport of herbicides. Minocha & Nissen (1985) showed that 2,4-D utilises a carrier system similar to that which transports IAA. Burton (1986) and Pipke *et al.* (1987) have provided evidence of a permease for glyphosate in a higher plant and a bacterium, respectively. In the former study, glyphosate uptake was inhibited by inorganic phosphate and amino acids, and displayed two saturable phases of uptake (apparent Km values of 5.8 and 50.8 μM). Burton (1986) concluded that the competitive nature of Pi inhibition of glyphosate and the similarities between Pi and glyphosate uptake kinetics

indicated that there may be a common permease for these two substances. Various inhibitors have been used to try and demonstrate the involvement of metabolic processes but care should be taken in interpreting such data. On physicochemical principles alone, the operation of a H^+-pump at the plasmalemma would enhance the uptake of weak acid herbicides without being directly involved with their absorption. Operation of this enzyme will maintain such compounds protonated on the outside of the membrane (thus maximising lipophilicity), while the inside of the cell, at a higher pH, would tend to increase the dissociation of the molecule (minimising lipophilicity and favouring retention in the cell). Also, the inside of the membrane will be negatively charged, further enhancing retention due to charge repulsion. Thus, demonstrating inhibition of uptake in the presence of metabolic inhibitors does not prove their direct involvement with the transfer of AI into the cell.

Uptake - endocytosis: Fluid-phase endocytosis (pinocytosis) involves uptake of extra-cellular material, usually in solution, by invagination of the PM. The vesicles so formed then discharge their contents inside the cell, the vacuole often being the main repository. This mechanism, by definition, is non-selective and therefore of little value in the prediction of herbicide activity, or in the design of molecules to utilise the process. However, receptor-mediated endocytosis involves a specific ligand-receptor interaction and there is potential for using this as a way of selectively introducing herbicide molecules into plant cells. This process has been well-characterised in animal cells for polypeptide hormones, lectins and antibodies but less so in plant cells (Horn *et al.*, 1989; Horn *et al.*, 1990). However it is thought that endocytosis pathways may exist in plant cells for compounds which are too polar to diffuse easily across the plasmalemma and/or too large to utilise membrane channels (Horn *et al.*, 1990). Water-soluble vitamins such as folate, vitamin B_{12} and biotin fit this category and Horn *et al.* (1990) showed that biotinylated macromolecules could be taken up by endocytosis.

Long-distance movement

Again, the relative involvement of the apoplast and symplast is important because both the velocity and direction of transport are different in these two 'compartments'. Long-distance apoplastic transport occurs in the xylem and has been adequately described previously (Ashton & Crafts, 1981). However, it is important to realise that the rate of flow of solution in the xylem is typically much greater than that in the phloem. This means that if any AI leaks from the phloem into the xylem, it will be transported a considerable distance, probably away from the intended site of action, before there is a chance of it diffusing back into the phloem.

Phloem translocation of herbicides

This topic has received a considerable amount of attention over the past five years. Research has progressed along two quite separate 'fronts' - that concerned with phloem physiology, in particular sucrose loading, and that concerned with pesticide uptake into sieve elements (SE) and subsequent systemicity. Both will be reviewed here, as both are crucial to the efficient transport of many post-emergence herbicides.

Phloem physiology

In order for the SE to accumulate sucrose in the amounts known to occur in the SE, there has to be a point (or points) at which increases in sucrose concentration occurs (Warmbrodt *et al.*, 1989). This is a fundamental issue having a direct bearing on: a) sugar loading and selectivity; b) routes of transport; and c) how the system is regulated *in vivo* - all of which, in turn, are relevant to herbicide uptake and systemicity.

a) Sugar loading and selectivity: It is only within the last three years that the sucrose transporter of higher plants has been isolated and characterised (Ripp *et al.*, 1988; Warmbrodt *et al.*, 1989). The permease was first identified in soybean cotyledon protoplasts as a 62 kDa protein which could specifically bind sucrose. In the latter report, an identical polypeptide was detected associated with PM proteins obtained from mature spinach leaves. Furthermore, immuno-gold labelling studies showed this protein to be localised specifically on the sieve-tube PM. This is convincing evidence that this protein is the sucrose carrier, or a major part of it. Full characterisation of this permease, in particular the architecture of the sucrose binding site, has obvious benefits for the design of systemic pesticides.

Many studies have shown that sucrose is the major sugar translocated in higher plants, although in certain plant families, sugar alcohols (*e.g.* mannitol) are the predominant translocate (Zimmerman & Ziegler, 1975). Furthermore, attempts to introduce sugars other than sucrose into the phloem have resulted in virtual complete failure (Baker, 1989). These findings imply that there is a high degree of specificity involved. Recent studies indicate that substrate recognition involves the relatively hydrophobic parts of the sucrose molecule, and hydrogen bonding between the C-3, C-4 and C-6 glucosyl hydroxyls with the protein carrier (Hitz *et al.*, 1986;

Card *et al.* 1986). However, this is not to say that there is *total* sucrose specificity as certain phenyl glucosides were also recognised by this carrier. Recently, Buckhout *et al.* (1991) have investigated sugar specificity using PM vesicles prepared from sugarbeet. They found that phenyl-α-thiopyranoside and phenyl-α-glucopyranoside were both recognised by the sucrose carrier, whereas phenyl-β-glucopyranoside was not. Furthermore, phenyl-α-thioglucopyranoside but not phenyl-α-thiomannopyranoside induced H^+ uptake into vesicles in a manner similar to sucrose. These data strongly suggest that: i) the fructose moiety has no specific function for recognition and transport by the sucrose carrier; ii) the α orientation of the anomeric carbon is critical for recognition; and iii) hydroxy groups on C-3 and C-4 of the glucose moiety are involved in binding to the carrier. This opens up the possibility that xenobiotics possessing these characteristics could be specifically phloem-loaded.

b) Transport routes: Two distinct routes have been proposed for the transfer of sugars from the mesophyll to the SE; an apoplastic route, in which sucrose is actively taken up from the apoplast surrounding the SE or SE-companion cell complex (SE-CC); and a symplastic route, in which sucrose moves via plasmodesmata between the cells of the mesophyll, bundle sheath parenchyma, then to the SE. The majority of evidence favours phloem loading taking place from the apoplast (Humphreys, 1988). However, the involvement of plasmodesmata should not be completely disregarded (Robards & Lucas, 1990). The patterns of translocation of the majority of systemic herbicides indicate that they have access to both phloem and xylem. Most are known to exchange, relatively freely between these two vascular tissues, the extent of transfer determining whether the compound is moved predominantly acropetally (in the xylem) or basipetally (in the phloem). Direction of transport is also governed by endogenous 'source-sink' relationships. Thus, in the SE, flow is from an assimilate 'source' (*e.g.* photosynthetic or storage tissue) to a physiological 'sink' (*e.g.* meristematic or storage tissue). There are many examples of herbicides with this type of distribution pattern (refs. in Devine & Hall, 1990); yet there are exceptions (refs. in Coupland, 1989). It would be naïve to expect a simple relationship between sucrose transport and that of the herbicide to occur all the time. By their nature, herbicides are phytotoxic and are likely to affect cell metabolism at some stage and disrupt 'source-sink' patterns. Also, herbicides have different physicochemical properties to those of sucrose and are, therefore, going to be absorbed differently. Furthermore, herbicide which leaks from the phloem (a characteristic of this tissue even for natural metabolites; Grimm *et al.*, 1990) is unlikely to be re-enter the SE as efficiently as is sucrose.

c) Herbicide regulation of phloem transport : Phloem loading could be perturbed by herbicides in several ways, in particular by affecting the H^+-gradient across the PM, or by altering the sucrose gradient. There are examples of herbicides influencing both of these processes. Many xenobiotics, including herbicides and fungicides, are known to affect lipid metabolism in plants (Harwood, 1988). The lipid environment of the PM ATPase is thought to have a regulatory function, so any changes in the lipid 'annulus' of the ATPase will also influence enzyme activity (Burden *et al.*, 1989). There may also be an effect on the enzyme itself, perhaps via a specific hormone receptor associated with the ATPase, *e.g.* 2,4-D (and related phenoxy acids) mimicking IAA action (Baker, 1985).

Herbicides which inhibit the supply of ATP to the SE will also reduce phloem translocation as this compound is essential for the maintenance of the H^+ gradient across the PM. Examples of herbicides with these properties include the hydroxybenzonitriles (Sanders & Pallett, 1985) and dinoseb (Gruenhagen & Moreland, 1971), inhibiting photo- and oxidative phosphorylation, respectively. A different mechanism, but with similar effects, is found with diclofop, a herbicide which depolarises the PM, acting as a specific, and rapidly-acting (within 5 min) H^+ ionophore (Wright & Shimabukuro, 1987).

At sink regions, unloading of sucrose is likely to be regulated by sucrose 'usage' which, in turn, may be under hormonal control (Humphreys, 1988). This has led to the use of compounds possessing plant growth regulator (PGR) activity to stimulate 'sink' activity and so enhance the transport of a herbicide applied simultaneously or separately (Basler, 1977; Waldecker & Wise, 1985; Coupland, 1989). Certain herbicides may produce a similar effect but in a different manner. Translocation of phytotoxic amounts of AI to the apical meristems (usually the strongest 'sinks') will kill these tissues, the result of which will be the stimulation into growth of adjacent meristems due to the removal of apical dominance. Coupland (1989) showed that glyphosate behaved in this way in *Elymus (Agropyron) repens*. These effects are highly dependent upon the concentration of AI in the internal tissues and any build-up of herbicide in 'sink' regions will eventually limit further transport to those areas due to phytotoxicity.

