Session 6A

Weed Control in Industrial and Energy Crops

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Papers	6A-1 to 6A-6
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REVIEW OF POLICIES RELEVANT TO NON FOOD CROPS

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ABSTRACT

Recent changes in common agricultural policy measures have opened up or encouraged opportunities for industrial and energy crop production. Changes in support levels for food crops and rules on rotational and non-rotational set-aside influence the relative economics of producing different crops as does the search and demand by industry for new raw materials.

INTRODUCTION

This paper outlines developments in UK and EC measures with implications for industrial and energy crops as at September 1993. The position may well have changed by the BCPC Conference in November 1993.

The last year has been an active one for policy affecting farming and the production of food and non-food crops: on the General Agreement on Tariffs and Trade (GATT), the Uruguay Round, the first to include extensive provisions on agriculture, needs to be completed by the end of this year. The Blair House Agreement concluded by the EC and US last November opens the way to a successful conclusion. The separate agreement concluded at Blair House on oilseeds for human consumption and industrial uses was adopted by EC Ministers in June. Individual farmers are having to come to terms with the new Arable Area Payments Scheme and the introduction of widespread set-aside.

INDUSTRIAL AND ENERGY CROPS: CURRENT POSITION

A wide range of industrial and energy crops can be grown on both rotational and non-rotational set-aside land. It is expected that the rules will be amended to allow sugar beet for industrial purposes to be grown on set-aside land but without any set-aside payment. EC production of oilseeds for industrial purposes is currently well below the level requiring corrective action under the Blair House oilseeds agreement.

Set-aside

As part of the 1993 price fixing it was agreed to increase the rate of payment for rotational set-aside land around 27% to about £320 per hectare. Also EC Ministers set the appropriate rate for non-rotational set-aside. Farmers have to set-aside 20% of land on which they are claiming area payments in all Member States except in the UK where 18% is to be permitted, subject to review after 2 years. Non-food crops, both annual or perennial, including short rotation coppice and miscanthus, can be grown on such land. More detailed rules on what non-food crops can be grown on set-aside land are described in detail in MAFF's two part explanatory guide on the Arable Area Payments 1993/94.

Other EC Measures

In 1992 a directive on the harmonisation of duties on vehicle fuels was agreed. One of its provisions means that liquid biofuels must be taxed at the same rate as the mineral fuels they replace. The only exception is for pilot plants using biofuels.

The EC Commission has also made a proposal, known as the Scrivener Directive, that biofuels be charged at a maximum rate equal 10% of the rate for the corresponding mineral fuel. Whilst this could make biofuels competitive with mineral fuels, the costs to the national exchequers would be very high and given reservations about the actual environmental benefits the proposal may not be a cost effective way of achieving them. So far the proposal has made little progress.

GOVERNMENT APPROACH: PRESENT AND FUTURE

The UK Government's approach towards the development of industrial and energy crop production includes the need for any measures to be cost effective, the economics to have been considered from production through to the market, GATT compatibility, avoiding distortion of the market for existing viable products and where environmental claims are made, looking at the total resources balance.

The UK Government has actively encouraged developments in industrial and energy crops through its various departmental research projects and by supporting joint Government/Industry funded research programmes, such as the LINK's Crops for Industrial Use Programme. This programme seeks to identify industrial uses for current and future crops, concentrating particularly on uses for fibres, starches, oils and proteins and to increase collaboration and discussion with industry and other interested parties.

NOVEL OIL, FIBRE AND PROTEIN CROPS IN UK - A FUTURE PERSPECTIVE

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ABSTRACT

Changes brought about by reform of EC Common Agricultural Policy and by free trade discussions in the world have released large areas of land for production of non-food crops in the European Community. At the same time novel developments in plant science in concert with traditional breeding have offered opportunities to produce new ideotypes for industrial crops and in some cases food crops too. Much field production technology is yet to be ascertained and exploitation may be constrained by international agreements and prices. Additionally, there is an inadequate understanding of world market demand for renewable industrial crops which hampers their development.

INTRODUCTION

Since the late 1980's a series of changes at world, EC and to a lesser degree at state level have combined to modify trade in a number of leading agricultural commodities and these, concomitant with scientific developments involving both traditional concepts and novel approaches to breeding and crop improvement, have combined to offer new opportunities for Agriculture to produce materials which have prime uses as industrial biostocks. There have also been concurrent developments in the potential for Agriculture to produce crops as prime sources of energy for heating or generating of electricity but these are not discussed in this paper.

CHANGES IN AGRICULTURE

The Uruguay round of discussions under General Agreement on Tariffs and Trade (GATT) and reform of Common Agricultural Policy (CAP) in the European Community (EC) have been in progress for a number of years and have led to changes in structure of the EC cereals, oilseeds and protein markets. These changes in turn have altered the potential to exploit a number of crops. Ramifications for individual crops and rotations are :

Set-aside

EC has a current set-aside potential of approximately 6.0 million hectares. Set-aside regulations began in 1992/93 financial year; they affect EC Member States and crops upon which aid is claimed. Initially only rotational set-aside operated. In UK this meant a 15% of intended cropping area of oilseeds, proteins or cereals being set aside as "fallow" or put to non-food crop uses. During August 1993 non-rotational set-aside at 18% of intended crop

area was introduced; rotational set-aside continues. (MAFF, 1993). Additionally linseed (Linum usitatissimum) will be included in these regulations from 1994.

Commodity Pricing in EC

Under CAP reform, aid has become area rather than tonnage based and commodities world-priced rather than market price supported. Original ADAS estimates made in 1992/93 (ADAS, 1992) suggested that oilseed rape price per tonne would decline from approximately £270 to approximately £115. Similarly cereal prices would fall to between £90 and £100 per tonne. Clearly these changes affect input cost : benefit and the introduction of set-aside offers alternative opportunities for weed control - and creates some new problems too. Currently currency fluctuations have modified price projections.

Crops for non-food use, grown on set-aside land receive no specific aid and are therefore sold at prevailing world, or in reality because of CAP regulations, prior agreed contract price. This situation has major repercussions upon which species and which of their products can be developed in EC Agriculture to compete in a world market since many EC production costs (eg; labour) tend to be higher than in less industrially developed parts of the world.

Oilseeds

In EC, oilseeds are defined as oilseed rape, soyabean and sunflower. Linseed is not classified as an oilseed *per se*.

The Blair House Agreement will limit EC oilseeds area to 5.138 million hectares and if that area is exceeded, constraints on aid or production will follow.

In EC, oilseeds trigger set-aside although the land released can be used to produce crops for non-food (ie industrial) use, including oilseeds. Since oilseeds for industrial use are annual or biennial species requiring rotation, for reasons of agronomy and pest and disease control, then clearly they could only be grown on rotational set-aside. Moreover the Blair House Agreement limits EC oilseeds meal production (for animal feeds) on set-aside, to 1 million tonnes of soya-bean equivalent. Hence, potentially at least, there are limits on the extent to which EC oilseeds can be expanded for non-food use, even if economically viable.

Cereals

In EC, cereals include cereals for grain or forage (usually silage) production. All trigger a set-aside requirement.

Pulses

In EC, pulses include dried peas, (Pisum sativum) field beans (Vicia faba) and sweet lupins (Lupinus spp.).

All of these proteins trigger set-aside requirements when grown as aid-receiving species in EC.

INDUSTRIAL OR FOOD CROPS

The changes in structure of aid systems and current market structures offer the potential to produce both industrial and food crops, but because of Blair House Agreement (presuming its total implementation in EC) industrial crops perhaps offer the greatest opportunities. This is especially so when the need for renewable resource is taken into consideration.

LAND AREA POTENTIAL FOR INDUSTRIAL CROPS

On the presumption that all industrial crops will be grown on set-aside (this is a broad presumption since, for example, high erucic acid rapeseed can also be grown as a 'normal' oilseed) potential areas for exploitation in UK are :-

From Cereals c. 0.5 million ha *
From Oilseeds c. 0.15 million ha **
From Pulses c. 0.02 million ha **

* based upon ASI data published 23 July 1993 (Anon, 1993a)
** based upon EC working docs (Anon, 1993b)

MARKETS FOR INDUSTRIAL CROPS

Whilst the opportunity to use land freed from food production for industrial crops is welcomed, the market realities are very complex and trading in a world market at world price will lead to abrupt changes in supply and demand; Agriculture will need to adapt to this phenomenon.

For example, under current EC legislation there is support for fibre flax production under EC Regulation 1164/89. As arrangements stand, produce surplus to market demand is bought from the market and stored. Recently there has been considerable oversupply and, if surpluses were not supported there would be a collapse in price followed swiftly by a collapse in production and, in turn this would create a potential shortage.

Anecdote suggests an oversupply of lauric acid-providing oils on world markets in the near future. If that surplus materialises then the opportunities for EC or UK growers to produce lauric acid from cool temperate crops will be severely limited. Moreover there will be a marked but understandable reluctance for those funding research into or development of crop species to invest in such circumstances.

The trend towards world market supply and pricing obviously offers opportunities to all, but at the same time increases risk in many sectors of agriculture. These risks could be reduced by a substantial and penetrating analysis of likely market requirements and likely crop developments on a world scale, projecting as far as possible into the future and certainly in excess of 10 years.

Oil Crops

Gray (1993) reported world oil and fat use in 1990 - 91 to be 81.5 million tonnes of which 61 million tonnes was plant-derived. Approximately 12% of the total market was in the chemical industries. A further analysis of that 12% (10.5 million tonnes) is shown in figure 1.

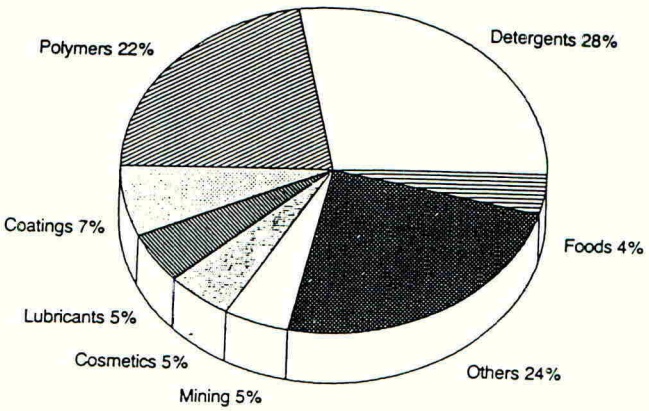

Fig 1 : Oil Crops : Markets in Chemical Industries

After Gray (1993)

Starches/Sugars

Data on a world basis for starches and sugar use in industry from agriculturally produced crops has not been ascertained. However Kleinhanss (1987) suggested a total of approximately 1.75 million tonnes per annum being used in EC10 (ie EC excluding Spain and Portugal).

Annual Fibre Crops

Insofar as N W Europe is concerned, and especially UK, potential annual fibre crops are flax and hemp. The former has already been identified as an area of overproduction; the latter is a new crop in many areas (eg The Netherlands and UK) and precise end market demand requires critical elucidation. Markets include fibre for paper production (various paper types and fineness), fibre for weaving into textiles, fibre for use in construction industry materials, and pith for use as a bedding.

OPPORTUNITIES FOR NOVEL SOURCES OF INDUSTRIAL MATERIALS FROM PLANTS IN EUROPE

Since a very large number of species have been identified as possible sources of material (eg Kleiman & Spencer, 1982), discussion is limited to examples rather than descriptions of each.

Sourcing Novel Products

The rapid advancement in molecular biology and techniques associated with production of transgenic species have begun to offer new routes for plant breeders to develop new genotypes. Initially it was considered that transgenic and molecular biological technologies would replace traditional breeding methodologies but experience has shown that the latter are essential to development of the former to full farm crop level.

Nevertheless, these technological breakthroughs now offer added opportunities to develop new plant types and 3 opportunities are now available to breeders:-

i) modifying existing species by addition of alien genes to confer a desirable product characteristic (eg short chain fatty acids from oilseed rape).

ii) modifying an existing species with desirable characteristics so that the species can be grown in an area to which it was not initially adapted (eg introduction of genes which induce cold tolerance)

iii) traditional pedigree breeding, but including chromosome doubling and similar 'traditional' methodologies.

Many researchers favour the approach outlined in i) for development of novel oils since oilseed rape (Brassica napus) can be readily manipulated. Whilst this capability is well proven there is however the need to adapt the capability of science to the biology of the plant and agricultural field and factory practice. Since oilseed rape is a partial out-pollinater; sheds large numbers of viable seeds at harvest and these have high persistence potentially appearing as volunteers over many years; is a small black round seed regardless of fatty acid composition and is therefore difficult to identify separately from other rape seed of different quality, there will be a potential need to zone or restrict production and develop techniques that prevent volunteers or which kill volunteers of one rapeseed type within another and to isolate crops. Without these capabilities it is probable that rapeseed cannot be envisaged as the universal recipient of genes for production of novel products.

Oils

Castor (Ricinus communis) - Ideally a cool temperate strain of castor would offer a large opportunity to EC producers. Bonjean (1991) identified imports of 50 000 tonnes per annum of castor oil for EC industry. Developments have not yet occurred to allow progress of this crop into Northern Europe but clearly this species is a potential target for development.

Oilseed rape - Oilseed rape has been identified as a 'universal recipient' of alien genes, some of which could be introduced to modify fatty acid composition of oil or modify enzyme systems to produce basic products like polyhydroxy butyrate. However the problems of isolation of crop and produce outlined earlier will probably limit the viability of this universal solution to novel products. Estimates suggest 40 000 ha of industrial rapeseed in UK for 1993 harvest (MAFF, 1993) of which 16,500 ha were high erucic rapeseed.

Crambe (Crambe abyssinica) - This species has potential to produce approximately 38% oil with 50 - 60% erucic acid in that oil. It has been developed as a crop in parts of USA (eg Idaho) where it matures satisfactorily in warm temperate conditions. Further development is continuing in Southern Europe and a breeding programme is progressing in The Netherlands. Whilst small pilot plots of crambe have been grown in UK it is currently not a reliable enough crop for the climate. Estimates made in 1990 suggested an EC requirement of approximately 20,000 tonnes per annum of high erucic acid oil but current indications, suggest that 1993 contracts for production substantially exceed this amount. Novel uses in lubrication could explain this increase.

Honesty, (Lunaria annua). This species has the potential to produce long chain fatty acids, particularly erucic acid (see "crambe" above) and vernolic acid. It is currently under agronomic development in Germany, The Netherlands and UK.

Cuphea species. Considerable interest has centered on the genus Cuphea as a source of short/medium chain length fatty acids, some of which would have uses in detergent industries. Personal experience suggests that the genus is not yet well adapted to cool temperate climates, nor yet to agricultural production on a field scale. Roath (1990) suggests the most likely species for development as a source of lauric acid is C. viscosissima. Statistics (FEDIOL, 1992) show that 71,000 tonnes of lauric acid oils were processed during 1991 in EC; they originated from copra (ie coconut) and palm kernel.

Meadowfoam (Limnanthes alba) - The crop has established in Oregon, USA, under a growers' association, whereas it is still under development in Europe. L. alba is an entomophilous species and suffers from poor pollination if conditions are cool or damp at flowering (June/July) in UK. This has hampered European development and development of self pollination mechanisms is an urgent need. American experience shows L. alba to produce a large proportion of its oils as C20 or C22 chains; these have considerable potential in production of synthetic rubbers, lubricating oils and perhaps polymers. However, oil price will need to be competitive with that of oils from high erucic rapeseed, crambe and honesty.

Coriander (Coriandrum sativum). This species is already adapted to Northern European climates and is grown as a herb and as a flavouring agent for gin. However, its oil also provides petroselinic acid, an 18 carbon molecule which can be cleaved for industrial chemical uses. Further development of the crop could be justified, but research to overcome germination/dormancy problems in seed may be a prerequisite.

Fibres

Several arable crop species produce fibres although for Northern Europe, hemp (Cannabis sativa) seems the only tenable primary fibre producing species in the short term. Kenaf (Hibiscus cannabinus) may offer potential for warm temperate climates.

Hemp - This plant can only be grown in Britain under licence from the Home Office. Varieties licensed are all proven to have low THC (tetra hydro cannabinol) content; it is THC which causes narcotising effects where hemp is used as a drug. Early evidence suggests hemp to be a useful fast-growing annual plant with the potential to produce long, strong fibre with a number of uses, as outlined earlier, and with potential for use of its by-products for bedding. Markets are still unproven. Hemp is already grown as an industrial crop in the Le Mans and Troyes areas of France and is under development in The Netherlands.

Starches/Sugars

Starches and sugar are produced naturally by a wide range of established crop plant species. Though not novel, these include sugar and fodder beet (Beta vulgaris), chicory (Cichorium intybus) and potato (Solanum tuberosum). In the cases of sugar from sugar beet and starch from potatoes, production is predominantly for food use and as such is governed by EC regulations, (eg; 1785/81 Common organisation of the markets in the sugar sector).

The European Commission have agreed in principle that sugar beet for non-food use can be grown on set-aside land but precise details have yet to be announced. Additionally, the EC Sugar Regime is due for reform although because of its complexity reform has been deferred until mid-1994 at earliest. Clearly, however production of sugar for food consumption appears out of step with demand and grower returns, at least in EC, are much higher than those from other arable crops.

Undoubtedly, if EC maintains its area of sugar beet there will be a substantial surplus of sugar, for industrial use. This use includes production of a range of alcohols, plastics, enzymes, general organic and pharmaceutical materials.

POTENTIAL TO PRODUCE FOOD CROPS ON AIDED LAND

For some years constraints have been laid upon food production within EC, as a budgetary measure or more recently as a market-directing measure and, GATT, as Blair House Agreement, has been a further development.

Nonetheless, there are opportunities to produce foods in EC deficit presuming the overriding regulatory constraints are not exceeded. Unfortunately many opportunities occur in the oilseeds and protein sectors and in the former at least potential for development may be constrained according to implementation of EC rules in the Iberian peninsular.

Food Oils

Recent evidence shows three potential opportunities.

Oilseed rape (B. napus & B. rapa) - currently it is suggested that rapeseed of low glucosinolate and low erucic acid character (commonly called '00') has a better fatty acid profile for human consumption, especially in monounsaturates, than sunflower. Hence a market expansion of rapeseed to replace sunflower is an option, especially in UK where sunflower is not a common crop.

Sunflower (Helianthus annuus). Notwithstanding comments above, scientific developments are beginning to make sunflowers an option for UK. Development has been assisted by the change in aid from a tonnage to an area basis. Official estimates show UK sunflower area to be less than 1000 ha but anecdote suggests a potential of 60 000 ha in southern England.

Edible linseed (commonly called Linola) is linseed with a modified fatty acid composition such that it is virtually identical to that of sunflower therefore offers a cool temperate crop with a warm temperate fatty acid profile. However the EC aid regime for industrial linseed will embrace edible linseed and any substantial development of it would trigger cuts in aid; as a food crop it could not be grown on set-aside land.

Proteins

There is a major deficit of proteins for animal feed in EC. In UK, markets for dried peas and field beans are currently bearish, perhaps stimulated by the recent weather disasters in USA.

However, the largest potential development area is that of lupins (Lupinus albus). Developments in France have produced early ripening, semi-determinate types and researchers in UK (Milford et al (1993)) have shown these types to have potential. Estimates, (Hazeldine, 1989) suggest a potential UK market of 1.25 million tonnes per annum for sweet lupin although any such expansion may be as a partial substitute for current proteins. Additionally area constraints and price cuts related to them may limit EC development of lupin. Pate et al (1985) showed lupin to have an almost ideal amino-acid composition.

Fibres from Food/Aided Crops

Whilst cereal straw is a well known source of fibre most development in recent years has taken place in use of linseed straw as a by-product from the oil crop. During 1993 the bulk of EC linseed was grown in UK, occupying 157,000 ha (MAFF, 1993). Gilbertson (1993) has shown linseed straw to have uses as long fibre per se and in composition boards used in the construction industry. He estimates that up to 200,000 tonnes of linseed straw per annum are currently wasted.

CONCLUSION

Scientific developments and changing markets have combined to change Agriculture, especially in EC and that change has opened up a number of new opportunities for arable crops in both the food and industrial crop sectors. Much is still unproven; development would be greatly assisted by definitive data on markets. Undoubtedly the changes that have occurred will have a major effect on what can be afforded by producers in terms of inputs. Moreover, some newly developed crops may not yet have a defined agronomy or readily available inputs, especially in the Agrochemicals sector.

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SHORT ROTATION COPPICE: A POTENTIAL NEW ENERGY CROP

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ABSTRACT

Short rotation coppice of willow and poplar has the potential to become a major non food crop in the UK as a result of studies funded through the Government's Renewable Energy Programme. Production trials with the crop have shown the ways in which the crop can be established, appropriate husbandry methods and the means by which the crop can be harvested.

As short rotation coppice is established from unrooted cuttings, rigorous weed control prior to planting and during the first season of growth is essential to prevent potentially lethal water competition. The DTI Wood as a Fuel Programme has investigated the means by which weed control is effected both pre and post planting. However, once canopy closure occurs the crop shades out competing weeds and further herbicide application is not required. After harvest, the vigorous re-growth from the established stools means that weed competition is unlikely to be a problem, and weed control may not be necessary. The only potential exception is ingression of specific weeds such as field bind weed which may require directed knapsack spraying.

In the future, even lower herbicide inputs to the crop may be possible by managing vegetation both pre and post planting.

INTRODUCTION

The UK Government's Renewable Energy Programme seeks "to stimulate the development and application of renewable energy technologies, wherever they have prospects of economic viability and environmental acceptability".

As a part of this programme, the production of renewable energy from crops has been studied since the early 1980's. This work has investigated both the utilisation of food crops as fuels and biomass grown specifically for energy, such as coppiced wood and C4 grasses. These crops can produce fuels to service many energy sectors. Esterified vegetable oils, and ethanol derived from cereals or sugar beet can be used as liquid fuels for transport. The relatively dry fuels such as wood can be burnt for heat and electricity generation, and the wetter biomass crops such as high yielding fodder crops can be anaerobically digested to methane which can be used for the same purposes.

All of these options are to a greater or lesser extent viewed as potentially "environmentally acceptable". Biofuels are all renewable and, in simple terms carbon dioxide neutral, that is the carbon sequestered during growth is the same as that liberated on

on utilisation. When used to replace a fossil fuel, a reduction in carbon dioxide emissions results thus helping to reduce global warming. These fuels are also typically low in sulphur extending this benefit to a reduction in acid rain emissions also. The magnitude of these benefits varies depending on how much fossil fuel input is required to produce and transport the biofuel compared with the energy contained in the fuel. This "energy ratio" also influences the economic viability of producing energy from crops.

Typically, the production of food crops requires high energy inputs. The addition of extra processing steps leading to fuel production further affects the energy ratio. In this way the production of the diesel fuel substitute rape methyl ester (RME or 'biodiesel') yields less than two units of energy for every unit expended in its production (Culshaw & Butler, 1992). On the other hand, studies on perennial crops grown for energy purposes such as coppiced willow or poplar indicate that only low inputs are required and as a result yield over twenty units of energy for each unit used in production, even with the low production efficiencies currently available (Foster 1993). Whilst it may be possible to reduce the inputs into food crops grown for energy and increase yields, it is unlikely that the order of magnitude difference between the energy ratios associated with the two cropping options can be greatly reduced.

This is a major reason why the perennial crops such as short rotation coppice and the C4 grasses have a greater chance of becoming "economically viable" and thus more worthy of support under the Renewable Energy Programme, and the production of fuels like RME are receiving less attention in the UK.

This paper will briefly describe the production of short rotation coppice, the production of which is now entering the commercial demonstration phase in the UK through the DTI's Farm Wood Fuel & Energy Project. In Sweden in excess of 6,000 ha of willow coppice is being grown. Current weed control practice will also be outlined.

SHORT ROTATION COPPICE

The use of coppicing techniques to produce fuel wood from deciduous trees is an ancient practice. Through the DTI's Wood as a Fuel programme these techniques have been adapted for modern energy production. Willow and poplar have been found to give high yields when grown at close spacing (10,000 tree/ha) on a rotation of 2 to 5 years as an alternative farm crop.

Short rotation coppice is established by planting unrooted cuttings 20-25 cm long into weed free ground in the spring. The requirements for ground preparation are similar to those for seed bed preparation, with rigorous weed control being essential. Planting can be by hand, or can utilise mechanisation such as modified cabbage planters or dedicated planting machines which have been developed in Sweden.

At the end of the first growing season the shoots are cut back to the ground to initiate coppicing, which is the stimulation of vigorous shoot production from the root or 'stool'. Willow produces a large number (up to 20) of stems of small diameter, while poplar

produces as few as 3 stems of thicker diameter. Being a clonal crop, the cut back shoots can either be utilised as a source of cutting material for subsequent plantings or to replace any failed cuttings. With falling cutting prices, mechanical cut back using a forage harvester may provide an early, if small, crop of fuel.

The coppice crop then requires no further input until harvest, which is carried out at any time from leaf fall to leaf set (October to March). The length of the crop rotation can be from 2 to 4 years depending on the mechanisation used for harvest. Annual growth increases linearly over this period (Potter 1990). In practice it is likely that the crop will be planted in a suitable rotation to ensure annual cropping and thus income. It is likely that coppice stools will retain high levels of productivity for at least ten harvests. Typically no herbicide or pesticide inputs are required between harvests.

The lowest cost harvesting option is to utilise an unmodified forage harvester fitted with a suitable header. These machines can accept stems with butt diameters of up to 50 mm, which make them suitable for willow at 3, and poplar at 2 years old, and produce high quality fuel wood chips in one pass. Other options for harvesting are currently considerably more expensive and involve harvesting the stems using specialist machines and removing them from the field for subsequent chipping. The advantage of this approach is that the stems are easily stored at the field edge and the wood dries over the summer with little dry matter loss. On the other hand, wood chips require careful storage to achieve sufficient drying to both prevent microbial breakdown of the wood and increase its net calorific value.

