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STUDIES INTO THE MODE OF ACTION OF
GLYPHOSATE

Submitted by
David J. COLE
for the degree of Ph.D.
of the University of Bath.
November, 1979

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ABSTRACT

The mode of action of glyphosate has been studied with a variety of plant material. Chlorophyll formation was particularly sensitive, but was unlikely to be due to an interference with chloroplast macromolecule synthesis. Protein and RNA synthesis by isolated chloroplasts were not inhibited in vitro although chlorotic growth contained reduced levels of rRNA. The induction of nitrite reductase, a chloroplast protein was repressed in peas but not to an extent which would suggest inhibition of de novo synthesis. The induction of nitrate reductase was stimulated, but this was only transitory. Effects of glyphosate could not be negated by metal ions or glycine.

Glyphosate inhibited the growth of both single node buds of *Agropyron* rhizome and wheat roots. $^{14}$C-glyphosate administered to single nodes was recovered from the buds principally unchanged and did not bind to cell walls or a protein/nucleic acid fraction. A metabolite was present, representing 10% of total $^{14}$C-activity. The syntheses of DNA, RNA and cellulose were inhibited to similar extents, due partly to inhibition of $^{14}$C-precursor uptake, notably in the case of DNA synthesis. The incorporation of $^{14}$C-leucine was inhibited markedly but that of $^{14}$C-phenylalanine was not greatly affected whereas the incorporation of $^{14}$C-protein hydrolysate was inhibited to an intermediate extent. These results indicated an inhibition of protein synthesis brought about by a diminution of the phenylalanine protein precursor pool. The induction of phenylalanine ammonia-lyase (PAL) was enhanced markedly in both single node buds and wheat root tips. In the latter case this was accompanied by declines in soluble protein and the rate of formazan reduction. Growth inhibition could not be alleviated by exogenous L-phenylalanine, mixed aromatic amino acids or PAL inhibitors. The specific activities of shikimic acid pathway enzymes, polyphenol oxidase and some hydrolytic enzymes characteristic of senescence were also enhanced. A 'classic wound response' was not elicited as the evolution of ethylene was not activated. The generation of ethane, however, an indicator of oxidative membrane lipid breakdown was stimulated, but the specific activities of microsomal marker enzymes were unaffected. Levels of these enzymes declined due to a reduction in the amount of microsomal protein present. Microsomal ATPases were not inhibited in vitro by glyphosate.

The toxicity of glyphosate would appear to be mediated at least partly by a decrease in free phenylalanine resulting in inhibition of protein synthesis and de-repression of PAL. The involvement of other factors is not excluded.
ABBREVIATIONS

AMPA  amino methylphosphonic acid
L-AOPP  L-α-aminoxy-β-phenylpropionic acid
ATP  adenosine triphosphate
ATPase  adenosine triphosphatase
Bis  NN'-methylenebisacrylamide
CPM  counts per minute
CTP  cytosine triphosphate
2,4-D  2,4-dichlorophenoxyacetic acid
DNA  deoxyribonucleic acid
DNOC  2-methyl-4,6-dinitrophenol
DPM  disintegrations per minute
EDTA  ethylenediamine tetra-acetic acid
GLC  gas-liquid chromatography
glyphosate  N-(phosphonomethyl)glycine
GTP  guanosine triphosphate
HEPES  4-(2-hydroxyethyl)-1-piperazine ethane sulphonlic acid
IAA  indoleacetic acid
IDPase  inosine diphosphatase
IRGA  infra-red gas analysis
leu  leucine
MCPA  4-chloro-2-methylphenoxyacetic acid
MES  2-(N-morpholino)ethane sulphonic acid
NADH  reduced nicotinamide dinucleotide
NADP  nicotinamide dinucleotide phosphate
NADPH  reduced nicotinamide dinucleotide phosphate
NEDD  N-naphthylethylenediamine dihydrochloric acid
PAL  phenylalanine ammonia-lyase
phe  phenylalanine
Pi  inorganic phosphate
POPOP  1,4 di[2-(5-phenyloxazolyl)]benzene
PPO  2,5-diphenyloxazole, polyphenol oxidase
PVP  polyvinylpyrrolidone
RNA  ribonucleic acid
RNase  ribonuclease
SDS  sodium dodecyl sulphate
SORase  shikimate:NADP oxidoreductase
sp. act.  specific activity
TCA  trichloroacetic acid
TEMED  NNN'N'-tetramethylethylene diamine
TES  N-tris-(hydroxymethyl)-methyl-2-amino-ethane-sulphonic acid
TLC  thin layer chromatography
Tricine  N-[(tris hydroxymethyl)-methyl glycine]
Tris  2-amino-2-(hydroxymethyl)propane-1,3-diol (tris)
tryp  tryptophan
ty  tyrosine
UDP  uridine diphosphate
UTP  uridine triphosphate
ABSTRACT

The mode of action of glyphosate has been studied with a variety of plant material. Chlorophyll formation was particularly sensitive, but was unlikely to be due to an interference with chloroplast macromolecule synthesis. Protein and RNA synthesis by isolated chloroplasts were not inhibited in vitro although chlorotic growth contained reduced levels of rRNA. The induction of nitrite reductase, a chloroplast protein was repressed in peas but not to an extent which would suggest inhibition of de novo synthesis. The induction of nitrate reductase was stimulated, but this was only transitory. Effects of glyphosate could not be negated by metal ions or glycine.

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Introduction
INTRODUCTION

1. THE ADVENT AND DEVELOPMENT OF HERBICIDES

The concept of the chemical control of weeds, as opposed to traditional manual and mechanical methods, was commercially introduced in the last years of the 19th century with the use of simple inorganic salts, following the discovery of the herbicidal properties of copper sulphate. The first novel organic compounds to be used in this manner were the dinitrophenols, introduced in 1932 with DNOC; and the beginnings of physiological selectivity came in 1940 with the discovery of the differential herbicidal properties of α-napthalacetic acid. The use of herbicides received its major impetus, however, later that decade with the discovery of 2,4-D, MPCA and other phenoxyacetic acids. It can be said that the selectivity that the advent of these compounds brought truly revolutionised methods of weed control and agricultural practice throughout the world. Since that time, the production of new herbicides has proceeded at an ever-increasing rate with herbicides now available for almost any specified task in agriculture and horticulture. In 1958, eleven herbicides were approved for use in the UK by the Agriculture Chemical Approvals Scheme (ACAS), (Fryer and Makepeace, 1978) and by 1979 this figure had increased to 80, a figure exceeded several times by the total number of commercial formulations available (Anon, 1979). Although many early herbicides such as the phenoxyacetic acids are still in widespread use, others, such as DNOC, have now been more or less completely supplanted.
Despite this progress, the search for new and more sophisticated herbicides continues, not least because the use of herbicides has in itself created new problems. The eradication of the once prevalent broad-leaved weeds in cereals, for example, has in the absence of competition enabled the establishment of grasses as the dominant weeds, which have proved more difficult to control, a problem compounded by the development of resistant strains.

2. GLYPHOSATE AND ITS PROPERTIES

2.1. Introduction

Glyphosate (N-(phosphonomethyl)glycine) is the active principle of the herbicidal formulation 'Roundup', introduced by the Monsanto Company. Its herbicidal properties were first described by Baird et al in 1971 and its activity against common couch (Agropyron repens L. Beauv) reported the following year by Baird and Begeman (1972). Among modern herbicides, glyphosate is particularly remarkable for combining outstanding herbicidal efficacy, due to its ability to undergo extensive translocation within the plant, with notable ecological safety. Special interest is merited in the biochemistry of glyphosate, however, since alone with a related compound glyphosine (N,N-Bis(phosphonomethyl) glycine; a promoter of yield in sugar cane) it represents a completely novel class of plant growth inhibitor. As a phosphonomethyl derivative of glycine, the structure of glyphosate is relatively simple and confers high polarity with a water solubility of 1% at 25°C (Fig. 1).

*hereafter referred to as Agropyron
Glyphosate is a non-selective, foliarly applied herbicide, but due to its translocatability its major use is in the control of perennial grasses which propagate mainly by vegetative means, either by rhizomes, e.g. *Agropyron repens*; stolons, e.g. *Agrostis stolonifera*; or tubers, e.g. *Cyperus rotundus*. The aerial parts of the plant are normally slow to develop visual symptoms of toxicity, in contrast to those of herbicides which act at the point of application e.g. paraquat, but new vegetative growth is inhibited with the eventual death of the plant. As such, glyphosate is currently the only herbicide which can offer complete kill of such weeds at economic dose rates.

2.2. Translocation

The translocation of glyphosate has been demonstrated by Haderlie (1975), Sprankle et al (1975c), Claus and Behrens (1976), and Wyrill and Burnside (1976). Isotopically labelled glyphosate, when foliarly applied, was shown to accumulate at the points of greatest metabolic activity throughout the plant. In the case of *Agropyron* the greatest physiological sinks were the apical buds of the rhizome, shown by Claus and Behrens (1976) to be the sites of initial and greatest accumulation of foliarly applied $^{14}$C-glyphosate. At doses which were below that required for
complete kill of all rhizome buds, the distribution of $^{14}$C along the rhizome formed a gradient, with greatest accumulation in nodes at the apical end of the rhizome and least activity in nodes at the mother plant end. This distribution correlated with the inhibition of bud development. It thus appeared that after the initial accumulation of glyphosate in the apical bud to a toxic level, subsequent death abolished apical dominance, releasing the penultimate bud from dormancy which in turn became the largest sink. In this way accumulation of glyphosate worked back along the rhizome to the mother plant.

The pattern of translocation and accumulation of glyphosate with respect to physiological sinks would tend to suggest movement in the phloem; such herbicides being thought to conform to the mass-flow theory for assimilates (Ashton and Crafts, 1973). The efficacy of glyphosate should thus be greatest when applied to actively growing plants, having a maximum number of physiological sinks.

2.3. Applications of glyphosate

Glyphosate as 'Roundup' is formulated as the isopropylamine salt and has now been approved in the UK for several uses (Anon, 1979), most importantly for the control of *Agropyron* and other perennial grasses before or after cropping. This is particularly useful in combination with the cultural practice of minimum tillage and direct drilling. Other approved uses included the clearance of waste ground, verges or pasture before resowing, the control of weeds in fruit orchards and coniferous forests and plantations, and of aquatic weeds in water courses and lakes. In 1979, glyphosate became available as a domestic weed killer in
the UK, marketed by Murphy as 'Tumbleweed'.

2.4. Ecological Aspects

The ecological safety of glyphosate is characterised by low or negligible toxicity to wildlife and microorganisms, rapid microbial breakdown and strong binding in the soil. Mammalian toxicity is very low, with an LD<sub>50</sub> for oral toxicity in rats of 43200 mg kg<sup>-1</sup>. Rainbow trout have a tolerance in excess of 1000 ppm.

Several studies have indicated that glyphosate does not adversely affect soil microflora. Rueppel et al (1977) showed that glyphosate had no effect on the rate of evolution of <sup>14</sup>CO<sub>2</sub> from <sup>14</sup>C-sucrose in soil and population analysis showed no effect on total soil microflora. Marsh et al (1977) showed that glyphosate did not inhibit CO<sub>2</sub> production from soil and the production of NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> from soil was stimulated rather than inhibited. This study was carried out using glyphosate at 100 ppm, a level considerably in excess to that which may ever be expected in the field. In addition, it was found that glyphosate had no effect on microbial cellulytic activity as assayed by the degradation of straw and <sup>14</sup>C-labelled rye leaves (Grossbard and Cooper, 1974).

Degradation of glyphosate in soil has been demonstrated by Sprankle et al (1975b). Soil incubated with <sup>14</sup>C-glyphosate evolved <sup>14</sup>CO<sub>2</sub>, a process partially but not completely inhibited by the inclusion of sodium azide, a metabolic inhibitor. Rueppel et al (1977) reported that under both aerobic and anaerobic conditions, <sup>14</sup>CO<sub>2</sub> was rapidly evolved from <sup>14</sup>C-glyphosate at a rate comparable to the degradation of <sup>14</sup>C-sucrose. Breakdown did not occur
in sterile soils, with the conclusion that degradation of glyphosate was metabolic rather than chemical. One organic metabolite, aminomethylphosphonic acid (AMPA) was produced from \(^{14}\text{C}-\text{methyl labelled} \) glyphosate to a maximum of 15% total \(^{14}\text{C}\), but this also readily underwent degradation. Several other metabolites were detected, but these occurred to only very minor extents. Glyphosate was also degraded in water. Glyphosate loses activity when applied to the soil, as shown by Brewster and Appleby (1972), Upchurch and Baird (1972) and Sprankle et al (1975a). It has been concluded that glyphosate binds to soil particles via the phosphonic acid group, as it can be displaced by adding phosphate (Sprankle et al., 1975b). Binding was also increased by the addition of Fe\(^{3+}\) and Al\(^{3+}\). Hance (1976) from a study with nine soils concluded that glyphosate was adsorbed in a manner similar to that of orthophosphate.

3. THE CONTROL OF **AGROPYRON REPENS** AND OTHER PERENNIAL GRASSES

3.1. Ecology of Agropyron

The introduction of a herbicide for the reliable control of perennial weeds is of considerable significance. *Agropyron* is now regarded as the most pernicious weed in the UK and other temperate regions by virtue of its ability to propagate swiftly by underground rhizomes. Baker (1965) has listed advantageous characteristics for the 'ideal weed' which include vigorous vegetative growth and an ability to regenerate from severed portions of rootstock. These properties are ably demonstrated by *Agropyron*. Buchholtz (1962) has reported that one ha can
Plate 1: *Agropyron repens* (L.) Beauv.
contain 4273km of rhizome, a bud every 2.5cm giving a total of 161.4m buds. Most rhizome buds remain dormant if undisturbed and it has been estimated that in untilled land 95% of buds will never germinate (Johnson and Buchholtz, 1962). Fragmentation of the rhizome, however, will break apical dominance with the subsequent proliferation of new independent plants and under competition-free conditions, one bud can give rise to 90m of rhizome in one year (Hay, 1962). Crop yield can be reduced by competition for sunlight, water and nutrients and possibly by the production of allelopathic substances from the rhizome (Osvald, 1947; Kommedahl et al., 1959; Welbank, 1963). *Agropyron* can also act as host for fungal diseases of crops (Muenscher, 1955; King, 1965).

### 3.2. Chemical control

Prior to the advent of glyphosate, an acceptable degree of control could be achieved using one of several herbicides (Fryer and Makepeace, 1978). However, complete kill was rarely possible, dose rates were high and the residual nature of these herbicides necessitated a considerable lapse of time after application before crops could be sown. Also, control of *Agropyron* by conventional herbicides has often necessitated a programme of repeated application over a period of several years.

Sodium chlorate in the past has been used at a rate of 120-250kg ha\(^{-1}\) but has now generally been superceded. Control with TCA can be achieved using a recommended dose rate of 33kg ha\(^{-1}\). Herbicides which have mainly been used for control of *Agropyron* are dalapon and aminotriazole. Complete kill may be attained at high dose rates, but the
prohibitive cost has meant the employment of lower rates which give an acceptable level of control. Recommended dose rates for dalapon are 9.5-16kg ha$^{-1}$ and for aminotriazole 4.5kg ha$^{-1}$ and the two compounds can be mixed. Paraquat can be used to repeatedly burn off the foliage, progressively exhausting the rhizome.

In contrast, glyphosate is recommended for use with *Agropyron* at 1.44kg ha$^{-1}$, a dose rate which gives excellent control. Indeed, Harvey and Potts (1978) have demonstrated that effective control can be attained using 50% of the recommended dose. Residual herbicide is negligible and crops may be sown very soon after application, provided sufficient time is allowed to lapse before tillage to allow effective translocation. Dowland and Tweedy (1972) and Behrens and Elakkad (1974) have reported that in pot-grown plants, allowing the foliage to remain intact for 6-8hr and 8-12hr respectively was sufficient to enable complete kill. In the field complete kill can be achieved in 24hr (Brockman et al., 1973), but Monsanto advise a period of 7-14 days between application and cultivation.

3.3. Control of other perennial grasses

Although *Agropyron* is particularly sensitive to glyphosate, many other weeds can be effectively controlled (Table 1). The tropical and sub-tropical grass *Cyperus rotundus* L. is acknowledged to present the world's most serious weed problem, having been reported in more areas than any other (Holm et al., 1977). The efficacy of glyphosate on *Cyperus Rotundus* has been evaluated by Bairó et al. (1971), Zandstra et al. (1974) and Suwunninamek and Parker (1975). Some other important perennial grass
Table 1

Perennial weed control by glyphosate
(from Roundup technical bulletin, Monsanto, 1977)

<table>
<thead>
<tr>
<th></th>
<th>Stage of growth at treatment is very important</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(a) Susceptible at 1.08 to 1.8 kg ae/ha</strong></td>
<td></td>
</tr>
<tr>
<td>Agropyron repens</td>
<td>Mentha spp.</td>
</tr>
<tr>
<td>Agrostis gigantea</td>
<td>Mercurialis perennis</td>
</tr>
<tr>
<td>Agrostis stolonifera</td>
<td>Pennisetum clandestinum</td>
</tr>
<tr>
<td>Arthrotheca spp.</td>
<td>Phalaris arundinacea</td>
</tr>
<tr>
<td>Dactylis glomerata</td>
<td>Phragmites communis</td>
</tr>
<tr>
<td>Eragrostis curvula</td>
<td>Poa pratensis</td>
</tr>
<tr>
<td>Eragrostis plana</td>
<td>Pteridium aquilinum</td>
</tr>
<tr>
<td>Festuca pratensis</td>
<td>Rubus spp.</td>
</tr>
<tr>
<td>Glyceria maxima</td>
<td>Rumex spp.</td>
</tr>
<tr>
<td>Lolium italicum</td>
<td>Sorghum halepense</td>
</tr>
<tr>
<td>Lolium perenne</td>
<td></td>
</tr>
<tr>
<td><strong>(b) Susceptible at 2.16 to 2.88 kg ae/ha</strong></td>
<td></td>
</tr>
<tr>
<td>Alisma plantago</td>
<td>Paspalum spp.</td>
</tr>
<tr>
<td>Artemisia vulgaris</td>
<td>Plantago lanceolata</td>
</tr>
<tr>
<td>Carex spp.</td>
<td>Plantago major</td>
</tr>
<tr>
<td>Cirsium arvense</td>
<td>Potentilla anserina</td>
</tr>
<tr>
<td>Digitaria scalarum</td>
<td>Ranunculus repens</td>
</tr>
<tr>
<td>Epilobium hirsutum</td>
<td>Sonchus arvensis</td>
</tr>
<tr>
<td>Festuca rubra</td>
<td>Stachys palustris</td>
</tr>
<tr>
<td>Heracleum sphondylium</td>
<td>Taraxacum officinale</td>
</tr>
<tr>
<td>Juncus effusus</td>
<td>Tussilago farfara</td>
</tr>
<tr>
<td>Lotus corniculatus</td>
<td>Typha latifolia</td>
</tr>
<tr>
<td>Panicum maximum</td>
<td>Urtica dioica</td>
</tr>
<tr>
<td><strong>(c) Susceptible at 3.24 to 3.96 kg ae/ha</strong></td>
<td></td>
</tr>
<tr>
<td>Aegopodium podagraria</td>
<td>Ottolochia nodosa</td>
</tr>
<tr>
<td>Arctium spp.</td>
<td>Potentilla reptans</td>
</tr>
<tr>
<td>Cyperus spp.</td>
<td>Ranunculus bulbosus</td>
</tr>
<tr>
<td>Daucus carota</td>
<td>Symphytum spp.</td>
</tr>
<tr>
<td>Imperata cylindrica</td>
<td>Trifolium repens</td>
</tr>
<tr>
<td>Mikania cordata</td>
<td></td>
</tr>
<tr>
<td><strong>(d) Susceptible above 4 kg ae/ha</strong></td>
<td></td>
</tr>
<tr>
<td>Alopecurus pratensis</td>
<td>Equisetum arvense*</td>
</tr>
<tr>
<td>Asparagus spp.</td>
<td>Hedera helix</td>
</tr>
<tr>
<td>Carduus crispus</td>
<td>Oxalis latifolia</td>
</tr>
<tr>
<td>Convolvulus arvensis</td>
<td>Scirpus maritimus</td>
</tr>
<tr>
<td>Convolvulus sepium</td>
<td>Senecio jacobea</td>
</tr>
<tr>
<td>Cynodon dactylon</td>
<td>Prosopis fracata</td>
</tr>
</tbody>
</table>

*Stage of growth at treatment is very important.*
weeds against which glyphosate is effective are *Imperata cylindrica* (Dickens and Buchanan, 1975), *Cynodon dactylon* (Baird et al., 1971; Jordan, 1977), *Cyperus aesculente* (Stahler et al., 1975) and *Cirsium arvense* (Baird et al., 1971; Haderlie, 1975; Saidak and Marriage, 1976).