Herbicides will also affect sugar transport if they reduce the supply of photosynthate to the SE. Two examples from the literature illustrate different ways in which this can occur. Glyphosate limits phloem

transport by inhibiting ribulose 1,5-bisphosphate carboxylase, so reducing CO_2 assimilation and the supply of sugars to the phloem (Servaites *et al.*, 1987). Chlorsulfuron, however, does not inhibit photosynthesis but appears to reduce sucrose export from the leaf (Devine *et al.*, 1990). In both cases, sucrose is prevented from entering the SE, reducing the build up of SE turgor, and thus inhibiting transport, not only of assimilate, but of the herbicide also. It is often difficult, therefore, to predict what the final outcome will be after treatment with herbicides having this type of action. On the one hand, there is a phytotoxic effect, on the other, an inhibition of herbicide transport.

Herbicides might affect sugar loading via a direct effect on the sucrose transporter itself. This is purely hypothetical, although the fact that certain amino acids could relieve the inhibition of phloem transport due to chlorsulfuron (Bestman *et al.*, 1990), might have been due to an effect on the sucrose permease, or an associated protein.

d) Membrane disruption: Disruptive effects on sugar transport would also be found if any of the cell membranes involved in sugar loading were damaged. Formulation components, such as surfactants, have this ability (Coupland, 1989), although their mobility in plants is usually quite limited (Silcox, 1988) and, therefore, they are likely to have only a local, perhaps limited effect. Nevertheless, for those surfactants and organic solvents which spread well and penetrate into the plant, there is the potential for more widespread membrane damage with concomitant effects on reduced phloem, and herbicide transport (Towne *et al.*, 1987; Stevens & Bukovac, 1987; Coupland, 1989).

Herbicide entry into the phloem

Research into uptake of herbicides (as well as other pesticides) into the phloem has also achieved major successes over the past few years. Several studies have culminated in producing a mathematical model which accounts for many of the effects of the physicochemical properties on the systemicity of xenobiotics in plants (Kleier, 1988; Hsu *et al.*, 1988; Grayson & Kleier, 1990). The model describes phloem systemicity in terms of a concentration factor (C_f) which is defined as the ratio of the concentration of AI in the phloem sap, some distance away from the site of application, to that in the apoplast at the treatment zone. The C_f depends upon the physicochemical properties of the AI, principally lipophilicity (usually described by K_{ow}), the pKa, and various plant parameters. In essence, this model predicts:

a) For a given pKa, there is an optimum permeability which enables the compound to enter into, and be retained in, the phloem (*i.e.* maximising phloem mobility).

b) For non-acidic compounds, the optimum permeability is around log K_{ow} -1.

In this review, I shall regard most of the plant parameters as relatively unimportant (*e.g.* plant length, SE radius, sap velocity, etc. [see Kleier, 1988]). Indeed, they may be regarded as 'constants', being the same for all applied compounds in any one situation. However, certain aspects of the plant's physiology and the permeability characteristics of the SE (or CC?) membrane (presumably the PM) are not modelled accurately, if at all. The model assumes a steady-state distribution of AI in the vascular tissues in different parts of the plant. Clearly, this does not occur, as reference to any publication describing the distribution of herbicides in plants will verify. Another assumption is that the concentration of AI in the apoplast surrounding the phloem at sites remote from the application areas is zero. Again this is not substantiated by experimental evidence. Firstly, many phloem-mobile compounds (*e.g.* glyphosate [Coupland & Caseley, 1979]; 2,4-D and clopyralid [Turnbull & Stephenson, 1985] and fluazifop [Derr *et al.*, 1985]) are excreted from plants in root and guttation exudates, which implies that diffusion from the phloem into the surrounding apoplast occurs at several different sites. The concentration of AI in the apoplast at these distal sites, therefore, cannot be zero. Secondly, most foliage-applied herbicides are known to exchange between the phloem and xylem vascular tissues (refs. in Coupland, 1989), indicating a dynamic, rather than a steady-state situation. Lastly, the model does not provide for any metabolism or compartmentation of the AI, processes which would effectively make the compound unavailable for transport. With regard to the permeability of AI through the phloem membrane(s), this is assumed to be directly related to lipophilicity as: $\log P = m.\log K_{ow} + g$; where P is the permeability of AI through the membrane, and m and g are related in some way to membrane properties. Clearly, this is only a very general approximation since it implies that increasing the K_{ow} would result in increased permeability. As the above model shows, this is not so, and water solubility is obviously an important limiting factor. This sensitivity of the model to the characteristics of the membranes which are involved in transfer of AI into the SE

was graphically illustrated by Hsu & Kleier (1990) by considering a hypothetical compound with a pKa of 5 and a *log* K_{ow} of 3. C_f values for this compound varied over at least 3 orders of magnitude, depending upon which membrane attributes, taken from the literature, were used. It is obvious that a better understanding of the permeability characteristics of the SE PM (and perhaps that of the CC) is necessary before the model can be improved.

Nevertheless, these weaknesses should not affect the ability to rank test compounds in order of their relative phloem mobilities for any given plant/experimental system. How does the above model compare with published results? Firstly, it is difficult to see how a model which describes uptake of xenobiotics specifically into phloem SE cells can be substantiated by the results of experiments in which these compounds are applied to plants in various ways, in a variety of formulations, and in all manner of experimental systems (Chamberlain *et al.*, 1986; Lichtner, 1986; Bromilow *et al.*, 1987; Chamberlain *et al.*, 1987; Hsu *et al.*, 1988). Yet, in many cases, predicted C_f values agree quite well with experimental results (Grayson & Kleier, 1990); there are, however, a few notable exceptions. In the work by Chamberlain *et al.* (1986), 4-(1-naphthoxy)-butanoic acid was predicted to be non-phloem mobile ($C_f = 0$), yet its concentration in the phloem exudate was found to be approximately 2.5 times that of sucrose applied simultaneously. Lichtner (1986) investigated a series of acidic and non-ionised compounds and found that their phloem mobilities (relative to sucrose, M_{suc}) ranged from 3.4 x 10^{-3} to 6.8 x 10^{-6}. Their predicted values, however, ranged over 1 order of magnitude only. Furthermore, D-sorbitol was predicted to rank second highest in the series, whereas, experimentally, it was only sixth with a M_{suc} of 2.1 x 10^{-5}. This highlights a very important discrepancy in the model, that there is no provision for any physiological influence on mobility, apart from that due to the pH differential between the SE and the apoplast. If Lichtner had performed the experiment using apple, sorbitol would have ranked by far the highest with a $M_{suc} \approx 3.2$, 5 orders of magnitude greater than that obtained with soybean. This is because in apple, D-sorbitol is the preferred translocated carbohydrate (Webb & Burley, 1962). These examples show that the above model can only be relied on to provide approximate indications of phloem systemicity and that, depending on the experimental conditions, there will inevitably be compounds which do not 'fit'.

Concluding remarks and future studies

It is impossible, using whole plant systems, to investigate in any detail the mechanisms of herbicide uptake. Isolated cell systems, or membrane vesicles, are the best, perhaps only way of achieving this precision. The pharmacokinetic approach of Ooka & Balke (1990) should be extended to herbicides other than the sulfonylureas to determine the relative importance of diffusion, ion-trapping, and carrier-mediated mechanisms. More research is needed to understand the mechanisms responsible for herbicide detoxification and compartmentation in plants, specifically into vacuoles. Making more AI available for transport would be a very effective way of improving the systemicity of existing compounds. The unified model by Kleier (1988) is a significant step forward in defining and predicting the phloem systemicities of herbicides in plants. Based on this, and with continued improvements in the understanding of herbicide action at enzyme reaction centres, it may soon be possible to:

i) alter the molecular structure of existing, poorly translocated compounds to optimise their physicochemical properties with respect to uptake into SE cells. However, it should be realised that there will be a limit to the amount of 'molecular manipulation' that can be performed before loss of herbicidal activity occurs;

ii) produce molecules having the 'right' physicochemical properties for uptake into the SE, perhaps incorporating a glucose-like moiety, and screen them for herbicidal properties. Interestingly, a new class of herbicide, which is carbohydrate-derived and based on a polycyclic, sugar-like structure, has recently been described (Henzell *et al.*, 1990; Blattner *et al.*, 1991). Although the biochemical mode of action has not been determined, these compounds are apparently effective on meristematic tissues, such as shoot apices and developing seeds, which may reflect their efficient translocation;

iii) design molecules having the required configuration for binding to the reaction centre of the target enzyme plus a 'herbicidally-inert' part of the molecule, the sole purpose of which is to confer those properties needed for uptake into the SE. In this respect, recent progress in characterising the sucrose transporter should benefit the molecular design of systemic compounds.

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PROSPECTS FOR THE BIORATIONAL DESIGN OF CROP SELECTIVE HERBICIDES

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ABSTRACT

The prospects for rational design of crop selective herbicides based on knowledge of herbicide metabolism is explored. Four examples of metabolism-based selectivity are described, and structure/selectivity data are presented that suggest that prior knowledge of these metabolic pathways would not have led researchers more easily to the commercialized analogs. The role of substituents distal from the actual site of metabolism in determining susceptibility of the herbicide analog to metabolism is illustrated. We conclude that while early knowledge of metabolic pathways can help focus analoging programs, the primary successful strategy for optimizing crop selectivity will, for the foreseeable future, remain broad-based chemical synthesis and testing.

INTRODUCTION

The need for pest control in world agriculture is inescapable and will increase as agricultural productivity attempts to keep up with forecast population increases on today's arable land base (Borlaug, 1990; Econ. Res. Ser., 1989). However, modern pest control materials must be compatible with legitimate requirements for environmental and food safety. This presents a major challenge to agrochemical researchers seeking to discover chemical and biological pesticides having ideal attributes of efficacy, crop selectivity, environmental properties, toxicology, and low cost. New research approaches are being explored including the *de novo* design of agrochemicals based on biochemical knowledge, often called the "biorational" or "rational design" approach. In principle, biorational design should someday be applicable to the initial discovery of a new agrochemical class and its subsequent customization for crop selectivity and desired environmental properties. This paper addresses the current prospects for biorational design of metabolism-based crop selective herbicides. Our approach will be to describe the selective metabolism of several herbicides and then to consider, in retrospect, whether *advance* knowledge of that metabolic capacity would have easily led to the final commercial herbicide.