From trials carried out to date, the average yield of coppice is 11 dry tonnes/ha/y, with the range being 8-19 dry tonnes/ha/y (Potter 1990, Tabbush 1993). These results were obtained by research foresters using unimproved clones, on relatively poor land. More recent results from the Farm Wood Fuel and Energy project indicate that farmers growing the latest clones on better quality land may achieve yields close to 15 dry tonnes/ha/y (Buckland personal communication). In the future, clonal selection and breeding could increase these yields to around 30 dry tonnes/ha./y (Rook, 1991), though for planning purposes 21 dry tonnes/ha./y is the preferred figure. Interestingly, field trials have shown these yields to be insensitive to the application of fertilisers (Mitchell *et al* 1993). This is not surprising given that harvest is after leaf fall, and the chemical composition of wood being predominantly carbon, hydrogen and oxygen. However, some soils may require fertilisation during the course of the 20-30 year life of the crop to maintain yields.

Coppice derived fuel wood is utilised in the form of chips which allows the fuel to be fed automatically into a suitable combustion plant. This equipment is available over a range of sizes suitable for large scale domestic use up to that suitable for industrial space or process heating. In the near future, large scale commercial electricity generation projects may be constructed utilising conventional steam cycle or gasification technology fed by coppice derived fuel wood. In a recent White Paper (Energy Paper Number 60) shows how this option may become a major route for electricity generation providing that both the crop production and high efficiency utilisation technologies can make the transition from the research phase to commercial deployment.

Figure 1a. Annualised standing value of a SRC crop yielding 11dt/ha/y with increasing energy prices.

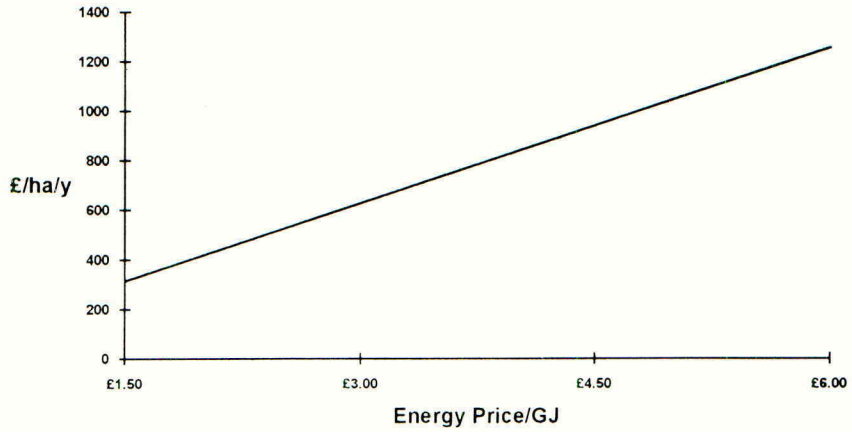
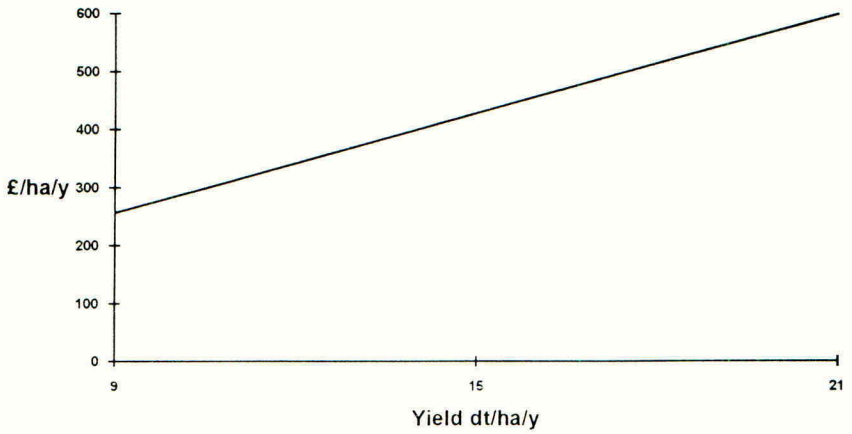


Figure 1b. The effect of incresing yields on the standing value of a SRC crop sold at an energy price of £1.50/GJ



The economics of coppice production are characterised by the high establishment costs which are incurred some three to four years in advance of the first harvest (Maryan 1991). This is a major disincentive to growers and will require grant aid to cover the bulk of the establishment costs, at least during the preliminary stages of market development. However, after first harvest, the coppice crop appears to require no further grant aid throughout its 20 to 30 year life.

Figure 1a indicates the value /ha./y of standing crop yielding 11 dry tonnes/ha./y at a range of energy values. A figure of £1.50/GJ is paid for coal by the large power generators and is one of the lowest energy prices in the UK, whilst £7.00/GJ for propane

gas, is currently one of the most expensive sources of energy. Figure 1b shows the effect of increasing yields on standing crop value/ha./y based on an energy price of £1.50 /GJ. From these values harvesting cost equivalent to £50/ha./y (based on a 3 year harvesting cycle) and storage and transport costs of £69/ha./y must be deducted.

WEED CONTROL IN COPPICE CROPS

As short rotation coppice is planted as unrooted cuttings, the crop must be kept rigorously weed free during the establishment phase to prevent water competition. Failure to do this will lead to poor cutting survival and significantly reduced crop yields. Weed control on ex-arable land is easier than ex-grassland - this is due to the increased density of perennial weeds in the grassland. However, once the crop is established and canopy closure occurs further weed growth is suppressed - although spot control of creeping thistle (*Carduus arvense*), perennial nettle (*Urtica dioica*) and bind weeds (*Convolvulus* spp) may be necessary.

Weed control can be achieved by both mechanical and chemical means. To date the chemical methods have been substantially more effective. Control programmes which have been used fall into four distinct phases. These are: pre planting foliar acting herbicides, post planting soil acting residual herbicides, follow up foliar acting herbicide in first season and post cut back at the end of year one. Table 1 lists the herbicides for use at these times.

1) Pre planting treatment

The most effective control of annual and perennial weeds in this crop is glyphosate which can be used prior to harvesting cereal crops. If establishment is to occur on grassland, a summer treatment with 14-D Dicamba and triclopyr can be effective. Control of annual weeds immediately prior to planting is important and can be achieved with low close glyphosate, glufosinate or (if rain is imminent) paraquat.

2) Post planting residual treatment

This is more difficult as there are few herbicides with off label approval to which willow and poplar are resistant. Simazine has been the most cost effective product but does not have the necessary approval for use in coppice crops. On most sites mixtures of herbicides provide the best control. Weed control will be greatly improved if the weed seed population of the site is assayed initially. Trials to establish the tolerance of poplar and willow to post-emergence to herbicides have been conducted by Dr D Clay of Avon Vegetation Management on behalf of the DTI through ETSU (Clay 1993).

3) Follow-up treatments

Herbicides which are suitable in this situation are listed in Table 1 and include: clopyralid for thistle (*Carduus* spp) control and fluazifop - butyl for couch-grass (*Elymus repens*), both of which are selective and can be applied across the crop (Clay et al 1990, Clay and Dixon 1993). If weeds do emerge in the crop consideration should be given as

to how important weed control is as herbicides can damage the emerged crop. If mechanical or hand weed control is not an option then the use of low level chemical control will be preferable (even if crop damage occurs) to allowing the weeds to smother the crop. However, indiscriminate spraying of non selective herbicides can be disastrous.

4) Post cut back treatments

The most effective treatment post cut back and pre canopy closure has been the application of amitrole and simazine (Clay *et al* 1990). Willow appears resistant to this mix, however, poplar can be susceptible post bud burst. Later treatments are damaging and thus careful inter row treatments should be used. Further information on crop safety treatments is currently being obtained through DTI funded research via ETSU.

Once established, the extensive root network produced by the crop makes it less vulnerable to lethal competition by weeds. However, at this stage the combined effects of water competition and shading severely reduce the growth rate of the trees and will thus limit both the achievement of canopy closure and reduce the first yield. After harvest, the coppice stools are sufficiently developed to enable vigorous growth and early canopy closure despite the presence of weeds. It is unlikely that further weed control is required during the life of the project unless specific weeds become a problem by interfering with harvest, for example field bind weed (*Convolvulus arvensis*) and couch grass (*Elymus repens*). These will most likely be controlled by directed knapsack spraying (see Table 1).

THE SCOPE FOR REDUCING HERBICIDE INPUTS

The scope for reducing herbicide inputs is limited to a combination of reducing dosage directly using new low dose products, or the development of herbicide resistant species. Since adequate information on reduced dosage of herbicides is unavailable this is not at present a credible option. The development, release and approval of new products takes time, as do the benefits that might accrue from the use of genetic engineering techniques. Hence it must be concluded that the options for reduced dosage of herbicide are currently limited.

Non chemical weed control

1) Mechanical weed control has been employed in short rotation coppice but with restricted success. The development of relevant machinery and appropriate testing is required.

2) Planting through mulches offers much promise but is also subject to problems. The retention of moisture in the soil by mulches leads to enhanced growth - but "ponding", "toxic suppression" and mechanical damage can lead to a reduction in growth. Furthermore, the use of polyethylene film or wood chip mulches increases establishment costs significantly and can be unsightly.

Table 1 List of herbicides approved for use in short rotation coppice. (Clay,1993).

Active Ingredient	Product Name	Type of Activity	Use Situations
Amitrole	Weedazol TL[a]	Foliar-acting	Overall pre-planting. Directed between rows. Overall after cutting back willow
Asulam	Asulox	Foliar-acting	Directed spray onto weeds
Clopyralid	Dow Shield[b]	Foliar-acting	Overall or directed spray on emerged weeds
Cyanazine	Fortol[b]	Foliar and soil-acting	Overall post-planting spray
Dichlobenil	Casoron G	Foliar and soil-acting	Overall granular application on established plantations in winter
Fluazifop P-butyl	Fusilade 5[b]	Foliar-acting	Overall application on emerged grass weeds
Glufosinate	Challenge	Foliar-acting	Overall pre-planting. Directed spray on emerged weeds
Glyphosate	Roundup	Foliar-acting	Overall pre-planting. Carefully directed spray on emerged weeds
Isoxaben	Flexidor[b]	Soil-acting	Overall post-planting spray
Metazachlor	Butisan S[b]	Soil-acting	Overall post-planting spray
Paraquat	Gramoxone 100	Foliar-acting	Overall pre-planting. Directed spray on emerged weeds
Pendimethalin	Stomp 400[b]	Soil-acting	Overall post-planting spray
Propyzamide	Kerb	Soil-acting	Winter treatment as overall spray or granules 9 months post-planting or later
Simazine	(various)[a]	Soil-acting	Overall post-planting spray. Overall after first year cutback
Triclopyr	Timbrel	Foliar-acting	Carefully directed spray to emerged weeds

[a] Off-label approval applied for [b] Off-label approval

3) Planting through Non- Competitive Ground Cover appears to be a promising option and DTI funded research through ETSU is currently underway (by Avon Vegetation Research). However, preliminary results suggest that establishment is poor and the cost of preparing the ground cover is expensive. Only time will tell if more work can prove the feasibility of this approach.

CONCLUSION

Clearly coppice has the prospect of becoming a valuable renewable energy crop once appropriate markets for wood fuel have been established. However, in order to achieve robust, low cost crop production, an essential prerequisite, more research will be needed.

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WEED CONTROL IN MISCANTHUS AND OTHER ANNUALLY HARVESTED BIOMASS CROPS FOR ENERGY OR INDUSTRIAL USE

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ABSTRACT

There is potential for using surplus land to produce so-called biomass for use as fuel or industrial raw material. The cultivation of trees for this purpose in the UK is being evaluated, and a programme of work on crops is now under way. Miscanthus, a perennial grass, is a promising candidate and this is used as an example to consider weed control problems, the potential of the crop to become a weed and the costs and benefits of weed control. Weeds may cause problems in the establishment phase, but an established crop should smother them because of its vigorous growth. The current lines of Miscanthus are infertile, so there is little chance of the plant becoming a weed, but this may not be the case with seed producing candidate crops. The destruction of established plantations needs to be investigated. Weed control is unlikely to reduce the energy balance of the crop significantly but, even with incentives, the gross margin is likely to be small and the cost of inputs will have to be constrained to keep the crop profitable.

INTRODUCTION

The considerable interest in using land that has been set aside from food production for growing crops to produce so-called biomass as a source of fuel for electricity generation or as a raw material for industrial processes has been explored in Speller (1993) and Speller & Harvey (1992). In summary, there is potential for the use of crops for energy capture, but existing crops in their present form may not produce high enough dry matter yield or content, especially if grown with modest or low inputs. Work on novel systems of tree cultivation for high biomass yield has been under way for some time and the current situation is summarised by Foster (1992). Novel crop species are now being screened and one plant, Miscanthus, appears to offer particular promise. This paper examines the problems of weed control in biomass crops in general, and Miscanthus in particular.

THE NEED FOR WEED CONTROL

The essential requirement of a crop for fuel is high yield at a high dry matter content. Perennial growth may also be an important characteristic (Anon., 1991). Miscanthus is a perennial grass which is native to Asia and is currently cultivated in Europe as a garden ornamental. It appears to have many of the characteristics of a good fuel crop. Its potential is now being evaluated throughout Europe and limited experience in the UK has given an indication of the likely weed control problems of a crop of this type. The extent to which

weeds could reduce Miscanthus yield by competing for light and nutrients has yet to be evaluated, but their effect may depend on the stage of growth of the crop.

At present, the recognised, high yield potential, lines of Miscanthus can only be reproduced vegetatively, by rhizome cuttings or micro-propagation. Lines that produce fertile seed may be easier, and certainly cheaper, to establish, but Miscanthus seed is strongly heterozygous (Andrews & Gilbert, 1992) and flowering may constrain total biomass yield. It, therefore, appears that vegetative propagation will be normal practice in the medium term. This carries the risk of the spread of weed contamination in the propagation medium, and, by way of example, there was a significant presence of annual meadow grass (*Poa annua*) in micro-propagated stocks of *Miscanthus sinensis* imported from Germany this year for experimental work at four UK research institutes (M.J. Bullard, personal communication).

The removal of competitive perennial weeds, especially grasses such as couch (*Elymus repens*), is a pre-requisite to the establishment of Miscanthus. It is ADAS experience that there is a considerable post-transplanting shock, especially in the case of micro-propagated plants. At Arthur Rickwood this year, this resulted in the near-total loss of green leaf area in two micro-propagated lines of *M. sinensis*. This poses two problems. First, in spite of the use of stale seedbed techniques, weeds have the opportunity to grow rapidly in the face of limited competition from a crop under stress. This is especially the case on the high organic soils at Arthur Rickwood, where previous work has shown that 1400 weeds/m² can be expected to emerge each spring (May, 1984). Secondly, the opportunities for herbicide use are limited because it is doubtful that the crop could take the extra stress of any damage from herbicide application. Thus, hoeing or mechanical weeding appear to be the only effective current options for weed control through this difficult phase. Once the crop has recovered, and this year that took until mid-July, it is likely to be strong enough to take a herbicide application. However, any weeds that missed the mechanical weeding will be well developed and the use of residual herbicides will not be favoured by the dry conditions that often prevail in mid-summer. Rhizome cuttings or transplanted whole plants, whilst showing transplanting shock in comparison to their undisturbed counterparts, do appear strong enough for herbicide application immediately post-emergence. *Miscanthus sacchariflorus* material established at Arthur Rickwood last year was treated twice with a bromoxynil/fluroxypyr/ioxynil mixture, tank mixed with MCPA on the first occasion and clopyralid on the second, followed by an application of 3.4 l/ha atrazine, without any sign of damage. However, micro-propagation is likely to be the preferred method of large-scale propagation because of its ability to generate new stocks rapidly and the fact that plants can be held more easily whilst unfavourable planting conditions prevail. Clearly, if Miscanthus is to become cultivated widely, there is a great need for work on weed control in this phase of the crop.

Established Miscanthus plants emerge in late March and early April and grow rapidly, especially in late May and June. The period between harvest in February or March and emergence gives a good opportunity for the use of a total herbicide such as paraquat or glyphosate. This could be mixed with a residual product to give lasting control. Once the crop grows away it competes very strongly, but the effectiveness of this competition depends on its age. From establishment, Miscanthus spreads by rhizome development and takes about three to four years to reach full crop cover. Assuming a clean start in the spring following the use of a post-harvest herbicide, a fully established crop will smother weeds because of its vast rate of growth. For example, the second year crop of *M. sacchariflorus* at Arthur Rickwood

had attained a height of 1.24 m by 4 June 1993. This, of course, does have the drawback that the window for weed control is very limited. Those managing the Arthur Rickwood crop estimate that it was too tall for a conventional sprayer by 19 May 1993 (J.B. Kilpatrick, personal communication). Plant spacing will affect the competitiveness of Miscanthus in the establishment phase. A plant spacing of 0.5m will give very rapid complete ground cover, but the cost of 20,000 plants/ha may be too expensive. On the other hand, spacings of 1.0m or greater will take longer to produce complete ground cover. In Ministry of Agriculture, Fisheries and Food (MAFF) funded work on the yield potential of *M. sacchariflorus* at Arthur Rickwood, Buckfast Abbey, Devon and Rosemaund, Herefordshire, plants spaced at 0.5m were able to compete effectively with weeds in this, the second, year, but plants at a spacing of 1.0m had not achieved full ground cover by late July and weed competition was present. In view of crop height, hand weeding was the only viable option. Clearly, weed control problems are likely to be an important issue when selecting the optimum spacing of Miscanthus or other perennial crops. In the case of annual crops, growth in the early stages when light levels are high will make a substantial contribution to yield, so weed competition or shock from herbicide application could have a very significant effect.

The current lines of Miscanthus in experiments start to senesce in the autumn, either naturally or with the onset of frost. The dead leaves tend to fall off once they have dried out and evidence from established plots at ADAS Starcross suggests that they may form a useful weed-suppressing mulch. The canes dry out over winter and it is hoped they will reach dry matter contents in excess of 80% by harvest in February or early March, although a target of genetic development will be a range of cultivar maturity to give some spread of the harvest period. High levels of weed contamination, apart from having an effect on yield, could reduce the dry matter content, causing problems with combustion or spoilage of stored material. Drying would then be necessary, adding to the cost of growing the crop and reducing the energy balance. Once the cover of the leaves is lost, some development of weeds seems inevitable, but rapid growth at that time of the year is unlikely, and reduction in dry matter from late developing weed contamination does not appear likely to be a major problem.

Access to herbicide products might be a problem for all novel crops. Miscanthus and other grasses are likely to be tolerant of many of the vast range of selective herbicides available for grass crops. Broad-leaved crops may have a smaller range of materials available. In both cases, there will have to be significant areas grown before agrochemical companies feel it worthwhile undertaking the necessary work to extend label recommendations. (The use of herbicides on ADAS experiments has been done under an experimental permit.) Finally, all the comments made about perennial crops assume that we will be dealing with the current range of arable weeds, but it is possible that tall plantation crops may stimulate the development of a new weed flora that is adapted to low light intensities and further development of herbicides will, thus, be necessary.

THE COSTS AND BENEFITS OF WEED CONTROL

In the absence of a market for biomass fuel, modelling the economic performance of Miscanthus is extremely difficult. However, it has been suggested (Rutherford & Bell, 1992) that a Miscanthus plantation established at a plant population of 10,000/ha, with the plant cost amortised at 8% over ten years, would have production costs of £36/t assuming a yield of 20

t/ha d.m.. Sprays were estimated to cost £30 out of total variable costs of £530. Set-aside payments were not taken into account and would lower production costs per tonne.

The market price for biomass fuel is likely to be dictated by the price of other fuels such as coal and oil and will be a factor of its relative calorific value. This relationship was considered by Rutherford and Bell (1992) and, updating their figures by assuming a crude oil price of $17 per barrel and an exchange rate of £1 to $1.50, the "energy-equivalent" value of biomass is now £26.75/t. This is somewhat lower than the production cost of Miscanthus quoted above and emphasises that some further stimulus, such as set-aside aid and/or financial inducements for the use of fuels of non-fossil origin, is likely to be necessary to create a viable enterprise. Even so, the crop gross margin is likely to be modest and there is not likely to be a large scope for expenditure on weed control and other inputs.

Effect on the energy balance

There is a fundamental concern that the energy yield of a crop grown for fuel should exceed the energy put, either directly or indirectly, into growing it. This is a subject in its own right and extremely complex insofar as it is difficult to find common start and end points for comparing agricultural systems. Unpublished ADAS work by Ellis and Heath, funded by the Department of Trade and Industry (DTI), looked at the energy put into crops in terms of the total energy used to produce the physical inputs and the energy used in the process of application. They incorporated the energy used in the manufacture of equipment in terms of an amount per hectare related to depreciation. To create the energy balance, they took the energy value of the crop at harvest and assumed that it was stored on the farm. Whilst the limitations of this approach could be debated, it does represent a fairly straightforward way of comparing different crops. On this basis, they calculated an energy input of 16,572 MJ/ha in the establishment year of a Miscanthus crop and an input of 20,989 MJ/ha to an established crop producing 20 t of material/ha at a d.m. content of 85%. Taking a first year yield of 6.5 t/ha (based on experience with *M. sacchariflorus* raised from rhizome cuttings at Arthur Rickwood and Rosemaund), they suggest an energy output of 97,500 MJ/ha and a output input ratio of 5.9:1. In the case of the mature crop, the output is 300,000 MJ/ha and the ratio 14.3:1. The figures in Table 1 were derived by applying the values for application and mechanical weeding used by Ellis and Heath, and the energy contents of individual herbicides published in Green (1976) to the type of weed control programme outlined above. It is clear from this that herbicides are likely to make a small contribution to the total energy cost of cultivating Miscanthus or any other biomass crop and their financial cost is, thus, likely to be the factor that limits their use.

CONTROLLING THE CROP

Miscanthus may be relatively expensive to establish, but it is hoped that a plantation will last for fifteen years or more. It is difficult at this stage to predict the factors that may lead to the decision to destroy a plantation, but rotational considerations, cultivar improvement, disease or pest attack could prompt the need to remove a crop. There is a concern that crops may simply lose vigour and, thus, yield at some point, but this cannot be evaluated until we have some long-term experience of Miscanthus as a crop. A fully established plantation of Miscanthus is likely to have a very well developed network of rhizomes. On the evidence of

TABLE 1. Energy use in a weed control programme for Miscanthus.

Material or Operation	Timing	Quantity	Unit energy value[1]	Application Energy MJ	Total MJ/ha
paraquat	Pre-planting	0.6 kg/ha	460 MJ/kg	266	542
Mechanical weeding	Post-planting	2 passes	240 MJ/ha		480
MCPA	Post-planting	1.5 kg/ha	130 MJ/kg		195
atrazine	Post-planting	1.7 kg/ha	190 MJ/kg	266	589
glyphosate	Post-harvest	1.44 kg/ha	454 MJ/kg	266	920
Total Year One					2726
Mechanical weeding	Post-emergence	1 pass	240 MJ/ha		240
MCPA	Post-emergence	1.5 kg/ha	130 MJ/kg		195
glyphosate	Post-harvest	1.44 kg/ha	454 MJ/kg	266	920
Total Year Two					1355

[1] Source: Green (1976)

our current experience, the mat of rhizomes is likely to occupy the top 30 cm of soil, or even more. Physical removal of such a quantity of material is likely to be extremely difficult, although small areas may be treated in this way to obtain material for propagation, so removal by herbicide is likely to be the principal option. Application of glyphosate in April or May when the crop is growing actively and is small enough to allow the passage of a sprayer may be the initial treatment. In view of the likely volume of rhizome material, it seems reasonable to postulate that re-treatment at intervals throughout the year may well prove necessary, prior to autumn ploughing. This approach would preclude cropping in that year, but aerial spraying of glyphosate in the previous autumn may be an alternative that allows immediate re-cropping after harvest.

The crop as a weed

Current problems with crops as weeds has already prompted some concern about the potential for Miscanthus to become a weed, and this is an important question about any novel species that may be cultivated as a crop. The principal advantage of Miscanthus is that the genetic lines under test at the moment do not produce fertile seed. Any spread will, therefore, be by vegetative means and the most likely sources would seem to be the remnants of old plantations, or the spread of crops into field boundaries and neighbouring fields. The former problem can be tackled either by the use of selective herbicides, and it seems that the specific graminicide fluazifop-P will control Miscanthus (J. Braunholtz, personal communication), or by the use of glyphosate through spot treatment or techniques such as weed-wiping. The problem of spread from plantations maybe slightly more problematical and is likely to require the use of a cultivated strip around the edge and the periodic use of herbicides within the strip. However, it should be stressed that Miscanthus does not appear to spread rapidly, so frequent treatment may not be necessary. It is important not to understate the potential of plants to become weeds, and study of crop destruction and control of the crop as a weed will both be necessary early stages of a research programme of any novel crop. This will be particularly important in the case of novel species that set seed and it is not inconceivable that we could

find a species that has high potential as a crop, but cannot be exploited because it is impossible to control.

CONCLUSIONS

Whilst there are no specific data on the effect of weed competition on the yield of Miscanthus in the UK, it is clear that weeds have the potential to present problems, particularly during the establishment of this and other potential fuel crops. If Miscanthus is shown to have good yield potential in the UK, weed control studies will be an important part of the next phase of the research programme. The current, infertile, lines of Miscanthus under test do not appear to pose a threat as a potential weed problem, but research on plantation destruction will be necessary. The potential of plants to become weed problems must always be considered carefully and adequate control measures devised at an early stage in their development as novel crops. Finally, whilst herbicides would not appear to reduce the energy balance of fuel crop production, financial gross margins are likely to be small and weed control will have to be undertaken within a tight budget.

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ANTIBODY PLANTS AS NOVEL NON-FOOD CROPS

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ABSTRACT

It has been recently shown that plants can produce functional intact antibodies and also the very versatile single chain F_V antigen-recognising antibody derivative. These have been targeted to the cytosol or intercellular spaces where they are functional and stable. Ultimately, full spatial and temporal control of antibody accumulation is likely to be possible. No special efforts have been made to optimise yields but levels of accumulation are towards the upper limits of recorded transgene product accumulation. Antibody-producing crop plants thus appear to represent a means of producing large quantities of antibodies and derived immunoreagents much more economically than existing methods. Factors relevant to the types of crop which may be exploited as antibody plants including yield and location of antibody accumulation, kinds of antibody, extraction and downstream processing, genetic and commercial containability are considered in terms of potential medical, industrial, consumer-product and plant protection applications.