4. THE MODE OF ACTION OF HERBICIDES

Herbicides can generally be said to act through one of three main functional areas, namely energy supply, growth and structural organization. In most cases, herbicides act specifically at points peculiar to the physiology and metabolism of plants, for example photosynthesis and hormonal growth control.

Photosynthetic inhibitors form the major class of herbicides and as more than half the currently used herbicides have been shown to inhibit photosynthesis these have been most widely studied. Photosynthetic inhibitors act either on the Hill reaction by inhibiting photosynthetic electron transport, e.g. the ureas such as monuron and diuron and the triazines, e.g. simazine and atrazine, or by acting as electron acceptors, i.e. the bipyridiliums, e.g. paraquat which divert electrons from the reduction of NADP in PSI. These compounds are somewhat unusual in that the free radical produced by this reduction is reoxidised in the presence of O$_2$ to regenerate the original ion, available again as an electron acceptor. Reoxidation in addition forms reactive radicals such as superoxide (O$_2^-$) which attack lipid membranes, initially those of the chloroplast and then of the rest of the cell. Free radical generation in this way is now thought to be a major
contributing factor in the toxicity of paraquat and similar reactive ions have been implicated in the action of Hill reaction inhibitors.

Conversely, few herbicides act by inhibiting respiration. These are mainly the dinitrophenols, e.g. DNOC and dinoseb which uncouple respiratory electron transport by destroying the proton differential across the mitochondrial membrane. The hydroxy-benzonitriles, e.g. ioxynil are also uncouplers, but in addition can inhibit the Hill reaction. Biosynthetic inhibitors have not found wide use as herbicides. Protein synthesis is the target of the α-chloroacetamides, e.g. propachlor. Here Cl reacts readily with nucleophilic groups such as thiols, resulting in covalent bonding and it has been suggested that these compounds may act by alkylation of the amino group of amino acyl t RNA's (Jaworski, 1969). Alidochlor has been shown to inhibit the incorporation of 14C-malonate into lipids (Mann and Pu, 1968) and the thiolcarbamates, e.g. EPTC and di-allate have been implicated as inhibitors of lipid synthesis, particularly of epicuticular waxes. Amino-triazole inhibits carotenoid synthesis with the subsequent photo-destruction of chlorophyll (Burns et al., 1971). Several other compounds are known to act similarly, e.g. dichloromate. The N-phenyl carbamates, e.g. propham and barban act through mitotic inhibition, but most compounds acting on growth are the synthetic auxins: the phenoxyalkanoic acids, e.g. 2,4-D and MCPA and the benzoic acid derivatives, e.g. 2,3,6-TBA. Application of these herbicides promotes rapid growth of the root and stem, resulting in premature senescence of
leaves and secondary roots. At present it is believed that auxins act at two separate metabolic points, influencing both transcription by controlling RNA polymerase and cell wall loosening and deposition, by for example enhancing β-glucan synthetase activity (Cherry, 1976). The mode of action of herbicides has been extensively reviewed by Hilton et al. (1963), Moreland (1967), Ashton and Crafts (1973), Corbett (1974) and Audus (1976), but although a wealth of information now exists on the modes of action of many established herbicides, those of many more including the more recent additions is poorly understood.

5. THE MODE OF ACTION OF GLYPHOSATE

5.1. GLYPHOSATE AND THE AROMATIC AMINO ACID PATHWAY

Initial work on the mode of action of glyphosate was published in 1972 by Jaworski who found that the toxicity of glyphosate could be alleviated to varying degrees by the concomitant administration of aromatic amino acids, or some of their precursors, intermediates of the shikimic acid pathway. Glyphosate inhibited the growth of fronds of *Lemna gibba*, an effect completely abolished by the addition of equal concentrations of phenylalanine, tyrosine and tryptophan. The addition of phenylalanine alone reduced inhibition by 50%. Growth inhibition of the bacterium *Rhizobium japonicum* was reduced by 50% with the addition of a mixture of phenylalanine and tyrosine and in both species lesser degrees of alleviation were found with the use of some shikimic acid pathway intermediates, i.e. phenylpyruvic acid, p-hydroxyphenylpyruvic acid, prephenic acid and chorismic acid (*Rhizobium*); and phenylpyruvic acid,
phenyllactic acid and chorismic acid (*Lemna*). L-Proline also caused some alleviation in *Lemna*.

From these results Jaworski speculated on the interference by glyphosate at some point in the pathway of the biosynthesis of aromatic amino acids, proposing specifically chorismate mutase and/or prephenate dehydratase as likely inhibition sites. The hypothesis was upheld by the effect of glyphosate on levels of amino acids in *Lemna*. It was noted that whereas the level of phenylalanine decreased, almost all other protein amino acids increased in concentration. In particular, the increase in the level of tyrosine was such that the ratio of tyrosine: phenylalanine was doubled by glyphosate. The increase in other free amino acids could be expected if protein synthesis was inhibited due to a deficiency of a single amino acid. A build up of tyrosine may be expected if an inhibition of prephenate dehydratase caused an increased flow of carbon from prephenate to tyrosine (see Fig. 2). Jaworski envisaged 2 forms of chorismate mutase (Gilchrist, 1972), specific for the pathways of phenylalanine and tyrosine. Inhibition of chorismate mutase activity of the phenylalanine pathway could also be consistent with increased tyrosine production, through diversion of chorismic acid metabolism. Furthermore, high concentrations of tyrosine caused symptoms in *Lemna* similar to those of glyphosate and an excess of tyrosine could be expected to cause feedback inhibition of one or more enzymes in the pathway. However, although plants contain three isoenzymes of chorismate mutase (Woodin et al., 1978) two are inhibited by both phenylalanine and tyrosine and a third shows no sensitivity. There is thus no differential sensitivity of
Figure 2: The shikimic acid pathway for aromatic amino acid biosynthesis (from Yoshida, 1969).
any isoenzyme to phenylalanine and tyrosine (Woodin and
Nishioka, 1973). Subsequent literature, however, has
shown the involvement of glyphosate in this area to be
rather more complicated, and Jaworski's original hypothesis
has not been unequivocally upheld. Reversal by adding
exogenous aromatic amino acids has not always been
demonstrated, being confirmed by Haderlie (1975, Haderlie
et al., 1977). Tymonko (1978) and Gresshoff (1979), but
not by Brecke (1976) or Duke and Hoagland (1978). A
possible interpretation of these conflicting results is that
potential reversal agents were not effectively taken up
into the tissues, or that the hypothesis cannot be
universally applied. Since it is unlikely that a herbicide
exhibits fundamental species differences in mode of action,
the literature deserves close analysis.

Haderlie (1975, Haderlie et al., 1977) studied the
mode of action using cultures of carrot and tobacco cells
and found that toxicity was partially reversed by the
addition of combinations of two aromatic amino acids,
greatly by the addition of all three and completely by
using casein hydrolysate. However, amino acid analysis
showed that the level of free phenylalanine was not
depressed, though levels of some other amino acids
increased significantly. An increase in total amino
nitrogen was accounted for by elevated levels of arginine,
ornithine, histidine, glutamate, glutamine and \( \text{NH}_3 \). A
similar pattern was also shown by Nilsson (1977) in an
amino acid analysis from sprayed plants of spring wheat.
In work by Haderlie, however, no rapid inhibition of \(^{14}\text{C-}
leucine incorporation or decrease in protein levels was
apparent, nor were there any significant differences in the rates of incorporation of $^{14}$C-phenylalanine and $^{14}$C-leucine (Haderlie et al., 1977). Nevertheless, the metabolic effects exerted by glyphosate appeared to be direct, since the addition of aromatic amino acids did not influence the rate of uptake of $^{14}$C-glyphosate into the cells. A carrot cell line resistant to p-fluorophenylalanine had partial resistance to glyphosate, but apparently only because these cells took up less glyphosate. These had levels of free phenylalanine six times that of ordinary cells, but there was no evidence to suggest that resistance to glyphosate was by virtue of this (Haderlie, 1975).

Studies by Tymonko (1978) yielded much more coherent results. By using cells isolated from soybean leaves, protein synthesis was shown to be the most sensitive biosynthetic function of several studied and was accompanied by a significant decrease in the level of soluble protein, by 26% and 41% after one and two days respectively. Free amino acid pools increased after one day with a breakdown pattern similar to those of Haderlie et al (1977) and Nilsson (1977). Phenylalanine levels, along with those of some other amino acids, began to fall after two days. The effects of glyphosate on growth, protein synthesis (see also Tymonko and Foy, 1973) and protein levels in bean leaf cells could be reversed, notably by a mixture of phenylalanine and tyrosine. Aromatic amino acids added individually were rather less effective. Also, as was the case with the work of Jaworski (1972) alleviation was obtained with some shikimic acid pathway intermediates, but only those occurring subsequent to chorismate mutase.
The most effective agent was prephenic acid, reducing the inhibition of protein synthesis from 57% to 19%. Phenylpyruvic acid, the immediate precursor of phenylalanine was more effective than p-hydroxyphenylpyruvic acid the immediate precursor of tyrosine. Inhibition of growth and protein synthesis was accompanied by an increase in the level of free amino acids, due largely to NH₃, arginine, glutamine, asparagine and aspartate. However a decrease in the free pools of phenylalanine and tyrosine was not observed until two days. Reversal was also obtained by Roisch and Lingens (1974) using E. coli. In an attempt to localise the site of action, these workers studied the in vitro effect of glyphosate on several isolated enzymes from the aromatic amino acid biosynthesis pathway. Two such enzymes were inhibited, deoxyoxoarabinoheptonic acid 7-phosphate synthetase and dehydroquinic acid synthetase. The former enzyme, catalysing the initial reaction of the pathway, namely the condensation of phosphoenol pyruvate and erythrose-4-phosphate, was inhibited by 50% at 10⁻²M glyphosate, a concentration which has dubious in vivo significance. However, neither chorismate mutase, prephenate dehydratase or prephenate dehydrogenase were inhibited in vitro.

The reversal phenomenon was not evident in experiments performed by Brecke (1976) in which the growth of plants of Zea mays and P. vulgaris in solutions culture were used. Aromatic amino acids singly or in combination did not alleviate toxicity when applied in the root nutrient solution simultaneously with or prior to the administration of glyphosate. Glyphosate did not prevent the uptake of
phenylalanine since this itself was toxic at the highest concentration used. More evidence that glyphosate is involved in the metabolism of amino acids was published by Ekanayake et al. (1979), who found that in treated plants of Panicum repens L. Beauv, the total amino acid content of the rhizome rose whilst phenylalanine was absent. Tyrosine was one of the amino acids which displayed an increase. Gresshoff (1979) found that the growth inhibition of E. Coli, Chlamydomonas reinhardi, suspension cultures of carrot and soybean and roots of Arabidopsis thaliana seedlings could all be alleviated by phenylalanine and tyrosine, or casein hydrolysate, but shikimate and chorismate were not active reversal agents.

Puke and Hoagland (1979) demonstrated that glyphosate markedly enhanced the level of the inducable enzyme phenylalanine ammonia-lyase (PAL) in dark-grown maize roots, thus introducing the possibility that the free phenylalanine pool decreased due to an enhanced rate of deamination rather than an inhibition of its formation. However, they also showed that protein levels did not decline and whereas the addition of aromatic amino acids produced a partial repression of glyphosate-induced extractable PAL, the toxicity of glyphosate as measured by root fresh weight was in fact enhanced. These workers postulated that the enhancement of PAL activity mediated an accumulation of toxic phenolic end products. Using an assay for total hydroxyphenolic compounds this was found not to be the case (Hoagland et al., 1978). An analysis of free amino acids again showed elevated levels of basic amino acids, although others were at lower levels than controls. The levels of both phenylalanine and tyrosine were reduced. In a subsequent
report, similar phenomena have been shown to occur in glyphosate treated dark-grown soybeans (Hoagland et al., 1979), whilst in light-growth soybeans, glyphosate and light were synergistic in promoting PAL activity (Duke et al., 1979). Holländer and Amrhein (1979) have reported that glyphosate, rather than promoting the level of phenolic compounds in fact markedly reduced the levels in hypocotyls of buckwheat, a phenomenon reversed by L-phenylalanine, together with partial alleviation by chorismic acid, phenylpyruvic acid and trans-cinnamic acid. The accumulation of free phenylalanine precipitated by the administration of the PAL inhibitor L-a-aminoxy-β-phenylpropionic acid (L-AOPP) was reduced by glyphosate. The possibility that glyphosate inhibited the shikimic acid pathway derived support from the fact that the conversion of 14C-shikimic acid to phenylalanine was markedly inhibited. However, Duke and Hoagland (1979) reported that whilst L-AOPP abolished the free phenylalanine deficit caused by glyphosate in soybean seedlings, growth inhibition was only slightly relieved.

In general, the literature discussed here suggests the likelihood of an inhibition of the shikimic acid pathway at some point between the metabolism of shikimic acid and chorismic acid (Fig. 2), resulting in a deficit of free phenylalanine. Inhibition before chorismate mutase would be unlikely since this would also inhibit the formation of tryptophan, not an effective reversal agent. The observations of Duke and Hoagland (1979) would indicate that such an inhibition may not fully explain the mode of action.
5.2. Chelation as a mode of action

Phosphonic acids are known chelating agents (Carter et al., 1976) and it is possible that glyphosate may exert its action by complexing biologically important divalent cations within the cell. If so, toxicity should be alleviated by the addition of an excess of cations, however Jaworski (1972) was unable to demonstrate such a phenomenon. The in vitro inhibition by glyphosate of deoxyoxo-arabino-heptonic acid-7-phosphate synthetase and dehydroquinic acid synthetase observed by Roisch and Lingens (1974) was removed by the addition of Co^{2+}, a known cofactor for the former but not the latter enzyme (Srinivasan and Sprinson, 1959; Sprinson et al., 1962a,b). The complexation of glyphosate with metal ions is certainly strong enough to reduce its efficacy and in particular iron, zinc, aluminium and calcium have been shown to antagonise glyphosate in tank mixes, (Wills, 1973; Philips, 1975; Sandberg et al., 1976; Hanson and Rieck, 1976) whilst adding EDTA and other complexing agents increased the activity of glyphosate (Turner and Loader, 1978). Sprankle et al (1975c), however, did not find any antagonism when glyphosate was sprayed with iron.

5.3. Other metabolic effects

A variety of effects of glyphosate on biochemical and physiological processes have been reported in a diversity of plant material, i.e. whole plants, leaf discs, isolated cells and cell cultures.

Work by Abu-Irmaileh (1977) on plants of Cyperus rotundus showed that the responses of attached leaves to glyphosate were slow. The carotenoid content of leaves
declined by 39% after 96 hrs, with a similar value for catalase activity, although chlorophyll levels decreased to a lesser extent (Abu-Irmaileh and Jordan, 1978) implying photoxidation of chlorophyll. However, Ali and Fletcher (1978) found that in maize seedlings, carotenoid content of leaves did not decline although chlorophyll levels did. Abu-Irmaileh (1977) postulated an oxidative action of glyphosate on leaves, since the anti-oxidant a-tocopherol (Vit.E) apparently antagonised the action of glyphosate on leaves of Sesbania. Whole plants of Phaseolus vulgaris showed increased rates of ethylene and CO₂ evolution, with a slight increase in cellulase activity of abscission zones (Abu-Irmaileh et al., 1979). Most of these effects, however, took several days to manifest themselves, and it is thus doubtful if they can be directly related to an initial mode of action.

Haderlie (1975, Haderlie et al., 1977) examined several aspects of metabolism in carrot and tobacco cell cultures. No rapid effect was observed on RNA or protein synthesis but there was a significant reduction in DNA synthesis as measured by the incorporation of ¹⁴C-thymidine. This appeared, however, to be due to non-availability of substrate, since uptake was similarly inhibited. It was found that glyphosate caused an initial increase in nitrate reductase activity, but this soon fell to sub-control levels (Haderlie, 1975). Brecke (1976) used leaves of P. vulgaris and suspensions of cells enzymatically isolated from them. Leaf discs from treated plants showed reduced rates of RNA and protein synthesis, photosynthesis and respiration as determined by studies with labelled precursors, RNA synthesis being inhibited before protein
synthesis. However, isolated cells from pre-treated leaves showed reduced absorption of labelled uridine, leucine and glucose, which may again have been the limiting factor in these functions. Biosynthetic effects were also investigated by Tymonko (1978) who found that in cells isolated from soybean leaves, glyphosate caused a marked inhibition of protein synthesis. RNA synthesis was inhibited to a lesser extent, and lipid synthesis was the least sensitive of these processes, being inhibited to a similar extent as photosynthesis and respiration. The metabolite of glyphosate, aminomethylphosphonic acid (AMPA) also affected protein and RNA synthesis, although was only about half as effective. In the case of glyphosate, the inhibition of these processes could be reversed by the addition of aromatic amino acids, but inhibition by AMPA was not.

Studies on uptake of glyphosate by Brecke (1976) showed that whereas leaf discs readily absorbed $^{14}$C-glyphosate, cells subsequently isolated from them contained no activity. Similarly single cells did not appear to absorb $^{14}$C-glyphosate. This led to the idea that the effects observed on metabolic processes were due to an interaction with glyphosate at the cell membrane. In Brecke's work, the most rapid effect observed was on inhibition of absorption of $^{86}$Rb and $^{32}$Pi. Cell membrane integrity appeared not to be affected as leakage of electrolytes and $^{86}$Rb from isolated cells was negligible. From these and observations that glyphosate decreased the leaf angle and stomatal resistance in bean leaves, Brecke (1976) postulated that glyphosate might interfere with K$^+$ pump mechanisms, possibly K$^+$-ATPases in the cell membrane.
Shaner (1978) has reported that glyphosate inhibited transpiration in bean plants, apparently due to stimulation of stomatal closure rather than affecting the water potential of leaf cells (Shaner, 1978; Brecke, 1976). Subsequent work by Shaner and Lyon (1979) demonstrated that glyphosate cause stomatal cycling with a period of about 40 mins in bean leaves, possibly as a result of changes in internal water stress. This could not be explained purely as a result of the inhibition of K⁺ accumulation within guard cells, since accumulation would be necessary for stomatal opening. The onset of stomatal cycling was preceded by decreases in leaf conductance (50%) and net photosynthesis (by about 35%) after 6 hrs.

In contrast to Brecke's results with uptake of ¹⁴C-glyphosate, cultured carrot cells readily accumulated the labelled compound (Haderlie, 1975) and from a number of ultrastructural reports it would seem evident that glyphosate does enter the cell. In leaf discs from sprayed plants of *Agropyron*, Campbell et al (1976) found that the chloroplast envelope was disrupted, along with swellings of the rough endoplasmic reticulum and vesicle formation. Hull et al (1977) reported the disruption of chloroplasts in leaves of *Prosipis juliflora* and Hoagland and Paul (1978) found that after dipping leaves of *Cyperus rotundus* in glyphosate, chloroplasts displayed membrane disorganization with swelling and eventual bursting. This was followed by changes in mitochondrial appearance and tonoplast structure. The related compound glyphosine also causes chlorosis and an examination of the cell ultrastructure in emerging bleached leaves of maize seedlings by Croft et al (1974) showed swollen chloroplasts, with reduced levels of grana and
thylakoids. From these observations on photosynthetic cells, it seems clear that chloroplast damage occurred prior to other membrane effects. Chlorosis of new growth after herbicide application indicates that glyphosphate may selectively inhibit some aspect of chloroplast development. Croft et al (1974) showed that bleached emergent leaves of maize seedlings treated with glyphosate contained reduced levels of 70S ribosomes and chloroplast ribosomal RNA, whereas the counterpart cytoplasmic components were not affected.