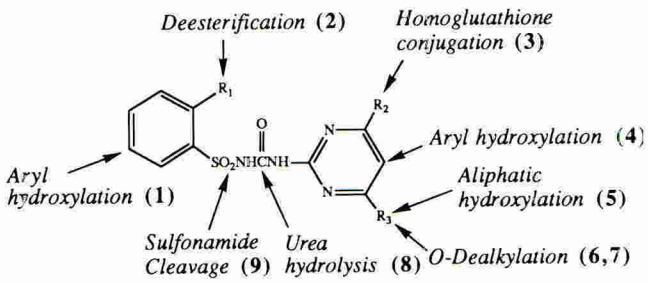
CROP SELECTIVITY MECHANISMS

The basis for crop selectivity is now well understood for many herbicides and some generalizations are possible. Inherent sensitivity differences at the active site between weeds and crops is a relatively rare selectivity mechanism. The best examples of this mechanism come from the cyclohexanedione and aryloxyphenoxy grass herbicides where broadleaf crop (and weed) tolerance results from the natural insensitivity of broadleaf acetyl CoA carboxylase, the target site of these herbicides in grass species (Rendina, 1988; Walker, *et al.* 1988). Special examples of this mechanism come from mutagenized or genetically-engineered crop varieties which contain an insensitive form

of the target site (e.g. Sebastian, *et al.*, 1989). However, most modern herbicides owe their inherent crop selectivity, at least in part, to rapid metabolic inactivation of the herbicide in the crop plant (see Hatzios and Penner, 1981). Many chemically-diverse herbicides are metabolized by different crop and weed species with consequent plant tolerance. We've chosen to simplify this discussion by focusing on the sulfonylurea herbicides because, 1) members of this herbicide class have general chemical and physical property elements in common and the same mode of action, allowing relevant comparisons, 2) many crop selective analogs have been commercialized, and 3) the metabolic bases for many of these selectivities have been elucidated (For reviews, see Beyer, *et al.*, 1987; Brown and Kearney, 1991; Brown, *et al.*, 1991; and Brown, 1990).

The sulfonylureas are a large chemical class containing potentially tens of millions of herbicidally-active analogs. To date, 17 commercial products or advanced candidates have been discovered for selective weed control in numerous crops including wheat, barley, oats, rice, maize, canola, soybeans, flax, sugarbeets, tomatoes and potatoes (see Brown and Kearney, 1991; Brown, 1990). In every case where the mechanism has been reported, plant metabolism has been the basis for this selectivity. Table 1 summarizes

TABLE 1. Plant metabolism of crop-selective sulfonylurea herbicides (Adapted from Brown and Kearney, 1991)



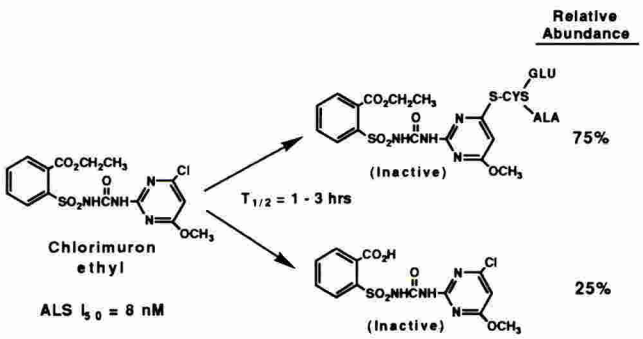
METABOLIC REACTION	SELECTIVE HERBICIDE	PLANT SPECIES	REF.
(1)	Chlorsulfuron	wheat, barley	Sweetser, *et al.*, 1982
	Metsulfuron	wheat, barley	Anderson, *et al.*, 1989
(2)	Chlorimuron	soybean	Brown and Neighbors, 1987
	Thifensulfuron	soybean, wheat	Brown, *et al.*, 1990; Cotterman and Saari, 1989
(3)	Chlorimuron	soybean	Brown and Neighbors, 1987
(4)	Nicosulfuron	corn	Du Pont Unpublished
(5)	Chlorsulfuron	flax	Hutchison, *et al.*, 1984
(6)	Bensulfuron	rice	Takeda, *et al.*, 1986
	Thifensulfuron	wheat	Cotterman and Saari, 1989
(7)	Ethametsulfuron	oilseed rape	Du Pont Unpublished
(8)	Thifensulfuron	wheat	Cotterman and Saari, 1989
	Chlorsulfuron	barley	Du Pont Unpublished
	Metsulfuron	barley	Du Pont Unpublished
(9)	Thifensulfuron	wheat	Cotterman and Saari, 1989

the known metabolic transformations of sulfonylureas in plants, and it is clear that a wealth of information exists, offering the possibility that this science has grown to the point where biorational design of selective analogs can be based on this knowledge. However, experience shows that the susceptibility of a compound to these metabolic pathways is dependent on more than just the presence of the chemical functionality which directly undergoes transformation. (See, for example, Frear and Swanson, 1970; Guddewar and Dauterman, 1979.) The substrate specificity of the metabolizing enzymes leading to plant selectivity can be quite strict, with specificity being determined by structural features remote from the site of metabolism. The remainder of this paper will present 4 examples of such specificity which will illustrate the difficulty of rationally-designing herbicidal analogs which will be readily metabolized by known pathways in crops.

SOYBEAN METABOLISM OF CHLORIMURON ETHYL

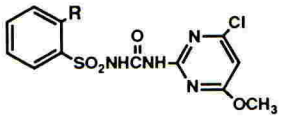
Chlorimuron ethyl is a commercial sulfonylurea herbicide for broadleaf weed control in soybeans (*Glycine max*). Soybean tolerance results from rapid metabolism by the pathways shown in Fig. 1 (Brown and Neighbors, 1987). The major metabolite is the homoglutathione (hGSH) conjugate, formed through the action of a hGSH transferase, while the minor metabolite is the deesterified free acid. Both metabolites are herbicidally-inactive and inactive *in vitro* against the target site, acetolactate synthase. Conjugation of chlorimuron ethyl to hGSH proceeds through an aromatic nucleophilic displacement reaction which depends on the electronically-polarized pyrimidinyl chlorine/carbon bond and the favorable leaving properties of the chlorine group. Note that this chemical arrangement is quite similar to the triazinyl chlorine of atrazine, which readily undergoes aromatic nucleophilic displacement by glutathione in corn (see Hatzios and Penner, 1981). This points out a specificity for this reaction between species; although both chlorimuron ethyl and atrazine are chemically-susceptible to conjugation to GSH and both corn and soybeans have GSH-S-transferase activity, chlorimuron ethyl is not readily metabolized in corn and atrazine is not readily metabolized in soybeans, with consequent high sensitivity of these crops to these herbicides. Thus, while it is a reasonable biorational design strategy to construct herbicide analogs which are chemically-susceptible to known plant metabolic reactions, that a specific crop (or weed) will actually "recognize" and metabolize the analog is not readily predictable.

FIGURE 1. Soybean metabolism of chlorimuron ethyl (adapted from Brown, *et al.*, 1991).



Further, within a single plant species there can be a high degree of specificity among analogs having very similar chemical susceptibility to a metabolic reaction. For example, Table 2 shows the metabolic rates and relative soybean sensitivities of several chlorimuron ethyl analogs, all of which are very similar in *chemical* susceptibility to conjugation to hGSH. Note the strong influence of the relatively remote *ortho* - substituent on the rate of metabolism. For example, the *o*-carboxymethyl analog is only slowly metabolized and is highly injurious to soybeans while the ethyl (chlorimuron ethyl) and propyl esters are readily metabolized, primarily to the hGSH conjugate. Furthermore, the non-ester analogs shown in Table 2 are representative of hundreds of other 4-chloro-6-methoxy pyrimidinyl sulfonylureas which are not soybean-tolerant even though each of those analogs has the susceptible heterocyclic moiety. This example illustrates the difficulty of working from metabolic first-principles in the design of crop selective herbicide.

TABLE 2. Soybean metabolism of chlorimuron ethyl analogs (from Brown, *et al.*, 1991)

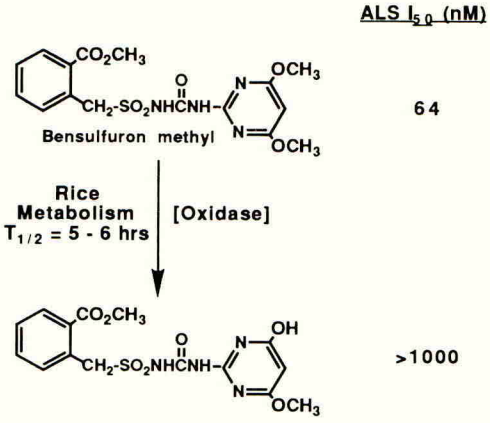


R-Group	Metabolic T1/2 (h)	Soybean Response
$-CO_2CH_3$	16	Sensitive
$-CO_2CH_2CH_3$	1 - 4	Tolerant
$-CO_2CH_2CH_2CH_3$	1 - 3	Tolerant
$-SO_2N(CH_3)_2$	12	Sensitive
$-SO_2CH_3$	20	Sensitive

RICE METABOLISM OF BENSULFURON METHYL

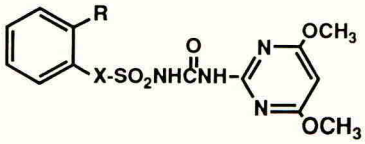
Bensulfuron methyl is a rice-tolerant herbicide for broadleaf and sedge weed control at 25 - 70 g a.i./ha. As shown in Fig. 2, rice plants metabolize bensulfuron methyl with a half-life of 5 - 6 hrs to the 4-hydroxy-6-methoxy-pyrimidinyl derivative which is completely inactive against ALS. Conjugation to glucose does not occur, possibly due to the tautomerization to the keto form of this metabolite. This *o*-dealkylation reaction is thought to proceed through hydroxylation of the methoxy substituent to form the unstable formyl adduct which decomposes to the hydroxylated metabolite with loss of formaldehyde (Takeda *et al.* 1986) .