INTRODUCTION

As a prelude to discussing the progress and prospects of the production of antibodies in plant crops, a brief review of relevant aspects of the molecular structure of an immunoglobulin G (IgG) antibody (the class most utilised so far in antibody engineering) may be helpful.

The IgG antibody is Y shaped and is composed of two pairs of identical polypeptides, two heavy chains and two light chains. The two heavy chains lie close together for part of their length (linked by disulphide bonds) and are separated for the remainder of their length thus forming the basic Y shape of the IgG antibody. Each of the two shorter light chains associate with one of the arms of the Y and are linked to the heavy chain near the branch of the Y by disulphide bonds.

It is of course the ability of an antibody to recognise its cognate antigen with exquisite specificity which forms the basis for its usefulness in the present context and this ability to recognise resides entirely in the tips of the Y. Each tip is identical and each of these two antigen-recognising sites (Fv domains) is composed of regions of the heavy and light chain which are variable in structure (V_H and V_L respectively). It is this variation in structure which allows specific interactions with antigens. The remainder of the antibody molecule is not at all involved with the recognition of antibody and in the animal is concerned with other aspects of the immune response. Thus if the end of the Y is cleaved off by a suitable proteolytic enzyme to leave a V shape, this V shaped antibody derivative retains the antigen-recognising characteristics of the parent antibody.

Very significantly in the present context, it is possible to isolate genes coding for antibody heavy and light chains and to identify within them the regions coding for the functional domains - including the variable regions. With this information, recombinant genes may be constructed which encode antibody derivatives which

contain the variable regions of the heavy and light chains and fully retain the antigen-binding capacity of the parent antibody. A much-studied and particularly promising antibody derivative is composed of the variable regions linked by a synthetic peptide bridge and is known as the single chain variable fragment (scFv)

Thus recombinant, antigen-recognising polypeptides can be derived from the much larger native antibody by dispensing with regions not involved in antigen recognition. The small size and relative structural simplicity of the recombinant derivatives resulting from the absence of the constant regions offer advantages over native antibodies.

Since they have been derived from larger polypeptides it is not surprising that it is also possible to add to these recombinant antibodies, protein domains which carry desirable functions. Such domains include affinity handles to facilitate purification, toxic moieties for use in magic bullet applications (Kreitman,1989) or enzyme functions for use for example in the production of recombinant immunotherapeutic or diagnostic reagents. Many such bifunctional fusion proteins have been produced.

At the present time the most convenient source of antibody genes with particular desired properties is an cultured animal (hybridoma) cell line secreting an antibody which possesses the desired properties. In the event that a suitable hybridoma line does not exist the generation of a suitable cell line is time-consuming and sometimes difficult. Fortunately, recent remarkable advances in antibody engineering have permitted the development of techniques for the generation of very large libraries of random combinations of heavy and light chain variable regions from which recombinant antibodies possessing desired characteristics may be selected (Winter & Milstein, 1991). It would appear then that the selection of recombinant antibodies with virtually any desired binding characteristics will soon be a relatively straightforward process and innumerable strategies utilising them, including those discussed below which involve plants, have become realistic propositions.

FUNCTIONAL ANTIBODY PRODUCTION IN PLANTS; CURRENT STATUS

Intact immunoglobulins.

The first report of functional intact antibody production in plants involved a two step process in which tobacco plants were transformed with either complete light chain or complete heavy chain genes (Hiatt *et al.*, 1989; Hiatt, 1990; Hiatt & Mostov,1993). Individual plants were shown to accumulate either heavy or light chains. When such plants were sexually crossed the resulting progeny accumulated functional antibody outside the living protoplast in the plant cell wall (i.e. in the apoplast) which was indistinguishable in binding characteristics from the parent antibody. (Hein *et al.* 1991) The yield of antibody was around 1% of the total protein, amongst the highest reported levels of heterologous proteins accumulated in transgenic plants. Since no particular efforts were made to optimise accumulation it seems likely that even higher levels of accumulation are possible. In animal cells producing antibodies, assembly and disulphide bond production involves co-translational insertion into the endoplasmic reticulum and includes the participation of a chaperonin, protein disulphide isomerase and finally glycosylation. These findings indicate that analogous processing can occur in plants.

When similar experiments were carried out with truncated heavy and light chain genes in which the sequence coding for the signal sequence which directs

the heavy and light chain polypeptides to the endoplasmic reticulum (ER) was deleted no such accumulation was observed.

In contrast, During *et al.* (1990) found that the expression in tobacco of an IgM gene, in which the native signal sequence was replaced by the coding sequence of the barley a-amylase secretion signal sequence, resulted in the accumulation of antibody in the ER and particularly surprisingly, in chloroplasts. Taken along with the work of Steiger *et al.* (1991) in which transient expression following injection of IgM genes into the nucleus of *Acetabularia* resulted in the accumulation of functional antibody in the cytoplasm the conclusion appears to be that secretion into the ER is not an absolute prerequisite for the production of functional intact antibody in plants. It is possible that the observed discrepancies are dependent on the particular properties of individual antibodies or antibody classes.

In summary then although the rather demanding assembly requirements of at least some antibodies indicates that targeting to specific locations may be problematical it is clear that plants are indeed capable of assembling and accumulating complete intact antibody molecules to substantial levels.

Antibody fragments and fusion derivatives.

Owen *et al* (1992 a; 1992b.) have demonstrated that tobacco plants are capable of producing an scFv antibody derivative which is functional in the plant cytosol as well as following extraction from the plant. The scFv is derived from an antibody (AS32) which recognises a highly conserved epitope of the photoregulatory protein phytochrome A which is located in the plant cytosol. Phytochrome A mediates in the perception of light in de-etiolation and light stimulated germination. Tobacco plants transformed using an *Agrobacterium*-mediated system with an scFv gene derived from AS32 under the control of the CaMV 35S promoter exhibited aberrant light stimulated germination and de-etiolation. This demonstrated that the scFv had interacted with and interfered with the function of phytochrome *in vivo* - a process which may be termed immunomodulation.

Firek *et al.* (1993) fused the AS32 scFv to the signal sequence of the tobacco pathogenesis related protein PR1a which directs secretion to the apoplast. As predicted the signal sequence targeted the scFv to the extracellular space of the plant. The scFv was functional and stable in this apoplastic location. Furthermore the levels of accumulation were around 0.5 -1.0% of the total soluble leaf protein, an order of magnitude increase over the cytoplasmic accumulation levels. Cultured cells derived from these plants secreted scFv to the culture medium to levels around 50% of the protein in the medium in which it was functional and stable for long periods.

As noted above one of the major advantages of the scFv is the facility to add functional domains to produce bi or even multifunctional fusion proteins. To assess the characteristics of plants with reference to the production of this class of versatile and potentially valuable reagents we have fused AS32 to staphylococcal protein A, a much used affinity reagent, and to B-glucuronidase (GUS) and alkaline phosphatase, valuable reporter enzymes. Preliminary evidence indicates that tobacco plants transformed with chimaeric AS32 fusion genes accumulate polypeptide fusions in which protein A and GUS moieties are both functional.

The levels of accumulation of the AS32/protein A fusion are substantially higher than the levels of AS32 alone. This may indicate that the fusion of the scFv with the particularly robust protein A may have enhanced the stability of the AS32

moiety or enhanced correct polypeptide folding during synthesis. Regardless of the explanation of this effect the ability to increase the level of antibody accumulation in the cytoplasm by fusion with protein A (and possibly other equivalent fusion partners) is likely to be useful.

In summary then the evidence so far is that plants are capable of accumulating substantial levels of functional scFv antibody derivatives and fusions and that it is possible to direct these recombinant antibodies to either the cytoplasm or apoplast where they are both functional and stable. With the use of appropriate gene sequences directing sub-cellular targeting, tissue-specificity and inducibility, complete spatial and temporal control of antibody accumulation should eventually become possible.

WHY PRODUCE ANTIBODIES IN PLANTS?

In broad terms there are three reasons for producing antibodies and their derivatives in plants:

a) large scale production - antibody farming;
b) antibody mediated modification of antigen activity in or by the living plant;
c) the study of protein targeting and assembly mechanisms in plants -

Reasons a) and b) are discussed below. Reason c) will not be further considered here.

a) Antibody farming.

Monoclonal antibodies are currently produced for a very wide range of commercial applications. Therapeutic agents for example which consist of a drug, isotope or toxin conjugated to a cell-type specific antibody (in the future probably more often to a recombinant antibody derivative .e.g. scFv) are used in the treatment of cancer and the diagnosis of cardiovascular diseases. The estimated value of such therapeutic reagents in 1994 is in the region of $1000M. In addition to therapeutic use, the exquisite specificity of monoclonal antibodies has been exploited in a range of affinity purifications and in a large number of diagnostic kits such as those employed for home pregnancy testing.

Current methods for the production of monoclonal antibodies usually involves the complex and expensive procedures of hybridoma cell growth and antibody purification or the isolation of antibody from ascites fluid produced in animals (a procedure which is increasingly seen as unacceptable). There is therefore considerable interest in developing convenient and economical heterologous systems for the production of antibodies and their derivatives. Candidate expression systems for this role are bacteria, yeast, insect cells and of course plants.

As described above plants are capable of producing intact antibodies and a range of recombinant derivatives and have a particular advantage where very large scale production of industrial quantities are envisaged. It is difficult to imagine a more economical protein production system than a crop plant; requiring only sun, soil and water. This view is particularly convincing if the antibody plant is a version of an existing crop plant for which husbandry is well understood and for which harvesting, storage and processing infrastructure already exists. Perhaps the ultimate scenario would involve the extraction of the antibody from a fraction of a processed crop which currently goes to waste. Extraction of antibody from the aqueous extract discarded in the production of starch from potatoes is an example of this ideal situation.

It is also possible that targeting of antibodies to protein storage locations in plants such as seeds may prove to be a feasible proposition. The accumulation of functional antibodies in seeds has particular attractions in terms of storage and transport and possibly even as a convenient way of producing and distributing passive immunity vaccines.

Although at the present time the transformation of existing crops, particularly the monocots, is not routine, rapid progress is being made and it is certain that in the near future the vast majority of crops will be accessible as potential antibody plants. An unrelated advantage of plants as a source of antibodies is in the area of consumer acceptability. At least at the present time the exploitation of genetically manipulated plants appears to be more acceptable to the general public than genetically modified animals or micro-organisms.

Obviously in the end the viability of antibody farming will depend entirely upon the market for a particular antibody and the economics of production and this in turn will vary from application to application. Further studies on the levels of extractable yield attainable in the possible locations in suitable crop plants are required but it is clear that where very large quantities of antibody are required antibody farming as a non-food crop use of land is a realistic possibility. Indeed it is likely that the economical availability of large amounts of antibodies and derivative immunoreagents would allow the development of industrial processes and consumer applications hitherto proscribed by the current cost of producing 'industrial' quantities of antibodies.

b) Antibody mediated modification of antigen activity in or by the living plant.

Quite separate from the use of plants as factories for the production of antibodies there are many ways in which the ability of plants to express antibodies may be usefully exploited. The work described above in which the expression of an anti-phytochrome scF_v alters the perception of light by phytochrome establishes the principle of immunomodulation in plants. There are innumerable applications of this approach - for example the temporally and spatially controlled endogenous expression of antibody derivatives against, for example enzymes, regulatory proteins, and (inaccessible to the antisense approach) non-direct gene products and small regulatory molecules such as the plant growth substances, will permit modulation of virtually any aspect of plant growth and metabolism for biotechnological purposes or for the study of plants. For example the inhibition of an enzyme beyond a branch point in metabolism might be used to divert the plant's resources towards a desired product, It is also possible to endow plants with entirely new enzymic activities not found in nature by the expression of catalytic antibody derivatives.

One area in which the expression of antibody derivatives is likely to find early application is in plant protection. The possibility of generating novel forms of virus resistance by transforming plants with antibodies which recognise and interfere with virus replication or transmission from cell to cell or plant to plant is being actively investigated. A major attraction of this approach is that only the addition of a single gene is required and the characteristics of an existing cultivar for which processing procedures are established and for which a known market exists will not be significantly altered. Strategies to provide protection against bacterial, fungal, nematode and other pathogens are also possible.(During *et al.* 1993). Simple recognition of a pathogen component will not always be sufficient to interfere with the activities of the pathogen. It will sometimes be necessary for a pathogen-recognising antibody to carry with it some toxic function. The bifunctional fusion of an scF_v which recognises a fungal surface epitope with a wall degrading

enzyme provides an example of this. Secretion of the fusion to the plant apoplast would probably be required and wound induced production would provide additional sophistication.

The production of herbicide resistance is another possibility. Plants expressing an scF_V which recognises and masks a functional epitope of a herbicide would be expected to be resistant to that herbicide. Similarly the secretion to the cell wall of an scF_V which binds a herbicide and prevents it from entering the cell would also be expected to give resistance to that herbicide.

More speculative perhaps the possibility of using antibody plants as environmental cleanup agents. Plants producing antibodies in the apoplast with a high affinity for a particular pollutant could chelate and concentrate the pollutant. The plants could then be collected and disposed of appropriately.

WHAT KINDS OF ANTIBODIES ARE CANDIDATES FOR ANTIBODY FARMING?

As noted above the exploitation of plants in the production of antibodies is most attractive where very large scale production of antibodies is envisaged. There are three areas in which very large amounts of antibodies could be utilised, industrial medical and what may be termed consumer applications.

Two basic kinds of industrial application may be envisaged. In the first the specificity of antibodies could be used in affinity purification processes to collect a low abundance valuable product from very large volumes thus allow ing exploitation of otherwise uneconomically low yields. The second kind of application would involve removal of undesirable components. Decaffeination of coffee or the removal of herbicide residues from fruit juices are possible applications.

Antibody therapy involving say anti-cancer cell antibody/toxin fusions can require the use of large quantities of immunoreagent. The expense of such therapies could be reduced, by the production in plants of a suitable non-immunogenic recombinant derivative of either human or animal antibody.

Oral hygiene is an area where it has been suggested that antibodies would find consumer product applications (Hiatt & Ma, 1992). For example a mouthwash which contained antibodies which prevented the establishment of *Streptococcus mutans*, a bacterium implicated in causing dental caries, though eminently marketable, will require a more economical source of large amounts of antibody than is currently available. In such applications the high consumer acceptability of plant products may be of particular significance.

Especially in therapeutic and consumer product applications it is likely that rigorous purification of antibody will be required. The affinity of the antibody for its antigen, if sufficient quantities of antigen were readily available, could of course be exploited here but it is possible that the fusion of the antibody to an affinity tag would prove to be generally applicable and therefore convenient. If necessary a cleavage site could be included so that the affinity tag could be removed if required.

WHICH CROP PLANTS ARE LIKELY TO BE USED IN ANTIBODY FARMING?

At the present time this is quite a difficult question to answer in other than general terms. Not enough is known about the yields, stability and downstream processing of antibodies and derivatives in plants. It may be that unexpected characteristics of particular crops will be of overwhelming significance in deciding which crops are most suitable for antibody farming. Only further research can

resolve these imponderables. Furthermore some of the most obvious possibilities, maize and the other monocot crops, are not yet amongst those that are routinely transformable.

Of the currently readily transformable crops potato is a good candidate as an antibody plant, in which an antibody targeted to the tuber would remain hydrated and for which facilities for large scale growth, harvesting, storage and processing already exist.

Targeting of antibody, resulting in seed storage in legumes for example represents an entirely different strategy which it is likely would allow substantial yields. However it is not yet known whether antibodies would be recoverable from the dehydrated seeds. If it turns out that they are then the seed provides a very convenient package for harvest, transport and storage.

SPECIAL REQUIREMENTS OF ANTIBODY CROPS

Since it is likely that in the short term all antibody crops will be immediate derivatives of existing crops there will already exist a great deal of information on the growth, plant protection and weed control measures. However the nature of the antibody product may influence the choice of appropriate weed control measures. If for example the antibody is ultimately destined for human consumption then some herbicides might be inappropriate because of the downstream purification problems they could present. On the other hand the high value of the antibody product would permit the use of more expensive weed control measures than would be economical with the "parent" plant.

As with all transgenic crops the question of genetic containability must be addressed. Potato which is propagated vegetatively presents few problems from the point of view of genetic containability whereas outbreeders might be unacceptable as transgenic crops for field growth since they would be likely to release the recombinant gene to the environment. It is possible that in the short term it may be necessary to grow and perhaps store and transport antibody crops in defined area under licence. Problematical though this may appear numerous applications of transgenic plant technology are in precisely the same situation and there seems little doubt that generally acceptable solutions will ultimately be forthcoming.

A second aspect of containability relates to the protection of commercial property and although this may be a difficult problem once again it will be no more so than with any other field grown transgenic plant.

If plants which are not current crops are ultimately found to be the most suitable for antibody production then the weeds from which they might suffer and he weed control measures that they might require are a matter for future research.

CONCLUSION

It has already been shown that plants are capable of producing intact antibodies and recombinant antibody derivatives and their fusions in substantial quantities and a start has been made towards the objective of permitting spatially and temporally regulated antibody accumulation. Modified existing crop plants or possibly entirely new crops could be used for the economical production of large quantities of recombinant immunoreagents. This would render practicable a wider application of existing technologies and could also permit the realistic design of entirely new medical and commercial strategies requiring very large amounts of recombinant antibody-based reagents. The indications are that these antibody

plants will constitute a new class of industrial crop and although no special problems with weed control are envisaged, research will be required to ensure the compatibility of weed control measures with antibody production.

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THE PROBLEMS OF DEVELOPING HERBICIDE RECOMMENDATIONS FOR INDUSTRIAL AND ENERGY CROPS

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ABSTRACT

The introduction of new herbicides is the culmination of a large amount of toxicology and efficacy studies. It is unthinkable that any agrochemical manufacturer would introduce a herbicide for other than a major world crop. With existing herbicides a chemical manufacturer might consider extending existing recommendations to another crop provided the acreage was a minimum of 100,000 ha in the United Kingdom and with similar sized markets in other EC countries.
For most industrial and energy crops the cost of determining herbicide tolerance will have to be born by public funding or by the co-operative action of the growers.
Approval under the Control of Pesticides Regulations can be obtained via "off-label Approval" route.
The most suitable trials for determining herbicide efficacy on these crops are discussed. Much will depend on the similarity of the industrial or energy crop and existing agricultural crops for which recommendations already exist.

INTRODUCTION

HERBICIDE EVALUATION

The aim of herbicide evaluation is to produce satisfactory commercial products that will be reliable and safe to use. There are four major participants involved in the development of a herbicide: (1) the Pesticide Industry as innovator, developer and supplier; (2) the Registration Authority acting for the government to protect the user and the public; (3) advisory and research specialists concerned with extension work and introducing new techniques; (4) the grower acting both as host for trials and also the prime user of herbicides.

Herbicide development is the application of a herbicide in such a manner as to allow an analysis of the tolerance of both crop and weeds to the herbicide with respect to all the known variables to which the herbicide will be exposed in use. The most important variables are: (a) dose; (b) formulation; (c) compatibility; (d) taint and crop quality; (e) timing; (f) application; (g) crop biotype; (h) environment; (i) weed response; (j) geography and husbandry; (k) fate of herbicide in soil - effects on following crops.

The data produced by such analysis must ultimately be interpreted into a label recommendation relating to herbicidal efficacy and crop tolerance.

THE PRODUCTION OF DATA

Virtually all data from which herbicide recommendations can be made is generated in trials of one kind or another. It is important in the planning stage that the correct types of trial are proposed. They must yield as much information as possible whilst ensuring that unnecessarily complicated experiments are not used. Cost will be a factor in designing trials and it is necessary to appreciate that different trial types will yield only limited types of data. Trial types can be classified as follows: (1) Glasshouse and post tests; (2) field screens;

(3) Logarithmic trials; (4) small plot replicated trials applied by precision sprayer; (5) large plot replicated trials applied by commercial sprayer; (6) non-replicated trials applied by commercial sprayer; (7) grower applied trials.

The relative usefulness of the different trials types shown above is summarised in Table 1.

TABLE 1

	Variable under investigation	Trial type						
		1	2	3	4	5	6	7
(a)	Rate of use & phytotoxicity	√	√	√	√	√		
(b)	Formulation	√			√			
(c)	Compatibility	√			√			
(d)	Taint and crop quality					√	√	√
(e)	Timing			√	√	√		
(f)	Application					√	√	√
(g)	Crop biotype		√		√			
(h)	Environmental effects	√					√	√
(i)	Weed response				√	√	√	√
(j)	Geography and husbandry					√	√	√
(k)	Soil residues		√			√		√

The table shows that one type of trial is capable of yielding considerably different types of data to others. As a general rule there is a progression in time from glasshouse studies through to grower applied trials. The time scale involved will depend on the nature of the herbicide and weeds controlled. For a novel compound one must consider a time scale of up to seven years (Roberts, 1982).

INDUSTRIAL AND ENERGY CROPS

The development of new herbicides is motivated by the need of agrochemical manufacturers to generate profit from sales to users. It is now accepted that the costs of undertaking such development are very high due to the cost of toxicology studies and registration, product testing and construction of manufacturing plant. The rational of such development strategy is based on developing uses on major world crops e.g. soya, wheat, cotton, maize and rice (Hance & Holly, 1990). Typical costs for development for a new herbicide on a major crop is in the order of 40 million dollars. It is inconceivable therefore a new herbicide would ever be developed for any of the crops being developed for industrial and energy use.

The lack of new herbicides does not however preclude the development of existing herbicides for industrial and energy crops. Herbicides that are currently in use have the attraction of being registered for other crop uses. This therefore avoids the need for the generation of data on toxicology or ecological aspects. Operator exposure will also not need evaluation provided the same formulation can be used and applied by the same methods as on the existing product label.

Where existing herbicides can be considered for industrial and energy crops even the efficacy aspects of its use are well established and may not need further evaluation. The closer the candidate crop is to an existing agricultural or horticultural crop the less need there is to do trials on the control of weeds.

The greatest concern when developing herbicide recommendations for industrial and energy crops is that of safety of the herbicide to these crops. As with weed control the closer the candidate crop is to an agricultural or horticultural crop for which the herbicide is recommended the less the risk of phytotoxicity to the industrial or energy crop. Even if there is no direct botanical relationship between a candidate crop and a label recommended crop considerable guidance to safe herbicides can be gained if the candidate crop and label recommended crop are in the same botanical family or are morphologically identical.

TABLE 2

The Use and Relationship of Industrial and Energy Crops to Existing Crops is as Follows:

FUEL CROPS	BOTANICAL FAMILY
Coppice (Salix & Populus spp.)	- Salicaceae
Elephant Grass (Miscanthus sinensis)	- Gramineae
Whole crop cereal	- Gramineae
FIBRE CROPS	
Hemp (Cannabis sativa)	- Moraceae
Flax (Linum usitatissimum)	- Linaceae
OIL CROPS	
Oilseed Rape for methylester	- Cruciferae
for erucic acid	
for lauric acid	
Linseed (L. usitatissimum) industrial oils	- Linaceae
Linola - oleic acid	- Linaceae
Sunflower (Helianthus annuus) oleic acid	- Compositae
Crambe maritima - pharmaceutical oil	- Cruciferae
Honesty (Lunaria biennis) - pharmaceutical oil	- Cruciferae
Cuphea spp. - lauric acid	- Lythraceae
Gold of pleasure (Camelina sativa) - pharmaceutical oil	- Cruciferae
Caraway (Carum carvi) - pharmaceutical oil	- Umbelliferae
Borage (Borago officinalis) - linoleic acid	- Boraginaceae
Evening Primrose (Oenothera biennis) - linoleic acid	- Onagraceae
Naked Oats - pharmaceutical oil	- Gramineae
ALCOHOL FUEL ADDITIVES	
Potatoes - ethanol	- Solanaceae
Sugar beet - ethanol	- Chenopodiaceae
Cereals - ethanol	- Gramineae
PROTEINS	
Lupins	- Leguminoseae

The use of existing herbicide recommendations on similar crops must be the first consideration when trying to find herbicides for industrial and energy crops.

It must not however be assumed that the uses will automatically be acceptable and trials will be needed to confirm the crop safety of any such recommendations.

The potential for using existing herbicides can also be restricted by any difference in the agronomy of the industrial and energy crop compared to the agricultural crop.

There is also the complication of whether the industrial or energy crop is going to be grown on rotational or non rotational set aside land. The terms for growing such crops on set-aside land are very specific and also cover the use of pesticides (MAFF 1993). Special written permission is required to use herbicides on set-aside land but even this will exclude the use of residual soil acting herbicides. It is a further condition that written records must be kept of any pesticides applied to set-aside land, in agreement with the general requirements of the Control of Pesticides Regulations, 1986.

The restrictions for herbicide use on set-aside land could be sufficiently serious to jeopardise the growing of some crops on this type of land. The need to keep crops free from weeds during the establishment phase on set-aside land will require considerable management skills. Maximum opportunity will need to be taken of cultural weed control measures and even the use of mechanical weed control post-emergence of the crop using the new types of "weed-rippers" developed for organic farming. In addition the use of inter-row hoes is a practical consideration.

POTENTIAL HERBICIDES

Salicaceae

Both Poplar and Willow used in short rotation coppice have been subject to considerable research for suitable selective herbicides. The control of germinating weeds is with simazine (Scott, 1980) and post-emergence weed control with glyphosate (Lawrie, 1989) is well established. Other selective residual herbicides are described in the literature (Parfitt et al, 1992) and results on pot grown plants treated with foliar-acting herbicides (Clay, 1993). Whilst the residual herbicides are excluded from set-aside land there is a good potential number of foliar-acting herbicides in addition to glyphosate to control most potential weed problems, for example clopyralid for dicotyledonous weeds and cycloxydim and fluazifop-p-butyl for graminaceous weeds. This is summarised in the Forestry Commission Research note 201.