The effects of glyphosate on photosynthesis and respiration have been studied more extensively than other cell functions. Respiration has generally shown to be not greatly affected initially, as evidenced by work with whole plants (Sprankle et al., 1975c), roots and isolated cells (Brecke, 1976) and yeast cells and isolated mitochondria (Hanson and Rieck, 1975; Haderlie, 1975). Ali and Fletcher (1978) found that in foliarly treated maize seedlings respiratory activity of root tips, as measured by formazan reduction was reduced to 50% after 6 hrs. Since, by definition, respiration is ultimately inhibited in cell death, this probably merely indicated the speed with which glyphosate was moving in the plant. Haderlie (1975) and Sprankle et al (1975c) have shown photosynthetic CO\textsubscript{2} uptake to be more sensitive, and studies with Scenedesmus have shown an inhibition of O\textsubscript{2} evolution (van Rensen, 1974). In isolated spinach chloroplasts pre-incubation with glyphosate caused an inhibition of electron transport with diquat and DCPIP acceptors (van Rensen, 1974). Croft et al (1974) found no inhibition of chloroplast electron
transport by glyphosine.

Recently, glyphosate has been shown to be a potent inhibitor of auxin transport in isolated coleoptiles, an investigation prompted by the fact that glyphosate causes extensive tillering at sub-lethal doses. Baur (1979) found that glyphosate inhibited the basipetal movement of $^{14}$C-IAA in maize coleoptiles, applied to the apex from an agar block. Transport in this way was completely inhibited at $7.5 \times 10^{-5}$M glyphosate and by approximately two thirds at $7.5 \times 10^{-7}$M glyphosate. In addition, a build up of $^{14}$C-IAA was observed at the basal end of the coleoptile, which together with a lack of diffusion of $^{14}$C-IAA into agar blocks at either end of the coleoptile indicated that glyphosate had caused binding of IAA within the coleoptile. Cotton coleoptiles were less responsive and a similar phenomenon could only be elicited upon pre-incubation with glyphosate for 24 hrs. This was correlated with the fact that whole plants of cotton were less sensitive to glyphosate than those of maize. Since it had been previously shown that ethylene inhibited auxin transport (Beyer and Morgan, 1969), Baur postulated that glyphosate may induce the formation of ethylene. As mentioned earlier, a stimulation of ethylene production by glyphosate was demonstrated by Abu-Irmaileh et al. (1979). Other effects of glyphosate reported were influences on protease and phosphatase activities in *Lemna* (Hoagland, 1978) and some genetic modification of pollen mother cells in sprayed plants of *Secale cereale* (Boyle and Evans, 1974).
6. APPROACHES TO STUDYING THE MODE OF ACTION OF HERBICIDES

The mode of action of herbicides has been investigated using whole plants, isolated organs, isolated protoplasts, cell culture, isolated organelles and ultimately in vitro reactions. With whole plants, functions such as growth and CO₂ exchange can be studied. Alternatively, treatment of whole plants can be followed by isolation of those areas where symptoms are seen to develop. Once the target organs of the plant have been identified, these can if required be isolated prior to treatment. This approach requires smaller scale experiments, facilitating replication and hence uniformity and reproducibility. Because the herbicide is applied directly to the organ, effects can be discerned more rapidly due to the abolition of transport which may have been required in the whole plant; experimental times are thus shorter. Great advantages over this approach can however be achieved with the use of suspensions of cells isolated from plant material, e.g. leaves and fruit, or true cultures of dividing cells. This results in metabolically uniform cells which can also take up the herbicide quickly enabling effects to be determined rapidly. An important advantage is that material can be manipulated easily and accurately in the manner of bacterial suspension cultures, enabling a much higher standard of reproducibility. The use of this material does, however, present some disadvantages and is not particularly useful if the compound being investigated is normally metabolised in the plant from an initially inert precursor to an active compound at a point removed from the site of action, or if the herbicide interferes with the transport of assimilates, hormones, etc.
In addition, there are important differences between these two types of cell. Enzymatically isolated single cells have been used previously in mode of action studies by Boulware and Camper (1972), Porter and Bartels (1977), Ashton et al (1977) and Rafii et al (1979). Both Brecke (1976) and Tymonko (1978) have studied the mode of action of glyphosate using cells isolated from mature leaf tissues, yet there is abundant evidence that when applied to the leaf glyphosate is readily translocated to meristemic sites and does not accumulate in cells of mature tissues. Since glyphosate does not appear to enter stationary cells in the whole plant it seems dubious to use these for mode of action studies. Cells isolated from mature leaves are specialised photosynthetic cells, and being stationary may lack some processes associated with dividing cells. In particular, DNA synthesis and processes associated with cell extension and expansion would be present only at low rates and the cells would generally have a lower rate of molecular turnover.

The concept that a compound that affects only meristemic cells would inhibit cultures of dividing cells rather than stationary cells has been discussed by Gressel and co-workers. These have shown that dikegulac, a growth regulator which like glyphosate accumulates only in apical areas with negligible effect on other areas of the plant (Arzee et al., 1977) inhibited the incorporation of $^{14}$C-leucine in dividing cultures of Solanum nigrum L. to a much greater extent than in similar but stationary cells (Zilkah and Gressel, 1978). It was later found that dikegulac interacted with the cell membrane, inhibiting the uptake of
The leakage of a pre-loaded dye in response to dikegulac was less rapid in stationary cells than in dividing cells (Zilkah and Gressel, 1979). It is possible that in a similar way these two different cell types would exhibit differences in sensitivity to glyphosate and thus the choice of material for mode of action studies with this herbicide is of particular importance.

7. AIMS OF THE THESIS

The mode of action of glyphosphate has in the past been investigated with a diversity of plant material of varying degrees of suitability. The approach here has been to take into account the known areas of glyphosate accumulation although in a context that can be related to the whole plant. In Section 1 of the Results and Discussion, the growth response both of wheat seedlings and single node rhizome buds of *Agropyron* is presented together with some other processes and the effect of various external factors either with respect to the mode of action or the performance of glyphosate. Section 2 represents an attempt to clarify the reasons for inhibition of chlorophyll formation, a notable characteristic of glyphosate. Here, use has been made of etiolated pea explants together with some *in vitro* studies. Most of the work is reported in Section 3 and has been concerned with the characterisation of some biochemical effects of glyphosate in buds of isolated *Agropyron* rhizome single nodes and root tips of wheat seedlings. The purpose here has been two-fold. Firstly, to expand upon the interaction of glyphosate in the biosynthesis and/or metabolism of aromatic amino
acids, suggested by previous workers to be a major site of action and secondly to investigate effects on some other biochemical processes unrelated to aromatic amino acid metabolism, but which could represent alternative sites of action. Also included is an examination of the fate of $^{14}C$-glyphosate within the single node bud.

Previous work on the mode of action of glyphosate has been characterised by the use of excessively high concentrations of glyphosate, commonly of $10^{-3}$M or greater, a level at which many processes are bound to be affected. Since considerable inhibition of growth can be obtained at $10^{-4}$M, lower concentrations have been used here in order that the range of processes affected can be narrowed to facilitate the identification of those likely to be primarily concerned with toxicity.

Each area of work involving related experiments has been discussed separately and major points brought together in a concluding discussion.
Materials & Methods
MATERIALS & METHODS

1. PLANT MATERIAL

Several species and organs thereof were used in the execution of this work.

1.1. *Pea* (*Pisum sativum* L. var. Meteor)

Seeds were germinated in trays of Levington Universal compost and grown in continual darkness at 22°C. Apical explants of 10cm were harvested after nine days.

1.1.1. Studies on the induction of nitrate reductase and nitrite reductase: For studies into the effect of glyphosate on enzyme induction in greening explants, inhibition of glyphosate at $2 \times 10^{-4}$M was allowed for 24hrs in darkness. Explants were then transferred to glyphosate containing $10^{-3}$M KNO$_3$ and illuminated at 5.25Wm$^{-2}$. Enzyme activities and chlorophyll levels were determined daily for 5 days. For studies on the effect of glyphosate on pre-greened explant trays of 9-day etiolated peas were illuminated at 10Wm$^{-2}$ for 24hrs, after which explants were harvested and allowed to imbibe $2 \times 10^{-4}$M glyphosate for a further 24hrs at 5.25Wm$^{-2}$. Explants were then transferred to glyphosate containing $10^{-3}$M KNO$_3$, and enzyme activities and CO$_2$ fixation determined after 24 hrs.

Ribosomal RNA was isolated from explants pre-treated with glyphosate and subsequently illuminated for five days.

1.1.2. Material for isolated chloroplasts: Chloroplasts for *in vitro* incorporation studies were isolated from greenhouse-grown plants. Seeds were germinated in Levington Universal compost and plants were grown under mercury vapour lamps with a photoperiod of 14hrs at a mean temperature of
23°C. Apical leaves were harvested from newly emergent plants, at 7-12 days.

1.2. **Mung bean** (*Phaseolus aureus*)

Seeds were germinated on damp vermiculite and grown in continual darkness at 21°C for 5-days.

1.2.1. **Studies on the formation of chlorophyll**: Apical 5cm explants were allowed to imbibe glyphosate at a range of concentrations for 24hrs in darkness. Explants were then transferred to a light cabinet at 5.25Wm⁻² and the chlorophyll content of the first leaf pairs was determined after a further 24hrs. Where metal ions were included, glyphosate was used at 2 × 10⁻⁴M.

1.3. **Flax** (*Linum usitatissimum var Reina*)

Seeds were sown on damp vermiculite in crystallising dishes for 7 days at 5.25Wm⁻² at a temperature of 23°C.

1.3.1. **Studies on CO₂ exchange**: Cotyledons detached from 7-day old plants were floated on solutions of glyphosate and incubated at the same light intensity for 24hrs in 4cm petri dishes. Rates of CO₂ exchange were then determined. Where metal ions were included, glyphosate was used at 10⁻⁴M.

1.4. **Agropyron repens** (*Common Couch, Headington Clone 31*)

Single node fragments were obtained from rhizome collected from outdoor stockpots. Plants had been established for several seasons, were consequently pot-bound and thus the rhizome was easily unwound. Rhizomes were fragmented into nodes of 2.5cm with the bud centrally placed. Apical areas of rhizome where buds were not viable, were discarded. Nodes were selected for uniformity and fitted at each end
with 2 cm lengths of autoanalyser tubing, through which after randomisation, water, glyphosate or other appropriate solutions were syringed (Plate 2). Single nodes were normally exposed to glyphosate for 24 hrs prior to the assay of processes. Specific experimental details are given in legends to data.

1.5. Wheat (*Triticum vulgare* vars Bouquet and Atou)

1.5.1. Solution Culture: For most experiments, seedlings were grown in a simple solution culture. Seeds were germinated at 22 °C in darkness on filter paper moistened with $2 \times 10^{-3} \text{M CaSO}_4$. After 2 days, seedlings were transferred to nylon mesh rafts supported on $2 \times 10^{-3} \text{M CaSO}_4$ in crystallising dishes by expanded polystyrene beads. After a further 24 hrs, rafts were transferred to fresh dishes containing CaSO$_4$ with or without glyphosate, which was normally used at $5 \times 10^{-4} \text{M}$ (Plate 3). Processes were examined using excised 0.5 cm root tips. Details of other growth methods are given in the legends to data, where appropriate. The growth of the two varieties showed a similar response to glyphosate.

2. USE OF GLYPHOSATE

In all experiments, the free acid of glyphosate was used, of technical grade, 96.7%. In studies with labelled glyphosate, (methyl-$^{14}$C)-glyphosate was used, sp. act. 1.95 mCi mmol$^{-1}$.

3. REPLICATION

Experiments were normally replicated three times. The variation in response of *Agropyron* rhizome single nodes
Plate 2: Single nodes of *Agropyron* rhizome

Plate 3: Solution culture of dark-grown wheat seedlings
necessitated the use of large numbers of nodes, usually fifteen. Errors, where given represent standard deviation.

4. QUANTITATIVE DETERMINATIONS

4.1. Chlorophyll

The chlorophyll content of leaf pairs of mung beans or apical pea buds was determined by soaking in 80% acetone at -20°C. After five days, chlorophyll was determined according to Arnon (1949):

\[
\text{Chlorophyll a (\mu g cm}^{-3}) = 12.7 (A_{663}) - 2.69 (A_{645}) \\
\text{Chlorophyll b} = 22.9 (A_{645}) - 4.68 (A_{663}) \\
\text{Total chlorophyll} = 20.2 (A_{645}) + 8.02 (A_{663})
\]

4.2. CO$_2$ exchange

CO$_2$ uptake or evolution was determined using a Grubb Parsons Infra-red Gas Analyser (IRGA) linked to an open circuit gas-flow system. CO$_2$ uptake or evolution was calculated from the equation:

\[
y = \frac{x \cdot Cf}{M \cdot 10^{-6}}
\]

where \( y \) = rate of CO$_2$ uptake or evolution (cm$^3$ min$^{-1}$)

\( x \) = dark recorder reading (evolution) or difference between light and dark recorder readings (uptake)

\( Cf \) = calibration of one IRGA division

\( M \) = chart recorder/IRGA magnification factor.

4.3. Determination of Protein


Reagents: Sol.A 2g Potassium sodium tartrate
100g Sodium carbonate
500cm$^3$ 1N NaOH
made up to 1dm$^3$ with water.
Sol.B  2g Potassium sodium tartrate
  1g Copper sulphate
  10cm³ 1N NaOH
  made up to 100cm³ with water, prepared daily.


The protein extract was made up to 1cm³ with water and 0.9cm³ sol.A added. This was heated to 50°C for 10 mins and allowed to cool to room temperature before the addition of 0.1cm³ sol.B. After standing for 10 mins 3cm³ sol.C was rapidly added using a vortex mixer. The solution was heated for a further 10 mins at 50°C and after cooling, the absorbance was read at 650nm. A calibration curve was prepared using bovine serum albumin standard.

4.4. Determination of Total Soluble Sugars (Dubois et al., 1956)

Twenty x 0.5cm root tips were ground in 4cm³ cold 80% ethanol and the homogenates centrifuged at 10,000 x g for 10 mins. 1cm³ 5% phenol was added to 1cm³ of supernatant and mixed, after which 2.5cm³ conc. sulphuric acid was rapidly added using a vortex mixer. After mixing well and cooling to room temperature, absorbance was read at 490nm. A calibration curve was prepared from glucose standards and results were expressed as µg equivalents of glucose.

4.5. Determination of ethylene and ethane

Ethylene and ethane were determined by GLC using a Pye Unicam GCD chromatograph with an activated alumina column at 125°C.

Gas flow rates were: Nitrogen 10cm³/15 secs
  Hydrogen 15 psi
  Air 10 psi
4.6. Extraction and Separation of Ribosomal RNA

(Loening & Ingle, 1967)

Ribosomal RNA was extracted and purified from pea buds by a phenol/detergent method (Loening and Ingle, method B), and subjected to SDS polyacrylamide gel electrophoresis.

4.6.1. Extraction: 5g of apical leaves were ground with sand in an ice-cold mortar and pestle with 10.2cm³ of a grinding medium of 10⁻² Tris, pH 7.6, containing 5 x 10⁻² M NaCl and 0.5% naphthalene 1:5 disulphonate (Pfaltz and Bauer). After thorough grinding 6cm³ of 18% 4-aminosalicylic acid (Aldrich) was added together with 1.8cm³ 10% tri-isopropynaphthalene sulphonic acid (Eastman). After further mixing, the homogenate was passed through 4 layers of butter muslin, to which was added 18cm³ of a phenol mixture consisting of phenol containing 10% m-cresol and 0.1% 8-hydroxyquinoline. The mixture was thoroughly shaken and centrifuged at 2000 x g for 10mins at 4°C. The upper aqueous layer was retained and NaCl added to 0.5M. 20cm³ phenol mixture was added, and the mixture shaken and centrifuged as before. The process was repeated a third time and the final aqueous layer was diluted 2-fold with cold ethanol and stored at -20°C for 30mins to allow precipitation of the RNA. The precipitate was sedimented by centrifugation at 5000 x g for 10 mins and redissolved in 0.15M acetate buffer, pH 6.0 containing 0.5% SDS. RNA was reprecipitated by the addition of 2 volumes of cold ethanol, storage at -20°C and recentrifugation. The sediment was dissolved in cold 80% ethanol and precipitated a third time. The final sediment was dried under nitrogen.
4.6.2. **Electrophoresis:** A 2.4% acrylamide solution was prepared by adding 5cm³ of stock monomer solution (95:5 acrylamide: Bis) to 26cm³ of electrophoresis buffer (6 x 10⁻³M Tris phosphate, pH 7.7 containing 2 x 10⁻⁴M EDTA). The solution was de-oxygenated by bubbling nitrogen through for 10 mins. Polymerisation was initiated by added 25μl TEMED and 0.25cm³ freshly prepared 10% ammonium persulphate. 3cm³ was gently pipetted into each running tube, avoiding the formation of bubbles. Buffer was layered over the gel to the top of the tube, taking care not to disturb the interface. The bottom of the tubes were sealed by placing squares of dialysis membrane over the grommets of the top reservoir of the unit and passing the tubes through. The bottom of the tubes were immersed in water. Tubes were initially permanently siliconised to facilitate extraction of gels by rinsing in a 5% solution of dimethyldichlorosilane in chloroform.

Gels were pre-electrophoresed for 30 mins at 5mA per tube with a running buffer containing 0.2% SDS. 1mg RNA was dissolved in 1cm³ buffer containing 5% sucrose together with a trace of bromophenol blue as solvent front marker. A 25μl sample was loaded on the gel under the reservoir and electrophoresis was carried out at 5mA per tube. Components were identified by comparison with *E. Coli* 16s and 23s rRNA units. Gels were scanned at 265nm in a Pye-Unicam SP1800 scanning spectrophotometer using a SP1809 densitometer attachment.

4.7. **Scintillation counting**

All ¹⁴C samples were assayed using scintillation cocktails of three types:

† BCL Limited
(a) Toluene scintillant: 5.5g PPO and 0.1g POPOP per dm³ toluene.

(b) Triton X-100 scintillant: 5.5g PPO and 0.1g POPOP per dm³ toluene:Triton X-100 (2:1).

(c) Acidified Triton X-100 scintillant: Triton X-100 scintillant plus 0.5M HCl (9:1).

Whatman GF/A discs were counted in scintillant a, aqueous samples in scintillant b and solubilised samples in scintillant c. Toluene was either redistilled or of scintillation grade. Vials were counted in Packard Tri-Carb or Philips liquid scintillation spectrometers. All results were corrected for quenching.

5. RECOVERY OF ¹⁴C-GLYPHOSATE FROM SINGLE NODE BUDS

Single nodes were treated with 10,000 DPM ¹⁴C-glyphosate in 25 μl 10⁻⁴M ¹²C-glyphosate administered at each end and incubated for 48 hrs at 22°C in darkness. Buds were excised and processed in a number of ways to achieve the following fractions, using 15 buds per fraction.

5.1. Total TCA precipitate

Buds were ground in cold 5% TCA, filtered onto Whatman GF/A discs, washed 3 times with TCA, once with 80% ethanol, dried and counted in toluene scintillant.

5.2. Cell Walls

Buds were ground in ice-cold water and cell wall material was deposited by centrifugation at 2,000 x g for 10 mins. The supernatant was decanted and retained and the pellet washed and recentrifuged. The process was repeated and the resuspended cell wall material was washed onto GF/A discs and rinsed 3 times with water followed by
80% ethanol. Discs were dried and counted.

5.3. Reprecipitated soluble contents

The combined supernatants from the cell wall isolation procedure were made to 5% with TCA and stored overnight at 2°C. The solution was filtered onto GF/A discs, washed and counted as before.