FIGURE 2. Rice metabolism of bensulfuron methyl (adapted from Brown, 1990)



The ability of rice plants to metabolize the dimethoxypyrimidine heterocycle of this sulfonylurea herbicide would suggest that many analogs having this moiety would be rice selective. In fact, many hundreds of dimethoxypyrimidine sulfonylureas have been synthesized at several companies with only a very few showing adequate rice tolerance. A few examples are shown in Table 3. Bensulfuron methyl has a methylene group in the sulfonylurea bridge; removal of that single methylene group completely eliminates rice selectivity. Also shown in Table 3 is one of many examples where this metabolic reaction is sensitive to the moiety in the *ortho* position of the benzene ring. The methylsulfonyl analog of bensulfuron methyl is not rice tolerant. Thus, as for soybeans, rice herbicide metabolism is highly dependent on structural features remote from the site of metabolism.

TABLE 3. Rice tolerance to bensulfuron methyl analogs



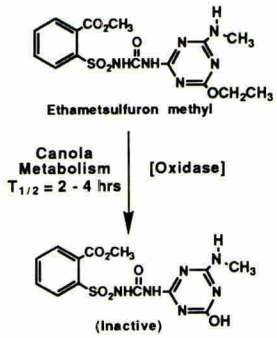
Structure		Rice Injury (%)		Weed Control (%)[c]
R	X	62 g/ha	31 g/ha	31 g/ha
$-CO_2CH_3$	$-CH_2-$[a]	0 - 10	0	75 - 90
$-CO_2CH_3$	--	90 - 100	70[b]	85[b]
$-SO_2CH_3$	$-CH_2-$	50	40	90

[a] Bensulfuron methyl
[b] Applied at 4 g a.i./ha
[c] Average of 9 broadleaf weed species

CANOLA METABOLISM OF ETHAMETSULFURON METHYL

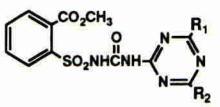
Ethametsulfuron methyl (formerly DPX-A7881) controls broadleaf weeds in spring oilseed rape *Brassica napus*, (canola). This selectivity extends to the control of wild mustard,*Sinapis arvensis* (formally *Brassica kaber*), a weed so closely related to *B. napus* that they can readily cross (Hutchison, *et al.*, 1987). This selectivity is again based on differential metabolism, and Fig. 3 shows that canola plants rapidly O-dealkylate the 4-ethoxy group of ethametsulfuron methyl.

FIGURE 3. Canola metabolism of ethametsulfuron methyl



However, the specificity of this reaction is again found to be quite sensitive to other structural features. Table 4 shows that canola plants do not rapidly O-dealkylate the methoxy analog of ethametsulfuron methyl, showing an apparent preference for higher alkoxy analogs. Also, the 6-methylamino substituent plays an important "steering" role in the recognition of ethametsulfuron methyl as a substrate; the 4-ethoxy-6-methyl analog is not canola tolerant. Thus, while advance knowledge that canola could catalyze O-dealkylations could focus synthetic efforts, considerable synthetic exploration would be required to find a combination of substituents that would actually be "recognized" as a substrate by this plant.

TABLE 4. Canola tolerance to ethametsulfuron methyl analogs



Structure		Canola Injury (%)		Weed Control (%)[b]	
R_1	R_2	64 g/ha	16 g/ha	64 g/h	16 g/ha
$-NHCH_3$	$-OCH_2CH_3$ [a]	0-10	0	95	80
$-NHCH_3$	$-OCH_3$	100	90	100	85
$-CH_3$	$-OCH_3$	100	100	100	95
$-CH_3$	$-OCH_2CH_3$	100	100	100	90

[a] Ethametsulfuron methyl

[b] Avg of 8 broadleaf weeds

NICOSULFURON METABOLISM BY MAIZE

Our last example is based on nicosulfuron, a postemergence herbicide for grass (and some broadleaf) weed control in maize. Maize plants rapidly metabolize nicosulfuron to the herbicidally-inactive 5-hydroxypyrimidinyl derivative, which is subsequently conjugated to glucose (Fig. 4). However, from a rational design perspective, this pathway would provide little guidance. Many pyrimidinyl sulfonylureas are chemically-susceptible to 5-hydroxylation, yet maize tolerance is exceptionally rare among the many hundreds of pyrimidinyl sulfonylureas tested. Commercial examples include chlorimuron ethyl, bensulfuron methyl, sulfometuron methyl, and pyrazosulfuron ethyl, each of which has a susceptible pyrimidine heterocycle and yet which are highly injurious against maize (see Brown, 1990; Brown and Kearney, 1991 for structures). As shown in Table 5, susceptibility to hydroxylation of the pyrimidine ring is quite dependent on substituents remote from this site of metabolism. For example, the direct benzene analog of nicosulfuron is much less maize-tolerant, indicating a role of the pyridine nitrogen in conferring recognition of nicosulfuron as a substrate for pyrimidine hydroxylation. However, even within a series of pyridine analogs we again find a very distinct influence of the *ortho* substituent. Note that the *o*-carboxymethyl ester and the *o*-methylsulfonyl pyridine analogs are quite injurious to maize, even though the effect of these substituents on the overall physical properties of these analogs is relatively minor.

FIGURE 4. Maize metabolism of nicosulfuron.

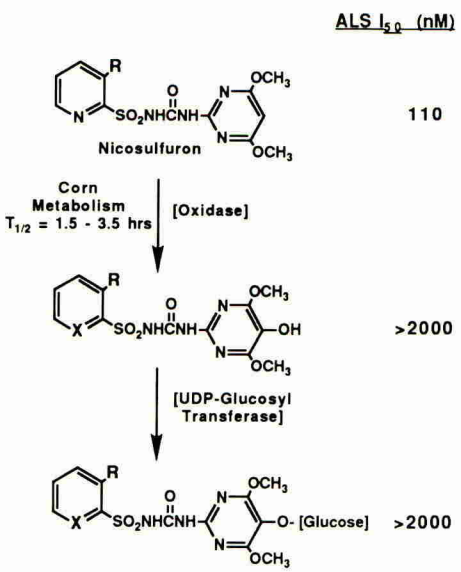
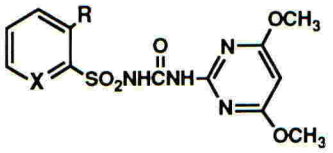


TABLE 5. Maize Tolerance to Nicosulfuron Analogs



Structure		Maize Injury (%)		Weed Control (%)	
R	X	50 g/ha	10 g/ha	BL[b] 10 g/ha	GR[c]
O ‖ -C-N(CH_3)$_2$	-N[a]	0-5	0	70	90
O ‖ -C-N(CH_3)$_2$	-CH-	60-70	40-50	70	75
O ‖ -C-OCH_3	-N-	100	95	100	95
-SO_2CH_3	-N-	95	80	95	90

[a]Nicosulfuron, formerly DPX-V9360
[b]Avg. of 3 broadleaf weed species
[c]Avg. of 5 grass weed species

CONCLUSIONS

The goal of rational pesticide design is to reduce the cost and time required to discover new agrochemicals through the application of our increasing knowledge of pest and crop biology. In principle, rational design strategies should be applicable to *de novo* lead discovery as well as optimization of important attributes such as crop selectivity, toxicology, and environmental properties. This paper has explored the current prospects of rationally-designing crop selective herbicides based on knowledge of metabolic processes in plants. Our approach has been to take current knowledge of selective metabolism and to consider in retrospect whether advance knowledge of that pathway would have easily led to the actual commercialized herbicide. Our conclusion is that simple knowledge of a metabolic capability in a crop is inadequate to provide exact synthetic guidance. There is clearly a very distinct substrate specificity associated with plant xenobiotic metabolizing enzymes. While this advance knowledge can allow more specific focus of synthetic efforts, the role of thorough, broad-based synthetic exploration will remain paramount for the foreseeable future. In fact, detailed elucidation of the structure/selectivity relationships for a crop metabolic reaction will *require* the very synthesis of numerous analogs that this approach seeks to circumvent.

However, there is value in elucidating the metabolic bases for crop selectivities of herbicide leads. As mentioned above, such knowledge gained early in a program can help focus analoging programs to insure discovery of the best candidate. Also, this information can presage potentially adverse interactions with other agrochemicals

known to interfere with such reactions or provide guidance for herbicide safener discovery programs. Also, general knowledge of metabolic reactivities of certain substituents can be factored into other synthesis programs as a means of improving the odds that crop tolerance might be obtained. However, significantly more knowledge will be required before true rational design of crop selective herbicides becomes a common process. As shown in this paper, much more knowledge of the substrate specificities of plant xenobiotic metabolizing enzymes is required as well as the role of isozymes which may have different specificities. It will also be necessary to understand how metabolic capability and specificity may vary as a function of seedling age or growing conditions. Of course, success in elucidating the substrate specificity of the crop metabolizing enzyme must be matched by insuring that similar systems in target weeds do not recognize the proposed molecules as substrates. Some progress is being made, especially in unraveling the enzymology of plant xenobiotic metabolizing enzymes. We suggest that a multidisciplinary approach involving broad-based synthesis, weed science, biochemical investigation, and computer-assisted quantitative structure-selectivity efforts will be the key to success in efforts to discover the crop selective herbicides of the 21st century.