Gramineae

Elephant grass differs from other graminaceous crops in being perennial. Establishment has been from rhizome cuttings. Weed Control in the establishment phase is essential. Broad-leaved weeds can be controlled using existing cereal post-emergence herbicides. There appears to a problem with *Poa annua* (Speller, 1993). Candidate compounds such as isoproturon and chlorotoluron may well give satisfactory control of this weed and prove tolerant to the crop.

The other crops in this group are existing cereal crops. Weed control in these crops will be no different to that in agricultural cereals apart from the need to clarify herbicidal effects on fresh and dry matter production.

Cruciferae

There is a wide range of agricultural cruciferous crops with a diverse range of herbicides available with reasonable levels of selectivity. In the case of oilseed rape there is a direct read-

across from the agricultural recommendations. In the case of the novel cruciferous crops e.g. Crambe, Honesty and Gold of Pleasure, trials will need to be done to establish the safety of existing herbicides.

Candidate herbicides are as follows:

Residual Herbicides	Foliar Herbicides
aziprotyrne	carbetamide
chlorthal-dimethyl	clopyralid
cyanazine	desmetryn
metazachlor	sodium monochloroacetate
pendimethalin	cyloxydim
propachlor	diclofop methyl
tebutam	fluazifop-p-butyl
trifluralin	glyphosate (pre-harvest)
TCA	

Linaceae

The linseed crop is becoming well supported with herbicide recommendations. It would be reasonable to expect flax crops to be similarly tolerant to the same recommendations

Currently the following herbicides are recommended on linseed:

Residual Herbicides	Foliar Herbicides
cyanazine	bentazone
tri-allate	bromoxynil
trifluralin	clopyralid
linuron	MCPA
	diclofop-methyl
	metsulfuron-methyl
	sethoxydim
	diquat (pre-harvest
	glufosinate ammonium (pre-harvest)
	glyphosate (pre-harvest)

Compositeae

The only crop in this group is sunflower. Recommendations for herbicides on this crop are limited in the United Kingdom but are extensive where the crop is well established, such as France (ACTA, 1993)

Potential herbicides include the following:

Residual Herbicides	Foliar Herbicides
linuron	carbetamide
monolinuron	fluazifop-p-butyl
metalachlor	diquat (pre-harvest)
pendimethalin	
prometryne	
propyzamide	
terbutryne	
trifluralin	

Umbelliferae

Caraway is a novel crop and there would be need to carry out selectivity trials with all potential herbicides. These would be selected from those already approved for use on other umbelliferous crops - carrots, parsley and parsnips.

These can be listed as follows:

Residual Herbicide	Foliar Herbicides
chlorpropham	alloxydim-sodium
linuron	fluazifop-p-butyl
metoxuron	sethoxydim
pendimethalin	diclofop-methyl
petanochlor	
prometryn	
linuron	

Salanaceae, Chenopodiaceae

Where potato and sugar beet crops are being grown for industrial uses there are no differences from existing agricultural varieties and all current herbicide uses for the agricultural crops can be used for industrial potatoes and sugar beet.

Leguminoseae

The crop that concerns us here is lupins being grown for protein purposes. The existing uses of herbicides on peas and beans would appear to be relevent. As beans are larger seeded than lupins they show higher herbicide tolerance than peas. It would therefore seem appropriate to follow current pea herbicide recommendations on this crop, which are summarised below:

Residual Herbicides	Foliar Herbicides
aziprotryne	alloxydim-sodium
chlorpropham	bentazone
cyanazine	diclofop-methyl
pendimethalin	glufosinate-ammonium (pre-harvest)
terbutryn	diquat (pre-harvest)
trietazine	sethoxydim
terbutylazine	

Moraceae (Hemp)
Lythraceae (Cuphea)
Boraginaceae (Borage)
Onagraceae (Evening Primrose)

In the case of the crops above there is no ready comparison with agricultural crops and no obvious herbicides that will be suitable. To find suitable herbicides growers will tend to use their initiative and try ad-hoc treatments. The result is that there is no commonly available weed control methods in these crops.

DEVELOPING HERBICIDE RECOMMENDATIONS

In the case of the Gramineae, Solanaceae and Chenopodiaceae the only trials required would be confirmatory large plot trials and farmer usage trials. Data requirements would be superficial and confined to plant health and general efficacy.

Where there are strong links between industrial/energy crops and agricultural crops more sophisticated trials would be required in order to check the difference between the tolerance of the industrial and energy crop and the agricultural counterpart. This would apply to the Cruciferae, Linaceae, Compositeae and Umbelliferae. Suitable trials would consist of replicated small plot trials with single and double doses. Three to four such trials would be required over a period of two years.

In the case of unrelated crops such as the Moraceae, Lythraceae, Baraginaceae and Onagraceae there is a need to start from a zero base.

This is best done using glasshouse pot trials and field screens. Glasshouse trials enable large numbers of compounds to be screened for crop safety. These techniques give good preliminary data (Makepeace, et.al., 1989) on herbicide tolerance but it must be re-assessed in field trials.

Field and outdoor pot trials can give very useful guidance on suitable recommendations. Pot trials have been used for the determination of selective herbicides in the Salicaeous crops, but these have been supported with field screening trials (Parfitt et al., 1992).

In the case of annual crops, field screens can also be very useful in gaining guidance on selective herbicides. Field screens mostly consist of crops and possible weeds, being planted in parallel rows. Candidate herbicides are then sprayed across the crop rows at varying doses. These trials are seldom replicated and more than one site can be set up in any one year. Such methods were commonly used for the development of vegetable herbicides and are still commonly used on peas, beans and cereals. A noteable use of this type of screen has been the use of field screens to examine the tolerance of wild flower species to established herbicides (Nowakowski, 1991, 1992). The disadvantage of field screen is that the trial site needs pre-treatment to reduce weeds and hand-labour to remove germinating endemic agricultural weeds.

Suitable candidate herbicides would then progress from the field screen to replicated trials.

COSTS OF DEVELOPMENT

As stated at the beginning of this paper, herbicide developments can only be funded where major use is contemplated. Where herbicides are already approved on other crops much of the development costs have been met. If one assumes that there is little or no need for efficacy evaluation the concern in developing herbicide recommendations for industrial and energy crops will be primarily that of crop safety. If this can be quantified a herbicide manufacturer may still be reluctant to put such a crop in this label as the crop acreage is too low to cover the costs involved.

TABLE 3 The Profit and Typical Costs of Some Herbicides

	Isoproturon 50% SC	Prometryne 50% WP	Ioxynil 225 g/l EC
Income			
Price to Grower	£3.65/l	£12.50/kg	£12.50/l
Price to Merchant:	£3.30/l	£10.10/kg	£10.10/l
Costs			
Pesticide Levy	0.04/l	0.12/kg	0.12/l
Labelling, formulation & packaing:	0.60/l	0.90/kg	1.20/l
Transport:	0.20/l	0.20/kg	0.40/l
Cost of active:	2.35/l	1.50/kg	3.25/l
Total Costs	£3.19/l	2.72/kg	4.97/l
Gross profit:	£0.11/l	£7.38	£5.13
Gross profit on 1,000 ha	£550.00 (at 5 l/ha)	£16,605.00 (at 2.25 kg/ha)	£14,364.00 (at 2.8 l/ha)
Maximum Agricultural Acreage	1.25m.ha	13,500 ha	12,000 ha

The major problem is the limited acreages involved.

Linseed can justify these costs as the crop is now grown on around 100, 000 ha. Most of the other industrial and energy crops are not grown on any significant scale.

Contracts for growing high erucic acid rape have been available for several years. Even so the acreage is only 14,000 ha. Evening Primrose is grown on little more than 500 ha. The cost and profit from the sale of herbicides varies. Some typical examples are shown in table 3.

From these figures it can be seen that a manufacturer of commodity herbicides, such as isoproturon, would not gain any benefit from developing a use on a minor crop until the acreage reached something in the order of 100, 000 ha. A manufacturer of a speciality herbicide such as prometryne or ioxynil may consider increased sales of as little as 1000 ha worth the costs.

What would be the cost of getting an "on-label" recommendation? They can be considered as follows:

TABLE 4
Some of the Direct Costs of Herbicide Development

Insurance cost of liability	£2,000/annum
Cost of trials	
glasshouse or field screen	£2,500
Field trials - 6 sites over 2 years	£18,000
Variety and or quality tests	£5,000
Registration fee - normal queue	£2,300
Cost of Registration and Overview	£2,000
Labelling and literature costs	£2,500
Total Possible Costs	£34,300

The costs above only reflect the direct costs of the herbicide development. When the indirect costs of the manufacturer are added to these costs it is unlikely that a manufacturer will want to recommend a herbicide on an industrial or energy crop until the acreage reached 50-100,000 ha or above.

REGISTRATION ASPECTS

All herbicide uses in the United Kingdom are subject to the Control of Pesticides Regulations, 1986. Before a herbicide can be used in any situation to control weed growth it must first be approved under the regulations.

Herbicide Recommendations that are approved for a manufacturer or distributor and will be included on the approved label will either have provisional or full approval. The product label will define the conditions of approval, namely the crop, the maximum dose, the latest time of application and any harvest interval necessary. Approved label recommendations exist for cereals, sugar beet, potatoes, linseed and sunflower.

Where no label recommendations exist growers, or their representatives, can apply for an "off-label" approval. Before an "off-label" use can be granted it is essential that the herbicide is approved in the United Kingdom for another use.

For an "off-label" approval, two major registration requirements for agricultural crops will not be required in the case of industrial and energy crops. Efficacy requirements are waived in the case of all off-label recommendations and as no edible products are generated from these crops there are no residue-data requirements.

Thus the application for off-label approval will not require the production of any scientific data. The off-label application fee of £460 per product per crop will be required. Where the need for an off-label approval is recognised as important and urgent by both growers, contract suppliers and hopefully ADAS. It is possible to apply for an "Emergency off-label" approval. This gets priority treatment in the "fast" queue, which can result in an approval 2-3 months after acceptance. Ordinary off-label approvals would take 12-18 months from acceptance before being granted.

THE WAY FORWARD

Where industrial and energy crops have achieved acreages of around 100,000 ha the crop is large enough to justify the herbicide manufacturers generating efficacy data and applying for on-label approvals. This is the case with linseed and to a lesser extent on sunflower.

For other crops, even including whole-crop-cereals and high erucic acid oilseed rape, the liability of possible adverse effects of herbicides on yield or quality is likely to deter any manufacturer from looking at on-label recommendation development.

This leaves these crops without interested support to pay for the necessary trials work to generate efficacy data for herbicides. Some crops attract research support from public funding such as short rotation coppice and Miscanthus. Other crops have no supporting agencies and there is little prospect of public funds. This is reflected in the government's negative attitude to the production of rape methyl-ester as bio-diesel.

In these circumstances the answer to funding must come from the growers. This has been very successful in the case of the Maize Growers Association and other associations such as the Leek Growers Association. What makes this type of co-operation viable for the growers

of industrial and energy crops is that for an off-label approval there is no requirements for data product and the crop-safety trials would be purely to investigate the safety of potential herbicides to individual crops.

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EFFECT OF HERBICIDE MIXTURES APPLIED TO NEWLY-PLANTED POPLAR AND WILLOW COPPICE

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ABSTRACT

Eight treatments with residual herbicide mixtures were applied after planting cuttings of two poplar and two willow cultivars. Crop growth at the end of the year had not been reduced by doses recommended for weed control in other crops or by three times those rates compared with hand-weeded controls. Herbicides well tolerated were isoxaben, metamitron, metazachlor, napropamide, pendimethalin, propyzamide and simazine.

Four herbicides and herbicide mixtures were applied in April or May to recently-planted cuttings after growth had started. Clopyralid, cyanazine, metazachlor and pendimethalin applied alone or in mixture in late April did not damage plants but May application of some of the mixtures, caused stunting after spraying and shoot weight reduction when assessed at the end of the first year. The experiment showed that early application of some of these herbicides treatments is needed to avoid crop damage.

INTRODUCTION

There is a need for effective herbicides for short-rotation coppice since annual and perennial weeds compete severely with the crop after planting (Parfitt *et al* 1992). Earlier experiments at Long Ashton Research Station showed that poplar and willow were tolerant to a number of residual herbicides applied alone or in mixture after planting cuttings (Clay *et al* 1990; Parfitt *et al* 1992). Tolerance to the same herbicides was tested in a further experiment reported in this paper.

Application of herbicides post-planting may be delayed by adverse weather by which time cuttings and weeds have started growth; there is a need to know the tolerance of the crops to herbicides applied at this stage. In a field experiment, four herbicides alone or in mixture were applied at recommended and three times recommended doses to poplar and willow cuttings in April and May after shoot growth had started. The results are reported below.

MATERIALS AND METHODS

The first experiment was carried out at Long Ashton Research Station on land adjacent to the earlier two experiments in the series (Parfitt *et al* 1992). Experimental design and lay-out, cultivars and herbicide formulations, treatments and application methods were the same as in those experiments. 25 cm long cuttings were planted on 3, 4 April 1991 and herbicides applied on 16, 17 April 1991. The herbicides and doses used were:- atrazine + cyanazine as 'Holtox' at 3.0 (Dose 1)and 9.0 (Dose 2) kg a.i./ha; isoxaben as 'Flexidor' at 0.13 and 0.38 kg a.i./ha; metamitron as 'Goltix WG' at 3.5 and 10.5 kg a.i./ha; metazachlor as 'Butisan S' at 1.25 and 2.50 kg a.i./ha; napropamide as 'Devrinol' at 2.25 and 7.75 kg a.i./ha; pendimethalin as 'Stomp 330' at 2.0 and 6.0 kg a.i./ha; propyzamide as 'Kerb 50 W' at 1.5 and 4.5 kg a.i./ha; simazine as 'Gesatop 500FW' at 1.5 and 4.5 kg a.i./ha. The mixtures used are listed in Table 1. The control treatment was kept as weed-free as possible by regular hoeing. Any weeds growing on treated plots were removed as seedlings.

Plant health was assessed at intervals during the growing season using a 0 - 7 scale where 0 = plant dead, 4 = 50% growth reduction, 7 = as healthiest control. Plant height was measured in June and shoot number, height and weight per plot recorded in the following January.

The second experiment was carried out at a field site with sandy loam soil at Claverham, Bristol. Land was ploughed in autumn 1991, cultivated in early 1992 and 25 cm long cuttings planted by hand at the end of March. Rows of poplar c.v. Beaupre, willow c.v. Bowles Hybrid and a discard row of poplar c.v. Fritzi-Pauley were planted alternately 0.5 m apart across the experiment, with cuttings spaced at 0.5 m along the rows. Plots consisted of single rows of 22 plants of each cultivar, and results were recorded from the central 18 plants. There were three replicates of each treatment; the experiment was set out in a randomized block design. The area received an overall spray of simazine at 1 kg a.i./ha 4 days after planting. The herbicides used were clopyralid as 'Dow Shield' at 0.22 (Dose 1) and 0.66 (Dose 2) kg a.e./ha; cyanazine as 'Fortrol' at 1.0 and 3.0 kg a.i./ha; clopyralid + cyanazine as 'Coupler' at 0.41 and 1.23 a.i./ha; metazachlor as 'Butisan S' at 1.25 and 3.75 kg a.i./ha and pendimethalin as 'Stomp 400' at 2.0 and 6.0 kg a.i./ha. The mixtures used are listed in Table 2. The herbicides were applied using an Oxford Precision Sprayer fitted with two flat fan Spraying Systems 8003 jets, at a pressure of 210 kPa giving a spray volume of 354 litres/ha. The first set of herbicide treatments was sprayed on 22 April. At the time of spraying the willows had two to three shoots per plant, 2-3 cm high; 50% of the poplars were dormant, the rest had one to two shoots per plant, 1-4 cm long. The second set of treatments was sprayed on the 18 May; the willow shoots were 15-20 cm long and the poplars 5-10 cm long.

Crop vigour and growth were assessed visually using the 0 - 7 scale. Maximum shoot height per plant was measured in July. The plots were harvested at the end of January 1993 and fresh weights of shoots were recorded on 1 February. Many plants, particularly of willow, had branched at 5 to 15 cm height giving shoots with four to six upright branches. The number of stems with this type of branching was recorded in January.

TABLE 1. Effect of herbicide mixtures on plant health and shoot weight.

		Visual health score 17/6/91				Fresh weight (kg/plot) Nov 91			
		Poplar		Willow		Poplar		Willow	
Herbicide	Dose	Fritzi*	Boelare	Bowles*	Dasycl*	Fritzi	Boelare	Bowles	Dasycl
atrazine +	1	5.70	5.50	5.93	6.18	1.31	1.38	2.12	1.84
cyanazine	2	5.03	5.43	6.23	5.95	0.81	0.85	1.45	1.33
metazachlor +	1	5.60	4.93	5.95	6.18	1.66	1.18	1.7	1.59
pendimethalin	2	5.63	4.95	5.73	5.95	1.22	1.38	1.88	1.35
metazachlor +	1	6.05	5.25	5.93	6.15	1.19	1.26	1.63	1.33
propyzamide	2	5.68	4.75	5.95	6.25	1.35	1.06	1.52	1.72
metazachlor +	1	5.48	4.98	5.95	6.53	1.21	1.08	2.01	1.60
metamitron	2	5.30	5.25	6.25	5.58	1.21	1.15	1.79	1.55
simazine +	1	5.38	5.23	5.98	6.10	1.28	1.23	1.98	1.34
propyzamide	2	5.38	5.28	6.13	6.25	1.26	1.18	1.42	1.43
isoxaben +	1	6.08	5.40	5.35	6.15	1.49	1.43	1.81	1.36
propyzamide	2	5.40	4.85	6.08	6.25	1.38	1.43	2.07	1.53
simazine +	1	6.13	5.63	6.08	6.60	1.36	1.27	1.98	1.61
napropamide	2	5.88	5.28	5.55	6.55	1.51	1.33	1.90	1.58
simazine +	1	6.18	5.25	5.80	6.00	1.21	1.19	2.00	1.60
prop + metaz$	2	5.20	4.75	5.95	5.85	1.55	1.41	1.52	1.37
CONTROL (hoed)		5.80	5.23	5.88	6.06	1.15	0.88	1.45	1.08
SED control v treated			0.704				0.388		

* Fritzi = Fritzi-Pauley, Bowles = Bowles Hybrid, Dasycl = x *dasyclados* (Wimm.)
$ prop + metaz = propyzamide + metazachlor

TABLE 2. Effect of herbicide treatments on plant health, shoot height and weight.

			Visual health score				Height (cm)		Fresh weight	
			21/5/92		25/6/92		30/7/92		(kg/plot)	
Date	Herbicide	Dose	poplar	willow	poplar	willow	poplar	willow	poplar	willow
April	metazachlor	1	6.7	7.0	6.0	5.7	137	158	7.98	7.34
		2	7.0	6.0	6.3	5.7	129	150	8.31	6.14
	pendimeth-	1	6.3	6.3	6.0	5.7	131	164	8.17	7.58
	alin	2	5.3	6.7	5.3	6.0	125	153	7.84	7.14
	cyanazine	1	7.0	6.3	5.7	5.3	128	149	8.64	6.69
		2	6.0	5.7	5.3	5.3	109	145	6.44	6.33
	clopyralid	1	7.0	7.0	6.0	6.0	125	159	7.34	7.13
		2	6.0	6.0	6.3	6.0	130	164	8.54	6.94
	metazachlor	1	6.3	6.7	6.7	6.7	145	175	8.60	8.35
	+ pendim*	2	4.3	4.7	5.3	5.7	121	156	8.40	7.60
	clopyralid	1	7.0	7.0	6.3	6.3	130	167	8.06	8.05
	+cyanazine	2	6.3	6.7	5.7	6.3	126	172	7.44	9.12
	cyanazine	1	6.3	6.3	5.3	5.7	127	147	7.43	5.86
	+ pendim	2	5.3	5.3	5.7	5.7	116	144	6.85	6.48
May	metazachlor	1	7.0	7.0	7.0	6.0	139	166	9.62	6.87
		2	7.0	7.0	5.0	5.0	116	159	6.60	7.42
	pendimetha-	1	6.0	7.0	4.3	5.3	100	149	5.14	7.57
	lin	2	6.0	6.7	4.3	5.0	108	146	5.93	6.37
	cyanazine	1	6.3	6.7	5.0	4.7	110	159	5.45	7.18
		2	6.7	6.7	3.7	4.3	85	127	4.08	4.17
	clopyralid	1	5.0	5.7	6.3	6.0	139	158	8.22	7.33
		2	5.0	5.0	5.0	6.0	120	163	6.74	7.13
	metazachlor	1	6.3	7.0	5.0	5.3	124	154	7.00	6.95
	+ pendim	2	6.0	6.0	4.3	4.3	117	143	6.52	6.26
	clopyralid	1	6.0	6.7	5.0	6.0	114	161	5.87	7.62
	+cyanazine	2	5.7	6.0	4.0	4.3	98	131	4.53	5.40
	cyanazine	1	5.0	6.0	3.3	4.0	72	115	2.91	4.36
	+ pendim	2	4.0	5.0	2.3	2.7	53	83	2.01	3.28
	CONTROL		7.0	7.0	5.0	6.0	117	163	7.06	7.55
SED	control v treated		0.32		0.52		11.8		1.17	
	treated v treated		0.37		0.60		13.6		1.35	

* pendim = pendimethalin

RESULTS

In the first experiment no adverse effects of the herbicide treatments on crop development were observed during the growing season or on weight of shoots recorded in January (Table 1). Shoot height measured in June and January was also unaffected by the herbicide treatments (data not shown). Yield on most herbicide treated plots was greater than on the hoed control.

In the second experiment metazachlor applications resulted in no adverse effects on either poplar or willow (Table 2). Pendimethalin caused slight stunting of shoot growth, particularly with May applications but there was no long term effect on growth. Cyanazine applied in both April and May caused leaf chlorosis and necrosis particularly the high dose applied in May. This treatment resulted in appreciable reduction in final shoot weight of both species. Clopyralid applied in May caused temporary shoot and leaf distortion on both species but effects were soon outgrown and there were no effects on final shoot weights. Mixtures of the higher doses of metazachlor and pendimethalin caused some stunting of growth of both species during spring but there were no reductions in final shoot weight. Application of clopyralid + cyanazine in April had no adverse effects but the May application led to vigour reduction and, with the higher doses, reduction in final shoot weight. Cyanazine + pendimethalin applied at both dates caused stunting of growth but there was no final growth reduction with the April treatment. Shoot weight of poplar and willow was reduced by the May applications, especially with the higher doses. There was some branching of stems at 5 to 15 cm above ground level on some plants on all plots, particularly willows; numbers of branched shoots were significantly greater ($p = 0.05$) with clopyralid and clopyralid + cyanazine treatments on willow (data not shown).

DISCUSSION

The appreciably greater growth on most herbicide treatments compared with the control was probably due to weed competition on the untreated plots early in the season. The first hoeing was delayed until late May by which time weed cover was about 40% (data not shown). The absence of adverse effects from the post-planting application of the residual herbicide mixtures confirmed results from the previous 2 years experiments at Long Ashton (Parfitt *et al* 1992) and at other sites (Williamson *et al* 1992). However in the 3 years in which these experiments were done rainfall in the month before and after spraying was near or below average. It is well established that heavy rain after applying some residual herbicides such as simazine can lead to crop damage particularly on light soils (Clay 1983). On other sites applications of simazine to newly-planted poplar and willow cuttings in 1993 when heavy rain occurred in the month after spraying did lead to some leaf damage later in the year but not to severe growth reductions (Clay, unpublished data). There is clearly a need for more information on the margin of tolerance of newly-planted crops to these herbicides in light soils.

The results of the experiment with applications to developing shoots showed that most treatments were tolerated at recommended doses. It is useful if herbicides applied at that time will control the seedling weeds likely to be present. Clopyralid, cyanazine and

metazachlor have post-emergence activity on some weed species. The results suggest that applications of single herbicides to growing shoots in spring may not have adverse effects on crop growth and that mixtures of the herbicides may be safe where shoots are less than 5 cm long. In further work mixtures of clopyralid with other herbicides were safe on young poplar and willow, but metazachlor caused some stunting of shoots (Clay & Dixon 1993). Shoot branching above 5 cm height can be caused by damage to the terminal bud from factors such as frost or insect attack. In the second experiment this effect was very variable but clopyralid appeared to cause branching of willow shoots. The branching was not particularly linked with growth reductions so effects on productivity may not be important although it could affect the quantity of material available if required for propagation. However the effect would not be reason to avoid the treatment given serious infestations of susceptible weeds such as *Cirsium arvense* (creeping thistle).

Cyanazine, clopyralid, isoxaben, metazachlor and pendimethalin products now have MAFF Off-label Approval for use in coppice in the U.K. (Ivens 1993), thus providing growers with a useful range of herbicides for post-planting weed control.

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Session 6B

The Biochemical Targets of Herbicide Action

Chairman	Dr K WRIGHT
Session Organiser	Dr K E PALLETT
Papers	6B-1 to 6B-5

PROTOPORPHYRINOGEN OXIDASE THE MOLECULAR TARGET SITE FOR PEROXIDIZING HERBICIDES.

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ABSTRACT

Protoporphyrinogen oxidase is the target enzyme for several classes of peroxidizing herbicides. This membrane-bound enzyme catalyses the last common step of the chlorophyll and heme biosynthetic pathways, the oxidation of protoporphyrinogen IX into protoporphyrin IX. Its inhibition leads to the *in vivo* accumulation of protoporphyrin IX the product of the reaction, a very potent photodynamic tetrapyrrole molecule which is responsible for the light-dependent phytotoxicity of these herbicides. The role and molecular properties of this enzyme, and the consequences of its inhibition will be considered. We will also discuss the reason why several classes of chemistry inhibit this enzyme and what the prospects are for further biorational design of new peroxidizing molecules.

INTRODUCTION.