5.4. Analysis of soluble 14C-glyphosate by TLC

Buds were ground in ice cold water and centrifuged at 20,000 x g for 15 mins. The supernatant was evaporated down to a known volume (~ 0.5 cm³) and sub-sampled to determine activity.

Two-dimensional TLC was carried out on microcrystalline cellulose plates (Kodak Eastman 13255) using phenol based and isobutyric acid based eluants respectively (Rueppel et al., 1977).

<table>
<thead>
<tr>
<th>Eluent I</th>
<th>84 cm³</th>
<th>90% aqueous phenol</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 cm³</td>
<td>H₂O</td>
<td>acetic acid</td>
</tr>
<tr>
<td>1 cm³</td>
<td></td>
<td>EDTA Na₄</td>
</tr>
<tr>
<td>37.2 mg</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Eluent II</th>
<th>84 cm³</th>
<th>NH₄OH (~ 18.1N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 cm³</td>
<td>Isobutyric acid</td>
<td></td>
</tr>
<tr>
<td>3 cm³</td>
<td>1-butanol</td>
<td></td>
</tr>
<tr>
<td>14 cm³</td>
<td>1-propanol</td>
<td></td>
</tr>
<tr>
<td>3 cm³</td>
<td>2-propanol</td>
<td></td>
</tr>
<tr>
<td>19 cm³</td>
<td>H₂O</td>
<td></td>
</tr>
<tr>
<td>48 mg</td>
<td>EDTA Na₄</td>
<td></td>
</tr>
</tbody>
</table>

Plates were spotted with 1,000 DPM. After development and drying, plates were divided into 100 rectangles, each of dimensions \( \frac{Rf_{II}}{10} \times \frac{Rf_{II}}{10} \).

The rectangles were placed in scintillation vials and eluted with 1 cm³ acidified water (~ 5 x 10⁻³ M HCl).
After 24hrs, 10 cm³ Triton X-100 scintillant was added and vials were shaken and stood for 24hrs. Vials were shaken again and counted.

6. UPTAKE AND INCORPORATION OF MACROMOLECULE ¹⁴C-PRECURSORS

Single nodes were fed solutions of glyphosate and incubated in darkness at 22°C. After 24hrs, solutions were removed and replaced by 25 µl of water at each end containing 0.025 µCi ¹⁴C-precursor. Nodes were re-incubated and the uptake and incorporation of precursors determined after a further 24hrs.

DNA, RNA, protein and cellulose synthesis were determined by the incorporation of these precursors respectively: [2⁻¹⁴C] thymidine, sp.act. 61mCi/mmol⁻¹; [2⁻¹⁴C] uridine, sp.act. 57.4mCi mmol⁻¹; [U⁻¹⁴C] protein hydrolysate, sp.act. 59mCi mAtom C⁻¹ or L-[U⁻¹⁴C] leucine, sp.act. 330mCi mmol⁻¹ or L-[U⁻¹⁴C] phenylalanine, sp.act. 513mCi mmol⁻¹; and D-[U⁻¹⁴C] glucose, sp.act. 316mCi mmol⁻¹. Sucrose uptake was determined using [U⁻¹⁴C] sucrose, sp.act. 381mCi mmol⁻¹.

6.1. Uptake

Excised buds were sliced and solubilised in 0.5 cm³ Soluene 350 (Packard) diluted to 50% with toluene, for 3-days at room temperature. Acidified Triton X-100 scintillant was added and vials counted after a further 24hrs.

6.2. DNA and RNA synthesis

Excised buds were ground in a small mortar and pestle with cold 5% TCA. Samples were filtered onto GF/A discs and washed three times with TCA. After an ether wash discs
were dried and counted in toluene scintillant.

6.3. **Protein synthesis**

The procedure was as above, but the TCA homogenates were deacylated by heating to 90°C for 15mins.

6.4. **Cellulose synthesis**

(Modification of the method of Izhar et al., 1969)

Excised buds were ground in ice-cold $5 \times 10^{-2} \text{M}^{\text{14C}}$-glucose. Homogenates were filtered onto GF/A discs and washed three times with cold glucose and finally with 80% ethanol. Discs were dried and counted.

7. **ENZYME ASSAYS**

7.1. **Nitrate Reductase**

2g leaf material was ground with sand and a cold mortar and pestle in 4cm$^3$ of medium consisting of $5 \times 10^{-2} \text{M}$ phosphate, pH 7.8, containing $5 \times 10^{-3} \text{M}$ EDTA, $10^{-2} \text{M}$ cysteine and 3% casein (Harel et al., 1977). The homogenate was filtered through 4 layers of muslin and centrifuged at 20,000 x g for 15mins. Enzyme was assayed according to Hageman and Flesher (1960) at 30°C in a 2cm$^3$ reaction mixture which contained:

- 1.0cm$^3$ 0.1M phosphate pH 8.6
- 0.5cm$^3$ containing 0.48mg NADH
- 0.2cm$^3$ 0.1M KNO$_3$
- 0.1cm$^3$ H$_2$O
- 0.2cm$^3$ supernatant.

The reaction was terminated after 10mins by the addition of 1cm$^3$ 0.1% sulfanilamide made up in 25% HCl, followed by 1cm$^3$ 0.02% NEDD. Samples were centrifuged at 2500 x g for 10mins and the absorbance of the supernatant read at 540nm against a blank in which NADH was replaced by
7.2. **Nitrite reductase** (Losada and Paneque, 1971)

Activity was assayed from the supernatant prepared for nitrate reductase at 30°C in a 2cm³ reaction mixture which contained:

- 1.0cm³ 0.5M Tris.HCl, pH 8.0
- 0.3cm³ 5 x 10⁻³M methyl viologen
- 0.2cm³ 2 x 10⁻²M NaNO₂
- 0.2cm³ supernatant.

The reaction was started by the addition of a 0.3cm³ of a freshly prepared solution of 500mg sodium dithionite in 20cm³ 0.29M NaHCO₃. After 30mins, the reaction was terminated by vigorous agitation until the disappearance of the blue colouration. A 50µl sample was diluted with 5cm³ water and 2cm³ of this was added to 1cm³ of sulfanilamide solution, 1cm³ NEDD and 1cm³ water. After colour development, absorbance was read at 540nm against a blank in which methyl viologen was replaced by water (maximum colour).

7.3. **Incorporation of ^1⁴C-leucine in isolated chloroplasts** (Ellis and Hartley, 1971).

7.3.1. **Isolation of chloroplasts**: Apical pea leaves (5g) were chopped with a razor blade to a fine mince with 2.5 x 10⁻³M HEPES pH 7.6 containing 0.35M sucrose, 2 x 10⁻³M EDTA and 2 x 10⁻³M sodium isoascorbate. This was then ground gently in a cold mortar and pestle and filtered through 4 layers of muslin. Cell debris was removed by centrifugation at 500 x g for 30s and the supernatant was centrifuged at 3000 x g for 4mins. The chloroplast pellet was suspended in 6 x 10⁻²M tricine, pH 8.3 containing 0.2M KCl and 6.6 x 10⁻³M MgCl₂. Both the grinding and resuspension media were previously steralised.
by filtration.

7.3.2. **Assay:** The reaction was carried out in a total volume of 0.5 cm$^3$ and contained:

- 0.36 cm$^3$ chloroplasts
- 0.1 cm$^3$ $5 \times 10^{-3}$ M glyphosate or H$_2$O (control)
- 40 µl containing 0.5 µCi L-$[\text{U}^{-14}\text{C}]$ leucine sp. act. 330 mCi mmol$^{-1}$.

Reaction mixtures were agitated in flat bottomed tubes in a water bath at 20°C, illuminated at 300 W m$^{-2}$. At regular intervals up to 50 mins, 50 µl samples were dropped into ice-cold 5% TCA. Deacylation was carried out by heating to 90°C for 15 min. After cooling, samples were filtered on Whatman GF/A 2.5 cm glass fibre discs and washed three times with cold 5% TCA, followed by rinses with 80% ethanol and bleaching with hydrogen peroxide. Discs were dried and counted in toluene scintillant. The reaction was linear to 25 mins and results are given for incorporation at 10 mins.

7.4. **RNA polymerase** (Bottomley et al., 1971).

7.4.1. **Isolation of organelles:** Apical pea leaves (5 g) were chopped using a razor blade to a fine mince with $5 \times 10^{-2}$ M Tris, pH 7.8 containing 0.4 M sucrose, $10^{-2}$ M KCl, $10^{-2}$ M MgCl$_2$, $4 \times 10^{-3}$ M mercaptoethanol and 25 µg cm$^{-3}$ chloramphenicol. The mince was gently ground to a slurry in a cold mortar and pestle and the homogenate filtered through 4 layers of muslin. Chloroplasts and nuclei were pelleted by centrifugation at 1000 x g for 4 mins and resuspended in grinding buffer. 2 cm$^3$ were layered onto discontinuous sucrose gradients 0.6 M-1.4 M made in 40 cm$^3$ tubes with 5 cm$^3$ each of 0.6 M, 0.8 M, 1.0 M, 1.1 M, 1.4 M sucrose
made up in the above buffer (Bottomley, 1970). Gradients were centrifuged at 2500 x g for 12 min in a 3 x 40 cm³ swing-out rotor. Chloroplasts were recovered from the 1.0 M layer and reconcentrated by sedimenting at 2000 x g for 5 min. Nuclei were recovered from the 1.4 M layer, to which was added 1 cm³ 12% Triton X-100, by centrifuging at 1000 x g for 5 mins. Etioplasts were isolated from apical buds of etiolated peas by a method similar to that for chloroplast isolation. Organelles were resuspended in 5 x 10⁻² M TES pH 8.3, containing 10⁻² M MgCl₂, 4 x 10⁻³ M mercaptoethanol and 25 μg cm⁻³ chloramphenicol. All solutions were sterilised by filtration prior to use. Buffer concentration was increased five times upon the original method to allow addition of glyphosate at 10⁻³ M without pH change.

7.4.2. Assay: The reaction was carried out in a total volume of 0.5 cm³ which contained:

- 0.4 cm³ chloroplasts, etioplasts or nuclei
- 50 μl 10⁻² M glyphosate or water (control)
- 40 μl containing 0.125 M ATP, CTP and GTP
- 10 μl containing 0.5 μCi ¹⁴C-UTP sp. act. 51 mCi mmol⁻¹

The reaction was agitated in a water bath at 25°C and 50 μl samples withdrawn at intervals up to 20 mins. These were dropped into an ice-cold solution of 5% TCA and 5 x 10⁻² M sodium pyrophosphate. Samples were filtered onto Whatman GF/A discs and washed three times with cold TCA/pyrophosphate, followed by one wash each of 80% ethanol and 80% diethyl ether. Discs were dried and counted in toluene scintillant. Results are given for an assay period of 5 mins.
7.5. **Phenylalanine ammonia-lyase** (modification of Zucker, 1968)

20 x 0.5cm³ wheat root tips or 10 *Agropyron* buds were ground in 4cm³ cold 0.1M borate, pH 8.8. All operations up to and including homogenisation were performed under green safe light. The homogenate was centrifuged at 20,000 x g for 15mins.

The reaction mixture contained:

- 1.5cm³ 0.1M borate, pH 8.8
- 1.0cm³ 3 x 10⁻²M L-phenylalanine (D-phe for blank)
- 0.5cm³ supernatant.

Assays were incubated at 30°C, and absorbance at 290nm was read after 1 or 2hrs. The amount of cinnamic acid produced was calculated from a standard curve. Activities are expressed in mUnits where 1 unit = 1μmol cinnamic acid produced per min.

7.6. **Shikimate:NADP oxidoreductase**

Fifty root tips were ground in 1cm³ 5 x 10⁻²M Tris-HCl, pH 7.4 with 1% PVP. The homogenate was centrifuged at 15000 x g for 20 mins.

Enzyme activity was assayed according to Kojima et al., (1969) in a 3cm³ cuvette containing:

- 2.3cm³ 0.2M Tris HCl, pH 8.6
- 0.4cm³ 10⁻³M shikimic acid
- 0.1cm³ 5 x 10⁻³M NADP
- 0.2cm³ supernatant

The reduction rate of NADP was followed at 340nm for 5mins. One unit of enzyme is that amount producing an increase in absorbance at 340nm of 0.01/min at room temp (23°C).
7.7. **Chorismate mutase**

Twenty root tips were ground in 3cm³ 0.2M Tris.HCl, pH 7.8 with 1% PVP. The homogenate was centrifuged at 15000 x g for 20 mins.

Enzyme activity was assayed according to Cotton and Gibson (1965). The reaction mixture consisted of 0.2cm³ supernatant added to 0.2cm³ of a solution containing 0.4μmol barium chorismate and 20μmol Tris.HCl, pH 7.8. The reaction was allowed to proceed for 45mins at 37°C and was terminated by the addition of 0.4cm³ 1N HCl. After heating for a further 10mins at 37°C, 3.2cm³ 1N NaOH† was added. Tubes were centrifuged for 10mins at 2800 x g and absorbance was read at 320nm immediately against a zero time blank. 1 unit is that amount of enzyme producing an increasing in absorbance at 320nm of 0.1/min at 37°C.

7.8. **Polyphenol oxidase**

Twenty root tips were ground in 4cm³ 0.1M phosphate pH 6.0, containing 10⁻³M EDTA. The homogenate was centrifuged at 15000 x g for 20 mins.

Enzyme was assayed by the addition of 1cm³ supernatant to 1cm³ 0.1M catechol, freshly prepared in buffer. The reaction was allowed to proceed at 37°C for 3hrs, after which absorbance was read at 500nm against a blank of reaction mixture stored on ice. 1 unit is that amount of enzyme producing an increase in absorbance at 500nm of 0.1/hr at 37°C.

† The addition of acid converts the prephenic acid product to phenylpyruvic acid, which absorbs at 320nm in alkaline solution.
7.9. Formazan reduction

Twenty root tips were incubated with 3cm³ 0.1% 2,3,5-
triphenyltetrazolium chloride hydrate in 4cm petri dishes
at 25°C in darkness. After 2hrs, root tips were ground in
2cm³ H₂O and extracted with 4cm³ ethyl acetate. After
centrifugation at 2800 x g for 10mins, the reduced dye in
the ethyl acetate layer was read at 486nm against an ethyl
acetate blank.

7.10. Azocaseinase

Fifty root tips were ground in 3cm³ 0.1M acetate,
ph 5.0 or 5 x 10⁻² M Tris-HCl, pH 7.0. The homogenate was
centrifuged at 15000 x g for 15 mins.

Proteolytic activity was assayed by adding 1cm³
supernatant to 0.5cm³ 1.5% azocasein (BDH) buffered to
either pH 5.0 or 7.0. The reaction was incubated for 3hrs
at 37°C and terminated by the addition of 0.5cm³ 20% TCA.
Tubes were centrifuged at 2800 x g for 10 mins, the super­
natant was diluted to 50% with 2N NaOH and the absorbance
at 430nm was read against a zero time blank. 1 unit is
that amount of enzyme producing an increase in absorbance
at 430nm of 0.001/hr at 37°C.

7.11. RNase

Thirty root tips were ground in 6cm³ 0.1M acetate
pH 5.0. The homogenate was centrifuged at 15000 x g for
15mins.

The enzyme activity was assayed by the addition of
1cm³ supernatant to 1cm³ RNA† made up in buffer. The reaction was incubated for 3hrs at 37°C and was terminated by adding 18cm³ cold ethanol. Residual RNA was removed by precipitating out at -20°C overnight and centrifugation at 5000 x g for 10mins. The absorbance of the supernatant was determined at 260nm against a zero time blank. 1 unit is that amount of enzyme producing an increase in absorbance at 260nm of 0.01/hr at 37°C.

7.12. Acid phosphatase

Twenty root tips were ground in 6cm³ 0.1M acetate pH 5.0 and the homogenate centrifuged at 15000 x g for 15mins. Soluble acid phosphatase was assayed from the supernatant. Lysosomal enzyme was obtained by resuspending the pellet in buffer and recentrifuging, after which the pellet was suspended in buffer plus 1% Triton X-100. The sample was recentrifuged and the supernatant assayed.

Enzyme activity was assayed by adding 1cm³ supernatant to 1cm³ 4 x 10⁻³M p-nitrophenyl phosphate. Soluble and lysosomal enzymes were assayed at 40°C for 30mins or 2hrs respectively, after which the reaction was terminated by adding 5cm³ 0.1N NaOH. Absorbance was read at 400nm and the amount of p-nitrophenol produced was calculated from a standard curve.

† Sigma type II is commercial, 85%. This contained appreciable amounts of ethanol-soluble material absorbing at 260nm and was removed by the following procedure: RNA was dissolved in water and ethanol added to 80%. This was precipitated by standing at -20°C followed by centrifugation at 5000 x g for 10mins. The pellet was redissolved in water and the process repeated twice more. The final pellet was dried under nitrogen.
7.13. **Membrane bound enzymes**

7.13.1. **Preparation of Mitochondrial and Microsomal fractions** (Hodges and Leonard, 1973): Five hundred root tips were ground in a cold mortar and pestle with 15cm³ of a grinding buffer consisting of $2.5 \times 10^{-3}$M Tris.MES, pH 7.5, containing $0.25$M sucrose and $10^{-3}$M EDTA. The homogenate was centrifuged at 13000 x g for 15 mins. The supernatant was gently decanted and centrifuged at 80000 x g for 30 mins. The pellet was resuspended in grinding buffer and recentrifuged at 80000 x g again for 30 mins to produce a washed microsomal pellet, which was washed and suspended in 2cm³ of a resuspension medium of $10^{-3}$M Tris.MES, pH 7.2 containing $0.25$M sucrose and $10^{-3}$M MgCl₂.

A mitochondrial fraction was obtained by resuspending the original 13000 x g pellet in 10cm³ grinding medium and centrifuging at 250 x g for 5 mins. 5cm³ of this supernatant was gently removed and centrifuged at 13000 x g again for 15 mins. The final pellet was suspended in 1cm³ of resuspension medium.

7.13.2. **Assays:**

7.13.2.1. **ATPases**: ATPase activities were assayed according to Leonard et al. (1973), but with a 50% reduction in assay volumes. A 50µl sample of membrane suspension was added to 0.45cm³ of a substrate solution containing $3 \times 10^{-3}$M ATP-tris⁺, 1.5 x

⁺ATP Na was converted to the tris salt by the following procedure: Zerolit 225 ion exchange resin (200/400 mesh) was added to 100cm³ of $3 \times 10^{-3}$M ATP Na in de-ionised water until minimum pH was reached (≈ pH 2.4). The solution was filtered and solid tris was added to ≈ pH 6.0. Solutions of this were buffered to either pH 6.0 with Tris MES or pH 9.0 with Tris. The substrate solution was completed with the addition of MgSO₄ ± KCl.
$10^{-3} \text{M} \text{MgSO}_4$ with or without $5 \times 10^{-2} \text{M} \text{KCl}$ (for monovalent ion stimulated activity, where necessary), in Tris.MES, pH 6.0 or Tris pH 9.0. Mitochondrial and microsomal enzymes were incubated at $37^\circ \text{C}$ for 10mins or 90mins respectively and the reaction was terminated by the addition of $0.5\text{cm}^3 10\% \text{TCA}$. Phosphaterelease was measured by the method of Tausky and Shorr (1953): A stock solution of 10% ammonium molybdate was prepared in 10N sulphuric acid. FSM reagent was prepared freshly by adding 10cm$^3$ of this to about 70cm$^3$ water and 5g ferrous sulphate and making up to 100cm$^3$. Pi was determined by adding 0.5cm$^3$ FSM reagent. Samples were centrifuged at 2800 x g for 10mins and absorbance read at 700nm before acid hydrolysis of residual ester.

7.13.2.2. IDPase: Enzyme was assayed as for ATPases, but with a substrate solution containing $3 \times 10^{-3} \text{M}$ IDP and $1.5 \times 10^{-3} \text{M} \text{MgSO}_4$ in $3 \times 10^{-2} \text{Tris.MES}$, pH 7.5.