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HERBICIDE DISCOVERY THROUGH RATIONAL DESIGN : SOME EXPERIENCES

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ABSTRACT

The use of biochemically inspired design as a means of discovering novel agrochemicals is reviewed for the non-specialist. At present we rely almost exclusively on knowledge or deductions about the chemical mechanism of the target enzyme to provide inspiration for the design of new inhibitors. To exemplify this approach, four case histories, involving pantothenate synthetase, ketol-acid reductoisomerase, pyruvate dehydrogenase, and glutamine synthetase, are outlined. Each project achieved different levels of success and together they serve to illustrate both the pitfalls and the potential of this approach. Although no commercial compound has yet been discovered solely through biochemical design, the scientific successes achieved suggest that such an approach is worthy of continued support. The prospects for the future are then reviewed with regard to the impact that the techniques of molecular biology and our increasing ability to visualise enzymes in three dimensions may have.

INTRODUCTION

The purpose of this paper is to provide a general review of the use of biochemically inspired design in the herbicide discovery process for the non-specialist. We will limit ourselves to discussing the design of enzyme inhibitors that might have potential as novel herbicides and not consider in detail the subsequent aspects of delivery and selectivity that are discussed in the following papers. It is worth bearing in mind that rational design embraces more than just the biochemical- or enzyme-directed approach since a rational approach is applied to all aspects of the herbicide discovery process, as far as possible. This is true even in cases where the initial discovery comes from a random lead and the subsequent development of the series is based on an empirical approach (Wright *et al.*, 1991).

The concept of biochemical design is not new and degrees of commercial success have certainly been achieved in the pharmaceutical industry (eg Silverman, 1988; Bey, 1989; Edwards *et al.*, 1989). A similar approach has also been employed within the agrochemical industry and has been part of our activities at Chesterford Park since the mid-sixties. Regrettably, no agrochemical product has yet been discovered solely by this approach but there have been scientific successes and we will outline in this review the real progress that has been made, by describing some examples from our own work and that of others. The prospects for the future will also be discussed, especially with respect to the potential impact that molecular biology and our increasing ability to visualise enzymes in three dimensions may have on the biochemical design process.

THE BIOCHEMICAL DESIGN PROCESS

The biochemical design process involves input from chemists, biochemists and, increasingly, molecular biologists. Two major steps are involved:

- choose a suitable biochemical target (eg. an enzyme);

- design and synthesise potent inhibitors.

Although many enzymes can be envisaged to be suitable targets, to date the decision on whether to initiate a project with a particular enzyme has greatly depended on chemical considerations. Unless potential inhibitors can be identified for synthesis based on the relatively limited approaches - generally involving knowledge about the mechanism of the enzyme - that are available at present, the project would be unattractive. The two steps in the design process are discussed below.

Choice of a suitable target

Deciding whether a particular enzyme plays an essential role in the well-being of a plant and is, therefore, a good target depends heavily on the available biochemical and physiological data. Key questions to address are the likely levels of enzyme present, the degree of inhibition required to significantly reduce the total flux through the pathway, and the effects that inhibition of the target enzyme will have on the plant. This type of data is often unavailable for many potential enzyme targets at present and we therefore have to extrapolate from the available current knowledge. Alternatively we could initiate the necessary, basic research, although this is only likely to be justifiable in a limited number of situations.

Enzymes that are established sites of action of known herbicides are, of course, attractive targets for further synthesis. This is one of the few situations where we can say with certainty that effective inhibition of the enzyme will lead to a herbicidal effect. Although there are examples where biochemical rationale has led to the synthesis of novel inhibitors of a known herbicide target, for example acetolactate synthase (Ciskanik and Schloss, 1986) and enol-pyruvyl shikimate phosphate synthase (Alberg and Bartlett, 1989), no biochemically designed inhibitor has yet been reported with levels of biological activity that approach those of the lead herbicide. We should not, however, be unduly put off by this poor track record as only a handful of enzymes are established as herbicide targets and even fewer lend themselves to biochemical design with the level of information currently available.

Design of potential inhibitors

The design of novel inhibitors clearly depends on the available biochemical information about the enzyme in question. For instance, information about known inhibitors is often a good starting point. Knowledge about the characteristics of the substrates, products, and any co-factors involved can also provide leads for synthesis. In practice the most fruitful approach to inhibitor design to date has been through a consideration of the chemical mechanism employed by the enzyme. From this type of information, stable analogues of the transition states or the proposed reaction intermediates can sometimes be envisaged. As well as being the most practical way forward, this strategy can yield highly potent and specific inhibitors with theoretical dissociation constants as low as 10^{-24}M, (Wolfenden, 1977). The potential and the pitfalls inherent in this approach to the discovery of novel herbicidally active compounds will be exemplified through the four case studies outlined in the next section.

EXAMPLES OF BIOCHEMICAL DESIGN

Pantothenate Synthetase

The starting point for our work on this project was the principle that a tetrahedral phosphorus atom can act as a stable mimic of the tetrahedral carbon formed as part of enzyme reactions involving nucleophilic attack on a carbonyl group (Bernhard and Orgel, 1959; Weaver *et al.*, 1977). We have used this strategy in a number of projects, stimulated by the publication by Biryukov *et al.* (1978) who devised inhibitors of aminoacyl-t-RNA synthetases by this means. Following our successful use of this strategy with glutamine synthetase (see later), pantothenate synthetase was chosen for investigation (Cornell C., *et al.* in preparation). Although pantothenate synthetase had not been demonstrated to be present in plants at the time of our work (and has still not been shown to be present), it seemed reasonable to suppose that plants would employ a similar route of pantothenate biosynthesis to that established for microorganisms. The enzyme had been purified essentially to homogeneity from *Escherichia coli* (Miyatake *et al.*, 1978) and we used a preparation from this source for all the biochemical investigations.

Based on the likely reaction mechanism shown in Figure 1, the most obvious target to synthesise was compound I. We hoped that compound I might also be adenylated by the enzyme to give a very close mimic of the reaction intermediate. This compound and the other two related analogues shown in Figure 1 were subsequently synthesised but none gave significant inhibition of the enzyme under a variety of assay conditions. The compounds were also essentially inactive in the biological screens.

Although we did not try to establish why our compounds were not inhibitors, there are, of course, several possible explanations for these disappointing results:

- Despite the close analogy with the proposed enzyme intermediate, our inhibitors were simply not a good enough fit to bind to the enzyme; for example the electron density around the phosphorus atom was wrong or the NH moiety is very important for binding.

- Adenylation of such inhibitors may well be required to allow effective binding to the enzyme and this may not occur.

- The enzyme may involve a "closed transition state" (see Kluger and Smyth, 1981) so that inhibitors based on the reaction intermediate may be unable to bind to the enzyme.

This result was obviously disappointing and the project was not progressed. The reason for outlining the work here is to demonstrate that, however good the theory seems, the practice may reveal its shortcomings. Biochemical design therefore has to be approached with realism with respect to the likely success rate.

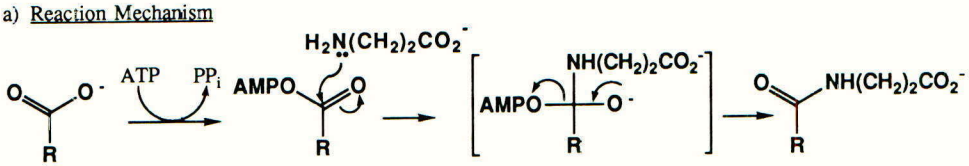
Ketol-Acid Reductoisomerase

The second case history involves work undertaken by Schloss and co-workers (Aulabaugh and Schloss, 1990; Schloss and Aulabaugh, 1990; Wittenbach *et al.*, 1991) on the enzyme ketol-acid reductoisomerase (KARI). This enzyme catalyses the next step to that of the established herbicide target acetolactate synthase in the biosynthesis of the branched chain amino acids. It therefore represents an attractive target in a pathway whose inhibition is already known to lead to herbicidal effects. The reaction mechanism of KARI is shown in Figure 2. From this mechanism, the oxalyl hydroxamate (compound IV in Figure 2) was proposed as a potential reaction intermediate analogue and was

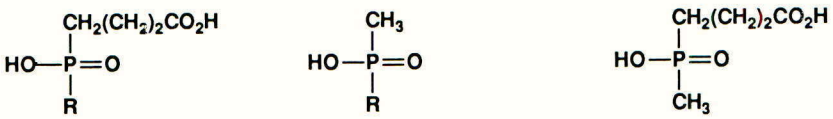
Figure 1 Pantothenate Synthetase: Likely Mechanism and Proposed Inhibitors

[R = $CH(OH)C(CH_3)_2CH_2OH$]

a) Reaction Mechanism



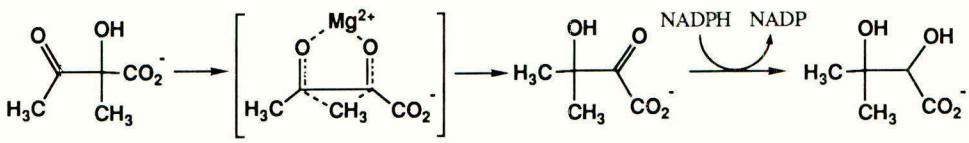
b) Proposed Inhibitors



I II III

Figure 2 Ketol-Acid Reductoisomerase: Mechanism and Inhibitors

a) Reaction Mechanism



b) Inhibitors

IV V

indeed shown to be a powerful inhibitor with an overall affinity of 22pM. Almost concurrently, Hoe 704 (compound V) was also reported as an inhibitor of this enzyme but with a much lower overall affinity of 0.8μM (Schultz *et al.*, 1988).