Peroxidizing herbicides like diphenyl ethers, exert their phytotoxicity through a light-dependent mechanism leading to peroxidative degradation of cellular constituents, especially membrane lipids (Matsunaka, 1969; Orr and Hess, 1982). In fact in treated tissues, these molecules cause an accumulation of protoporphyrin IX, a very potent photodynamic tetrapyrrole

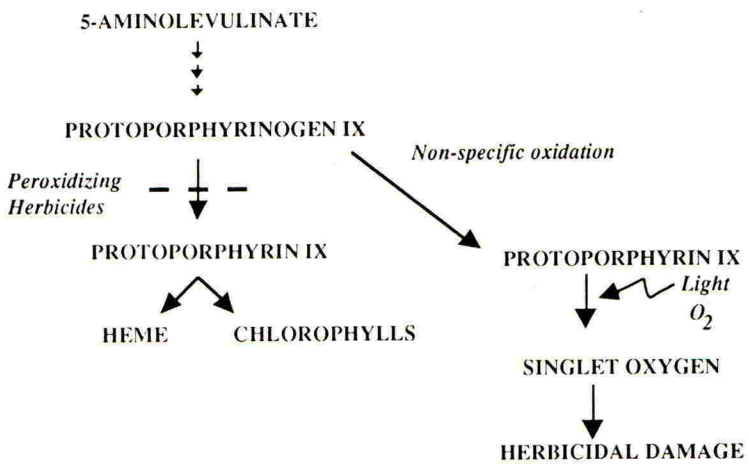


FIGURE 1 : Scheme of the light-dependent mode of action of peroxidizing herbicides.

molecule, which in the presence of light and oxygen gives rise to singlet oxygen (see Scalla and Matringe 1993). This abnormal accumulation of protoporphyrin IX was shown to be the consequence of the inhibition of protoporphyrinogen oxidase by these herbicides (Matringe *et al.*,1989; Witkowski and Halling, 1989), (Figure. 1).

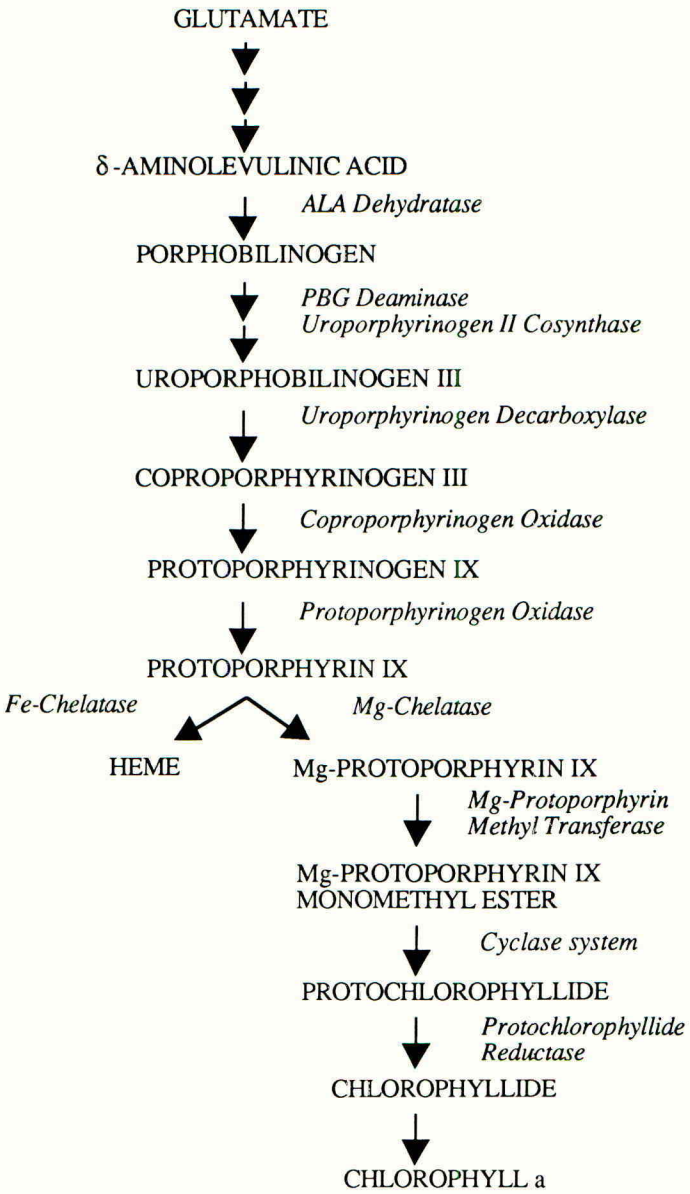


FIGURE 2 : Biosynthetic pathway of chlorophylls and heme.

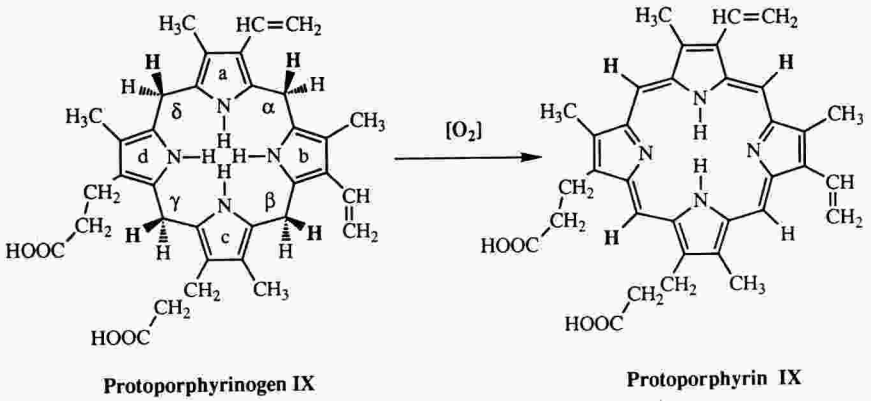
PROTOPORPHYRINOGEN OXIDASE.

Protoporphyrinogen oxidase (EC 1.3.3.4.) is a membrane-bound enzyme which catalyses the last common step of the heme and chlorophyll pathways (Figure 2), i.e. the six-electrons aromatic oxidation of protoporphyrinogen IX into protoporphyrin IX (Figure 3)

Although chemical oxidation of protoporphyrinogen IX into protoporphyrin IX was known to occur spontaneously at neutral pH, Sano and Granick (1961) and Porra and Falk (1964) were the first to propose the existence of an enzyme able to catalyze that oxidation. The existence of this enzyme was confirmed by its partial purification by Poulson and Polgase (1975). This reaction requires molecular oxygen as an electron acceptor in eukaryotic cells. In non-plant cells protoporphyrinogen oxidase is associated with the mitochondrial inner membrane. It has been purified to apparent homogeneity from bovine liver (Siepker *et al.*, 1987) and mouse liver (Dailey and Karr, 1987). It was found to be a monomer with a molecular weight of approximately 65,000 Dalton. Both enzymes were reported to be flavoproteins. The bovine enzyme contains a tightly bound FAD molecule and the mouse enzyme a FMN molecule (Siepker *et al.* 1987; Dailey, 1990). Concerning the plant enzyme, the situation is rather complicated since protoporphyrinogen oxidase has a dual subcellular localization (Jacobs and Jacobs, 1987; Matringe *et al.*, 1989). It is associated with the chloroplast, where it is involved in chlorophyll and plastidic heme synthesis, and with the mitochondria, where it is involved in the synthesis of the non-plastidic hemes. Partial purification of the plant enzyme from barley mitochondria and etioplasts was reported by Jacobs and Jacobs (1987). They found a 35,000 Dalton polypeptides in both cases with very similar properties. However, yeast and lettuce etiochloroplast enzymes were recently purified to apparent homogeneity as 55,000 Dalton polypeptides (Camadro *et al.* in preparation). Both appear as flavoproteins differing by the nature of the flavin associated with the protein. The yeast enzyme contains a FAD and the plant enzyme a FMN molecule. The lettuce enzyme has a pI of 6.5 and a specific activity of 9300 nmol protoporphyrinogen IX oxidized/h/mg protein. The Km for protoporphyrinogen was 0.3 µM (Camadro *et al.* in preparation).

MECHANISM OF ACTION.

At the present time, there is insufficient data to propose a model for catalyses. However, the occurence of a flavin at the active site of protoporphyrinogen oxidase may explain some catalytic properties of the enzyme. Jones *et al.* (1984), have studied the oxidation of protoporphyrinogen stereospecifically tritiated at the methylene bridges. These authors found



Protoporphyrinogen IX **Protoporphyrin IX**

FIGURE 3: Reaction catalysed by protoporphyrinogen oxidase : transformation of protoporphyrinogen IX into protoporphyrin IX.

that three hydrogen atoms from the methylene bridges are removed from one side of protoporphyrinogen while the fourth one is removed from the other side of the cyclic tetrapyrrole. Furthermore, the protons from bridges α and β (Figure 3) appeared to be removed through two distinct mechanisms. A model was proposed for protoporphyrinogen oxidation involving the removing of three hydrides and one proton. Thus, it is reasonable to suggest that while the flavin may be involved in hydride removal, another functional group on the protein, possibly a basic amino-acid residue may be involved in the fourth proton removal.

ACCUMULATION OF PROTOPORPHYRIN IX.

Surprisingly, *in vivo* inhibition of protoporphyrinogen oxidase by peroxidizing molecules leads to a massive accumulation of protoporphyrin IX which is normally produced by the enzymatical reaction. A similar protoporphyrin IX accumulation is described to be associated with a deficiency of protoporphyrinogen oxidase activity in the case of the human desease *Variegate porphyria* (Brenner and Bloomer, 1980). In this later case, it is generally assumed that unprocessed protoporphyrinogen IX molecules which accumulate as a consequence of the inhibition, diffuse out of their site of synthesis and react non-enzymatically with molecular oxygen to give protoporphyrin IX. This protoporphyrin IX is proposed to be sequestered in membrane structure devoid of chelatase activities, which would explain why, although functional, these chelatases seems unable to further metabolize the accumulating protoporphyrin IX. In agreement with this hypothesis, Lehnen *et al.* (1990), have shown that in diphenyl ether treated yellow plants, protoporphyrin IX accumulates outside the plastid, mainly in the plasmalemma. However, Jacobs *et al.* (1991), have shown that in intact chloroplasts treated by diphenyl ethers, protoporphyrinogen IX effectively accumulates and diffuses out of the chloroplasts, but this protoporphyrinogen IX was oxidized into protoporphyrin IX only in the presence of an enriched plasmalemma membrane fraction, by a diphenyl ether insensitive reaction. It seems that this oxidation occurs via a non-specific, diphenyl ether-insensitive oxidase associated with the plasmalemma, which could accept other porphyrinogen substrates like uro- and coproporphyrinogen (Lee *et al.* ,1993).

This observation raises several questions. How could protoporphyrinogen molecules leave the chloroplast? Is it a simple diffusion due to their accumulation, or a specific transport which have a physiological significance? The precise localization of protoporphyrinogen oxidase within the chloroplast revealed that this enzyme is associated with both membrane systems of the chloroplast *i.e.* the envelope and the thylakoids (Matringe *et al.*, 1992a). It's association with the envelope make the diffusion likely. However, some characteristics of the plant tetrapyrrole biosynthesis pathway would make the specific transport of protoporphyrinogen through the envelope physiologically significant. In fact although, mitochondria are able to perform their own heme synthesis since they possess protoporphyrinogen oxidase and ferrochelatase activities (Jacobs and Jacobs, 1987; Matringe *et al.*, 1989; Little and Jones, 1976), they seem unable to synthesize any δ-aminolevulinic acid (ALA) as they are apparently devoid of ALA synthetase (see Beale and Weistein, 1990). All the plant ALA is thus synthesized within the chloroplast from glutamate, and mitochondria must import a tetrapyrrole precursor for their own heme synthesis. The nature of the imported precursor remains to be determined. It could be protoporphyrinogen if in plant cells, contrary to non-plant cells, the cytosol is devoid of any soluble enzymatical steps involved in the transformation of ALA into protoporphyrinogen. However, the instability of protoporphyrinogen and the occurrence of these non-specific oxidase activities which give rise to the highly toxic protoporphyrin IX make that hypothetical mechanism rather unsafe for the plant.

It must also be noted that although protoporphyrinogen oxidase associated with the mitochondria is inhibited by these peroxidizing molecules, its role in protoporphyrin IX accumulation remain to be clarified.

ACTION OF PEROXIDIZING HERBICIDES ON PROTOPORPHYRINOGEN OXIDASE.

Protoporphyrinogen oxidase inhibition seems to be a general property of all the peroxidizing molecule inducing *in vivo* protoporphyrin IX accumulation tested so far *i.e.* diphenyl ethers, pyridine derivatives, pyrazoles derivatives, oxadiazoles, phenyl-pyrazoles, and N-Phenyl imides (Figure 4). The most active of these molecules *in vivo* are very potent

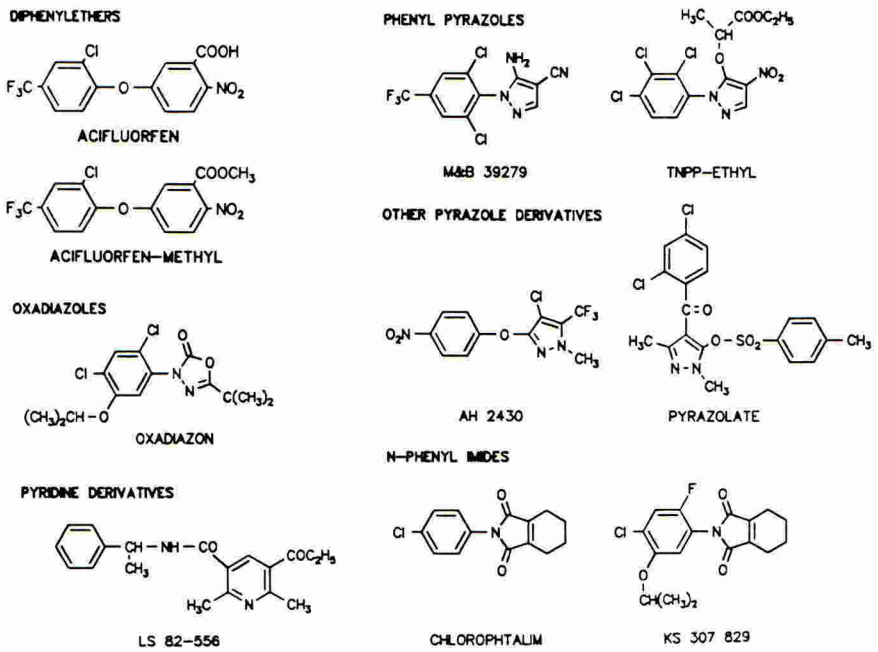


FIGURE 4 : Principal chemical families of protoporphyrinogen oxidase inhibitors.

inhibitors of protoporphyrinogen oxidase since they have I_{50} values on the nanomolar concentration range. In some cases, the inhibition of protoporphyrinogen oxidase seems dependent on the assay conditions. Jacobs *et al.* (1990) reported that barley protoporphyrinogen oxidase activities are much less sensitive to acifluorfen-methyl when glutathione was used in the reaction medium instead of dithiothreitol, and that in their conditions, the partially purified enzyme lost its sensitivity toward acifluorfen-methyl. In our assay conditions replacing dithiothreitol by glutathione did not change the sensitivity of protoporphyrinogen oxidase activities toward diphenyl ethers. Moreover, from our own observation, protoporphyrinogen oxidase from lettuce etiochloroplast purified to apparent homogeneity has similar sensitivity toward several peroxidizing molecules than the crude enzyme from corn etioplasts. Protoporphyrinogen oxidase appears thus to be the primary target of peroxidizing molecules.

Concerning the reversibility of the inhibition confusing results have been reported. Jacobs *et al.* (1990) found that barley protoporphyrinogen oxidase inhibition by acifluofen-methyl in presence of dithiothreitol could not be restored after dialysis and these authors suggested an irreversible binding of the inhibitors. Others (Nicolaus *et al.*, 1993) found a reversible inhibition of oxyfluorfen on corn etioplast protoporphyrinogen oxidase. By examining the question with binding experiments of a tritiated acifluorfen molecule we have shown that the binding of acifluorfen to pea and corn etioplast enzyme is totally reversible (Matringe *et al.*, 1992b).

In order to obtain an insight into the enzyme-inhibitors interaction the nature of the inhibition has been examined. It was shown that diphenyl ethers and N-phenyl imides exert a

competitive inhibition with respect to the substrate protoporphyrinogen on plant enzymes (Camadro *et al.*, 1991; Nicolaus *et al.*, 1993). The Ki values of this inhibition were found to be below nanomolar concentrations indicating a tight binding affinity of these inhibitors to the target site of the enzyme. The possibility for these molecules to share a common binding region on the enzyme was examined by binding experiments with a tritiated acifluorfen molecule on corn and pea etioplasts (Varsano *et al.*, 1990; Matringe *et al.*, 1992b). These studies revealed that acifluorfen binds to a single class of high affinity binding sites. The apparent Kd was found to be around 6-12 nM and the number of binding sites around 10 to 15 pmol/mg protein for corn etioplasts and around 30 pmol/mg of protein for pea etioplasts. Binding equilibrium was reached in about 1 min and was found totally reversible. All the other peroxidizing molecules tested *i.e.* oxadiazon, the pyrazole derivative M&B 39279 the pyridine derivative LS 82556, and the N-phenyl imide KS 307829 were found to inhibit the binding of tritiated acifluorfen. This was competitive for the first three inhibitors, indicating that their binding are totally exclusive, and for the later (KS 307829) inhibition was mixed competitive, indicating that its binding and that of acifluorfen are not totally exclusive, and that their binding sites are overlapping. Protoporphyrinogen IX the substrate of the enzyme was able to competitively inhibit the binding of acifluorfen in contrast to protoporphyrin IX the product which was found unable to prevent acifluorfen binding. This is in total agreement with the kinetic studies revealing a competitive inhibition with respect to the substrate, and confirm that the binding niche of all these peroxidizing molecules overlaps in some way the catalytic site of the enzyme. All these results give essential information for understanding enzyme/inhibitor relationships, and raise the question of the nature of the binding niche which could accept so many different chemical structures with a such high affinity.

WHY PROTOPORPHYRINOGEN OXIDASE IS AN EFFECTIVE TARGET?

The efficiency of protoporphyrinogen oxidase as a herbicide target can originate from several reasons and we could only postulate some of them. Firstly, as for many other herbicide targets which are membrane-bound enzymes, inhibition gives rise to peroxidative reactions. This reactions occur mainly at the level of membrane lipids, and could be propagated auto-catalytically which make them very toxic. Furthermore, protoporphyrin IX is a very potent singlet oxygen generator and the fact that *in vivo* its accumulation occurs outside the chloroplast is of central importance for its toxicity. The chloroplast contains many free radical scavengers and antioxidant systems such as carotenoids, ascorbic acid, glutathione, α-tocopherol...etc. Damage is induced in cells when the scavenging systems become overloaded. One thus could speculate that if protoporphyrin IX accumulation could occur inside the chloroplast a high accumulation would be needed to overcome the chloroplastidic pool of anti-oxidative systems. Since the oxidation of protoporphyrinogen IX into protoporphyrin IX occurs in the plasmalemma, much less protoporphyrin IX is needed to overload the antioxidative system present at this level. Moreover, plasmalemma is devoid of chelatase activity and thus protoporphyrin IX cannot immediately re-enter the tetrapyrrole pathway. It could be assumed that as in mammalian heme synthesis (see Dailey, 1990), a substrate channeling occurs for the conversion of protoporphyrinogen IX into protoporphyrin IX, thus only after a sufficient build-up of protoporphyrin IX concentration outside its normal metabolic channel will it re-enter the pathway. Further more, all the inhibitors are competitive with respect to the substrate and their inhibiton is reversible, thus, if protoporphyrinogen IX accumulates within the plastid, the effectiveness of enzyme inhibition would be reduced.

The regulation of the tetrapyrrole pathway in plant also could have some importance. Heme is known to feedback inhibit this pathway and protoporphyrin IX is required for heme synthesis. Therefore, in treated tissues heme level decrease and its feedback inhibition no longer occur. The carbons flow into the pathway increases and that could explain the very rapid and high level of protoporphyrin IX accumulation observed in treated plants.

The effectiveness of protoporphyrinogen oxidase as a target site is further highlighted by the absence, to date, of any resistance development, in spite of its long-term use in rice and soya crops. The existence of three different pools of enzyme (chloroplast envelope and thylakoid and the inner membrane of the mitochondria) and possibly several genes for protoporphyrinogen oxidase may have contributed to this absence of resistance. In addition, and probably more likely, the fact that all inhibitors tested to date are competitive with respect to substrate, mutations leading to resistance are likely to be lethal due to a less efficient enzyme.

Finally, protoporphyrinogen oxidase is a very low abundant protein and most of the inhibitors have a high affinity for this enzyme, therefore, these herbicides have the potential to be used at low doses in the field.

RELATIONSHIP OF CHEMICAL STRUCTURES TO INHIBITION OF PROTOPORPHYRINOGEN OXYDASE.

Only a few QSAR studies have been carried out to date using different series of molecules belonging to the same chemical family. In all these studies it appears that the lipophilicity plays an important role in the activity of the inhibitors (Nandihalli *et al.*, 1992; Nicolaus *et al.*, 1993). One could postulate that the membrane-bound nature of the target enzyme explain this lypophilicity requirement. It was repeatedly noted, for example, that acifluorfen which is a water soluble molecule is about 100 times less active *in vitro* than its methyl ester acifluorfen-methyl (Camadro *et al.*, 1991; Nandihalli *et al.*, 1992). However it is interesting to point out that this is true for plant and mouse enzyme but not in the case of the yeast (*Saccharomyces cerevisiae*) enzyme where acifluorfen and acifluorfen-methyl have similar I_{50} and Ki (Camadro *et al.*, 1991). It is possible that this discrepency originates from a different architectural organization of tetrapyrrole biosynthetic pathway in this yeast strain. In fact coproporphyrinogen oxidase, the previous enzyme, is localized in the cytosol and not in the mitochondrial inter-membrane space, loosely associated with the inner membrane, as it is in mamalian cells (see Dailey, 1990). Therefore the channeling of protoporphyrinogen from coproporphyrinogen oxidase to protoporphyrinogen oxidase in the lypophilic environment of the mitochondrial inner-membrane does not exist in this yeast strain, and the active site of its protoporphyrinogen oxidase could thus have a different orientation.

The steric effects which should reflect the architecture of the binding site have also been examined. Nandihalli *et al.* (1992), have compared the molecular and electronic properties of acifluorfen and protoporphyrinogen derived from semiempirical molecular orbital calculation. They have found that the maximum length and width axis of acifluorfen matched closely with the full length and one-half width of protoporphyrinogen molecule. The torsion angle of acifluorfen at the ether oxygen matched with the angle at the methylene bridge between two neighboring pyrrole rings. Their results also show that to be active, a molecule must be bicyclic and coplanar. Kohno *et al.* (1993) found that the X-ray structure and the molecular orbital calculation of some peroxidizing molecules have about the same size and match closely with the full length of protoporphyrinogen although these molecules have different chemical structures. Their results indicate also that the torsion angle between the two rings of these molecules have some influence on their phytotoxicity. Akagi and Sakashita (1993) have compared the quantum chemical calculation of different herbicidal compounds acting on different target enzymes, and found that the LUMO level of protoporphyrinogen oxidase inhibitors were similar and strikingly low. They thus postulate a role for LUMO in the activity of these compounds. By molecular field fitting they found an overall molecular similarity between oxyfluorfen (a diphenyl ether) and chlorophthalim (a N-Phenyl imide derivative) and a good LUMO and sterical features correspondance. From their results these authors suggest that mixed charge transfer and electrostatic interactions play a role in the binding of these compounds. This is in good agreement with what one could postulate on the functional mechanism of protoporphyrinogen oxidase.

FURTHER PROSPECTS.

Much more work are needed in order to understand the topography of the binding pocket of these inhibitors. As revealed by the studies of the topography of the D1 binding niche of PSII inhbitors, the availability of the amino acid sequence of the protein and of herbicide-resistant mutants provided inestimable information. At the present time, herbicidal-resistant mutant strain of *Chlamydomonas reinhardtii* (RS-3) has been selected and the mutation was reported to possibly be at the protoporphyrinogen oxidase level (Oshio *et al.*, 1993), this mutant strain will be used for molecular cloning of the resistant gene. A yeast mutant (CG122-6C) deficient in protoporphyrinogen oxidase activity was also described (Camadro *et al.*, 1982). It appears from immunological studies that this mutant strain contains an inactive protein of the same size and abundance as a wild type strain. This mutation not only abolished the catalytic activity of the enzyme but also prevented any binding of radiolabeled acifluorfen (Camadro *et al.*, in preparation). This may be due to a modification of the active site of the enzyme. This protoporphyrinogen oxidase deficient mutant is thus an invaluable tool for further studies on the enzyme structure and functions. It will also be used for molecular cloning of the yeast and plant gene by complementation techniques. Furthermore, the availability of purified yeast and plant enzyme, together with that of a photoaffinity-tritiated analog of acifluorfen pave the way for probing the binding site of these inhibitors through peptide mapping of the purified radiolabeled proteins.

All these further studies will provided a clearer view of the enzyme-inhibitor interactions which could be used for a biorational design of new inhibitors of protoporphyrinogen oxidase.

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PHYTOENE DESATURASE: A BIOCHEMICAL TARGET OF MANY BLEACHING HERBICIDES

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ABSTRACT

Inhibition of carotenoid biosynthesis, particularly interference with the enzyme phytoene desaturase (pds) has been known for many years as a target site of several successful commercial herbicides. There is a structural diversity of many of these herbicides and other known inhibitors, however at the present time we have only a limited knowledge of inhibitory mechanisms and indeed of the exact reaction mechanism of pds itself. This is due to several factors including the plastid membrane location of pds, its relatively low abundance and highly lipophilic nature of the substrate, which together provide extreme technical difficulties for its study.
However, recent developments in the molecular biology of carotenogenesis may overcome some of these difficulties and we can expect progress in understanding why so many diverse inhibitors are targeted towards pds. The aim of this review is to discuss the current knowledge of the pds reaction, the possible mechanism of inhibition, its consequences in the plant and the prospects for the design of new bleaching herbicides.