7.13.2.3. NADPH- and NADPH-dependent cytochrome C reductases (Leonard et al., 1973): The reduction of cytochrome C was measured at 550nm in a 3cm$^3$ cuvette which contained:

- $2.55\text{cm}^3 5 \times 10^{-2} \text{M phosphate pH} 7.5$
- $0.2\text{cm}^3 4.5 \times 10^{-4} \text{M cytochrome C}$
- $0.1\text{cm}^3 5 \times 10^{-2} \text{M NaCN}$
- 50µl membrane suspension.

The reaction was started by the addition of $0.1\text{cm}^3 3 \times 10^{-3} \text{M NADH or NADPH}$ and monitored for 5 or 10mins at room temperature ($28^\circ \text{C}$). The amount
of cytochrome C reduced was calculated using an extinction coefficient for cytochrome C of 18.5mM$^{-1}$ cm$^{-1}$.

7.13.2.4. β-Glucan_Synthetase (modification of Ray, 1973):

Enzyme activity was assayed by the incorporation of UDP-[¹⁴C] glucose into hot ethanol-insoluble material. The reaction mixture contained:

- 100µl membrane suspension
- 20µl containing 0.025µCi UDP-[¹⁴C] glucose, sp.act. 293mCi mmol$^{-1}$
- 10µl 0.5M MgSO₄.

The reaction was allowed to proceed at room temperature (28°C) for 2hrs and terminated by adding 0.5cm$^3$ ethanol. After the addition of 1-2mg carrier powdered cellulose and heating to 100°C for 2mins, solutions were washed onto GF/A discs and rinsed three times with 80% ethanol. Discs were dried and counted and zero time values subtracted.
Results & Discussion
RESULTS & DISCUSSION

1. THE INHIBITION OF GROWTH AND OTHER PLANT PROCESSES AND POSSIBLE INFLUENCING FACTORS

1.1. Results

The most obvious morphological symptom of glyphosate phytotoxicity is the cessation of growth. When wheat seeds were sown on filter paper in petri dishes, dark growth of both root and shoot subsequent to germination were inhibited over a range of concentrations. The rate of germination itself was inhibited at concentrations of glyphosate greater than $5 \times 10^{-4}$ M (Fig. 3). Inhibition of shoot growth became apparent at $5 \times 10^{-5}$ M, with an inhibition of fresh weight production of 50% occurring at approximately $10^{-4}$ M. The growth of roots was rather more sensitive to glyphosate and from the graph 50% inhibition would have been obtained at about $3 \times 10^{-5}$ M. In both cases, some enhancement of fresh weight production was observed at concentrations of $5 \times 10^{-6}$ M and lower, but this was marginal. Radical elongation of germinating flax seedlings was similarly inhibited by glyphosate (not reported here), however, in this case germination itself was not inhibited even at $2 \times 10^{-2}$ M.

Contrary to the results of Jaworski (1972); Haderlie (1975); Tymonko (1978) and Gresshoff (1979) working with other types of material, suppression of wheat seedling root growth was not abolished by the application of aromatic amino acids. This was investigated by selecting a concentration of glyphosate ($10^{-4}$ M) which gave appreciable, but not complete inhibition of root growth as measured by fresh weight. When wheat seeds were germinated with $10^{-4}$ M glyphosate,
Figure 3: EFFECT OF GLYPHOSATE ON ROOT (○) AND SHOOT (●) FRESH WEIGHT OF DARK-GROWN WHEAT SEEDLINGS.

Wheat seeds were sown on filter paper in 9cm petri dishes moistened with distilled H₂O (control) or solutions of glyphosate. Dishes were incubated in darkness at 27°C and root and shoot fresh weights determined after 4 days.
root growth was retarded to 15-30% of controls, varying between experiments. The inclusion in the germination solution of an equimolar mixture of L-phenylalanine, L-tyrosine and L-tryptophan at concentrations less than, equal to, or greater than that of inhibitor, i.e. 0.5, 1, 2, 5 and 10 times that of glyphosate, in no instance precipitated any alleviation, however slight, of root growth retardation by glyphosate. A typical set of results are shown in Table 2. No alleviation was observed either in a similar experiment in which L-phenylalanine alone was used as a potential reversal agent (Table 12). The concentrations of aromatic amino acids used had no effect on the growth of wheat roots in the absence of glyphosate.

When rhizomes of Agropyron are fragmented into single nodes which are nourished by feeding water through cut ends, the central bud is liberated from dormancy, with subsequent growth evident 1-2 days after fragmentation. This growth was inhibited by the administration of glyphosate. Agropyron buds were considerably more sensitive to glyphosate than were shoots of germinating wheat seedlings. At 10^{-3} M, glyphosate completely inhibited bud growth. Marginal growth was allowed at 10^{-4} M and an inhibition of about 30% was characteristic at 10^{-5} M (Fig. 4). However, this growth inhibition could not be alleviated by the simultaneous addition of aromatic amino acids (Table 3). When concentrations of mixed aromatic amino acids similar to those of Table 2 were used in conjunction with 10^{-4} M glyphosate, no reversal was evident. Aromatic amino acids alone had no effect upon bud growth, although marginal inhibition of growth may have occurred at 10^{-3} M.
### TABLE 2

**INHIBITION OF WHEAT ROOT GROWTH BYGlyphosate:**

**ADDITION OF AROMATIC AMINO ACIDS.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean root f. wt. (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>31.7 ± 7.5</td>
</tr>
<tr>
<td>10⁻⁴M glyphosate</td>
<td>5.2 ± 0.4</td>
</tr>
<tr>
<td>&quot; + 5 x 10⁻⁵M phe, tyr, tryp</td>
<td>7.0 ± 3.2</td>
</tr>
<tr>
<td>&quot; + 10⁻⁴M</td>
<td>6.7 ± 1.1</td>
</tr>
<tr>
<td>&quot; + 2 x 10⁻⁴M</td>
<td>6.6 ± 1.4</td>
</tr>
<tr>
<td>&quot; + 5 x 10⁻⁴M</td>
<td>6.6 ± 1.8</td>
</tr>
<tr>
<td>&quot; + 10⁻³M</td>
<td>6.9 ± 1.7</td>
</tr>
</tbody>
</table>

Seeds were germinated at 27°C in petri dishes on filter paper moistened with water, 10⁻⁴M glyphosate or glyphosate containing mixtures of L-phe, L-tyr and L-tryp. Root fresh weights were determined after four days in darkness.
Figure 4: EFFECT OF GLYPHOSATE ON GERMINATION OF AGROPYRON SINGLE NODE BUDS.

Single nodes were prepared (see Materials & Methods, 1.4) and fed solutions of distilled water (control) or glyphosate, using 15 nodes per treatment. Nodes were placed in petri dishes and incubated in darkness at 22°C. Bud length (●) and fresh weight (O) were determined after 5 days.
### TABLE 3

**INHIBITION OF AGROPYRON SINGLE NODE-BUD GROWTH BY GLYPHOSATE: ADDITION OF AROMATIC AMINO ACIDS.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean f. wt. (mg)</th>
<th>Mean bud length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>H₂O</strong></td>
<td>19.1</td>
<td>1.52 ± 0.4</td>
</tr>
<tr>
<td>5 x 10⁻⁵M phe, tyr, tryp</td>
<td>18.2</td>
<td>1.48 ± 0.59</td>
</tr>
<tr>
<td>10⁻⁴M</td>
<td>20.0</td>
<td>1.54 ± 0.41</td>
</tr>
<tr>
<td>2 x 10⁻⁴M</td>
<td>23.2</td>
<td>1.69 ± 0.40</td>
</tr>
<tr>
<td>5 x 10⁻⁵M</td>
<td>16.7</td>
<td>1.25 ± 0.62</td>
</tr>
<tr>
<td>10⁻⁴M glyphosate</td>
<td>4.3</td>
<td>0.39 ± 0.09</td>
</tr>
<tr>
<td>&quot; + 5 x 10⁻⁵M phe, tyr, tryp</td>
<td>3.8</td>
<td>0.32 ± 0.06</td>
</tr>
<tr>
<td>&quot; + 10⁻⁴M</td>
<td>3.4</td>
<td>0.30 ± 0.07</td>
</tr>
<tr>
<td>&quot; + 2 x 10⁻⁴M</td>
<td>4.0</td>
<td>0.32 ± 0.07</td>
</tr>
<tr>
<td>&quot; + 5 x 10⁻⁴M</td>
<td>4.0</td>
<td>0.37 ± 0.10</td>
</tr>
</tbody>
</table>

Single nodes were prepared (3.1.4) and fed distilled water, mixtures of L-phe, L-tyr and L-tryp, 10⁻⁴M glyphosate or glyphosate containing L-phe, L-tyr and L-tryp, using 15 nodes per treatment. Nodes were placed in petri dishes and incubated in darkness at 22°C. Bud fresh weight and length were determined after 5 days.
i. The influence of ammonium sulphate on growth inhibition

The field efficacy of glyphosate can be improved by the addition of ammonium sulphate ([NH₄]₂SO₄) in the tank mix formulation (Turner and Loader, 1972, 1975; Blair, 1973), a phenomenon observed with several water-soluble herbicides (Holly and Turner, 1979). The precise mechanism of this synergism is unknown but the inclusion of (NH₄)₂SO₄ is thought in some cases to improve the foliar penetration of the herbicide; Holly and Turner (1979) have shown that (NH₄)₂SO₄ enhanced the foliar uptake of ¹⁴C-MCPA. However, it was demonstrated by Suwunnamek and Parker (1975) that activation of glyphosate occurred if the two chemicals were applied at separate times, implicating a possible synergism within the plant.

An experiment was performed in which seeds of wheat were germinated in glyphosate at 5 x 10⁻⁵M with various concentrations of (NH₄)₂SO₄, or (NH₄)₂SO₄ alone. Glyphosate alone caused a marginal inhibition of shoot growth (Fig.5), but was clearly activated by the inclusion of (NH₄)₂SO₄; by 32% at 5 x 10⁻⁴M and about 50% at 10⁻³M. In contrast growth in the absence of glyphosate was enhanced. Activation by (NH₄)₂SO₄ was therefore not a phenomenon confined to uptake across the leaf cuticle, although root uptake could have been enhanced. The results suggest that intracellular synergism may occur although further experiments would be required to demonstrate this unequivocally.

ii. The influence of temperature on inhibition of single node bud growth

The activity of glyphosate against whole plants of Agropyron is improved at lower temperatures (Caseley, 1972) but, as with ammonium sulphate it is unclear whether the
Figure 5: EFFECT OF AMMONIUM SULPHATE ON INHIBITION OF WHEAT SEEDLING SHOOT GROWTH BY GLYPHOSATE.

Wheat seeds were sown on filter paper in petri dishes moistened with water or ammonium sulphate solution (O), or $5 \times 10^{-5}$M glyphosate alone or containing ammonium sulphate (●). Dishes were incubated in darkness at $22^\circ$C and shoot fresh weight determined after 5 days.
temperature influenced step in foliar uptake, transport or activity at the site of action. The experiment described here was set up to determine if the activity of glyphosate against *Agropyron* single nodes was potentiated at low temperature in a system in which cuticular penetration and to a large extent, transport were circumvented. The results show that growth of shoots from nodes which had initially been allowed to imbibe glyphosate at the same temperature prior to sowing out at a range of temperatures showed greater kill with decreasing temperature (Fig. 6). The activity of glyphosate at two concentrations under the high temperature regime was approximately double that under the low temperature regime, with an intermediate value for $10^{-4}$M glyphosate at the medium temperature, although the performance of $5 \times 10^{-5}$M glyphosate was similar for medium and low temperatures. The toxicity of glyphosate was thus altered in such a way as to suggest that activity at the site of action was temperature-influenced, although the possibility existed that rate of uptake of residual glyphosate by the bud from the nodal rhizome after washing and sowing may have been affected. One possible explanation of the influence of temperature on the efficacy of glyphosate at the node could be by increased metabolic degradation of glyphosate to a non-toxic form at elevated temperatures.

iii. Inhibition of chlorophyll formation

The appearance of chlorophyll in etiolated mung bean explants was considerably more sensitive to glyphosate than growth of wheat seedlings. When explants were allowed to imbibe glyphosate at a range of concentrations in the dark
Figure 6: Effect of temperature on performance of glyphosate against single nodes of *Agropyron* rhizome.

Rhizome was fragmented into single nodes which were placed on adsorbent paper moistened with distilled H$_2$O (control) or glyphosate and incubated in darkness at 22°C. After 24hrs, nodes were rinsed thoroughly with water and sown in trays of Levington Universal compost. Trays were placed in controlled environment rooms at high, medium and low temperature regimes; day/night temperatures were 26/16°C, 16/10°C and 10/6°C respectively. Daylength was 14hrs, light intensity 100Wm$^{-2}$ and relative humidity 90%. Shoot heights were assessed after controls had reached similar heights (~ 23cm).
for 24hrs and subsequently illuminated, chlorophyll formation was noticeably inhibited at $10^{-6}$M and greatly at concentrations above this, with almost complete inhibition at $10^{-4}$M (Fig.7). An inhibition of about 50% would have been obtained at about $5 \times 10^{-6}$M. The formation of chlorophylls a and b were inhibited to similar extents. A marginal stimulation of chlorophyll levels was observed at $10^{-7}$M.

iv. CO$_2$ exchange

Photosynthetic CO$_2$ uptake in detached flax cotyledons was sensitive and after an exposure period of 24hrs, was inhibited at concentrations greater than $10^{-5}$M (Fig.8). In contrast to this, respiration was not inhibited even at $10^{-2}$M glyphosate. At sub-lethal concentrations, a marked stimulation of photosynthesis was observed, with an almost doubling of the control rate at $10^{-7}$M.

v. Experiments with metal ions

Several types of experiment were conducted in an attempt to ascertain whether glyphosate acted by complexing with biologically important cations. In a biological system, chelating agents could be envisaged as competing with metal requiring enzymes for divalent cations, thereby causing enzyme inactivation. Provision of cations in excess could be expected to alleviate this inhibition. When flax seeds were germinated in petri dishes with glyphosate at $10^{-4}$M, radical elongation was considerably inhibited. Experiments using germinating seeds were set up in which a variety of biologically important cations were added with glyphosate, usually as sulphates, either singly or in combination, at a range of concentrations from $10^{-2}$M to $10^{-7}$M. In no instance was any alleviation of
Figure 7: CHLOROPHYLL FORMATION IN ILLUMINATED EXPLANTS OF MUNG BEAN.

(●) total chl, (○) chl a, (△) chl b.
Figure 8: CO$_2$ exchange in detached flax cotyledons: Effect of glyphosate on CO$_2$ evolution (○) and photosynthetic CO$_2$ uptake (●).
elongation inhibition detected; adding an excess of cations therefore did not inactivate glyphosate within the seedling, nor did this apparently occur in the germination medium with prevention of uptake.

More specific experiments were conducted in which the effect of addition of metal ions on the inhibition of photosynthesis and chlorophyll formation by glyphosate was investigated. In isolated flax cotyledons, glyphosate at $10^{-4}$M inhibited photosynthetic CO$_2$ uptake after 24hrs, but the addition of some cations known to be involved in photosynthesis, i.e. Fe$^{2+}$, Fe$^{3+}$ and Mn$^{2+}$ did not alleviate inhibition. It was noticed that a precipitate was sometimes formed in mixtures of glyphosate and Fe$^{2+}$, but this did not modify toxicity. Similarly, the inhibition of chlorophyll formation in etiolated explants of mung bean was not modified by the inclusion of Fe$^{2+}$, Fe$^{3+}$ and salts of other heavy metals. Details of all these experiments are given in Table 4. Since in all cases, results were completely negative, no quantitative data are presented.

Final investigations into the possibility of chelation were conducted to determine the effect of glyphosate in vitro on reaction velocities of some metal-requiring enzymes. Commercial preparations of three such enzymes were studied; hexokinase, having a requirement for freely dissociable Mg$^{2+}$ and 2 metalloenzymes, D-lactate dehydrogenase (D-LDH) and alcohol dehydrogenase (ADH), both having absolute requirements for bound Zn$^{2+}$. Addition of EDTA has been shown to inactivate D-LDH and addition of excess Zn$^{2+}$ caused reactivation (Curdel and Labeyrie, 1961). However, the addition of glyphosate to reaction media at $10^{-2}$M failed to retard the reaction rates of any of the enzymes. Also,
**TABLE 4**

**SOME PROCESSES INHIBITED BY GLYPHOSATE: ADDITION OF CATIONS.**

<table>
<thead>
<tr>
<th>Process Inhibited</th>
<th>Species</th>
<th>Glyphosate (M)</th>
<th>Cation</th>
<th>Conc. range (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root elongation</td>
<td>Flax</td>
<td>$10^{-4}$</td>
<td>$\text{Fe}^{2+}$, $\text{Fe}^{3+}$, $\text{Zn}^{2+}$, $\text{Cu}^{2+}$, $\text{Co}^{2+}$, $\text{Mn}^{2+}$, $\text{Mg}^{2+}$, $\text{Ca}^{2+}$</td>
<td>$10^{-2} - 10^{-7}$</td>
</tr>
<tr>
<td>Photosynthesis</td>
<td>Flax</td>
<td>$10^{-4}$</td>
<td>$\text{Fe}^{2+}$, $\text{Fe}^{3+}$, $\text{Mn}^{2+}$</td>
<td>&quot;</td>
</tr>
<tr>
<td>Chlorophyll formation</td>
<td>Mung Bean</td>
<td>$2 \times 10^{-4}$</td>
<td>$\text{Fe}^{2+}$, $\text{Fe}^{3+}$, $\text{Zn}^{2+}$, $\text{Cu}^{2+}$, $\text{Co}^{2+}$</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

* Administered singly or as a mixture
incubation overnight at 2°C of D-LDH and ADH with glyphosate was not inhibitory (Table 5). Glyphosate thus did not chelate free Mg²⁺ or remove bound Zn²⁺.

vi. Experiments with glycine

The similarity of glyphosate to some endogenous compounds has suggested an action through competition with such metabolites for enzyme active sites. The possibility that glyphosate acted as an analogue of glycine was investigated, again with germinating flax seedlings. Glyphosate at concentrations of 10⁻⁵M and greater inhibited radicle elongation, but the simultaneous admixture of glycine at concentrations less than equal to or greater than inhibitor concentration did not promote alleviation of toxicity. Some typical results are shown in Table 6.

1.2. Discussion

It has previously been reported that germination itself is not generally affected by glyphosate, although subsequent growth is. Quantitative data were presented by Haderlie (1975), showing that the germination rates of maize, *Setaria faberi* and soy-bean were not inhibited by 10⁻³M glyphosate. The elongation of radicle and hypocotyl of soy-bean was retarded at 10⁻⁴M by approximately two thirds, whereas the elongation of maize and *Setaria* coleoptiles was only marginally inhibited. Hoagland (1977) reported that 10⁻³M glyphosate inhibited the germination of seeds of *Rumex crispus*, but allowed germination of ten other crop and weed species, with subsequent growth inhibition. Egley and Williams (1978) showed that the germination rates of seeds of five weed species were unaffected by glyphosate. The differential sensitivity of maize and soy bean seedlings to
**TABLE 5**

**METAL REQUIRING ENZYMES AND GLYPHOSATE IN VITRO**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Ion requirement</th>
<th>Glyphosate (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexokinase</td>
<td>Free Mg(^{2+})</td>
<td>10(^{-2})</td>
</tr>
<tr>
<td>D-Lactate dehydrogenase</td>
<td>Bound Zn(^{2+})</td>
<td>&quot; *</td>
</tr>
<tr>
<td>Alcohol dehydrogenase</td>
<td>&quot;</td>
<td>&quot; *</td>
</tr>
</tbody>
</table>

* Also with overnight incubation with glyphosate at 4\(^{0}\)C.
<table>
<thead>
<tr>
<th>Glyphosate (M)</th>
<th>Radicle length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine (M)</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>6.16</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>3.85</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>1.20</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>0.95</td>
</tr>
</tbody>
</table>

Flax seeds were germinated on filter paper in petri dishes moistened with water, glycine or glyphosate containing glycine. Dishes were incubated in darkness at 27°C and radicle length determined after 4 days.
glyphosate was found by Haderlie to be due to differences in the rate of uptake. After 96hrs following inhibition, the accumulation of $^{14}$C-glyphosate by the radicle and coleoptile of maize was negligible, but was much greater in radicles of soy bean, the major sites of accumulation being the radicle tips. It thus appeared that at concentrations which did not prevent germination, but which did inhibit further growth, glyphosate did not enter until a certain stage of development was reached.