Compounds IV and V, however, showed only moderate levels of herbicidal activity, especially when compared with inhibitors of acetolactate synthase. This was, perhaps, particularly surprising for compound IV in view of its extremely potent inhibition of KARI *in vitro*. The uptake, movement and detoxification rates for Compound IV were subsequently shown to be comparable to those for thifensulfuron-methyl, an established inhibitor of acetolactate synthase (Wittenbach *et al.*, 1991). In addition, these authors also showed that more than 90% inhibition of the enzyme was achieved in treated plants. This suggests that KARI may simply be a poorer herbicidal target than acetolactate synthase. A possible reason for this may be that the KARI activity needs to be totally eliminated for an extended period of time in order to reduce the flux through the pathway sufficiently to kill the plant. Equally, as high levels of acetoin, the substrate for KARI, accumulate following inhibition of this enzyme (Schultz *et al.*, 1988; Wittenbach *et al.*, 1991), this implies that acetoin is not toxic to plants *per se*. This may be in contrast to the situation following inhibition of acetolactate synthase where the accumulation of the substrate for this reaction, α-ketobutyrate, has been proposed as a major cause of toxicity (LaRossa *et al.*, 1987). Although these authors have provided good evidence for the toxic role of α-ketobutyrate in bacteria, it is, however, much less certain whether the same situation applies in plants.

This case history serves to illustrate that, even if good biochemical inhibitors can be designed and synthesised, useful levels of biological activity will not necessarily follow. In many cases this will be due to poor uptake or rapid detoxification. However, as demonstrated here, projects can also apparently fail because inhibition of the chosen enzyme does not lead to a sufficiently toxic effect.

Pyruvate Dehydrogenase

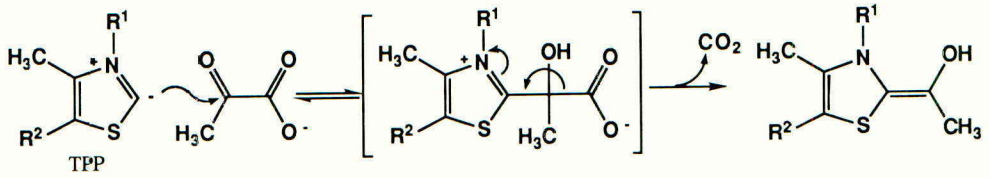
Our work on this enzyme was stimulated by a report by Kluger and Pike (1977) that a phosphorus compound (compound VI, Figure 3) resembling pyruvate was an effective inhibitor of pyruvate dehydrogenase. The mechanism by which compound VI may inhibit pyruvate dehydrogenase is shown in Figure 3.

We subsequently reprepared compound VI and showed it to be an effective plant growth retardant and decided to attempt the design and synthesis of more effective inhibitors as described in detail elsewhere (Baillie *et al.*, 1988). Compound VII was subsequently found to be a potent, time-dependent inhibitor of pyruvate dehydrogenase with a half-time of inactivation of about 12 minutes at 10μM. Further to this, compound VII was found to be an effective herbicide giving, for example, complete post-emergence control of some weed species at 700 grammes per hectare under constant environment conditions. The acetal derivative (compound VIII) subsequently emerged as the best material for field testing. Unfortunately, it was too damaging to crops at rates that gave reliable weed control and was therefore not a candidate for commercialisation.

Despite the lack of commercial success, this case history amply demonstrates that compounds synthesised from biochemical principles can give commercial levels of biological activity.

Figure 3 Pyruvate Dehydrogenase: Mechanism and Inhibitors

a) Reaction Mechanism



b) Proposed Reaction with VI

c) Other Inhibitors

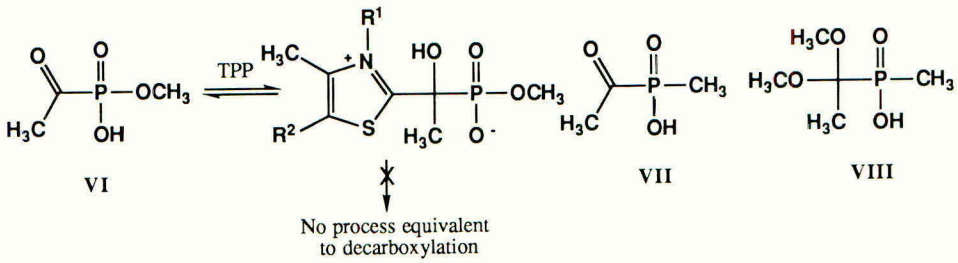
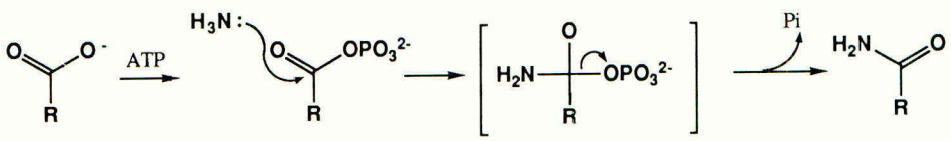


Figure 4 Glutamine Synthetase: Mechanism and Inhibition

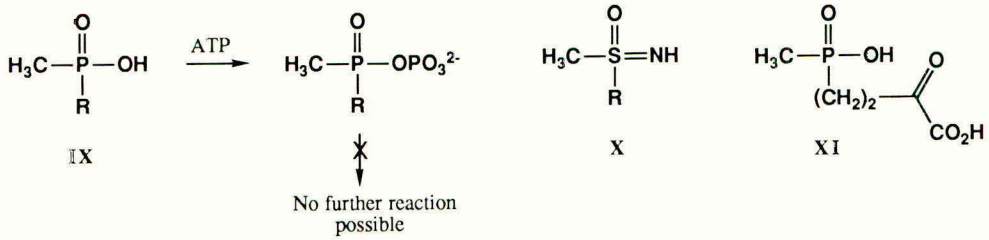
[R = $CH_2CH_2CH(NH_2)CO_2H$]

a) Reaction Mechanism



b) Proposed Reaction with IX

c) Other Inhibitors



Glutamine Synthetase

The final case history involves the enzyme glutamine synthetase which has an important role in inorganic nitrogen assimilation. The reaction mechanism of glutamine synthetase is shown in Figure 4. Based on the same principles outlined earlier for pantothenate synthetase we initiated a project that has been described in more detail elsewhere (Wright *et al.*, 1991). Compound IX was initially suggested as a potential inhibitor. By analogy with the known inhibitor methionine sulfoximine (compound X) we also hoped that phosphorylation might take place at the active site as shown in Figure 4. Searching the literature for the novelty of compound IX, we discovered that it had already been synthesised by Hoechst and patented as a herbicide (Rupp *et al.*, 1977). It had also been reported as a component of a herbicidal tripeptide isolated from a fermentation broth (Bayer *et al.*, 1972).

While it was encouraging to have predicted the activity it was disappointing to discover that the compound was owned by another company. We did, however, progress the project. In one approach we reasoned that plants are good at transamination reactions and might be able to produce compound IX from its keto acid analogue, and we therefore synthesised compound XI. We subsequently demonstrated that this was indeed the case (Wright *et al.*, 1991). Although compound XI did have commercial levels of activity, its development was discontinued for a number of reasons. Compound IX, however, fared better and has been commercialised by Hoechst as a total herbicide with the common name glufosinate.

This somewhat complicated case history does demonstrate, albeit in retrospect, that all the steps required for the biochemical design of a commercial compound have been carried out. Although no single research group took the project from concept to commercial reality, this example does provide real evidence that the whole process is possible.

PROSPECTS FOR THE FUTURE

These case histories serve to demonstrate that a biochemically based approach to the discovery of commercial herbicides can potentially succeed. Although commercial success has still to be achieved, we are firmly of the opinion that this approach does warrant continued effort and should be maintained as part of the discovery process. Clearly we need to strive towards a better success rate in the future and the main advances that are likely to help us to achieve this are discussed below.

With respect to establishing novel biochemical targets as potential sites of action for herbicides we can certainly expect significant steps forward in the future. One approach is through the study of plant mutants. Removal of the nutrient supplement after an auxotrophic mutant has been established can indicate the likely symptomology and speed of kill that might be expected if the mutated enzyme was to be blocked by the application of a herbicide. There have, however, been few if any reports of auxotrophs that have shown high toxicity upon removal of the nutrient supplement (McHale *et al.*, 1990; Negrutiu *et al.*, 1985; Schneider *et al.*, 1989). However, such studies are a little difficult to interpret at present as no mutant deficient in an established herbicide target has been reported with which to make comparisons. What we undoubtedly require is, for example, a mutant deficient in acetolactate synthase with which to validate the system. If such a mutant showed high toxicity upon removal of the nutrient supplement, this would give good grounds to treat with suspicion any enzyme for which mutants lacking the enzyme show only limited symptoms.

Investigation of whether a particular enzyme would be a good target can also be undertaken by the use of transgenic plants. The technology involved in regulating enzyme levels through the introduction of "anti-

sense" DNA is now becoming routine although it does, of course, depend on the availability of the plant mRNA sequence. The use of transgenic plants in the herbicide discovery process is well demonstrated by the recent report of tobacco with reduced levels of the enzyme phenylalanine ammonia-lyase (Elkind *et al.*, 1990). This enzyme could be considered as a potential herbicide target due to its role in lignification. However, although transgenic plants with reduced levels of the enzyme did show a number of visual symptoms, high toxicity was not evident. This may suggest that phenylalanine ammonia-lyase would not be a good herbicide target or, at least, would need to be inhibited to a higher degree to achieve a herbicidal effect. We can certainly expect to see many more examples of this approach in the future.