INTRODUCTION

The carotenoids are one of the most abundant groups of pigments in nature. They are present in all green tissues, as components of the chloroplasts, as well as being responsible for many of the yellow to red colours of fruits and flowers.

The inhibition of carotenoid biosynthesis has been studied for more than 40 years. The earliest investigations, using compounds that caused the accumulation of less unsaturated carotenoids, particularly phytoene, were aimed at elucidating the pathway. Since then a massive number of compounds that can act as bleaching herbicides have been discovered. There has been a substantial effort to understand the molecular mechanisms of inhibition and, as a result, design new compounds that are both specific and potent inhibitors of carotenogenesis.

At the present time, the inhibition of carotenoid biosynthesis, particularly by inhibitors of phytoene desaturase (pds), is considered to be a favourable target for herbicides for a variety of reasons. It is a plant specific target which gives potential toxicological benefits; it is predominately a meristematic target; it is associated with relatively low dose rates; and to date there is no evidence of the development of target site resistance in the field.

The aim of this review is to discuss our current knowledge of inhibitors of the phytoene desaturase (pds) reaction, the possible mechanisms of inhibition, its consequences in the plant and prospects for the design of new bleaching herbicides.

THE FUNCTIONS OF CAROTENOIDS IN HIGHER PLANTS

Carotenoids play vital roles in photosynthetic tissues. Probably their main function is the photoprotection of the photosynthetic apparatus. Unsaturated carotenoid molecules are able to quench both triplet chlorophyll and singlet oxygen. The former is produced under high light intensity, while the latter is formed by the transfer of energy from triplet chlorophyll to oxygen. Singlet oxygen is de-excited by carotenoids, thus preventing the accumulation of a highly reactive species of oxygen which causes rapid membrane peroxidation. All carotenoids with 9 or more conjugated double bonds are protective, but those with less, eg phytoene are ineffective (Foote *et al.*, 1970). Carotenoids are also accessory light-harvesting pigments (Frank and Codgell, 1993), precursors of the phytohormone abscisic acid and enable thylakoids to form partitions (Dahlin *et al.*, 1983). Therefore any compounds that alter the carotenoid content of the plastid, and particularly cause the accumulation of colourless carotenoids, will cause severe and usually lethal effects on the plant.

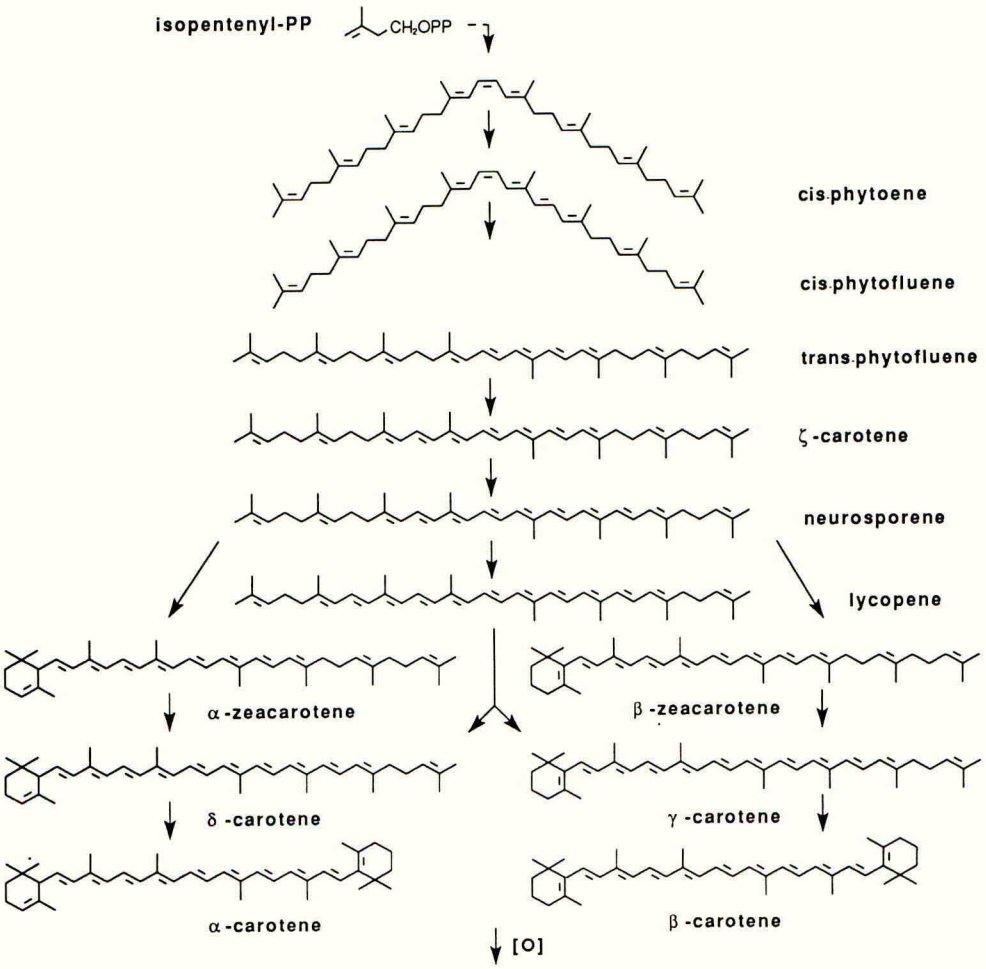
THE CAROTENOID BIOSYNTHETIC PATHWAY AND PROPERTIES OF PHYTOENE DESATURASE

All carotenoids are derived from the isoprenoid pathway, via isopentenyl pyrophosphate (Fig.1). The first reaction specific to carotenoid formation is the biosynthesis of phytoene from two molecules of geranylgeranyl pyrophosphate. In higher plants, the 15-Z isomer of phytoene is formed. The desaturation of phytoene occurs by a stepwise sequence of reactions to form phytofluene, ζ-carotene, neurosporene and lycopene. At each stage two *ANTI*-hydrogen atoms from adjacent functions are lost by oxidation to extend the chromophore by two double bonds (Fig. 1) and an isomerization of the 15-Z to the all-E isomer occurs at phytofluene stage in higher plants. Recent successes in the cloning of carotenoid genes from higher plants and cyanobacteria have now shown that two enzymes, encoded by different nuclear genes, are involved in the desaturation pathway. Pds catalyses the conversion of phytoene into ζ-carotene, whilst ζ-carotene desaturase (zds) converts ζ-carotene into lycopene (reviewed by Sandmann, 1991).

Cyclic carotenoids such as β-carotene are formed from the desaturated acyclic precursors such as lycopene (Fig. 1) and xanthophylls are formed by the introduction of oxygen functions such as hydroxy-and keto-groups.

Several crude cell-free preparations contain pds activity (reviewed by Bramley, 1985). Detailed studies on the desaturation sequence by daffodil chromoplast membranes have shown the reaction to require at least a terminal electron acceptor (oxygen) in addition to the individual enzymes (Mayer *et al.*, 1990). Other redox carriers could be used *in vitro* (Mayer *et al.*, 1992). Two segments of the desaturation pathway were found; one involving the conversion of 15-Z phytoene into 15-Z-ζ-carotene and requiring darkness and oxygen, whilst the second required a light-induced isomerization of the ζ-carotene (Beyer *et al.*, 1989). More recently, the phytoene desaturase of pepper chromoplasts has been purified and found to be a flavoprotein (Hugueney *et al.*, 1992). No necessity for darkness was established in this case. It is not yet known whether the pds in chloroplasts has similar properties to that in chromoplasts. No biochemical data have been reported on the higher plant ζ-carotene desaturase.

FIGURE 1.: An outline of the carotenoid biosynthetic pathway



THE INTRACELLULAR LOCATION OF CAROTENOIDS AND CAROTENOGENIC ENZYMES

Carotenoids are found within chloroplasts as part of the pigment-protein complexes in thylakoid membranes: but are also located in the chloroplast envelope (reviewed by Pallett and Young, 1993). The early enzymes up to the formation of phytoene are located in the stroma, or loosely bound to the thylakoid membranes (Kreuz *et al.*, 1982), but phytoene desaturase is membrane bound in chromoplasts (Kreuz *et al.*, 1982) and chloroplasts (Lutke-Brinkhaus *et al.*, 1983). A more recent study on the location of pds in spinach chloroplasts has found activity in both thylakoid and envelope fractions (Holford *et al.*, 1993).

TABLE 1: Possible target sites for inhibitors of phytoene desaturase

1.	Binding to the active site of the enzyme
2.	Binding to other domains of the enzyme
3.	Inhibition of pds gene expression
4.	Affecting the association of a multi-enzyme complex, eg phytoene synthase with pds
5.	Affecting membrane organisation
6.	Inhibition of electron transport associated with pds
7.	Affecting cofactor availability
8.	Prevention of targetting to the plastid or intraplastid location
9.	Affecting the carrier proteins involved in substrate transfer
10.	Inhibition of post-translational modifications

MECHANISMS OF INHIBITION OF PHYTOENE DESATURATION

A number of different, but often complementary techniques are used to study the inhibition of carotenoid biosynthesis. In general, they aim to pinpoint target sites, to discover which functional groups are prerequisites for inhibitor activity and to establish the physiological effects that result from the inhibition of carotenoid formation. A wide range of organisms has been used in such studies, as described by Bramley (1993).

As discussed in the previous section, a multitude of herbicides cause the accumulation of phytoene in plants cells. However, it should not be assumed that this is solely due to the inhibition of pds activity. Several other target sites for the molecules exist in the plant cell, as listed in Table 1, which would interfere with pds.

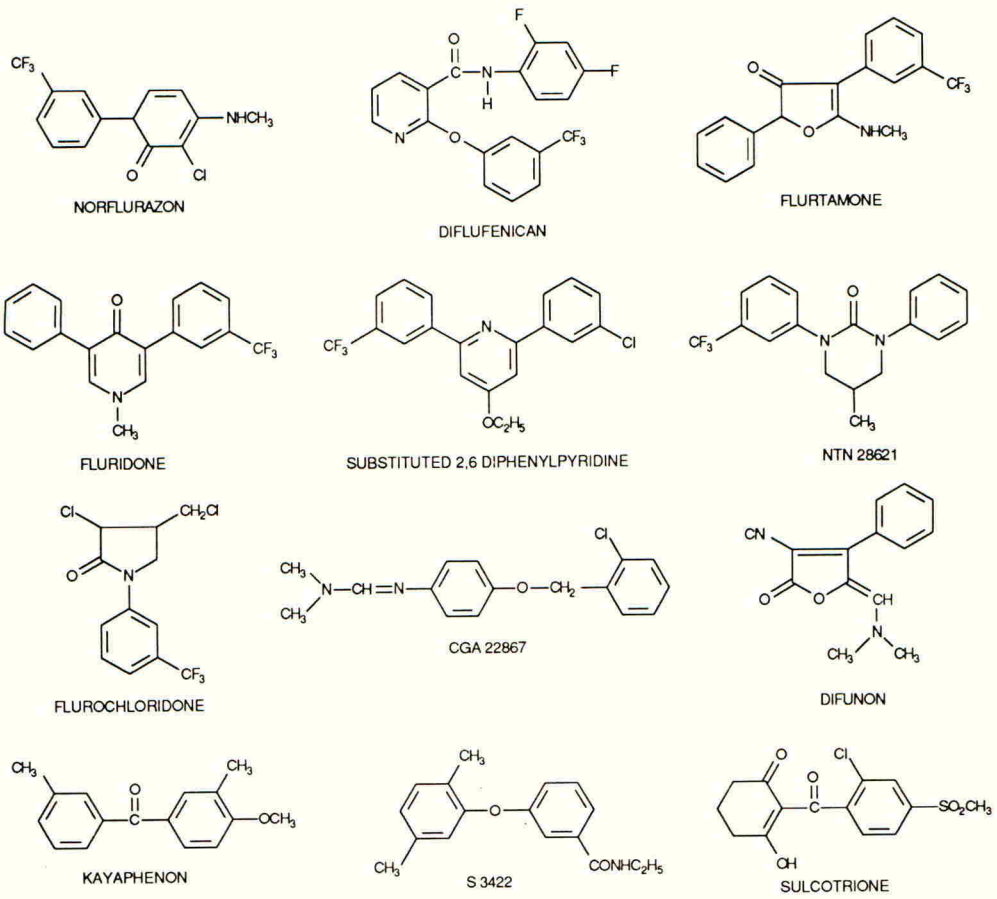
At present, it is impossible to investigate all the potential target sites listed in Table 1 for a single herbicide. Most progress has been made with *in vitro* studies, using crude cell extracts from cyanobacteria, pepper, spinach and daffodil (reviewed by Bramley 1993). In several cases, it has been possible to compare *in vivo* I_{50} data with *in vitro* K_1 values and to establish whether inhibition is reversible or irreversible. Further details can be found in the review of Sandmann and Böger (1989).

Reports on the mode of action of the new bleaching herbicide sulcotrione (Fig. 2) has indicated pds can be directly affected by inhibition of its cofactor availability (Schulz *et al.*, 1993; Prisbylla *et al.*, 1993). The triones or triketones cause phytoene to accumulate in treated tissues typical of other pds inhibitors (Mayonado *et al.*, 1989; Sandmann and Böger, 1990), but no direct inhibition of pds *in vitro* was detected (Schulz *et al.* 1993). This family of herbicides appears to inhibit p-hydroxyphenyl pyruvate dioxygenase preventing α-tocopherol and plastoquinone biosynthesis, with the latter proposed as an electron acceptor for phytoene desaturation.

THE INHIBITORS OF PHYTOENE DESATURASE

Examples of some of the many pds inhibitors discovered in the last 20 years are shown in Figure 2. These include commercialised herbicides and molecules which have not been, or have yet to be, commercialised. Evidence of inhibition is from accumulation of phytoene in treated cells and plant tissues and from inhibition of pds activity in enzyme preparations (for reviews see Sandmann and Böger, 1989; Bramley, 1993). There are also a number of other bleaching molecules eg, amitrole, pyriclor, J852;

FIGURE 2.: Examples of the inhibitors of phytoene desaturase, many of which are successful commercial bleaching herbicides. Sulcotrione is a new bleaching herbicide which does not inhibit pds directly.



dichlormate and LS80707 which cause phytoene accumulation *in vivo*. However, these are inhibitors of zds or lycopene cyclase (Sandmann and Böger, 1989; Barry and Pallett, 1990). The *in vivo* accumulation of phytoene is likely to be a consequence of inhibition at these later steps in carotenoid biosynthesis rather than any direct action on pds.

Several of these inhibitors do possess some structural similarities, ie *meta*-CF_3 phenyl ring substituents, carbonyl-containing heterocyclic rings, or alkyl amino functions. There are several reports of structure activity studies within the individual classes of inhibitors eg pyridazones, phenoxybenzamides, phenoxynicotinamides, diphenyl-pyridines, substituted 3(2H) furanones and tetrahydropyrimidinones (Babczinski *et al.*, 1990; Balasegaram *et al.*, 1991; Sandmann and Böger, 1989; 1993; Sandmann *et al*, 1992). These investigations, carried out on enzyme preparations or on whole cells, have given some indication of the optimum substitution within the various families but they do not seem to allow prediction of optimum substitution between families. Also, they have yet to reveal if these

classes of inhibitors share a common binding domain on pds or any information on mechanism of interaction with the target site.

Quantitative structure-activity relationships (QSAR) studies can be carried out on intact cells, whole plants or with enzyme preparations. The purpose of such investigations is to gain information on which functional moieties are necessary for inhibitory activity. Typically, a series of analogues is tested and the effectiveness of each compound related to physicochemical parameters such as lipophilicity and steric bulk. A QSAR correlation has been established for substituted 2-phenylpyridazinones (Sandmann and Böger, 1992). Steric factors did not influence inhibitory activities, but they were dependent upon lipophilicity and electronic parameters. The phenoxybenzamides containing a C_5 alkyl chain are the most inhibitory and the chain length is more important than lipophilicity (Sandmann and Böger 1992). It should be noted, however, that these studies were carried out on cyanobacterial cell extracts and the situation may be different in multicellular organisms with defined organelles.

Only limited information of the kinetics on the mechanism of inhibition has been obtained to date, due in part to the difficulty of studying pds *in vitro*. Norflurazon, fluridone, fluorochloridone and S3422 are reversible inhibitors and are non-competitive with respect to phytoene (Mayer *et al.*, 1989; Sandmann and Böger, 1989; Linden *et al.*, 1990; Kowalczyk-Schröder and Sandmann, 1992). Biochemical characterisation of cyanobacterial mutants selected against norflurazon revealed one mutant with cross-resistance to fluorochloridone (Linden *et al.*, 1990), indicating a possible common binding domain for these two inhibitors.

In conclusion based on current knowledge, the structural similarity of some of the phytoene desaturase inhibitors and the limited biochemical data allow us to speculate that at least some of these molecules share a common binding domain, analogous to the situation with the various classes of photosystem (PS) II inhibitors at the level of the 32 kD polypeptide.

CONSEQUENCES OF INHIBITION

The inhibition of pds leads to the prevention of the biosynthesis of coloured carotenoids causing the accumulation of the colourless phytoene. This causes the characteristic 'bleached' appearance of newly developing tissues (Barry and Pallett, 1988; 1989; Sandmann and Böger, 1989). The bleached tissue is completely devoid of chlorophylls which has provoked the view that with the absence of the photoprotective carotenoids, photosensitive chlorophylls undergo peroxidation upon synthesis (Sandmann and Böger, 1989; Sandmann *et al.*, 1991).

However, the proposed structural role for carotenoids (Dahlin *et al.*, 1983) indicates that carotenoid inhibitors do not simply induce photo-oxidation. Furthermore, hplc analysis of bleached tissues reveals no evidence of chlorophyll degradation products (Britton *et al.*, 1987; Barry and Pallett, 1988). It appears therefore that the inhibition of coloured carotenoid formation leads to an absence of normal chloroplast development and chlorophyll biosynthesis (Barry and Pallett, 1989). The reasons for the herbicidal effectiveness of phytoene desaturase inhibitors as herbicides is enhanced by a vast biosynthesis of metabolically inert phytoene which accelerates plant death by depletion of metabolic energy (Barry and Pallett, 1988). A peroxidative-based activity of pds inhibitors is likely in leaf tissue undergoing greening (photosynthetic development) or in already developed leaves at the time of treatment. Carotenoid turnover will be inhibited in these tissues. Initially, such leaves show little symptomology following herbicide treatment, although a large phytoene accumulation is evident (Barry and Pallett, 1988). Eventually,

chlorotic symptomology develops, typical of PSII inhibitors, due to a depletion of photoprotective carotenoids.

FUTURE PROSPECTS

Further inhibitors

It can be predicted with some certainty that further bleaching molecules will be discovered because of their ease of identity in screening procedures. Also, the variety of existing inhibitors indicates that others will be obtained and should they possess the desired efficacy and crop selectivity, they will be developed as commercial products. Confirmation of these molecules as pds inhibitors can be readily achieved by analysis for phytoene accumulation in bleached tissues. However, *in vitro* clarification will be essential, as evidenced by the triketone bleachers, in order to prove direct inhibition of phytoene or ζ-carotene desaturases.

Rational Design

Limited design is now feasible. Molecular modelling procedures coupled to QSAR will permit comparative studies of the inhibitors. However, rational design requires some knowledge of the binding site. At present, this is largely lacking. We have only rudimentary knowledge of pds structure, its reaction mechanism, cofactor requirements or its translocation and location into the plastid membrane. It cannot be assumed that all the classes of inhibitors share a common binding domain. Molecular biology of pds is now providing advances with several higher plant pds genes now cloned and sequenced (Bartley *et al.*, 1991; Hugueney *et al*, 1992; Pecker *et al.*, 1992). Their overexpression in appropriate vectors should facilitate knowledge of pds structure and properties. Alternatively, the high homology reported for the green algal and cyanobacterial pds with higher plant pds may obviate the need to study the higher plant system (Pecker *et al.*, 1992). However, advances in aspects of the biochemistry of pds will also be necessary to improve our understanding of the reaction mechanism and inhibition kinetics, if we are to be in a position to rationally design new inhibitors.

Herbicide Resistant Crops

Transformation of crops with mutated target site enzymes to obtain selectivity has already been achieved, eg acetolactate synthase (Hartnett *et al.*, 1990), EPSP synthase (Kishore *et al.*, 1992). Moreover, strains of the cyanobacteria *Synechocystis* and *Synechococcus* which have a mutated pds gene have been selected (Chamovitz *et al.*, 1991; Martinez-Ferez and Vioque, 1992). Transformation of crops with such mutated genes can be predicted to give rise to resistant crops in the future.

An alternative approach to obtain resistance has been recently reported (Misawa *et al.*, 1993). This involves transformation of tobacco with the bacterial desaturase gene (crtI) from *Erwina uredovora* which is insensitive to bleaching herbicides. Transformants were reported to show some resistance to both norflurazon and fluridone (Misawa *et al.*, 1993). If this approach proves to be successful, then resistance to the wide range of herbicidal pds inhibitors would seem feasible.

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ACETOLACTATE SYNTHASE; THE PERFECT HERBICIDE TARGET?

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ABSTRACT

Structurally diverse compounds act as herbicides through inhibition of acetolactate synthase (ALS), the enzyme catalyzing the first common step in the biosynthesis of branched chain amino acids. Commercial types are generally effective at low use rates, have both pre- and post-emergence activity and offer sufficient flexibility in their structure/activity to allow the development of variants for use in a wide variety of crops. Here we try to identify underlying features of the biochemistry which make ALS a much better target for herbicides than the other enzymes in the same biosynthetic pathway but, which, at the same time, seem to predispose its inhibitors to select resistant weeds relatively quickly.

INTRODUCTION

Herbicides which act by inhibiting ALS kill plants in a distinctive way. The symptoms first appear in the meristematic tissues where growth ceases soon after treatment. DNA synthesis is inhibited after a few hours (Ray 1982) and chlorosis and necrosis of young tissue soon follows with die back of the more mature parts of the plant taking a further 3-4 weeks. The discovery that sulfometuron-methyl-induced inhibition of the growth of bacteria was reversed in rich medium and, specifically, by mixtures of the three branched chain amino acids, first pointed to ALS being the primary site of action (LaRossa and Schloss 1984); the inheritance of herbicide-resistance as a result of specific mutations in the structural gene for ALS has since put the issue beyond question (cf Lee *et al* 1988). However, we still do not understand why inhibition of ALS should kill the plant rather than stunt its growth and, moreover, in the particular manner observed.

Inhibitors of amino acid biosynthesis do not all do the same thing. For example, glyphosate which inhibits the biosynthesis of aromatic amino acids and the triazole phosphonates which inhibit histidine biosynthesis (Hawkes *et al* 1993b) produce symptoms in treated plants which are distinct from each other and, even more obviously, are distinct from the ALS inhibitors. Glyphosate does, eventually, inhibit entry into mitosis (Vaughn and Duke 1986) but an *early* secondary effect on DNA synthesis and mitosis seems to be particularly associated with inhibition of ALS. The first event should be a reduction in the level of branched chain amino acids and pantothenate at the site of action in the leaves and meristems. If this somehow triggers the death of the plant then inhibitors acting elsewhere in the pathway should start the same cascade of physiological events and perhaps offer similar potential as herbicide targets. Alternatively, if the accumulation of some toxic substrate were important (cf protoporphyrinogen oxidase) then the potential for herbicidal utility should be limited to particular enzymes. Experimental herbicides which inhibit ketolacid-reductoisomerase (Schulz *et al* 1988; Aulabaugh and Schloss 1990), isopropylmalate isomerase (Hawkes *et al* 1993a) and isopropylmalate dehydrogenase (Wittenbach *et al* 1992) seem to kill plants in the same distinctive manner as the herbicides which inhibit ALS. This is encouraging and argues that plants die in the same way wherever the pathway is blocked. However getting herbicidal activity has, so far, required much higher use rates than for ALS. Here we examine why ALS should be a good target

for herbicides and, in particular, why it might be better than any of the other enzymes involved in the biosynthesis of branched-chain amino acids.

HERBICIDAL INHIBITION OF ACETOLACTATE SYNTHASE

This area has been reviewed a number of times (Hawkes *et al* 1989, Hawkes, 1989, Schloss 1990, Schloss and Aulabaugh 1990, Stidham 1991). Acetolactate synthetase is a non-oxidative thiamine pyrophosphate-containing decarboxylase. However, apart from thiamine thiazolone pyrophosphate, none of its rich variety of inhibitors seem to act mechanistically and Schloss has coined the phrase 'extraneous site inhibitors' to describe their action. Despite the non-redox nature of the catalytic reaction, the enzyme also retains a flavin close to the active site. Similarities in the sequence between the *ilv B*, *ilv G* and *ilv I* structural genes for ALS and the *pox B* gene encoding a thiamine pyrophosphate-dependent flavoenzyme, pyruvate oxidase, pointed to a likely common ancestor. Given the need to shield protons from the hydroxyethyl-thiamine pyrophosphate intermediate, evolution may have favoured the retention of flavin in ALS despite the loss of its redox role. Pyruvate oxidase transfers electrons to quinone electron acceptors in the membrane. Schloss *et al* (1988) speculated that, along with the flavin, ALS has also co-retained part of an ancestral 'quinone binding site' and, in support of this, showed that soluble quinones are quite potent inhibitors of isozymes I and III of ALS in bacteria. Quinone-binding sites (cf the plastoquinone site in PSII) may, in general, offer sites with susceptibility to a wide variety of structural types. In addition, (and possibly in close proximity) plant ALS also has sites which confer feedback regulation by leucine and valine. The possibilities created by inhibitors overlapping and bridging between the 'quinone' site, the regulatory sites and parts of the catalytic site, may well leave the enzyme almost uniquely susceptible to 'finding' inhibitors in random screens. In accord with this picture of a large binding region containing overlapping subsites, selection for resistant biotypes has identified mutant forms of ALS which encompass many variants of cross-resistance. In general, the evidence points to triazolopyrimidines and sulphonylureas sharing much of one subsite and imidazolinones and pyrimidyl oxobenzoic acids sharing much of another (Mourad and King 1992). However, there are individual alleles which confer resistance to only a few members of a single class of herbicide (Saxena and King 1988, Gabard *et al* 1989) and, at the other extreme, alleles which confer a high level of resistance across the range of herbicides and feedback regulators (Hattori *et al* 1993).