The failure of either L-phenylalanine or mixed aromatic amino acids to alleviate toxicity of glyphosate to germinating seedlings and single node buds would suggest that in these cases, either some other mechanism of toxicity other than depletion of the free phenylalanine pool was operative, or that irreversible damage was caused by glyphosate before entry of the amino acids. One difference between this work and experiments in which reversal was reported was that in the latter cases [growth of *Lemna* fronds (Jaworski, 1972), growth of carrot and tobacco cells (Haderlie, 1975; Haderlie et al., 1977), growth of *E. Coli* (Roisch and Lingens, 1974), protein synthesis in soy bean leaf cells (Tymonko, 1978) and growth of several species (Gresshoff, 1979)] material was used which was actively growing before application of glyphosate and reversal agents, whereas in the only other reported case of seedlings reversal was not observed, (Duke and Hoagland, 1978). It may well be then that in these cases of glyphosate action on developing meristems glyphosate interfered with processes which did not occur in material in which reversal was evident, although glyphosate certainly affected phenylalanine metabolism in root tips and single node buds with consequences
likely to cause cell death. These results are reported in Section 3.

The prevention of chlorophyll appearance by glyphosate as demonstrated in etiolated mung bean explants, is a property shared by several herbicides of unrelated structure. Achlorophyllous growth occurred under treatment with amino-triazole, haloxydine, pyriclor and Sandoz 6706 (Corbett, 1974; Wolf, 1977) and norfluorazon and fluridone (Bartels and Watson, 1978). In most cases, photooxidation of chlorophyll is thought to occur due to the inhibition of carotenoid synthesis. Carotenoid pigments are believed to provide a quenching mechanism for excited chlorophylls and in this way prevent photodestruction of chlorophyll. In the absence of quenching carotenoids the highly reactive states of oxygen thought to arise from excited chlorophyll may destroy chloroplast components (Krinsky, 1971, 1977). Several lines of evidence support action of these herbicides through inhibition of carotenoid synthesis rather than direct inhibition of chlorophyll synthesis. Illumination at low light intensity enabled the development of some chlorophyll, but this disappeared upon illumination at higher intensities (Bartels and Hyde, 1970; Burns et al., 1971; Bartels and Watson, 1978) and such chlorosis has been shown to be accompanied by the loss of 70s ribosomes and Fraction I protein (Bartels et al., 1967; Bartels and Hyde, 1970; Bartels and Watson, 1978). The inhibition of carotenoid formation was implicated by the build up of the precursor polyenesphytoene and phytofluene (Guillot-Saloman et al., 1967; Ben-Aziz and Koren, 1974; Bartels and Watson, 1978) indicating that reactions of carotenogenesis prior to or including cyclisation were inhibited. Some workers
have suggested that this is merely a manifestation of a more general inhibition of lipid synthesis (Guillot-Saloman et al., 1967; Hilton et al., 1971). Chlorophyll synthesis itself is not thought to be affected by these compounds, since Burns et al (1971) have shown that protochlorophyllide accumulation in darkness did not occur.

In this work, inhibition of chlorophyll appearance has been shown to occur in pre-existing tissue, which already contained carotenoid pigments and it is thus unlikely that chlorosis could be explained by inhibition of carotenoid synthesis. Conversely, aminotriazole applied to emergent tissue did not inhibit the appearance of chlorophyll, and chlorosis was obtained only by germination in the inhibitor (Bartels et al., 1967). However, when greenhouse grown plants of Agropyron were sprayed with glyphosate at sub-lethal doses, regrowth following defoliation was completely albinistic, evidently lacking carotenoid pigments as well as chlorophyll (Plate 4). Such tissue was capable of recovery with subsequent production of new green cells (Plate 5). When young pea plants were also sprayed with glyphosate, leaves emerging subsequent to treatment also lacked chlorophyll, but in this case were yellow, indicating that carotenoids were still present. In the case of emergent Agropyron growth then, synthesis of carotenoids was prevented and accompanied by photodestruction of chlorophyll. The presence of carotenoids, albeit as assayed visually in etiolated mung bean explants and emergent growth of pea would seem to suggest that in these cases an alternative explanation is required. This is considered further in Section 2.
Plates 4 + 5: Chlorotic regrowth from glyphosate treated plants of *Agropyron*

Greenhouse grown plants (6-weeks) were sprayed with glyphosate (Mon 0139) plus 0.2% Agral 90 surfactant with a pot sprayer at 0.2kg ha\(^{-1}\) and transferred to a controlled environment room at a day/night temp. regime of 16\(^0\)/10\(^0\)C. Foliage was removed at soil level after 24hrs and examples of chlorotic growth were photographed after 3-weeks.
The prevention of chlorophyll appearance is unlikely to be related to the mode of action at meristematic areas, as is the inhibition of photosynthesis demonstrated in detached flax cotyledons. A notable aspect of the results given in Fig. 8 is that evidently the metabolism of the cotyledons must have been disturbed in some way in order for photosynthetic CO₂ uptake to have been inhibited, but respiration remained completely unaffected, even at the highest concentrations used, thereby illustrating the basic unsuitability of such material for mode of action studies, apart from photosynthesis. Indeed, detached cotyledons floated on solutions of glyphosate under illumination retained their chlorophyll and healthy appearance for several days.

The comprehensive experiments carried out with metal ions seemed to indicate conclusively that chelation was not an important factor in mode of action. The chelating properties of phosphonic acids have been described by Carter et al. (1967) and the order of stability of complexes formed with both bidentate (e.g. glycine, 8-hydroxyquinoline) and quadridentate (e.g. EDTA) agents has been determined as Cu > Ni > Co > Zn > Fe > Mn > Mg (Mellor and Maley, 1947, 1948). Stronger complexes would therefore tend to be formed with the heavy metals rather than the alkali metals. There was no evidence from any of the experiments to suggest that glyphosate acted by the sequestration of biologically important cations. The effect of metal ions on the prevention of chlorophyll formation by glyphosate was investigated since chlorosis is a well-known symptom of mineral deficiency, particularly of iron and magnesium (Bogorad, 1966). Many endogenous compounds, such as
amino acids and organic acids have weak chelating properties, but complexes so formed would be transient in nature. Only compounds able to form strong complexes could be expected to exert deleterious effects.

The experiments with glycine indicated that glyphosate did not compete with glycine for sites on membrane carrier proteins or inside the cell. The possibility that glyphosate behaved as a glycine analogue in the inhibition of chlorophyll synthesis was considered by Dodge (personal communication), since the synthesis of δ-aminolevulinic acid, a precursor of chlorophyll a synthesis requires the condensation of glycine with succinyl CoA. Addition of glycine however did not cause alleviation of inhibition. Consideration of glyphosate as a possible analogue is important since it is known that phosphonomethyl derivatives of glycolytic intermediates can compete for enzymes (Dixon and Sparkes, 1974). One such analogue of phosphoenolpyruvate can be metabolised by enolase (Stubbe and Kenyon, 1972).

2. GREENING, ENZYME INDUCTION AND PLASTID PROTEIN AND NUCLEIC ACID SYNTHESIS

2.1. Results

1. **Chlorophyll synthesis and the induction of nitrate reductase and nitrite reductase**

   Since glyphosate inhibited the appearance of chlorophyll in etiolated explants of mung bean, apparently in the presence of carotenoids, it seemed likely that its
synthesis, rather than breakdown was affected, possibly due to interference in the synthesis of chloroplast macromolecular components. Although herbicides producing emergent chlorosis are thought to act by inhibiting carotenoid synthesis, many other compounds, notably antibiotics and nucleotide base analogues which inhibit protein and nucleic acid synthesis can prevent greening in plants (Wolf, 1977). Chloramphenicol is a well-known inhibitor of chlorophyll synthesis and has been shown to selectively inhibit chloroplast protein synthesis (Goffeau and Bracket, 1965; Spencer, 1965) rather than cytoplasmic protein synthesis (Eisenstadt and Brawerman, 1964) through binding to 70s ribosomes (Anderson and Smillie, 1966).

The enzyme nitrate reductase is generally considered to be located within the chloroplast (Ritenour et al., 1966; Dalling et al., 1972; Miflin, 1974). Because its induction could be repressed by chloramphenicol, it is thought to be synthesized on 70s ribosomes (Schrader et al., 1967), since the induction of the preceding enzyme in the nitrate assimilation pathway, nitrate reductase was not inhibited, although some disagreement exists (Stewart, 1972). A comparison of the effects of glyphosate on the induction of these two enzymes therefore appeared to be a useful way of determining whether glyphosate selectively inhibited the formation of chloroplast proteins. Results are shown in which nitrate reductase and nitrite reductase were induced in etiolated pea explants following treatment with glyphosate, either prior to or subsequent to illumination.

In explants which were allowed to imbibe $10^{-4}$M glypho-
sate for 48hrs before illumination, the subsequent development of chlorophyll was considerably limited (Fig.9). Thus, as in mung beans the appearance of chlorophyll was inhibited in pre-formed tissue containing carotenoid pigments. Nitrate reductase and nitrite reductase activities were induced by the inclusion of nitrate in the imbibition medium during the illumination period, but the responses of these enzymes to glyphosate were completely different. In the case of nitrite reductase, development of activity was repressed to a similar extent as chlorophyll production, over the 5-day greening period, but following the first day control activity developed in an approximately linear pattern. The activity of nitrate reductase in control explants developed to a maximum after 4-days illumination, with a decline after 5-days, whereas pre-treatment with glyphosate precipitated a much more rapid induction of enzyme resulting in a peak value 1-day after onset of illumination, following which activity decayed to sub-control levels.

Both enzymes could be induced much more rapidly in explants greened prior to treatment with glyphosate and nitrate and therefore initially photosynthetically competent, although a higher concentration of nitrate was found to be necessary to elicit induction. When pre-greened pea explants were fed glyphosate for 24hrs followed by glyphosate plus nitrate for 24hrs, the induction of nitrite reductase was progressively inhibited with increasing concentration of glyphosate, but photosynthetic CO₂ fixation was also inhibited to a similar extent (Fig. 10). Conversely, the induction of nitrate reductase showed an increase with concentration of glyphosate, although
Figure 9: Inhibition of chlorophyll formation (a) and the induction of nitrate reductase (NaR, b) and nitrite reductase (NiR, c) in illuminated pea explants; control (●—●), glyphosate pre-treatment (○—○). For experimental details see Materials & Methods 1.1.1.
**Figure 10:** Effect of glyphosate on the induction of nitrate reductase (●, a) and nitrite reductase (○, a) and levels of photosynthetic CO₂ uptake (b) in pea explants greened prior to treatment. For experimental details, see Material & Methods 1.1.1.
less marked. Higher concentrations of glyphosate were required to elicit responses similar to those obtained with $10^{-4}$M in greening explants (Fig.9), presumably since greening caused bud expansion, diminishing the sink status of the outer leaves, with the accumulation of less glyphosate.

The enhancement by glyphosate of nitrate reductase activity could be considered to be due to a stimulation of uptake of nitrate. This was investigated by studying the effect of glyphosate on the relationship between nitrate reductase induction and the inducing nitrate concentration. A definite optimum substrate concentration for induction could be observed (Fig.11), but with the inclusion of glyphosate, this optimum was somewhat lower, an observation which would support a theory of enhanced nitrate uptake. The enhancement of the saturation level of the enzyme is not easily explicable.

ii. Protein synthesis in isolated chloroplasts

In order to clarify the possibility that glyphosate inhibited chloroplastic protein synthesis the effect on in vitro $^{14}$C-leucine incorporation in isolated chloroplasts was examined. Isolated pea chloroplasts incorporated $^{14}$C-leucine under illumination linearly for approximately 25mins, but this process was not affected by the addition of $10^{-3}$M glyphosate as shown by Table 7. Results are given for an incorporation period of 10 mins.

iii. Polyacrylamide gel electrophoresis of ribosomal RNA from pea buds

Since several reports exist of 70s ribosome deficiency in chlorotic growth produced in response to herbicide treatment (Bartels et al., 1967; Croft et al., 1974; Bartels and Watson, 1978), or high temperature (Feierabend and
Figure 11: Variation of induction of nitrate reductase with nitrate concentration in pre-greened pea explants following treatment with glyphosate; control (●), glyphosate (○).

Pre-greened explants were placed in water or $10^{-4}$ M glyphosate for 24 hrs and transferred to similar solutions with KNO$_3$. Nitrate reductase activity was assayed after 24 hrs in KNO$_3$ with illumination at 5.25 W m$^{-2}$. 
TABLE 7

\[^{14}C\] - LEU INCORPORATION IN ISOLATED PEA CHLOROPLASTS: ADDITION OF GLYPHOSATE IN VITRO.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Incorporation CPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2710</td>
</tr>
<tr>
<td>+ $10^{-3}$ M glyphosate</td>
<td>2560</td>
</tr>
</tbody>
</table>
Schrader-Reichhardt, 1976) the effect of glyphosate on the rRNA content of pea buds prepared as for Fig. 9 was determined. Polyacrylamide gel electrophoresis of bud rRNA from explants illuminated for 5-days following glyphosate imbibition revealed that the level of the 16s chloroplast component was considerably lower (∼ 50%) than in control buds. Typical results are shown in Fig. 12. Electrophoresis of rRNA from etiolated buds prior to illumination showed that some 23s and 16s plastid components were present in the absence of light. Upon irradiation, synthesis of the 16s component was considerable in control tissue, but not so marked in the case of the 23s component, of which there was no discernible difference in levels between control and glyphosate treated material, although this was not completely resolved from the peak of the 25s cytoplasmic component. Overall reduction in the level of chloroplastic rRNA did not correlate with the reduction in amount of chlorophyll present, which was by about 80% (Fig. 9).

iv. RNA polymerase in isolated organelles

The possibility that glyphosate directly inhibited the synthesis of plastid RNA was investigated by in vitro studies with RNA polymerase. In Table 8, RNA polymerase of isolated pea chloroplasts and etioplasts, assayed by the incorporation of $^{14}$C-UTP is compared with nuclear activity. In no case was any inhibition of activity observed when glyphosate was added at $10^{-3}$M. Indeed, a stimulation of incorporation was observed.
Figure 12: Polyacrylamide gel electrophoresis of ribosomal RNA from pea explants for 5-days illumination.

a) Control,
b) 10^{-4}M glyphosate,
c) control explants retained in darkness.
TABLE 8

RNA POLYMERASES OF ISOLATED PEA ORGANELLES:

ADDITION OF GLYPHOSATE IN VITRO.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$^{14}$C-UTP incorporation CPM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control $10^{-3}$M glyphosate</td>
</tr>
<tr>
<td>Chloroplasts</td>
<td>1972</td>
</tr>
<tr>
<td></td>
<td>3416</td>
</tr>
<tr>
<td>Etioplasts</td>
<td>1250</td>
</tr>
<tr>
<td></td>
<td>2167</td>
</tr>
<tr>
<td>Nuclei</td>
<td>1472</td>
</tr>
<tr>
<td></td>
<td>3028</td>
</tr>
</tbody>
</table>
2.2. Discussion

The induction of nitrite reductase was inhibited by glyphosate, but only to the extent of chlorophyll inhibition in greening explants (Fig.9) or CO₂ fixation in pre-greened explants (Fig.10). Since nitrite reductase activity is dependent upon photosynthetically reduced ferredoxin for electron donation (Paneque et al., 1964; Joy and Hageman, 1966) the activity of this enzyme would be expected to be limited by photosynthetic competence, which was observed here. However, the fact that induction was not repressed to any greater extent than CO₂ fixation would tend to indicate that photosynthesis was the sole factor governing nitrite reductase activity and that inhibition through repression of de novo protein synthesis was unlikely.

The stimulation of nitrate reductase (Fig.9) was similar to the observations of Haderlie (1975) who demonstrated a transient enhancement in maize plants, with subsequent rapid decay. Haderlie postulated that enhancement may have accounted for the increase in free amino nitrogen found in other material and that these elevated levels subsequently caused the decay of nitrate reductase. Since no other studies on pea explants were carried out here, the significance if any, of the changes in nitrate reductase can only be speculated on. The induction of nitrate reductase, a sensitive control point in the assimilation of nitrogen is known to be influenced by a variety of compounds which may enhance or initiate protein synthesis, or affect such factors as cell pH and membrane organisation (Beevers and Hageman, 1969; Hewitt et al., 1979). One other example of stimulation of nitrate
reductase by a herbicide is known, namely that of simazine. In maize plants, sub-toxic doses of simazine caused considerable enhancement of growth, with corresponding increases in protein and nitrate reductase levels (Ries et al., 1967). Stimulation of nitrate reductase was much greater and more sustained than observed here with glyphosate. The effect observed with glyphosate was essentially a toxic one, since it concurred with inhibition of chlorophyll formation and CO₂ fixation.

Simultaneous stimulation of nitrate reductase and repression of nitrite reductase could cause as accumulation of toxic nitrite, known to be a factor in the action of some herbicides (Klepper, 1974, 1975, 1976), although this could be expected to produce feedback inhibition of nitrate reductase. The ephemeral nature of the enhancement of nitrate reductase would make a large accumulation of nitrite unlikely. Since induction of nitrate reductase is thought to involve de novo synthesis (Beevers and Hageman, 1969) it is unlikely that glyphosate initially inhibited cytoplasmic protein synthesis.

The experiments with nitrite reductase induction did not determine conclusively whether de novo synthesis was inhibited and it was thus decided to carry out experiments with protein synthesis in isolated plastids. The fact that the incorporation of ¹⁴C-leucine was not inhibited by glyphosate indicated that there was no interference with amino-acyl-t-RNA formation or peptide elongation at the ribosome. This did not preclude the possibility that protein synthesis might be inhibited in vivo through component deficiency, or that inhibition might be due to
repression of chloroplast RNA synthesis.

The partial reduction in the level of chloroplast rRNA could have been a contributory factor in chlorosis, but is unlikely to be completely responsible. Considerable controversy exists as to the role of chloroplast RNA and protein in the formation of chlorophyll. Pollack and Davies (1970) showed that light induced chloroplast rRNA synthesis in pea was probably a pre-requisite of chlorophyll formation, supporting a hypothesis that enzymes and/or structural proteins involved in the synthesis and/or stability of chlorophyll are produced on chloroplast ribosomes. These workers moreover showed that chloramphenicol inhibited the production of chloroplast rRNA and chlorophyll to similar extents. Rifamycins, potent inhibitors of prokaryotic RNA polymerases were shown by Bogorad and Woodcock (1971) to inhibit the incorporation of $^{32}$Pi into chloroplast rRNA and also to inhibit RNA polymerase of isolated plastids. However, of two such inhibitors studied, only one prevented chlorophyll synthesis. Other instances implicating inhibition of RNA synthesis with resultant chlorosis have been described. Gressel and Cohen (1977) reported that dikegulac inhibited the incorporation of $^3$H-uridine by Spirodela fronds into chloroplast rRNA to a greater extent than the cytoplasmic counterparts. Shoots of maize grown at elevated temperature ($32^\circ$C) exhibited chlorosis of new growth, in the presence of light (Feierabend and Schrader-Reichhardt, 1976; Rademacher and Feierabend, 1976). Whilst this superficially appeared to be a case of photodestruction, no accumulation of carotenoid precursors was observed, and absence of 70s
ribosomes occurred in the dark as well as light, whereas herbicide induced destruction occurred only in the light (Bartels et al., 1967; Bartels and Hyde, 1970). However, glyphosate did not inhibit RNA polymerases in vitro and thus the reasons for deficiency of chlorophyll and partial deficiency of chloroplast rRNA remain unclear.

3. THE EFFECTS OF GLYPHOSATE ON MERISTEMS

3.1. The interaction of $^{14}$C-glyphosate with *Agropyron* single node buds

In later sections, the effects of glyphosate on a variety of processes in developing buds of *Agropyron* single nodes are described and it was considered salient to additionally characterise the behaviour of glyphosate in this system.