The design of new inhibitors depends on the biochemical information available and this, in turn, depends greatly on a plentiful supply of enzyme for study. Molecular biology has an essential role to play here in providing large amounts of enzyme by cloning and overexpression of the plant genes. A plentiful supply of the enzyme also opens up the prospects for obtaining three dimensional information about the enzyme, particularly through crystallisation and the acquisition of X-ray data. Probably the best example of the use of this type of data has been in the pharmaceutical industry with dihydrofolate reductase (Kuyper, 1990). Three dimensional information about an enzyme, preferably crystallised with an inhibitor or a substrate *in situ*, provides probably the only opportunity to pursue a predictive route of accessing interaction sites outside those involved in binding of the substrates, reaction intermediates, products, cofactors, and allosteric inhibitors of the enzyme.

We would certainly expect that the availability of information about the three-dimensional structure of agriculturally important enzymes to increase in the future. However, obtaining crystals suitable for X-ray analysis and collecting and interpreting the data are time consuming operations which are by no means routine at present. We would, therefore, only expect this approach to have an influence on the design process in the longer term. Implicit in all this type of work is the availability of interactive computer modelling facilities. This is an area that is also developing rapidly in terms of both handling three dimensional data as well as modelling from first principles (Vorpagel, 1987) and using the amino acid sequence of proteins to predict the likely conformation that the protein will adopt (for review see Jaenicke 1991).

In summary, therefore, we believe that biochemically inspired design does have a role to play in the discovery of novel agrochemicals. We also expect the process to be facilitated in the future through the application of the techniques of molecular biology and a steady increase in our ability to visualise enzyme in three dimensions. The challenge to us all will be to assimilate this new information into our current design strategies.

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ZEAXANTHIN AND ENERGY DISSIPATION IN PLANTS - A POTENTIAL *HERBICIDE* TARGET SITE ?

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ABSTRACT

The action of carotenoids in protecting the chloroplast from photodamage by scavenging singlet oxygen and quenching triplet state chlorophyll is well established. Recently it has become evident that the xanthophyll zeaxanthin is involved in a novel photoprotective process in higher plants. The production of zeaxanthin can occur rapidly *via* the enzymic de-epoxidation of violaxanthin and antheraxanthin (the xanthophyll cycle) or, over a longer period of time, through *de novo* synthesis. The presence of zeaxanthin in the thylakoid membrane has been clearly linked with the high-energy state quenching of chlorophyll fluorescence (**qE**) which, significantly, accounts for up to 90% of the dissipation of excess excitation energy.

By preventing the accummulation of zeaxanthin in the thylakoid, **qE** and, hence, photoprotection is much less effective. (e.g. in the presence of dithiothreitol which inhibits violaxanthin de-epoxidase). Secondary to their well known modes of action, other herbicides moderate the synthesis of zeaxanthin, for example; diuron inhibits violaxanthin de-epoxidase whereas paraquat induces zeaxanthin formation.

The early stages of carotenoid biosynthesis are a well known target site for a number of bleaching herbicides. The major photoprotective role of zeaxanthin in energy dissipation in the chloroplast makes this an additional attractive target site, particularly in combination with inhibitors of photosynthesis.

INTRODUCTION

The chloroplasts of higher plant are well conserved in their carotenoid composition. They generally contain the light-harvesting xanthophylls neoxanthin, violaxanthin, antheraxanthin, lutein and zeaxanthin, together with the reaction-centre bound ß-carotene. There are two well known processes whereby the carotenoids protect the photosynthetic apparatus (Siefermann-Harms, 1985). Firstly, they have the ability to quench triplet chlorophyll molecules, thus preventing the formation of singlet oxygen and other highly reactive radical species. Secondly, they can scavenge for singlet oxygen and

these other reactive oxygen species directly. The action of carotenoids, principally ß-carotene, in these processes is essential for the tolerance of stress conditions and ultimately the survival of the plant (Young and Britton, 1989).

More recently a novel protective process, quite distinct to those described above, involving the carotenoid zeaxanthin has been observed in higher plants and algae. The production of zeaxanthin has been implicated in the quenching of chlorophyll fluorescence in these organisms. The xanthophylls, violaxanthin and zeaxanthin are associated with the photosynthetic pigment-protein complexes and can be converted enzymatically *via* an intermediate antheraxanthin in the xanthophyll (or violaxanthin) cycle (Figure 1.). The de-epoxidase which catalyses zeaxanthin formation has a low pH optimum (pH 5.0) and is thought to be located on the lumenal side of the thylakoid membrane. The epoxidase responsible for the reverse reaction has a neutral pH optimum suggesting its presence on the stromal side of the membrane. Thus, zeaxanthin formation is promoted in the presence of a trans-thylakoid pH gradient (ΔpH).

The potential for photoinhibitory damage is present in situations where the light energy absorbed by leaves exceeds the rate at which photosynthesis, and hence de-excitation, can take place. In higher plants, under conditions where incident light exceeds photosynthetic capacity, a number of mechanisms for the dissipation of excess chlorophyll excitation are observed. The harmless dissipation of this excess energy in the thylakoid membranes, primarily at Photosystem II (PSII), of higher plants is thus an important regulatory and protective process. It is important to recognise that such dissipation of excitation energy is essential during the growth of a plant under field conditions, particularly under stress conditions (e.g. chilling-enhanced photoinhibition). The most significant of these dissipation processes is that associated with the formation of the trans-thylakoid gradient (Δ pH), and is termed high-energy state quenching (**qE**). This can form and relax in a matter of minutes. **qE** has been reported to be important in the control of PSII yield and as a protective agent against the damaging effects of light.

The role of the carotenoid zeaxanthin in **qE** formation in higher plants has recently come under extensive study in our laboratories (Rees *et al*, 1989; Noctor *et al*, 1991; Ruban *et al*, 1991) and in that of Demmig-Adams (see Demmig-Adams, 1990 for review). In this paper we examine the potential of energy dissipation (**qE**) as a herbicide target site, through the inhibition of zeaxanthin formation in the chloroplast.

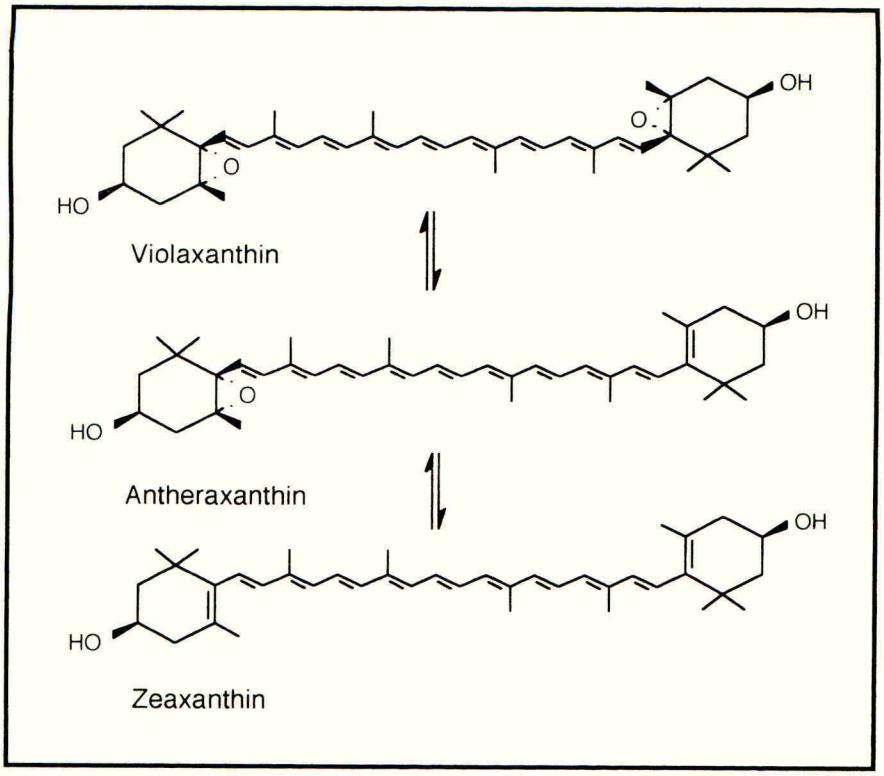

FIGURE 1. The xanthophyll-cycle carotenoids found in higher plants; violaxanthin, antheraxanthin and zeaxanthin. The de-epoxidation reactions leading to the formation of zeaxanthin are generally promoted in the light or under stress conditions.

MATERIALS AND METHODS

Spinach plants were grown for 4-6 weeks in greenhouse conditions under supplemented light with a 12h photoperiod. After 24h dark adaptation, leaves were removed and floated with their petioles in (a) water or, (b) 3.00 mM Dithiothreitol (DTT). Chloroplasts were isolated from these leaves using the procedure described in Noctor *et al* (1991).

Room temperature chlorophyll fluorescence characteristics were measured on isolated chloroplasts using a Walz fluorimeter (Rees *et al*, 1989; Noctor *et al*, 1991). Leaf and chloroplast samples for subsequent pigment analysis were frozen immediately in liquid nitrogen. Throughout this paper q9-aa (the

fractional quenching of 9-aminoacridine fluorescence) has been used to provide an estimate of ΔpH. The ΔpH can be defined as the difference in pH between the intrathylakoid space and the external medium.

Pigments were extracted in redistilled ethanol and transferred to diethyl ether. Samples were dried under a steady stream of nitrogen and stored at -20°C prior to analysis. The carotenoid composition of leaves and chloroplasts was determined by reversed-phase high-performance liquid chromatography. A Spherisorb ODS2 (25.0 x 0.46 cm) column was used on a solvent gradient of 0-100% ethyl acetate in acetonitrile / water (9:1) at a flow rate of 1.0 ml min^{-1}. This chromatographic system resulted in the near-baseline separation of lutein and zeaxanthin. Detection was made by a Hewlett Packard 1040A diode-array detector at 447 and 455 nm. Individual pigments were quantified by integration at their λ max.