Inhibitors of plant acetolactate synthase all seem to be 'slow-binding'. In general, inhibition can be fitted to a model where an initial enzyme/ inhibitor complex slowly (0.05 min^{-1} in the case of pea enzyme) isomerizes to a more tightly bound complex. However the model may be misleading. The imazapyr-inhibited pea enzyme did not recover activity in the expected way after it was exchanged free of excess inhibitor (Hawkes 1989). Moreover, it was shown, using radiolabelled imazapyr and chlorsulfuron, that these inhibitors could physically dissociate from the barley enzyme without any corresponding recovery of activity (Durner *et al* 1991). We proposed that the slow process (0.05 min^{-1}) might not correspond to the formation of a tight complex but, rather, to an irreversible shift down to a conformation of the enzyme with little or no catalytic activity (noting that this need not imply that the polypeptide be more than usually sensitive to proteolysis which it clearly isn't; cf Shaner and Singh 1991). However, the story gets more complicated. Shortly after treating plants with imidazolinones the amount of ALS activity which could be extracted declined dramatically (i.e. in accord with what happens *in vitro*). However, at spray rates resulting in a similar overall herbicidal effect, sulphonylureas caused a much lesser diminuation in enzyme activity and, in mixture, appear to oppose the effect of imidazolinones (Shaner *et al* 1990). Clearly, more work is needed to better define under what conditions ALS loses its activity and to understand the process involved.

In summary, two properties could mark out ALS as an unusual herbicide target. Firstly it seems susceptible to a wide variety of structures and, secondly, the enzyme may be peculiarly labile to even the transient binding of reversible inhibitors.

OTHER TARGET ENZYMES IN BRANCHED CHAIN AMINO ACID BIOSYNTHESIS

The experimental herbicide, HOE 704 (2-methylphosphinoyl-2-hydroxyacetic acid) inhibits ketolacidreductoisomerase (KARI), the enzyme which follows ALS in the common pathway to the three branched-chain amino acids; it closely resembles a transient intermediate in the isomerisation. Inhibition is time-dependent and (perhaps uncertain) final Ki values of 0.8, 0.25 and 0.8 µM have been estimated (Schulz *et al* 1988, Hawkes and Edwards 1989) versus the enzyme from *E. coli.*, yeast and plants (the latter being only a value derived from Schulz *et al*'s IC_{50} value versus carrot enzyme and the Km value for acetolactate reported by Dumas *et al* (1989) for the spinach enzyme). The dissociation rate of HOE 704 from the yeast enzyme (the 'off rate') is *c.* 0.1 min^{-1}. Inhibition of the growth of duckweed was reversed by branched chain amino acids and 2-acetolactate (acetoin) was shown to accumulate in treated plants (Schulz *et al* 1988). HOE704 is not an impressive herbicide (1-2 kg ha^{-1}) but, neither is its apparent Ki value (0.8 µM) all that low in comparison to the Km values of 25 and 37 µM reported (Dumas *et al* 1989) for 2-acetolactate and 2-acetohydroxybutyrate. On the basis of these data you would not conclude that KARI is a bad target and, on the contrary, might even conclude that it is a good one in search of a better inhibitor..

However, workers at DuPont synthesised N-isopropyl oxalylhydroxamate (IpOHA) as a reaction-intermediate analogue. This indeed turned out to be an extremely potent slow-binding inhibitor of KARI from *E. coli* with a final Ki value of 22 pM and a t½ for dissociation from the magnesium enzyme of 6 days (Aulabaugh and Schloss 1990). Surprisingly, it was, if anything, a less effective herbicide than HOE 704. Using radiolabel, it was shown that IpOHA was taken up and translocated no worse than the sulphonylurea, thifensulfuron methyl. It penetrated chloroplasts *in vitro*, inhibited the plant enzyme and was somewhat more stable to metabolism than thifensulfuron. Exposure to relatively low levels (< 1 ppm) of IpOHA eventually reduced the level of extractable KARI activity in cultured maize cells by more than 90%. However, a further 10 fold increase in the concentration of IpOHA was then necessary to either cause a substantial accumulation of acetoin (derived from 2-acetolactate) or to produce any significant reduction in growth (Wittenbach *et al* 1990). These data indicated that KARI is intrinsically a poor target for herbicidal inhibition.

Two herbicides are known to act as specific inhibitors of leucine biosynthesis. O-isobutenyl oxalylhydroxamate is a potent inhibitor (Ki 5 nM) of isopropylmalate dehydrogenase from peas and a herbicide at *c.* 0.4 kg ha^{-1}. Leucine alone was shown to reverse the inhibition of pea root growth (Wittenbach *et al* 1992). The nitronate ion of 1-hydroxy-2-nitro-cyclopentane-1-carboxylic acid which resembles the *aci*-carboxylate intermediate in the reaction of isopropylmalate isomerase proved to be an excellent inhibitor of the enzyme from yeast (Ki *c.* 0.6 nM). The pKa value of the nitronate anion is around 7 and the compound is therefore mainly in the inhibitory form at physiological pH. It is herbicidal at 1 kg ha^{-1} and inhibition of the growth of carrot cells was shown to be specifically reversed by leucine (Hawkes *et al* 1993a).

There are no reports of herbicides inhibiting other steps in branched-chain amino acid biosynthesis. Such inhibitors as are known may not be potent and/or specific enough to test the potential herbicidal utility of these sites. Schloss and Aulabaugh (1990) suggested that there might be significance in the fact that auxotrophic plant lines which lack either threonine deaminase or dihydroxyacid dehydratase activity are known but that none have been described which lack either ALS or KARI. Perhaps

auxotrophs with lesions at potential herbicide targets are always too lethal to be recovered (and reveal themselves as the mutants you *don't* get) while the deaminase and the dehydratase might offer *no* potential at all? However, given that exogenous amino acids are an effective antidote for the herbicides themselves, it seems most likely that mutants representing the same block should also be recoverable by this expedient. Overall there is insufficient data and, in some plants at least, the missing auxotrophs may correspond to steps where there is more than one isozyme. Occam's razor probably still favours the view that an indefinite 100% block anywhere in the pathway should kill plants in the same way and that understanding the difference between good sites and poorer ones will be mainly a question of quantifying what happens when a less than complete block is applied for a limited period.

THE MOST *EFFECTIVE* SITES FOR HERBICIDAL INHIBITION?

What determines the intrinsic efficacy of inhibition at the different sites in branched chain amino acid biosynthesis? For a good herbicide, we expect...

Something irreversible, which will lead to plant death, that happens on the same time-scale as the inhibitor (or its effect) persists at the site of action.

Based on the similar visible effects on plants, any *early* irreversible event which discriminates ALS from the other sites of action is unlikely to be at the physiological level. For ALS, a good candidate is the apparent inhibitor-induced inactivation of the enzyme itself. This would mean that ALS inhibitors need persist at the target site for a relatively short period in order to produce an eventual herbicidal effect.

Substrate accumulation should not act negate inhibition.

In general, while the substrate concentration ([S]) remains below the Km (perhaps the usual starting condition, Fersht 1984), accumulating [S] will eventually restore the initial flux following a transient fall in response to inhibition. This re-adjustment will be quicker if the block is upstream of a point subject to negative feedback regulation. Competitive inhibitors can be much less effective than non-competitive types because they raise the effective value of the Km (i.e. $Km' = Km\ (1 + [I]/Ki)$) and ensure that [S] can continue to accumulate without reaching saturation. Therefore, unless you get early damage (while the system is still adjusting to inhibition), competitive inhibitors will fail without there being some physiological (or mass action) limit on the extent to which the enzyme's substrate can accumulate. Note, however, that very potent competitive inhibitors (with Ki values in the pico-molar range) dissociate too slowly from the enzyme for accumulating substrate to be effective in overcoming inhibition.

Because of its position in mainstream metabolism, it seems unlikely that the concentration of pyruvate would increase greatly in response to inhibition of ALS. 2-ketobutyrate, on the other hand, should accumulate freely (particularly since a fall in the concentration of isoleucine would relieve the feedback inhibition of threonine deaminase). Pyruvate and 2-ketobutyrate compete for the hydroxyethylthiamine pyrophosphate form of ALS to make either 2-acetolactate for valine and leucine biosynthesis *or* 2-acetohydroxybutyrate for isoleucine biosynthesis. An unbalanced accumulation of 2-ketobutyrate over pyruvate would only further add to the inhibition of leucine and valine biosynthesis and, potentially therefore, further amplify the effect of the block. Since the herbicides which inhibit ALS are, in general, mixed or non-competitive type inhibitors, we would not, in any case, expect an accumulation of 2-ketobutyrate to be effective in opposing inhibition.

Since ALS is subject to feedback regulation and catalyses a reaction which is close to irreversible in the forward direction, any block at the following enzyme, KARI,

would be expected to result in the accumulation of 2-acetolactate and 2-acetohydroxybutyrate. This should oppose the effects of the competitive inhibitor, HOE 704, but, with a t½ for dissociation measured in days, would not be effective in displacing IpOHA from enzyme it was already bound to.

Substrate accumulation is likely to be an important factor in limiting the herbicidal efficacy of O-isobutenyl oxalylhydroxamate and 1-hydroxy-2-nitro-cyclopentane-1-carboxylate, the competitive inhibitors of isopropylmalate dehydrogenase and isopropylmalate isomerase. The forward reaction of the first step in the leucine pathway, isopropylmalate synthase, is strongly favoured and both α- and β-isopropylmalate are likely to accumulate as the result of a subsequent block. The rates at which the two inhibitors dissociate from isopropylmalate isomerase and isopropylmalate dehydrogenase (*c.* 0.1 min^{-1} in each case) are not so slow as to prevent their displacement by an accumulation of these substrates.

Good sites are where the accumulating substrate is toxic.

This is the converse of substrate accumulation *negating* inhibition. It has been argued that inhibition of ALS might be effective in killing plants because of the toxic effects of accumulating 2-ketobutyrate. However, *via* feedback regulation of threonine deaminase, it might then have been expected that isoleucine alone should reverse ALS herbicide-induced inhibition of growth (which it doesn't).

There should be the least excess of activity versus the flux required.

It makes sense for regulated points in pathways to be rate limiting, and, rather like a coupled assay, for the subsequent steps to have an excess of enzyme activity. This is consistent with the idea that 'mid-pathway' enzymes should normally operate at substrate concentrations considerably below the Km (Fersht 1984) and also the common observation in antisense mRNA experiments that expression may need to be decreased by as much as 80% before effects at the end of the pathway become obvious. Targets which are regulated at the transcriptional level may be particularly poor if derepression of the enzyme can oppose inhibition. Amino acid biosynthesis inhibitors perhaps share the advantage that they should all *eventually* reduce protein synthesis and minimise enzyme resynthesis (although, the *de novo* synthesis of some enzymes initially *increases* in response to amino acid stringency) .

In corn culture, 90% inhibition of KARI induced a modest increase in [2-acetolactate], while, above this threshold, a much steeper rise was observed (Wittenbach *et al* 1990). The threshold probably corresponds to the point where the concentrations of branched chain amino acids first starts to fall and to thereby relieve the feedback inhibition of ALS. With no effect up to 90% inhibition there must indeed be a good deal more KARI activity than needed. It is difficult to make the comparison with ALS. On the basis of the activities which can be extracted from plants, ALS is not obviously less abundant than other enzymes in the pathway. However, given relatively high Km values (further raised by feedback inhibition; cf Hawkes *et al* 1989), it probably operates as rate limiting *in vivo*. Given that heterozygous herbicide resistant plants survive treatment, it is likely that sustained inhibition by as much as 50% is not lethal.

The physical concentration of targets should not be so high as to set high minimum use rates for potential herbicides purely on the basis of stoichiometry

A typical activity for ALS in a seedling extract is, perhaps, *c.* 4 nmol min^{-1} mg^{-1}. Assuming a kcat similar to bacterial enzyme (Schloss and Van Dyk 1988), a single herbicide-binding site per subunit, a subunit molecular weight of *c.* 65 kD (Singh *et al* 1992), 10 mg of protein $gram^{-1}$ of leaf tissue and that 50% of this is soluble you arrive at an estimate of *c.* 12 nM [ALS]. Using the data for spinach KARI (Dumas

et al 1989), the internal enzyme concentration in the leaf comes out surprisingly high at *c.* 220 nM (the other enzymes such as isopropylmalate dehydrogenase generally seem to be little more abundant than ALS). 'Imazapyr' is taken up and translocated relatively well and therefore makes a good (near 'best case'?) standard for relating foliar spray rates to herbicide concentrations within the plant. Stidham and Shaner (1990) reported that 4 hours after a foliar spray with 100 g ha^{-1} of imazapyr, the internal concentration of the herbicide within a maize leaf was 570 nM. On this basis, 12 nM [ALS] translates to a minimum use rate of *c.* 2.2 g ha^{-1} (i.e. for 100% inhibition of ALS and a herbicide with the same foliar uptake and transport properties as imazapyr). This is comfortingly close to other estimates (cf Wittenbach *et al* 1992). The corresponding answer for KARI comes out at 40 g ha^{-1} (or twice this for a racemic mixture such as HOE 704). While the absolute numbers in this sort of calculation are undoubtedly wrong, comparisons using ALS as an internal standard are probably useful.

By itself, the absolute *amount* of KARI would thus seem unlikely to set the apparent limit on herbicidal activity observed at a kg or more. However, combined with other factors, it could be significant and, for example, a racemic mixture with a single active enantiomer which accumulated at the site of action only 10% as well as imazapyr would be set a minimum use rate of 800 g ha^{-1}. However, the simple arguments become a great deal more complex when the distribution of herbicide and target enzyme, both throughout the plant and at the subcellular level, are taken into consideration. To what extent do different herbicides concentrate and kill at the meristems (perhaps only meristematic concentrations matter)? At the chloroplast level, enzyme concentrations are much higher than calculated per gram of leaf tissue. Nevertheless, it seems most appropriate to calculate concentrations on the basis of the compartment within which the herbicide is distributed (this assumption depending on free exchange between subcompartments). Presumably acidic herbicides will concentrate in the chloroplast; alternatively, might some be esterified and deposited into the vacuole? It is important to recognise that any number of complications are possible.

HERBICIDE RESISTANT WEEDS

Starting with the selection of resistant prickly lettuce by chlorsulfuron, it has become evident that ALS herbicides are prone to selecting resistant weeds relatively quickly (Reed *et al* 1989). Most resistant biotypes contain an altered ALS enzyme. It has become clear that many variants of the enzyme with different patterns of cross-resistance to herbicides are possible. Given that the 'extraneous' inhibitor site is also largely extraneous to function it is also not surprising that many of these mutants are associated with only a marginal cost in 'fitness' (e.g. a slight decrease in kcat or, perhaps, relief from feedback control cf Saari *et al* 1992). Given the underlying biochemistry, it is not surprising that resistant biotypes start out relatively frequent in the population, can be selected quickly and will be difficult to get rid of. Because of cross-resistance, it is also likely that widespread use of existing ALS herbicides will shorten the useful lifetime of any new types. Although requiring hopelessly high use rates, as reaction intermediate analogues, the inhibitors of the other sites in the pathway do at least offer virtual immunity from such 'active-site' based mechanisms of weed resistance.

SUMMARY

The main points which make ALS a good target for herbicides are 1) it has an unusual 'quinone' site making it susceptible to a wide variety of inhibitors 2) it appears peculiarly labile to even the transient binding of reversible inhibitors 3) inhibition is not opposed by substrate accumulation 4) there is physically little enough of it to set a low minimum use rate (2 g ha^{-1}) and 5) *perhaps* a lesser degree of

inhibition is required to produce an effect at the end of the pathway. As far as it is possible to tell, the other enzymes in the pathway each have some good and some bad points as potential herbicide targets. So, for example, isopropylmalate isomerase and isopropylmalate dehydrogenase are probably not so abundant in plants as to set high minimum use rates but, in each case, substrate accumulation would oppose the effects of the competitive inhibitors described in the literature. Non-competitive inhibitors at these sites might be effective but are probably harder to find. There is data to support the idea that KARI is a bad target because 1) there is at least 20 fold more KARI activity than required to support the flux through the pathway 2) substrate accumulation will act to oppose inhibition (which might matter for a competitive inhibitor such as HOE 704 but is not a convincing argument for the very slow binding oxalylhydroxamates) and 3) the physical abundance of the enzyme sets a likely mimimum use rate of *c.* 40 g ha^{-1}. The latter may not seem high but could be significant in combination with other factors. Thus, for example, racemic compounds which accumulated at the site of action only 20% as well as imazapyr (our 'good' standard) would be set a minimum use rate of *c.* 0.4 kg ha^{-1}.

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THE NOVEL MECHANISM OF ACTION OF THE HERBICIDAL TRIKETONES

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ABSTRACT

The benzoylcyclohexane-1,3-diones, the triketones, are potent bleaching herbicides that also inhibit mammalian *p*-hydroxyphenylpyruvate dioxygenase (HPPD, EC 1.13.11.27). HPPD catalyzes the formation of homogentisic acid (HGA) from *p*-hydroxyphenylpyruvate (HPPA). NTBC (2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione), a representative triketone, is used to treat tyrosinemia type I patients. The pharmaceutical application is based on preventing buildup of hepatotoxic intermediates of tyrosine catabolism. In plants, HPPD is a component of the biosynthetic pathway to plastoquinone (PQ) and α-tocopherol. NTBC or sulcotrione (ICIA0051, 2-(2-chloro-4-mesylbenzoyl)-1,3-cyclohexanedione) treated plants accumulate tyrosine and PQ levels fall dramatically. Bleaching of *Lemna gibba* by triketone treatment is reversed by HGA. Plant HPPD, like mammalian sources, is inhibited *in vitro* by NTBC. These biochemical effects provide evidence that the triketone herbicidal mechanism is HPPD inhibition leading to a deficiency of a key co-factor, most likely plastoquinone.

INTRODUCTION

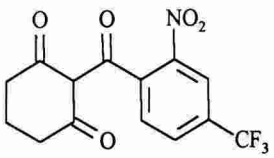
The triketones, originally discovered and optimized at the Western Research Center of Zeneca Ag Products (Michaely & Kratz, 1986, 1988), are a novel class of potent herbicides. Herbicidal triketones (TKO) have broad spectrum activity on grass and broadleaf weeds in both pre-emergence and post-emergence applications and have selectivity in corn. The herbicidal activity has been reported for sulcotrione (Beraud *et al.*, 1991) and SC-0774

(Wilson & Foy, 1990), as well as other members of the triketone class of herbicides (Maier & Valentin, 1988).

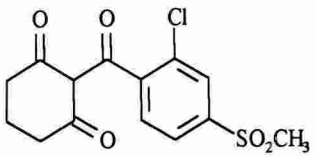
Triketone treated plants are bleached with reduced chlorophyll and carotenoids and elevated phytoene levels (Soeda & Uchida, 1987; Mayonado *et al.*, 1989). These results are similar to commercial bleaching herbicides such as norflurazon, and seemed to implicate phytoene desaturase inhibition as the triketone target site. However, this hypothesis was speculative and did not fully explain all the symptoms produced by the triketones. The triketones are more active on new growth, while phytoene desaturase inhibitor effects are greater on older leaves. This difference was attributed to activity at a different stage of plastid development (Mayonado *et al.*, 1989). Also inconsistent was the lack of *in vitro* activity on phytoene desaturase. This discrepancy, combined with the unique structure and physical properties of the triketones, led to a proposal for bioactivation (Sandmann *et al.*, 1990). While these theories were consistent with the above observations, we considered a novel site of action. Our decision and direction were guided by biochemical effects of triketones in animal systems. We describe here studies on a new bleaching mechanism of action.

These mechanistic studies have utilized the representative triketones, NTBC and sulcotrione (Figure 1). NTBC is a potent bleaching herbicide and inhibits mammalian HPPD, an important enzyme in the catabolism of tyrosine. HPPD catalyzes the formation of HGA from HPPA. Knowledge of this inhibition has lead to a treatment for tyrosinemia type I patients with NTBC. Tyrosinemia type I is a lethal hereditary disorder characterized by a deficiency of a distal tyrosine catabolic enzyme, fumarylacetoacetase (Linblad *et al.*, 1977). This deficiency leads to accumulation of hepatotoxic intermediates such as succinylacetone. NTBC prevents the buildup of toxic tyrosine metabolites and represents the only effective pharmacological therapy for tyrosinemia type I patients (Ellis *et al.*, 1992; Linstedt *et al*, 1992). Thus, the discovery of a unique herbicidal mechanism of action was based on our understanding of triketone effects on mammalian HPPD.

FIGURE 1. Structures of NTBC and Sulcotrione.



NTBC



Sulcotrione

MATERIALS AND METHODS

Reversal experiments were performed using *L. gibba* leaflets (6 per well) maintained in 2 ml wells of a 6 x 4 microtiter plate. Half strength Murashige and Skoog Plant Salt Mixture containing 0.1 mg/ml carbenicillin was used as medium. Herbicide and HGA were added at the concentrations shown in Table 1.

Plants were grown in a sandy loam soil in glasshouses and watered to maintain maximum growth. At the time of post-emergence treatment, corn, milo and *Setaria viridis* were in growth stage 13 (Tottman, 1987); *Ipomoea hederacea* was in growth stage 11; *Abutilon theophrasti* was in growth stage 12. *Cyperus esculentus* was treated pre-emergence. Compounds were dissolved in 50/50 acetone/water solvent with 0.5% v/v Tween 20 and sprayed at 400 l/ha using an 8002E even flat fan nozzle. At harvest, plants were cut at the soil line and frozen immediately on solid CO_2. Frozen samples were homogenized in liquid nitrogen and stored at -80° C until analysis. Free amino acids were extracted with 70% aqueous ethanol, reacted with *o*-phthalaldehyde-3-mercaptopropionic acid and analyzed by hplc (Schuster, 1988). Amino acid levels were quantified by comparison to authentic analytical standards using an external standard method. Samples were pooled for each treatment and, except where noted, analyzed in triplicate. Results are expressed as nmole per gram of plant (frozen homogenate). Significant differences from time matched controls were evaluated by Student's t-test following an analysis of variance, $p<0.05$.

Plastoquinone was measured in plant extracts using a modified hplc procedure (Tevini *et al.*, 1981). Plant homogenate was extracted with $CHCl_3/CH_3OH$ (2:1) and interfering substances were removed by separation on a silica cartridge. PQ absorbance at 255 nm was measured after gradient chromatography with CH_3OH/H_2O on a RP-8 column. Ubiquinone was used as an internal standard to correct for differences between injection amounts. The identity of the PQ peak was confirmed by mass spectral analysis. Plant HPPD activity was measured by $^{14}CO_2$ evolution (Fellman *et al.*, 1972). The corn enzyme was partially purified from etiolated tissue (D.O. Adams, *et al.*, manuscript in preparation).

RESULTS AND DISCUSSION

In vivo studies

Tyrosine metabolism in mammals is a catabolic process, whereas in plants it leads to the biosynthesis of PQ and α-tocopherol (Schultz, *et al.*, 1985). Evidence that triketone HPPD inhibition occurred in plants came from reversal studies with *L. gibba*. In this assay the triketones produced bleaching symptoms similar to higher plants. HGA was able to fully prevent the bleaching produced by triketones (Table 1). No reversal by HGA was seen with known phytoene desaturase inhibitors such as fluridone. These results suggest that triketone bleaching is a consequence of HPPD inhibition.

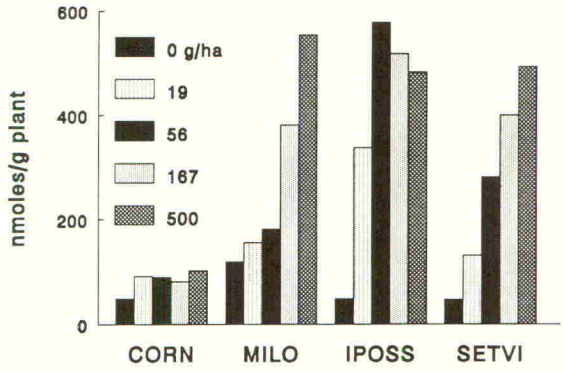
TABLE 1: Effect of HGA on bleached *L. gibba*.

	Rate (ppm)	% GI	% Bleaching	+ HGA (90 ppm) % GI	% Bleaching
sulcotrione	10	20	60	0	0
	1	20	60	0	0
fluridone	350	40	60	20	60
	40	0	60	0	20

% GI = percent growth inhibition vs. untreated control

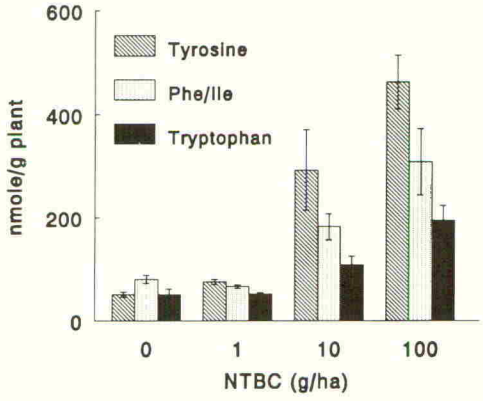
To further characterize the biochemical consequences of these herbicides in plants, the biosynthetic pathway from tyrosine to PQ was studied. Tyrosine, the precursor of HPPA, was measured in four plant species: corn, milo, *I. hederacea* and *S. viridis*. Four days after NTBC treatment, tyrosine levels were elevated compared to untreated control tissue (Figure 2). The increase was rate dependent and species specific. *I. hederacea* was the most sensitive of the four species tested, showing a substantial increase, even at the lowest rate of NTBC. Grass species, while less sensitive, showed rate dependent elevation in tyrosine levels. Corn, which is tolerant to the triketones, showed very little response to NTBC even at high rates.