3.1.1. Results

Initially the pattern of distribution of $^{14}$C-glyphosate in the single node was determined in order to compare the content of the bud with that of the rest of the node. Single nodes were treated with $10^{-4}$M $^{12}$C-glyphosate containing $^{14}$C-glyphosate through cut ends and after 48hrs incubation the distribution of activity throughout the node was assayed by removing the bud and fractionating the node into ten lengths each of 0.25cm. These sections and the bud were then counted separately. The distribution of activity is plotted in Fig.13. Apart from the two-end sections which were in contact with the bathing solutions activity in the nodal rhizome was low until the centre sections were reached. Here, most of the activity was present in the fifth section from the basal end of the node, from which the central bud
Figure 13: Distribution of $^{14}$C-glyphosate in Agropyron single nodes.

Single nodes were prepared and fed 5000 DPM $^{14}$C-glyphosate (2500 DPM at each end) and incubated in darkness at 22°C. After 24hrs, buds were excised and nodes fragmented into 10 x 0.25cm sections, nos. 1-10 from proximal to distal end. Buds and segments were solubilised in 0.5cm$^3$ Soluene 350:toluene (1:1) for 3-days at room temperature after which acidified Triton X-100 scintillant was added and samples counted.
arose. Indeed this section contained approximately 50% of the activity in the rhizome, discounting the two end sections. In contrast, the bud itself contained only low activity, approximately 13% of that of the fifth section and less than any other single section of the node. Although results are not presented per unit weight, buds were of approximately the same size and weight of a single node section.

The movement of glyphosate into the bud may involve an ATP-dependent transport mechanism in the plasma membrane of bud cells, since glyphosate is highly polar, resembles some endogenous compounds and may also move in the whole plant in an energy dependent manner. This hypothesis was tested by studying the accumulation of $^{14}$C-glyphosate in single node buds in the presence of sodium azide, a respiratory uncoupler (Table 9). At concentrations greater than $10^{-6}$M, azide inhibited the accumulation of $^{14}$C-glyphosate in the bud, but only to a limited extent. Even at $10^{-2}$M, accumulation was reduced by only approximately 40%. At concentrations at which azide should be an effective metabolic inhibitor glyphosate still accumulated to a large extent and thus, in this system at least, appeared to enter the bud mainly by a process of simple diffusion.

The status of $^{14}$C-glyphosate taken up into the bud was determined by the use of several treatments. The inhibition of growth, characteristic of glyphosate could involve binding to macromolecular components thereby inhibiting translation, transcription or peptide elongation. In this case, $^{14}$C-glyphosate would be associated with a TCA precipitate from bud material. However a repeatedly washed ice-cold 5% TCA precipitate from whole excised buds
TABLE 9

EFFECT OF SODIUM AZIDE ON UPTAKE OF $^{14}$C-
GLYPHOSATE BY AGROPYRON SINGLE NODE BUDS

<table>
<thead>
<tr>
<th>NaN₃ (M)</th>
<th>DPM per bud</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>104.5 ± 37.4</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>112.4 ± 47.7</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>89.3 ± 49.0</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>76.7 ± 42.7</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>96.2 ± 50.0</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>64.8 ± 29.5</td>
</tr>
</tbody>
</table>

Single nodes were prepared and fed 5000 DPM $^{14}$C-glyphosate (2500 DPM at each end) in $10^{-4}$M $^{12}$C-glyphosate containing a range of concentration of NaN₃. Nodes were incubated in darkness at 22°C. After 24 hrs, buds were excised, solubilised in 0.5cm³ Soluene 350:toluene (1:1) for 3 days at room temperature after which acidified Triton X-100 scintillant was added and samples counted.
of single nodes, contained only 1.4% of the total uptake of activity (Table 10). A cell wall fraction prepared by repeated washing and centrifuging of buds ground in ice-cold water contained 1.0% of total uptake. Since the total TCA precipitate contained cell wall material, most of this activity was associated with the cell walls. Most of the total uptake (99%) was accounted for in the combined water supernatants from the cell wall preparation. When a sample of this was made to 5% with TCA, no radioactivity was associated with the precipitate. Thus most of the activity was present in a water-soluble unbound form. The nature of the $^{14}$C activity in the aqueous extract was determined by two dimensional TLC as described by Rueppel et al (1977). Chromatography of standard $^{14}$C-glyphosate yielded a single spot in the origin quarter of the plate, although separation of small amounts of $^{14}$C-contaminants by a single solvent system has been reported (Sprankle et al., 1978). Chromatography of the bud extract yielded two radioactive spots (Fig.14). The spot corresponding to glyphosate contained most of the activity but the second, more mobile in the isobutyric acid based eluent accounted for an average of 10% of the total loaded activity. By comparison with Rf values from Rueppel et al (1977) this spot was tentatively identified as glycine or aminomethyl phosphonic acid (AMPA) as development with a phosphorous detection reagent was not carried out.

3.1.2. Discussion: The limited accumulation of $^{14}$C-glyphosate in the single node bud compared with that of the nodal section from which it arises, raises the possibility that glyphosate may influence bud development indirectly from the
### Table 10

**Association of $^{14}$C-glyphosate with cell fractions of *Agropyron* single node buds.**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>DPM</th>
<th>% Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Uptake</td>
<td>13740</td>
<td>100</td>
</tr>
<tr>
<td>TCA precipitate</td>
<td>194.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Cell walls</td>
<td>141.2</td>
<td>1.0</td>
</tr>
<tr>
<td>Precipitated soluble cell contents</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Soluble contents</td>
<td>13800</td>
<td>98.6</td>
</tr>
</tbody>
</table>
**Figure 14:** Two-dimensional TLC of glyphosate.

a) Aqueous bud extract from $^{14}$C-glyphosate treated *Agropyron* single nodes,

b) Glyphosate and some hypothetical metabolites,

Taken from Rueppel *et al* (1977).
rhizome material, conceivably by inhibiting nutrient transport into the bud since the concentration used, $10^{-4}$M was sufficient to bring about complete kill. Alternatively, the accumulation of glyphosate by the bud may have been sufficient to have caused toxicity. A useful corollary to this experiment would be to similarly investigate $^{14}$C-distribution in nodes of intact plants, supplied foliarly with glyphosate.

The experiments on distribution of $^{14}$C-glyphosate in cell fractions showed that inside the bud glyphosate existed in a free form, predominantly unmetabolised. The small amount of activity bound to the cell wall fraction would be unlikely to have any significance. Richards and Slife (1979) have recently shown that organelles isolated from leaf cells by differential centrifugation contained only minor amounts of the total cell $^{14}$C-glyphosate. Generally, glyphosate has not been shown to be extensively metabolised in plants. Wyrill and Burnside (1976) showed that in *Asclepsis syriaca* L. and *Apocynum cannabinum* L. negligible metabolism of $^{14}$C-glyphosate occurred. In *Convolvulus arvensis* some metabolism to $^{14}$C-AMPA was found, but never greater than 15% of activity (Sandberg, 1978). Sprankle et al (1978) found 7% and 11% recoverable $^{14}$C as AMPA in leaves and shoots respectively, together with minor amounts of glycine and sarcosine, although these were also found to be contaminants of stock $^{14}$C-glyphosate. In avocado fruit, $^{14}$C-glyphosate remained largely unmetabolised; after 10-days 3.8% was given off as $^{14}$CO$_2$ and 0.36% as $^{14}$C-AMPA (Hasegawa et al., 1977). Lund-Høie (1976) however, found that in plants of spruce which were resistant to
glyphosate, rapid evolution of $^{14}\text{CO}_2$ took place, which may have partially accounted for resistance. With this exception, the results from *Agropyron* buds here are in agreement with other reports that glyphosate is persistent within the plant with only marginal degradation, the major product being AMPA, the known microbial metabolite of glyphosate (Rueppel et al., 1977). It is not proven in any of these cases that the production of $^{14}\text{CO}_2$ or $^{14}\text{C-AMPA}$ was due to plant metabolism rather than microbial breakdown. The production of $^{14}\text{C-AMPA}$ in *Convolvulus* observed by Sandberg (1978) is thought to be microbial in origin (Calder, personal communication). Certainly large microbial populations are associated with subterranean plant organs and bacterial metabolism may have occurred before the solution of $^{14}\text{C-glyphosate}$ entered the cut end of the single node. Further investigations to resolve this issue would involve surface sterilisation of nodes, autoclaving of tubing and the use of an antibiotic in the glyphosate solution. Differences in metabolism rates may also have been observed without the inclusion of a toxic dose of $^{12}\text{C-glyphosate}$ ($10^{-4}\text{M}$) as used here. The experiment detailed here was also only concerned with metabolism at the site of action and did not take account of the possibility that in the whole plant, glyphosate may undergo metabolism at some point removed from the site of action.

3.2. **Uptake and Incorporation of $^{14}\text{C-macromolecular Precursors in Agropyron single node buds**}

3.2.1. **Results:** The effects of glyphosate on some processes fundamental to the division and expansion of cells within single node buds are shown in Fig.15. The incorpo-
ration of $^{14}$C-labelled thymidine, uridine, protein hydrolysate and glucose as indicators of DNA, RNA, protein and cellulose synthesis respectively was inhibited after pre-treatment with glyphosate for 24hrs. Inhibition was considerable at $10^{-4}$M and almost complete at $10^{-3}$M, although the synthesis of DNA appeared to be rather less sensitive than the other processes. Glyphosate at $10^{-5}$M had only a small effect on incorporation of $^{14}$C-uridine, protein hydrolysate and glucose with respect to uptake, whilst the incorporation of $^{14}$C-thymidine was actually stimulated. The uptake of $^{14}$C-glucose was also stimulated at this concentration. The uptake of all precursors at $10^{-4}$M and $10^{-3}$M was substantially inhibited and therefore effects on synthesis were due in part to non-availability of substrate. To facilitate comparison of data, incorporation results of Fig.15 have been pooled in Fig.16a and expressed as inhibition of incorporation relative to uptake inhibition in Fig.16b. This clearly shows that in the case of DNA synthesis inhibition was completely due to inhibition of $^{14}$C-thymidine uptake at $10^{-4}$M and considerably at $10^{-3}$M, whereas for RNA, protein and cellulose synthesis, inhibition of precursor uptake accounted for approximately 50% of incorporation inhibition at $10^{-4}$M. The uptake of $^{14}$C-sucrose, a likely major requirement of developing buds was not inhibited to such a marked extent as the other labelled compounds and was only reduced at $10^{-3}$M (Fig.17).

3.2.2. Discussion: Since each experiment with precursor uptake and incorporation was carried out with a different batch of material, the differences in the behaviour of these
Figure 15: Uptake (●—●) and incorporation (○---○) of 
14C-macromolecular precursors in Agropyron 
single node buds.
Figure 16: Incorporation of $^{14}$C-macromolecular precursors in Agropyron single node buds.

a) Total incorporation,

b) inhibition of incorporation as % of precursor uptake

$\bullet \longrightarrow \bullet$ $^{14}$C-thymidine, $\circ \cdots \circ$ $^{14}$C-uridine, $\triangle \longrightarrow \triangle$ $^{14}$C-protein hydrolysate, $\square \cdots \square$ $^{14}$C-glucose.
Figure 17: Uptake of $^{14}$C-sucrose by *Agropyron* single node buds.

( ● ) per bud, ( ○ ) per mg. of f.wt.
processes for $^{14}$C-uridine, protein hydrolysate and glucose are not considered to be significant. However, the behaviour of DNA synthesis with respect to uptake of $^{14}$C-thymidine clearly was different from the other functions examined. Inhibition of DNA synthesis occurred largely due to the inhibition of $^{14}$C-thymidine uptake whereas for other processes, a more direct effect was exerted in the cell. The biosynthetic functions thus differed in sensitivity, DNA synthesis being much less sensitive than RNA, protein and cellulose synthesis. It thus appeared that cell expansion, as characterised by the incorporation of $^{14}$C-glucose was more sensitive to glyphosate than the prior process of cell division, characterised by the incorporation of $^{14}$C-thymidine. No biosynthetic process however was more markedly affected than others and thus a direct effect of glyphosate on any of these processes was unlikely.

The substantial inhibition of precursor uptake could have been due to either reduction of the sink status of the bud or a direct effect on uptake across bud cell membranes. This would imply that in rhizomes at least, the toxicity of glyphosate may be mediated in part by the prevention of uptake of stored nutrients from the internodal rhizome into the bud, quite apart from inhibitory effects in the cell. This hypothesis is substantial by the large build up of $^{14}$C-glyphosate in the central section of nodal rhizome with respect to the bud itself (Fig.13); glyphosate may thus regulate the flow of nutrients into the bud from outside. In this case one of the most important nutrients would be expected to be sucrose, as a
carbohydrate source, but the uptake of $^{14}$C-sucrose was not greatly affected.

3.3. PAL and Related Effects

3.3.1. Results: The discovery that glyphosate enhanced the level of phenylalanine ammonia-lyase (PAL) in maize roots (Duke and Hoagland, 1978) prompted the investigation of PAL induction in single node buds. Buds excised from single nodes which had been fed water for 24 hrs following fragmentation and incubated in darkness contained a surprisingly high level of activity with a mean of 5.3 units per mg protein (Fig. 18), a period at which growth was not yet discernable. When fed glyphosate activity was influenced only at $10^{-3}$ M, with an approximate doubling of activity either on a protein, bud or fresh weight basis. In nodes in which buds were excised at 48 hrs, glyphosate was removed after the initial 24 hrs and nodes were reincubated with water, thus using the same protocol as experiments on precursor uptake and incorporation. Although the control level of activity per mg of protein did not change, activity per bud increased, due to bud growth. In buds treated with glyphosate at $10^{-3}$ M, PAL activity increased to over 3 times that of the control and activity induced at $10^{-4}$ M was even higher at 5 times the control level. Glyphosate at $10^{-5}$ M had little effect. Increases were not quite so great on a bud basis, since retarded buds at high glyphosate concentrations contained less soluble protein. This higher induction of PAL at $10^{-4}$ M than $10^{-3}$ M after 48 hrs meant that either $10^{-3}$ M was a supra-optimal concentration, at which PAL was not induced so efficiently or that a higher value had been reached at some point between 24 and
Figure 18: Phenylalanine ammonia-lyase (PAL) in *Agropyron* single node buds.

a) mUnits PAL per bud,  
b) mUnits PAL per mg protein; (●) 24hrs, (○) 48hrs.
48hrs, which subsequently declined. If the enhancement of PAL mediated the toxicity of glyphosate the latter would be expected, since growth retardation was greater at $10^{-3}$M than $10^{-4}$M.

If this in vitro assay reflected in vivo activity the pool of free phe may be expected to be depleted with resultant inhibition of protein synthesis. If labelled phe were to be added, the incorporation into protein should occur to a much greater extent than for any other protein amino acid. Table 11 shows results from an experiment in which equal amounts (moles) of $^{14}$C-phe and $^{14}$C-leu were fed to different sets of single nodes after pre-treatment with $10^{-4}$M glyphosate. The incorporation of $^{14}$C-leu relative to uptake displayed marked inhibition by glyphosate, but the incorporation of $^{14}$C-phe was inhibited to a minor extent only. This was in contrast to the results of Haderlie (1975), in which there was no difference in the rates of incorporation of $^{14}$C-phe and $^{14}$C-leu in glyphosate treated carrot cells. An intermediate figure of inhibition was obtained here by feeding $^{14}$C-protein hydrolysate which contained phe amongst other amino acids. These results suggest that the pool of free phe was reduced by glyphosate and the experiment can be regarded as an indirect method of assessing the relative levels of free amino acids in the bud. The inhibition of protein synthesis by a deficit of free phe may have caused a build-up in the levels of other protein amino acid pools, e.g. that of leu. The addition of $^{14}$C-leu would mean dilution of this inside the bud by an abnormally high endogenous pool thereby diluting the amount of $^{14}$C-leu which would still be able to be incorporated.
TABLE 11

INHIBITION OF INCORPORATION OF $^{14}$C-AMINO ACIDS BY GLYPHOSATE IN *AGROPYRON* SINGLE NODE BUDS

<table>
<thead>
<tr>
<th>$^{14}$C-AMINO ACID</th>
<th>% INHIBITION OF INCORPORATION, PER BUD$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucine</td>
<td>68</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>11.5</td>
</tr>
<tr>
<td>Protein hydrolysate</td>
<td>30</td>
</tr>
</tbody>
</table>

$^*$ Compared to control and relative to uptake, at 10$^{-5}$M glyphosate.
Conversely, addition of $^{14}\text{C}$-phe would mean the presence of abnormally concentrated radioactivity due to the depleted endogenous phe pool, with a correspondingly high rate of incorporation. The administration of $^{14}\text{C}$-protein hydrolysate would enable some $^{14}\text{C}$-phe to be incorporated thereby abolishing the lesion with subsequent $^{14}\text{C}$-amino acid incorporation until another phe deficiency occurred. Since the inhibition of $^{14}\text{C}$-phe incorporation was only slight, the machinery of protein synthesis still appeared to be relatively intact and thus was not permanently debilitated.

The induction of PAL was also enhanced by glyphosate in root tips of wheat seedlings in solution culture although these were not so sensitive as single node buds. Root tips of 0.5cm showed a fairly constant level of PAL activity over a period of 6-days of 3-5mUnits per mg protein. When seedlings were transferred to $10^{-4}\text{M}$ glyphosate at the beginning of this period, PAL exceeded the control level after 2-days to a maximum of 2.7 times that of the control values after 4-days, after which activity decayed to that of the control (Fig.19a). Treatment with $5 \times 10^{-4}\text{M}$ glyphosate resulted in a much greater enhancement of activity. After 1-day, PAL activity developed to 6 times that of the control and after reaching a maximum value after 2-days, exhibited a slow decline. After 5-days however, activity was still nearly 3 times that of the control (Fig.19b). This enhancement was paralleled by a rapid drop in the level of soluble protein as assayed in the enzyme preparation, a 20000 x g supernatant containing truly soluble protein and microsomal protein. This was reduced after 1-day by 66.5% at $5 \times 10^{-4}\text{M}$ glyphosate.
Figure 19: Phenylalanine ammonia-lyase and soluble protein in wheat root tips.

a) $10^{-4}\text{M}$ glyphosate,
b) $5 \times 10^{-4}\text{M}$ glyphosate; (○·○) control, (●··●) glyphosate,
c) soluble protein (▲) $10^{-4}\text{M}$ glyphosate, (▲) $5 \times 10^{-4}\text{M}$ glyphosate.
(Fig.19c) and stayed at a similar level thereafter, although the protein content of control material decreased steadily accounting for the increase in % control after 3-days. The drop in protein content meant that PAL expressed per root tip showed only approximately half the increase as when expressed per mg protein. Expression on a protein basis is however a more valid figure for speculating on the metabolic consequences of such a PAL enhancement since a decrease in the level of protein would presumably lower the general level of metabolism in the cell accordingly. The protein content of root tips was affected to a much lesser extent at $10^{-4}$M glyphosate with significant reduction only after 3-4 days and after 5-days was still 74% of the control value (Fig.19c). Enhancement of PAL induction thus preceded protein decline.

An attempt to evaluate further the role of PAL enhancement in glyphosate toxicity was made by using the formazan reduction assay to determine the comparative respiratory metabolism of root tips. Depression of respiratory activity as measured by formazan reduction correlated well with the increase in PAL levels (Fig.20). At $10^{-4}$M glyphosate dye reduction fell only gradually, eventually to a value of about 50% of control after 4-days. At $5 \times 10^{-4}$M, very little activity was assayable after only 1-day (Plate 6). Over a time course therefore the patterns of decrease in formazan reduction and increase in PAL activity were very similar.

Reference to Plate 7 shows clearly that glyphosate affected only the tips of wheat roots. In control roots, tips were stained heavily with respect to the rest of the
Figure 20: FORMAZAN REDUCTION IN WHEAT ROOT TIPS.

(●) control, (○) 10⁻⁴M glyphosate, (△) 5 x 10⁻⁴M glyphosate.
Plate 6: Formazan staining of wheat root tips

Root tips (0.5cm) were excised from dark-grown seedlings pre-treated with glyphosate for 24hrs and incubated in 0.1% 2,3,5-triphenyltetrazolium chloride hydrate at 25°C in darkness for 2hrs.