RESULTS AND DISCUSSION

The effect of DTT on the quenching of chlorophyll fluorescence is clearly illustrated in Figure 2. In this experiment spinach leaves were pretreated with 3.0 mM DTT prior to the isolation of chloroplasts. This has the effect of reducing the degree of quenching of chlorophyll fluorescence in trace A, compared to the control trace B.

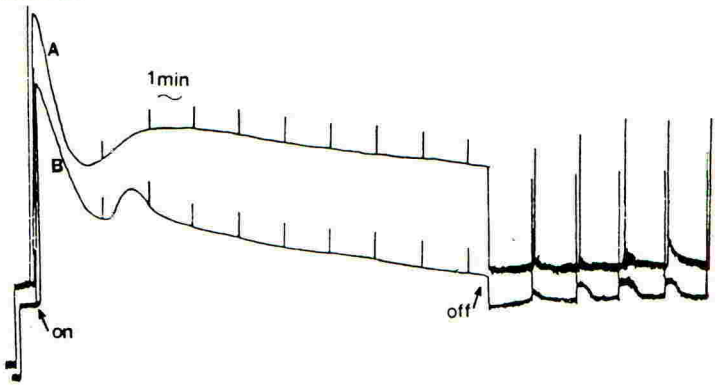

FIGURE 2. Traces of chlorophyll fluorescence for chloroplasts isolated from spinach leaves that have been preilluminated in the presence (A) or absence (B) of 3.00 mM DTT. The DTT-treated leaves and chloroplasts contained no zeaxanthin (A), whereas in the control tissues zeaxanthin accounted for 37-39% of the xanthophyll cycle carotenoids (violaxanthin + antheraxanthin + zeaxanthin).

The importance of the formation of zeaxanthin in the formation of qE, and hence the successful dissipation of excess excitation energy, is shown in Figures 3 and 4. A series of experiments were performed to show the relationship between the formation of qE and ΔpH (represented by q9-aa - the

quenching of 9-aminoacridine fluorescence across the thylakoid membrane) in chloroplasts containing either very low or high levels of zeaxanthin. Figure 3. shows this relationship for chloroplasts isolated from either pre-illuminated (zeaxanthin-containing) or dark-adapted (lacking zeaxanthin) spinach leaves.

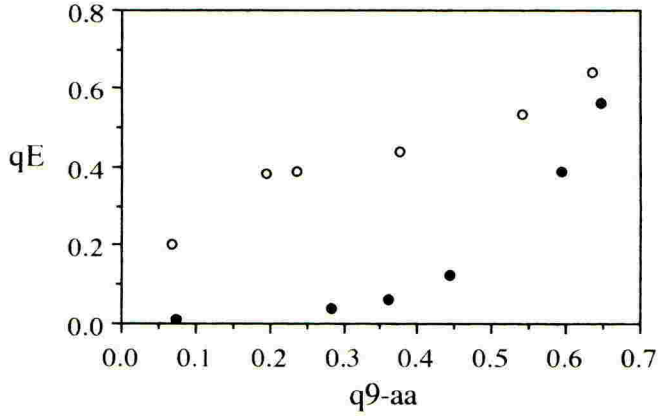

FIGURE 3. Effect of darkness (●) and preillumination (o) on the relationship between qE and ΔpH (q9-aa) in chloroplasts isolated from spinach leaves. This relationship was explored using changes in actinic light intensity. In the pre-darkened leaves zeaxanthin was present at between 0-6% of the total xanthophyll cycle carotenoids. Preillumination of leaves resulted in much higher levels of zeaxanthin (accounting for up to 39% of the xanthophyll cycle carotenoids) and a large shift towards the left in the curve.

Significantly, the relationship is shifted towards the left for the zeaxanthin-containing chloroplasts showing that in the presence of zeaxanthin the chloroplasts are able to achieve an effective level of energy dissipation at a much lower ΔpH. In the dark-adapted chloroplasts, the absence of zeaxanthin means that the ΔpH required to achieve the same level of energy dissipation may well be at a level that will be inhibitory to photosynthesis itself (Rees *et al*, 1989). The presence of zeaxanthin is not essential for and indeed does not appear to increase the maximal level of **qE** which is constant in both sets of chloroplasts. Rather, the presence of zeaxanthin appears to amplify **qE** formation. The molecular mechanism whereby zeaxanthin may act in promoting qE is currently under investigation and recent evidence has indicated that the transformation of violaxanthin into zeaxanthin induces or permits a configurational change in the light-harvesting complexes which affects **qE** (Ruban *et al*, 1991; Horton *et al*, 1991).

The link between zeaxanthin and a shift in the relationship between qE and q9-aa (ΔpH) is further strenghtened in Figure 4. In this experiment both sets of

chloroplasts were taken from leaves which had received the same level of pre-illumination. One set of leaves was treated prior to this with the sulfhydryl reagent dithiothreitol (DTT) which is known to inhibit violaxanthin de-epoxidase. The profile shown in Figure 4. for the chloroplasts isolated from leaves illuminated in the presence of DTT is similar to that obtained for dark-adapted material (Figure 3.). Again, there is a clear shift in the profile to the left in the untreated sample

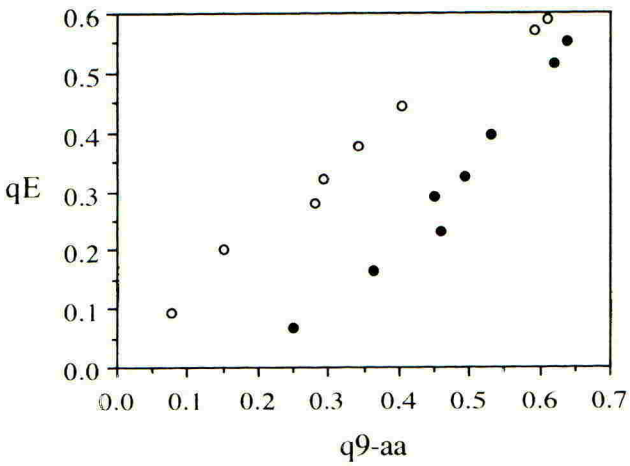

FIGURE 4. Effect of overnight treatment of detached spinach leaves with 3.00 mM DTT (●) on the ΔpH (q9-aa) profile for chloroplasts obtained from pre-illuminated spinach leaves. DTT-tretaed leaves and chloroplasts contained no zeaxanthin. Control leaves (o) were treated in a similar manner in the presence of distilled water and contained 39% zeaxanthin (percentage of xanthophyll cycle pigments).

Examination of the pigment composition of the leaves and chloroplasts showed that zeaxanthin formation was completely inhibited by pre-treatment with DTT. Importantly, recent work has shown that DTT has no deleterious effects on photosynthesis. These results have important implications for the plant, as the presence of zeaxanthin will allow energy dissipation through **qE** to proceed much more efficiently.

Similar *in vitro* relationships have recently been obtained for a number of weed species, including *Chenopodium album*, showing that the zeaxanthin-induced shift of the ΔpH profile is present in other higher plant photosynthetic tissues.

In addition to the short-term responses in pigment content described above, plants also show longer-term adaptation in their carotenoid contents to environmental conditions. This occurs through the *de novo* synthesis of violaxanthin and zeaxanthin from ß-carotene. Little is known, however, concerning the regulation of these biosynthetic processes. In a recent survey (G. Johnson, A.Young and P. Horton, unpublished data) we have determined that there is considerable inter-specific variation in the levels of carotenoids in field-grown plants. Specifically we have observed differences in (a) the dark levels of violaxanthin and antheraxanthin under control conditions and, (b) the ability of the plant species to produce zeaxanthin, and hence photoprotect, in response to conditions of light-mediated stress. This therefore suggests that the relative importance of zeaxanthin as a photoprotective agent may vary greatly between different plant species.

In a series of experiments investigating the effects of a number of herbicides on chloroplast pigments of intact plants it has been found that the formation of zeaxanthin may be affected by the presence of the herbicide (Young and Britton, unpublished data). The application of diuron or monuron to leaves completely inhibits violaxanthin de-epoxidase, preventing the formation of zeaxanthin in high light or in conditions of drought-mediated photoinhibition. This could serve to other compounds, notably paraquat, serve to promote the formation of zeaxanthin in treated leaves prior to the onset of radical-induced damage. Thus, treated leaves will form high levels of zeaxanthin in reduced light. It is not known how this occurs as paraquat will rapidly reduce the ascorbate pool which is essential for the de-epoxidase. The paraquat molecule itself may act as a donor for the de-epoxidation of violaxanthin. Certain of the diphenyl ether compounds (e.g. acifluorofen) have a similar, although less pronounced effect although it is not certain as to whether these compounds actively affect the de-epoxidase or epoxidase.

CONCLUSIONS

Inhibition or interference with energy dissipation processes within the chloroplast (especially **qE**), possibly through blocking the formation of zeaxanthin, may not in itself lead to the death of a plant, although the overall competitiveness of the plant may be severly reduced. A much more efficient approach to ensure rapid death of the plant may be to combine inhibition of zeaxanthin formation and hence reduce the efficiency of **qE**, with an inhibitor of photosynthetic electron transport.

Differences in the potential to synthesise zeaxanthin in different species may permit target site selectivity between crop and weed species. In addition to using this important protective process as a potential herbicide target site, the degree of natural variation in the carotenoid composition (especially in the xanthophyll cycle pigments) of plant populations consideration should also be given to the to the production of high-zeaxanthin-containing strains of crop plants by genetic engineering. In theory, these will show increased tolerance to

light-mediated environmental stresses through more efficient energy dissipation through **qE**.

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