FIGURE 2. Tyrosine levels in NTBC treated plants (n=1).



Analyses of the increases in free amino acids, while limited in the first experiments, included tyrosine and another aromatic amino acid, phenylalanine (Figure 3). In these earlier analyses, phenylalanine was not separated from isoleucine. However, subsequent improvement in the hplc method separated these amino acids and confirmed that phenylalanine alone was responsible for the increase. Rate dependent changes were not seen for other amino

FIGURE 3. *I. hederacea* aromatic amino acid levels 3 DAT with NTBC.



acids (data not shown). The analytical method was then adapted to complete the profile of aromatic amino acids of the shikimate pathway to include tryptophan. The sensitivity of *I. hederacea* to NTBC was also examined at lower rates and showed rate dependent changes in all three aromatic amino acids (Figure 3).

In contrast to the triketones, amino acid profiles in fluorochloridone treated *I. hederacea* did not show dramatic tyrosine elevation (Table 2). While NTBC increased tyrosine almost 10 fold, fluorochloridone treatment caused less than a 2 fold increase. Fluorochloridone is a representative phytoene desaturase inhibitor hence, these results suggest that tyrosine increases are a specific consequence of triketone bleaching. The role of tyrosine metabolism in triketone phytotoxicity is further demonstrated by analysis of whole plant PQ levels. PQ and β-carotene were decreased by both bleachers but the effect was slightly greater with triketone treatment (Table 2).

TABLE 2. NTBC and flurochloridone effects on biochemical changes in *I. hederacea.*

	nmole/g plant			integration units	
	Tyr	Phe/Ile	Trp	PQ	β-Carotene
control	40 ± 21[a]	61 ± 2	55 ± 6	118, 108[b]	80, 70
NTBC	513 ± 44*	209 ± 46*	207 ± 16*	26, 20	53, 35
fluorochloridone	99 ± 10*	246 ± 67*	340 ± 99*	58 ± 16	41 ± 10

NTBC 100 g/ha; fluorochloridone 1 kg/ha; * $p<0.05$; [a] mean ± s.d; [b] n=2

The time course of the biochemical changes produced by NTBC in *I. hederacea* differed. PQ levels fell much earlier than tyrosine accumulation. PQ was depleted within 2 days after treatment while aromatic amino acids did not increase until 4 to 5 days after treatment (tyrosine 10 fold; phenylalanine and tryptophan 3 fold; data not shown). The time course of the aromatic amino acid changes was somewhat variable and, on average, accumulation was seen 3 to 5 days after treatment.

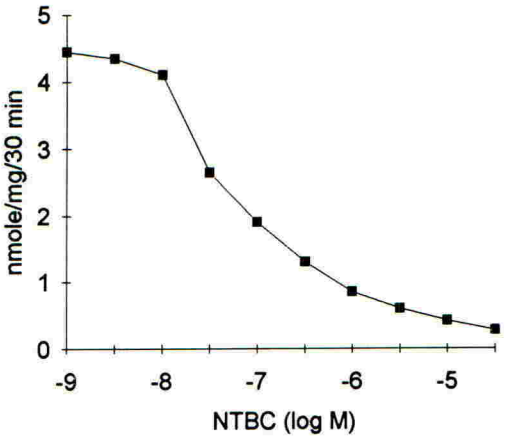
As shown by bleaching symptoms, the triketones are more active on meristematic tissue than mature leaves. Similar sensitivity was seen in the biochemical changes. Tyrosine levels were much higher (17x) and PQ and β-carotene were totally depleted in meristematic tissue from NTBC treated *I. hederacea*. Sulcotrione produced changes similar to NTBC on PQ, β-carotene and amino acid levels on treated *I. hederacea* and *A. theophrasti*. Biochemical changes were also noted in *C. esculentus* after pre-emergence application of either NTBC or sulcotrione. These effects were less than on the broadleaves, approximately 75% PQ decrease and 6 fold tyrosine increase (data not shown).

In vitro studies

NTBC is a time dependent, reversible, tight binding inhibitor of rat liver HPPD, IC_{50} 40 nM. In enzyme kinetic studies, NTBC is competitive with HPPA for the substrate binding site (M. K. Ellis, *et al.*, manuscript in preparation). Plant HPPD is also inhibited by NTBC.

The IC_{50} for the corn enzyme is 50 nM (Figure 4). Spinach HPPD showed similar sensitivity to NTBC. The kinetics of corn inhibition appeared competitive with the substrate (D.O. Adams, *et al.*, manuscript in preparation).

FIGURE 4. Corn HPPD inhibition by NTBC.



CONCLUSION

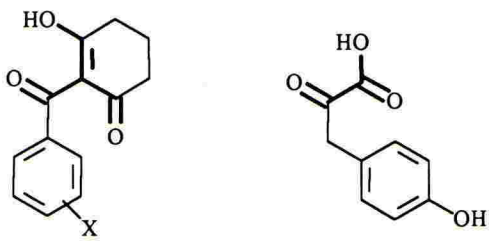
The observation that triketone herbicides inhibit mammalian HPPD has been extended to plant enzyme. NTBC has been shown to be a potent *in vitro* inhibitor of plant HPPD. Triketone herbicidal bleaching is due to this enzyme inhibition as demonstrated by reversal experiments using HGA on triketone treated *L. gibba*. This effect is specific for triketone bleaching since no reversal was seen with bleaching caused by fluridone, an inhibitor of phytoene desaturase. The consequences of HPPD inhibition include the massive accumulation of tyrosine and PQ depletion. Tyrosine increases were accompanied by similar but smaller changes of phenylalanine and tryptophan levels. This pattern of accumulation would be consistent with arogenate as a branch point in aromatic amino acid biosynthesis (Berlin *et al.*, 1989; Jensen, 1986; Siehl & Conn, 1988). The reversal studies suggest that herbicidal effects of the triketones are due to a deficiency of a critical component, such as PQ or α-tocopherol, rather than accumulation of aromatic amino acids. It has been proposed that quinones could serve as co-factors for the phytoene desaturase complex (Mayer *et al.*, 1990). Carotenoid and phytoene levels in triketone treated plants support this role for PQ. Triketones impair phytoene desaturase activity *in vivo*, but not *in vitro*, by co-factor depletion and thus indirectly block carotenoid biosynthesis. These results are supported by a recent report that HPPD is the target site of the triketones (Schulz *et al.*, 1993).

Herbicides such as fluorochloridone produce bleaching symptoms by inhibition of phytoene desaturase, resulting in the reduction in β-carotene and the elevation of phytoene. The reduction in PQ levels by fluorochloridone was not accompanied by dramatic tyrosine increase. In contrast, the triketones cause the complete loss of PQ in the meristem and the concomitant rise in aromatic amino acids, tyrosine in particular. This combination of effects,

resulting in the depletion of PQ, elevation of tyrosine and *in vitro* inhibition of HPPD is diagnostic of this new herbicidal mechanism of action and is thus termed the "triketone effect".

An important structural feature of the triketones is the tricarbonyl system. Triketones are competitive with the substrate HPPA for HPPD activity. A comparison is made between HPPA and the triketones in Figure 5 (structural similarities in bold). The plant growth retardant CGA 163'935 inhibits the α-keto glutarate coupled enzyme GA_{20}-3β-hydroxylase (Adams *et al.*, 1991) and is competitive with the enzyme co-factor, α-keto glutarate. The mechanistic similarities of α-keto acid dioxygenases such as HPPD and α-keto glutarate have been discussed (Abbott & Udenfriend, 1974; Hamilton, 1974; Pascal *et al.*, 1985). The implications from these comparisons is the potential for the tricarbonyl systems of the triketones to act as an α-keto acid mimic.

FIGURE 5: Structural similarities of tricarbonyl system and α-keto acids.



TRIKETONE HPPA

The molecular basis for the herbicidal activity of the triketones is in their ability to competitively bind with the active site of HPPD. This inhibition results in bleaching symptomology by the depletion of PQ and presumably indirect inhibition of phytoene synthase. The results described in this report demonstrate that HPPD is a new target site to which herbicide synthesis can be directed.

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IMIDAZOLE GLYCEROL PHOSPHATE DEHYDRATASE: A HERBICIDE TARGET

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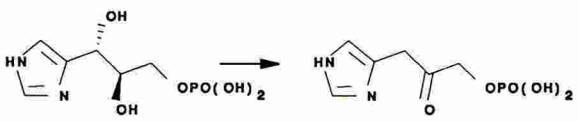
ABSTRACT

Phosphonic acid derivatives of 1,2,4-triazole inhibit imidazole glycerol phosphate dehydratase (IGPD) an enzyme involved in the biosynthesis of histidine. I is a potent (Ki 0.6 nM) slow-binding (koff 0.04 min^{-1}) inhibitor of the yeast enzyme and an effective herbicide. Herbicidal activity correlates with inhibition of IGPD; inhibition of the growth of maize cells is reversed by histidine. The yeast HIS 3 gene was overexpressed in *E. coli* and the recombinant IGPD was purified. Based on nmr studies, the pH-dependences of kinetic parameters and the relationship between structure and activity as either a substrate or an inhibitor it is proposed that 1) metal has no *direct* role in catalysis 2) the phosphate dianion of IGP acts as an internal base to remove the proton from the β carbon and 3) inhibitors mimic the diaza-fulvene intermediate from which this proton is removed.

INTRODUCTION

Triazole phosphonates (Cox 1983; Mori *et al* 1992 & Cox *et al* 1993) are broad spectrum, phloem-mobile herbicides, similar to glyphosate. I (see table 1) was discovered (Cox and Ridley, unpublished data) to inhibit IGPD, an enzyme, at one time, thought to be the target of the herbicide 'Amitrole'. IGPD catalyses the dehydration of imidazole glycerol phosphate (IGP) to imidazole acetol phosphate (IAP). The mechanism is unknown and, with no imine or carbonyl group α to the departing proton, must be unusual (unlike, for example, aconitase where the *aci*-carboxylate delocalises the incipient carbanion); the requirement for Mn^{2+} (Ames 1957) has seemed to argue for the involvement of metal. Most feasible mechanisms would generate the *enol* of IAP as the initial product; the final ketonization of any such enol must also be an enzyme-catalysed step (Moore *et al* 1993). IGPD has not, before, been purified to homogeneity in a fully active form. Details of the purification and characterisation of the yeast enzyme will be published in full elsewhere. Here we summarise the main points which bear on the present discussion of the action of the triazole phosphonates as mechanistic inhibitors of IGPD.

Figure 1 IGPD catalyses the dehydration of IGP to IAP



MATERIALS AND METHODS

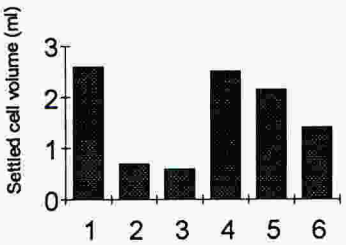
Synthetic methods will appear elsewhere (many are in the patents). IGP, chemically synthesized as described by Ames (1957), was mainly *anti* but also contained *c.* 15% of the (non-substrate) *syn* stereoisomer. The *anti* material was enantiomerically pure and completely transformed by the enzyme to IAP (based on ^{13}C and ^{1}H nmr). Assays at 30 °C in 0.1 M Hepes buffer at pH 7.0 containing 0.2 mM $MnCl_2$ were started with either

enzyme or IGP and stopped with alkali (to 1.2 M NaOH) to obtain time points up to *c.* 4 minutes. The extinction coefficient of the enol of IAP at 290 nM was *c.* 7500. For enzyme kinetics, a continuous assay was invented. 0.1M Hepes (pH 7.0) contained 0.1 mM pyridoxal phosphate, 0.1 M L-glutamate, 0.1 M NH_4Cl, 0.1 mM $MnCl_2$, 0.25 mM NADH and a large excess of pure IAP transaminase (overexpressed and purified from *E.coli*) and glutamate dehydrogenase (Sigma type III from bovine liver). Assays contained pure IGPD and were started with IGP or *vica versa* as appropriate. As expected, the dehydration of IGP resulted in quantitative oxidation of NADH up to absorbance changes of a unit or more (1 cm pathlength at 340 nm). With sufficient of the two coupling enzymes, initial lags were < 30 s and near-linear rates maintained for an hour or more. Ki values and 'off rates' (for inhibitor dissociation from the enzyme) of slow-binding potent inhibitors were determined (Schloss 1989) under conditions where 1) IC_{50} values (single enantiomer) were > 5 fold in excess of the enzyme (24 kD) concentration and 2) after enough time for inhibitor, enzyme and substrate to approach equilibrium (i.e. the same answer being obtained whether assays containing inhibitor were started with IGP or IGPD).

RESULTS AND DISCUSSION

I was a slow-acting, post-emergence herbicide giving total vegetative control at similar spray rates to glyphosate. Histidine (and its precursors) specifically reversed the inhibitory effect of I on the growth of maize cells (Figure 2). The herbicidal activities of various specific inhibitors correlated quite closely with their Ki values (cf table 1) versus IGPD.

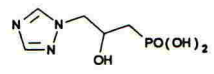
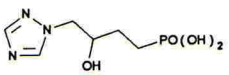
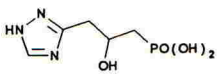
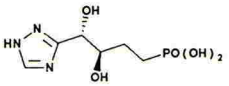
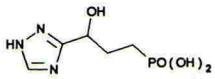
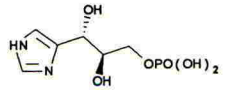
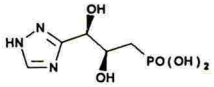
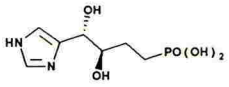
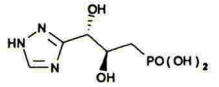
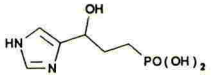
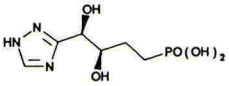
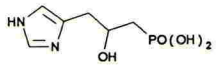
Figure 2 Inhibition of the growth of maize cells by I and specific reversal by histidine. Cells were grown in 10 ml of M & S medium at 24 °C. 1 = control, 2-6 contained I at 0.16 mM. 3 contained 0.5 mM each of val, leu and ileu, 4 contained 0.5 mM histidine, 5 contained 2 mM histidinol and 6, 0.5 mM histidinol phosphate.



The yeast HIS 3 gene (Struhl & Davis 1977) was overexpressed in *E. coli*. IGPD was induced as a peptide of the expected molecular weight (24 kD) on stained SDS gels. Electrospray mass spectroscopy indicated a molecular ion of the expected molecular weight (23834.5 after slight corrections to the published sequence) with perhaps 20% having lost the N-terminal methionine. As purified, in EDTA-containing buffer, *apo*-IGPD had no activity and chromatographed as a probable trimer (Mr 70 kD). The addition of a single equivalent per subunit of certain divalent metal cations (e.g. Mn^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+}, Fe^{2+} and Cd^{2+}) but not others (Mg^{2+}, Pb^{2+} and Ca^{2+}) caused the enzyme to assemble to a catalytically-active form of high molecular weight. Low angle X-Ray diffraction and light-scattering studies (Grabham *et al* 1993) indicated that the assembled enzyme has 24 subunits arranged in a particle with 432 symmetry and a molecular weight of 572 kD. Following assembly with excess metal and buffer exchange *via* gel filtration, *c.* 1.3 metal ions per monomer were tightly retained (Kd << 1 µM for most of the metal ions). We found no evidence for additional cofactors (organic phosphate) or significant amounts of metal other than that added during assembly. Mn-IGPD was the most active of the metallo-IGPD's with an activity of 65 µmol IAP formed min^{-1} mg^{-1} at 30 ° C and a Km of 0.1 mM for IGP at pH 7.0. Active-site titration and binding studies indicated a single

inhibitor binding site per enzyme subunit with one enantiomer of I being bound 20-30 fold more strongly than the other. From studies of the dipolar effects of the Mn^{2+} paramagnet on the T1 relaxation rates of protons (cf Dwek 1975) in water and in the competitive inhibitor, 1,2,4-triazole, we concluded that the metal in the enzyme is 1) buried in such a way that any water in its coordination sphere can exchange only slowly with the bulk water and 2) the metal is *c.* 12 Å away from triazole bound to the enzyme. Thus the metal seemed unlikely to be *directly* involved in either the binding of inhibitors or in catalysis (e.g. not the acceptor of the hydroxyl lost from IGP).

Table 1. SAR for inhibitors and substrates of IGPD. Compounds racemic except for IGP

Potent inhibitors	**koff** min^{-1}	**Ki** nM	**Inhibitors/ substrates**	**Vmax** % IGP	**Ki, Km** µM
 **I**	0.04	0.6	 **VII**	-	0.47
 **II**	< 0.01	<1.0	 **VIII**	not substr.	4.67
 **III**	0.36	11	 **IGP**	100	105
 **IV**	0.1	1.8	 **IX**	0.28	1500
 **V**	-	253	 **X**	not substr.	40
 **VI**	-	235	 **XI**	-	2900

The data in table 1 provided further clues to the enzyme mechanism. Inhibitors closely resembled the substrate and were, as you might expect, competitive with respect to IGP (as indeed are 1,2,4-triazole and phosphate). However, the most potent (I-V) were all a methylene unit shorter (C3) than needed to match the extended imidazole/ phosphate

distance of IGP. This suggested that C3 chain-length inhibitors might mimic a shortened intermediate in which a phosphorus oxygen acts as an *internal* base to remove the proton from the β-carbon (Widlanski *et al* (1989) made a similar proposal for dehydroquinate synthase). Activity as a substrate would then *require* a 4C (or equivalent) length chain. It is also notable that potent inhibitors were not, like the substrate, *anti* diols but *syn* diols, or preferably, β-mono-ols. This further suggested that these might mimic a catalytic intermediate which occurs *after* the elimination of the hydroxyl from IGP (Figure 4).

In place of imidazole, 1,2,4-triazoles were potent inhibitors but never substrates. For example, to compare the triazole, VIII, with the analagous imidazole, IX, we ran UV and nmr experiments over long periods using large amounts of enzyme (the enzyme being essentially stable) and would have expected to detect activity at < 1% of the level observed for IX. Given the profound differences in activity between imidazoles and triazoles, it seems likely that the heterocycle participates in catalysis and is not just a handle by which the enzyme recognizes the substrate. The mechanism proposed in Figure 4 starts with the formation of a high-energy diaza-fulvene intermediate *via* deprotonation of the imidazole and concomitant loss of the side chain hydroxyl from IGP. Since 1,2,4-triazoles (pKa *c.* 10) are more readily deprotonated than imidazoles (pKa 14) the triazole anion must be more stable and, presumably, less ready to push electrons into an exocyclic bond. This would account for 1,2,4- triazoles being poor substrates relative to imidazoles. On the other hand, we propose that 1,2,4-triazoles act as good *inhibitors* because, both C and N-linked, they are electronically similar to the diaza-fulvene.

The isosteric phosphonate analogue of IGP, IX, was a poor substrate with kcat *c.* 350 fold lower than for IGP and, at pH 7, a Km value some 15 fold higher. Replacing an electron-withdrawing oxygen with a methylene could raise the pKa of the proton on the β-carbon by as much as 3 units. The observed decrease in kcat is therefore consistent with any number of mechanisms in which proton abstraction is (partly) rate limiting.

The pH-dependence of kcat and kcat/Km can provide clues to enzyme mechanism *provided* that a chemical step in catalysis (rather than a substrate-induced change in conformation or product release) remains rate limiting over the range of assay conditions. We carried out nmr experiments (not shown) to discover how quickly various analogues of the substrate and product exchanged on and off of the enzyme. From the paramagnetic effect of Mn(II) in the enzyme on the T2 relaxation rates of protons (Dwek 1975) we discovered that 1,2,4-triazole (which has a Ki similar to the Km of IGP) exchanges at least a 100 fold faster than catalytic turnover. Triazole exchanged relatively quickly even in the tighter ternary complex it forms with phosphate. Together, triazole and phosphate cause a conformational change in the enzyme which, most probably (see below) also occurs in catalysis. Thus, overall, it seemed most likely that IGP is a 'non-sticky' substrate (with Km similar to its dissociation constant from the enzyme) and that neither a change in conformation nor the release of product (IAP is a very weak inhibitor) determine kcat.

Figure 3 shows the pH-dependence of kcat and kcat/ Km with IGP as substrate. The pKa's of free IGP were 5.75 and 7.2 which, on the basis of changes in nmr chemical shift, were assigned to (mainly) the phosphate and imidazole groups, respectively. Loss of a proton from a group with an apparent pKa near 7 governed a decrease in the Km (data not shown) and an increase in kcat/ Km (noting that the pKa's governing the curve for kcat/ Km were within 0.6 of a unit and therefore fully mixed). We suggest that IGP *binds* to the enzyme as the imidazole base (the pKa near 7 governing Km and kcat/ Km) and, for *catalysis*, a group in the enzyme needs be as the acid (the pKa of 7.5-7.7 in kcat/ Km and kcat) and the phosphate of IGP as the dianion (the pKa near 5.8 in kcat). Consistent with these speculative assignments, the pH optimum of kcat observed using the slow substrate, IX, was narrower and about a half a unit higher than for IGP (i.e. changes in the right direction but not as great as expected on the basis of the two-unit increase in the phosphonate pKa relative to the phosphate of IGP). The Ki values of 1,2,4-triazole (uncharged) and difluorophosphonate (dianion) remained relatively constant between pH 6.2

and 8.2 indicating that groups on the enzyme involved in inhibitor binding do not titrate over this range. On the other hand, the marked dependence on pH of the Ki values of the potent triazole phosphonate inhibitors indicated that these were much tighter bound as the phosphonate dianion. This is consistent with the proposition that they mimic a dianionic intermediate in catalysis. Overall, our assignments of pKa's remain entirely speculative but can, at least, be reconciled with a reasonable proposal for the mechanism.

Figure 3 pH-dependence of kcat/Km and kcat with IGP as substrate. A constant ionic strength mix of buffers was used. Symbols are separate experiments, lines the range of computer fits.

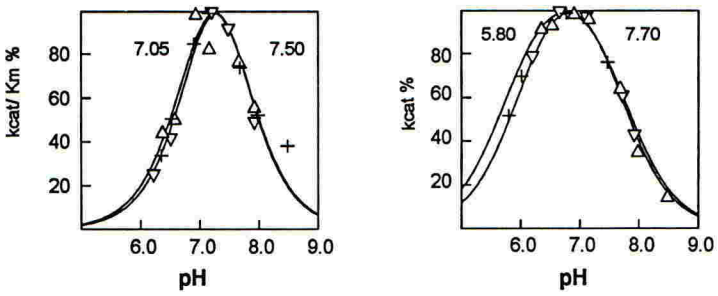
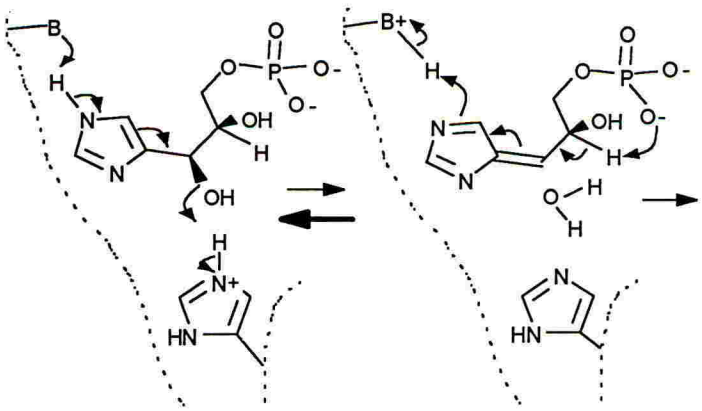


Figure 4 Possible steps in the mechanism of IGPD leading, via a diaza-fulvene intermediate, to the enol of IAP



A suggested mechanism as far as the *enol* of IAP is summarised in Figure 4 (the following steps being ketonisation and product release). The main features are 1) the metal is not directly involved 2) an acid group on the enzyme acts as the hydroxyl acceptor (e.g. perhaps a histidine with a pKa near 7.6 ?) and 3) the phosphate dianion (pKa 5.8 ?) acts as an internal base to remove the proton from the β-carbon. In the overall reaction, de-protonation at C2 triggers an irreversible rearrangement which displaces the initial, unfavourable, dehydration. The unstable diaza-fulvene intermediate would need to be tightly bound and it would make obvious sense for water to be excluded (nmr studies using ^{17}O label indicated that the enzyme does not catalyse exchange of solvent water with the hydroxyl group on the carbon next to the heterocycle in the slow substrate, IX); a localized hydrophobic environment would also help to drive the following stages of the reaction where charge separations are reduced. A number of lines of evidence suggest that such an environment might be created *via* a conformational change in the enzyme.

Subtle changes in the fluorescence of the tryptophan and in the enzyme-induced enhancement of the T1 relaxation rate of water protons indicated that probable reaction intermediate analogues (e.g. I, mixtures of 1,2,4 triazole with phosphate etc.) all caused a conformational change in the enzyme whereas 'ground-state' analogues (e.g. imidazole and phosphate) did not. Similarly, 1,2,4 triazole and phosphate were synergistic inhibitors whereas imidazole and phosphate were not. In addition, distance calculations based on nmr relaxation effects indicated that imidazole binds somewhat closer to the metal than does triazole. We suppose, therefore, that 1) IGPD is an example of 'Koshland-type' enzyme which wraps itself around the diaza-fulvene reaction intermediate and 2) potent inhibitors mimic this intermediate and induce the same protein conformational change as would normally occur in catalysis.

SUMMARY

Triazole phosphonates are a novel class of herbicides which act by inhibiting the biosynthesis of histidine. They are potent inhibitors of IGPD and, based on structure/ activity relationships and the pH-dependence of catalysis we have speculated on how their structure might be related to the catalytic mechanism. IGPD has a requirement for a divalent metal which, somewhat surprisingly, our nmr data (not detailed here) suggests to have no direct role in catalysis. A more definitive view of the enzyme mechanism awaits the synthesis of further potential alternative substrates (in particular, the imidazole analogue of V which we predict to *not* be a substrate) and a study of the primary isotope effects on kcat and kcat/ Km.

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