Plate 7: Formazan staining of whole wheat roots

Roots of seedlings pre-treated with glyphosate for 24hrs were incubated in formazan as above.
root, indicating a much higher rate of metabolic activity in this area. Tips of roots incubated in $5 \times 10^{-4}$M glyphosate were completely unstained for the apical 0.3cm although the rest of the root was stained to a similar degree as control roots. It is salient here to point out the difference between PAL experiments here and those of Duke and Hoagland (1978) who, with similar experiments with maize, used whole roots for the enzyme assay. A 2.8 fold increase in the level of PAL was demonstrated after 24hrs in response to $10^{-3}$M glyphosate, whereas here, by using 0.5cm root tips only, a 5-fold increase in PAL activity was found after the same time, using half the concentration. Also in the experiments of these workers, protein levels were not found to decrease in the presence of glyphosate and again it may be that the use of root tips only would have revealed similar changes as have been documented here.

The inverse correlation of PAL induction with formazan assay decrease does not necessarily indicate a central role for PAL in the mode of action of glyphosate. Enhancement of PAL induction may merely have been a concurrent symptom of toxicity. In order to test the hypothesis that PAL enhancement was responsible for toxicity, experiments were conducted with PAL inhibitors. PAL in vitro is subject to inhibition by a variety of compounds (Camm and Towers, 1973a, 1977) mainly allosterically by feedback inhibitors, e.g. $p$-coumaric acid, but notably by the immediate PAL product trans-cinnamic acid. PAL is also subject to competitive inhibition by D-phe. More recently, Amrhein and co-workers have reported that the amino-oxy analogue of L-phe, L-$\alpha$-
aminooxy-β-phenyl propionic acid (L-AOPP) competitively inhibited PAL with a $K_i$ value of $1.4 \times 10^{-9}$ M, the lowest value yet reported for a PAL inhibitor (Amrhein et al., 1976; Amrhein, 1978). Specificity of this inhibitor for the secondary product pathway was demonstrated by Amrhein and Hollander (1979) who showed that flower buds and seedlings imbibing L-AOPP developed normally but lacked anthocyanin. The effect of 3 PAL inhibitors, the product cinnamic acid, D-phe and L-AOPP on the toxicity of glyphosate was investigated by germinating wheat seeds in petri dishes with or without inhibitors. After 4-days, root fresh weight was considerably reduced by $10^{-4}$ M glyphosate, but the use of inhibitors at a range of concentrations of 0.5 to 10 times that of glyphosate gave no alleviation of growth inhibition (Table 12). Substrate addition also had no effect. These results thus throw doubt on an instrumental role for PAL in toxicity.

Possible mediating factors in the enhancement of PAL by glyphosate could have been the evolution of ethylene or the elevation of soluble sugar levels, 2 factors known to influence PAL. The evolution of ethylene was studied by transferring wheat seedlings to sealed flasks and analysing the atmosphere daily. Control seedlings evolved ethylene at a linear rate for 3-days during which time the production in the presence of glyphosate was slightly lower (Fig. 21). After 3-days, production from controls levelled off, but ethylene continued to be evolved at a linear rate with glyphosate treatment and was produced in excess of the control level after 5-days at $10^{-3}$ M and 6-days at $5 \times 10^{-4}$ M. Figures presented are for evolution per flask, but on a
Table 12

Inhibition of wheat root growth by glyphosate: addition of L-phenylalanine and PAL inhibitors

<table>
<thead>
<tr>
<th>Treatment (M)</th>
<th>Mean root f. wt. (mg)</th>
<th>L-phe</th>
<th>D-phe</th>
<th>Cinnamic acid</th>
<th>L-AOPP*</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-5}$M glyphosate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$+ 5 \times 10^{-5}$</td>
<td>13.1 ± 2.7</td>
<td>10.3 ± 2.9</td>
<td>12.9 ± 3.3</td>
<td>12.1†</td>
<td></td>
</tr>
<tr>
<td>$+ 10^{-4}$</td>
<td>10.2 ± 2.8</td>
<td>9.9 ± 0.8</td>
<td>12.8 ± 1.2</td>
<td>9.9</td>
<td></td>
</tr>
<tr>
<td>$+ 2 \times 10^{-4}$</td>
<td>9.4 ± 1.2</td>
<td>11.4 ± 0.9</td>
<td>10.4 ± 0.9</td>
<td>12.4</td>
<td></td>
</tr>
<tr>
<td>$+ 5 \times 10^{-4}$</td>
<td>9.9 ± 3.0</td>
<td>11.6 ± 2.3</td>
<td>14.0 ± 2.4</td>
<td>10.9</td>
<td></td>
</tr>
<tr>
<td>$+ 10^{-3}$</td>
<td>13.3 ± 0.4</td>
<td>10.7 ± 0.6</td>
<td>11.3 ± 1.7</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>$10^{-4}$M glyphosate</td>
<td></td>
<td>11.0 ± 2.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>33.8 ± 7.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Seeds were germinated at 27°C in petri dishes on filter paper moistened with water, $10^{-4}$M glyphosate or glyphosate containing either L-phe, D-phe, cinnamic acid or L-AOPP. Root fresh weights were determined after 4-days in darkness.

* Generously donated by Dr. J. S. Morley, ICI Pharmaceuticals Divn., Alderley Park, Macclesfield, Cheshire

† Not replicated due to limited amount of compound available.
Figure 21: EVOLUTION OF ETHYLENE BY WHEAT SEEDLINGS:

(●) control, (O) 5 x 10^{-4}M glyphosate, (△) 10^{-3}M glyphosate.

Seeds were germinated on filter paper moistened with 2 x 10^{-3}M CaSO₄ in petri dishes in darkness at 27°C. After 2 days seedlings were transferred to 100cm³ conical flasks (5 seedlings/flask) together with 3 cm³ 2 x 10^{-3}M CaSO₄ alone (control) containing glyphosate. Flasks were sealed with 'Suba-Seal' stoppers and incubated in darkness at 22°C. Atmospheric samples were taken at daily intervals and assayed for ethylene by GLC (see Materials & Methods, 3.5).
fresh weight basis, figures for evolution from treated seedlings would have been enhanced since growth was inhibited. Nevertheless, there was no initial stimulation in the production of ethylene which could have accounted for PAL activation.

Exogenous sugar has been shown to enhance PAL levels (Creasy, 1968; Amrhein and Zenk, 1971) which suggests that this may be a controlling factor in vivo. When measured as glucose equivalents the total soluble sugar content of root tips decreased upon treatment with $5 \times 10^{-4}$M glyphosate to approximately 50% of the control level after 2-days, thereafter staying at about that level. Thus no enhancement occurred which may have accounted for PAL stimulation.

3.3.2. Discussion:

3.3.2.1. The Control of PAL: PAL is the central enzyme in the connection of primary metabolism (shikimic acid pathway) with secondary metabolism, deaminating L-phe to form trans-cinnamic acid, the precursor of secondary compounds. As an important control point PAL is influenced by a variety of factors notably that of light via the phytochrome response but also wounding and infection. The properties of PAL have been recently reviewed by Creasy and Zucker (1974) and Camm and Towers (1973a, 1977).

The interpretation of the metabolic consequences of PAL stimulation by glyphosate are dependent upon the nature of the control mechanism of phenolic compound synthesis which is at present unresolved and under vigorous debate. The extraordinary sensitivity of PAL to a variety of stimuli has in the past led to
Figure 22: Total soluble sugars in wheat root tips

(○) control  (●) 5 x 10^{-4}M glyphosate.
the assumption of a direct effect on PAL, but more recent evidence suggests that this may not be so. At present, regulation of phenolic compound synthesis is thought to occur via 3 possible routes: by changes in end product pools, substrate pool or by a direct effect on PAL.

The view that the control of secondary product synthesis was brought about by direct regulation of PAL by external stimuli was supported by numerous reports that fluctuations in the level of extractable PAL concurred with similar changes in hydroxycinnamic compounds and flavonoids (Camm and Towers, 1973a, 1977). There are however, also many reports in which a correlation of this kind did not exist (Camm and Towers, 1973a, 1977; Margna, 1977). Margna has discussed reported changes in PAL with no fluctuation in end product levels and vice versa, cases in which similar changes occur but are not co-incident in time and cases of simultaneously opposing changes. In a survey of such references, Margna calculated the deaminating capacity of recorded PAL levels, concluding that in most cases this was far in excess of that necessary to account for the level of end products observed and thus extractable levels did not reflect *in vivo* activity. This excess would mean that any direct regulation of PAL would be unlikely to influence the utilisation of phe and a theory of substrate control was advanced from evidence that end product pools changed in response to exogenous phe.

A rather different theory has been proposed by
Engelsma (1978) in which the primary regulation point is proposed to be the end product pool. This is supported by extensive work on light-stimulation of PAL in gherkins and evidence from other workers. Accordingly, under conditions of low hydroxyphenolic synthesis a large pool of end products is present which causes feedback inhibition of PAL. Light causes a diminution of this pool, either by influencing compartmentation, or possibly by the conversion of cinnamic acid derivatives from the trans form to the less inhibitory cis form, thereby activating existing PAL, but also increasing extractable PAL. The resultant increase in the hydroxyphenolic pool causes eventual re-inhibition of PAL and a decline in extractable levels, since cinnamic acid derivatives have been shown to diminish extractable PAL as well as causing in vitro inhibition. Support for this theory also derives from the work of Amrhein (1978) who showed that the administration of the competitive PAL inhibitors α-aminoxyacetic acid and L-AOPP to gherkin hypocotyls caused a decrease in the level of hydroxyphenolic acids thereby stimulating PAL, effects negated by the use of cinnamic acid. A similar stimulation of PAL activity was reported by Szkutricka and Lewak with D-phe in seedlings of several species. Inhibitor stimulation did not occur in buckwheat however (Amrhein, 1978) and it was concluded that end product control was operative in gherkin but not in buckwheat. End product inhibition was also demonstrated by Lamb and
Rubery (1976) in potato tuber tissue in which the amount of extractable PAL was reduced by exogenous phe, but much more quickly by cinnamic acid and p-coumaric acid, indicating control by product rather than substrate. In the light of these results, the theory of Margna (1977) could be modified to one of product inhibition by substrate addition.

With regard to glyphosate therefore, the influence on this pathway may initially be by affecting the end product pool rather than directly on PAL, and an influence on compartmentation, possibly by altering membrane organisation may occur. A build-up of end products in response to glyphosate enhancement of PAL as discussed by Hoagland et al (1978) would in consideration of an end product control theory be unlikely at least with the kind of compartmentation in which PAL would be susceptible to feedback inhibition and repression. A significant accumulation of end products would only occur if free phe were continually available which is unlikely as work here has indicated. Continued protein synthesis would also be necessary for the production of post-PAL enzymes, although production of the reactive o-quinones can occur due to breakdown of compartmentation between substrate and enzyme, without new enzyme synthesis (Mayer and Harel, 1979). Toxicity of glyphosate by o-quinone accumulation would also imply that the administration of phe would enhance toxicity rather than reversing it. The operation of an end product control system could explain the beneficial effect of exogenous phe, although enhancement of toxicity in
this manner was reported by Duke and Hoagland (1978). The differential responses of gherkin and buck wheat to PAL inhibitors as described by Amrhein (1978) with the implication of species differences in control mechanisms may provide a clue to the discrepancies in reversal experiments with L-phe. It may be that in the instances in which glyphosate toxicity was reversed by adding substrate end product control was operative, but in the cases where reversal was not achieved, some other form of control was dominant. Since in this work, the toxicity of glyphosate to germinating wheat seedlings was not reversed by the addition of substrate, product or competitive inhibitors (Table 12) either some other form of control occurred or the inhibition of PAL was not sufficient to reverse toxicity. The latter seems more likely, since in soybean seedlings inhibited by glyphosate, L-AOPP abolished the phe deficit caused by glyphosate but reversed growth only to a slight extent (Duke and Hoagland, 1979). The likelihood exists that toxicity of glyphosate may be mediated through effects not directly related to PAL enhancement.

3.3.2.2. PAL induction: de novo synthesis or activation: The enhancement of PAL induction by glyphosate may be due to either de novo synthesis or activation of latent enzyme and work with conventional stimuli shows that both are possible. Isotopically labelled PAL has been isolated from tissue fed labelled amino acids after induction by light and wounding; similarly density labelled PAL has been recovered from material
fed with deuterium (Camm and Towers, 1973a; Kahl, 1978). In addition, metabolic inhibitors, particularly the protein synthesis inhibitor cyclohexamide have been shown to inhibit the appearance of PAL (Zucker, 1968; Kahl, 1978). The work of both Zucker and Engelsma has, however, implicated the existence of a proteinaceous inhibitor of PAL which inactivates PAL upon complexation but upon dissociation releases active PAL. Zucker (1968) showed that the decline in PAL activity in potato after initial induction could be inhibited by cyclohexamide suggesting the necessity of de novo protein synthesis for the inactivation of PAL. A similar phenomenon had been demonstrated by Engelsma (1967) in gherkin and it was further shown that low temperature treatment after this decline caused the re-appearance of cyclohexamide-insensitive PAL at subsequent normal temperature, implying that the low temperature-transfer treatment had caused dissociation of the PAL-inhibitor complex with the subsequent re-activation of PAL at the higher temperature (Engelsma, 1970). These results were duplicated by Attridge and Smith (1973) who moreover showed that in dark-grown gherkin seedlings cyclohexamide spectacularly increased PAL activity indicating the existence of a pool of enzyme maintained in a quiescent state by a factor requiring continuous protein synthesis. Induction of PAL in this manner required a much shorter lag phase than by blue light, an observation consistent with the hypothesis that light caused induction by de novo synthesis but cyclohexamide
induction was by activation of pre-existing enzyme. The enhancement of PAL induction by glyphosate could well be through de novo synthesis since in root tips treated with $10^{-4}$M glyphosate enhancement of activity occurred before the onset of protein decline (Fig.19). Activation via the inhibition of formation of a protein synthesis requiring factor is unlikely as the level of soluble protein did not decline rapidly, unless inhibition was of a specific protein only. The subsequent decline in PAL activity could have been due to the formation of an inhibitor requiring protein synthesis since at $10^{-4}$M glyphosate PAL activity declined after 5-days to the control level, a time at which metabolic activity was still considerable (Fig.20). Conversely, at $5 \times 10^{-4}$M glyphosate, PAL activity declined only slowly after peak activity was reached and metabolic activity was almost completely abolished after 1-day.

3.3.2.3. Possible factors mediating the induction of PAL:
Glyphosate could be envisaged as a chemical 'wounding agent', inducing the characteristic wound response of ethylene production followed by PAL induction in a similar way to physical wounding and pathogenic infection. The results showed that PAL induction was in fact not due to ethylene production since no initial stimulation of evolution was observed and as such a typical wound response was not seen. It was shown by Abu-Irmaileh et al (1979) however that plants of *Phaseolus vulgaris* sprayed with $2 \times 10^{-3}$M glyphosate produced ethylene at double the control
rate after 12hrs and this relative rate continued until a further increase after 72hrs. In addition, Baur (1979) suggested that the inhibition of $^{14}$C-IAA movement in maize coleoptiles by glyphosate may have been due to ethylene formation since ethylene itself had a similar effect (Beyer and Morgan, 1969). The failure of glyphosate to promote ethylene formation in wheat seedlings at high concentrations was surprising, in consideration of the range of stress conditions which have this effect, ranging from light, wounding, irradiation and infection to changes in temperature, water stress and orientation (Abeles, 1973; Morgan, 1976; Yang and Pratt, 1978). Herbicides have not been widely screened for the activation of ethylene evolution although the auxin analogues are well known enhancing agents (Morgan, 1976). The failure of glyphosate to produce ethylene is all the more unexpected since stimulation of ethylene normally occurs with PAL induction and is assumed to be an intermediate agent in enzyme induction since exogenous ethylene induces PAL (Camm and Towers, 1973). In the experiment described here, ethylene evolution was assayed from whole seedlings, and changes in the production from the root tips only may have been masked.

The involvement of sugar with PAL induction was demonstrated by Creasy (1968) who showed that in strawberry leaves the requirement for CO$_2$ fixation could be replaced by exogenous sucrose. Furthermore, Amrhein and Zenk (1971) found that the addition of
sucrose, fructose and glucose markedly stimulated PAL activity in buckwheat seedlings. Since sugars are the initial substrate of the shikimic acid pathway the effect of glyphosate on the level of endogenous soluble sugars was examined but no positive relationship with PAL was observed.

3.4. Shikimate: NADP oxidoreductase, Chorimate mutase and Polyphenol oxidase

The enhancement of PAL induction by glyphosate prompted further studies on other enzymes involved in phenolic compound synthesis in order to clarify the likelihood that PAL activation reflected increased activity of the shikimic acid pathway and secondary compound biosynthesis pathway.

3.4.1. Results: Two enzymes of the shikimic acid pathway were examined, shikimate: NADP oxidoreductase (SORase), which catalyses the conversion of shikimate to 5-dehydroshikimate coupled to the reduction of NADP; and chorismate mutase which transforms chorismic acid to prephenic acid, the precursor of phe and tyr. Both enzymes were readily demonstrable in wheat root tips and incubation in $5 \times 10^{-4}$M glyphosate increased the specific activity after 24hrs of both by about 160% (Table 13). Therefore although the enzymes were stimulated by glyphosate, this was not of the same magnitude as that of PAL induction.

The possibility of elevated phenolic end product synthesis as discussed by Hoagland and Duke (1978) and Hoagland et al. (1978), with the terminal formation of reactive o-quinones may involve the activation of polyphenol oxidase (PPO). In root tips of seedlings incubated in $5 \times 10^{-4}$M glyphosate, the level of PPO as assayed with catechol substrate was
TABLE 13

SHIKIMATE:NADP OXIDOREDUCTASE AND CHORISMATE MUTASE IN WHEAT ROOT TIPS.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Activity (units/mg protein) after 24 hrs</th>
<th>Shikimate:NADP oxidoreductase</th>
<th>Chorismate mutase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>4.46 ± 0.53</td>
<td>2.26 ± 0.12</td>
</tr>
<tr>
<td>5 x 10^{-4}M glyphosate</td>
<td></td>
<td>11.47 ± 0.15</td>
<td>5.94 ± 0.42</td>
</tr>
</tbody>
</table>
elevated by over 200% after 1-day and by 300% after 2-days (Table 14).

3.4.2. **Discussion**: Control of the shikimic acid pathway in plants is thought primarily to involve end product inhibition (Gamborg, 1966; Gibson and Pittard, 1968) in a similar way to that of the microbial pathway (Cotton and Gibson, 1965; Gibson and Pittard, 1968). Chorismate mutase in particular has been implicated as such a control point since chorismate represents the point of divergence of the pathway, serving as a substrate for both chorismate mutase thereby leading to tyr and phe synthesis and anthranilate synthetase leading to tryp formation. The regulatory properties of chorismate mutase have been demonstrated by Cotton and Gibson (1968) who showed that the pea enzyme was inhibited *in vitro* by phe and tyr and activated by tryp. Furthermore, Woodin *et al* (1978) reported that in a range of higher plants, 3 isoenzymes of chorismate mutase were separable, only 2 of which could be feedback inhibited. The existence of initially 2 isoenzymes in mung bean had previously been reported by Gilchrist *et al* (1972), later resolved to 3 by Woodin and Nishioka (1973). This work revealed that at least some of the isoenzymes were subject to inhibition by hydroxyphenolic metabolites such as caffeic acid (Woodin *et al*., 1978) and chlorogenic acid and p-coumaric acid (Woodin and Nishioka, 1973) as well as regulation by shikimic acid pathway end products. The control of chorismate mutase primarily by feedback inhibition was supported by evidence that phe and tyr did not repress the level of extractable chorismate mutase in plant cell cultures (Chu and Widholm, 1972). The enhancement by
## TABLE 14

**POLYPHENOL OXIDASE IN WHEAT ROOT TIPS**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Units/mg protein</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24-hr</td>
<td>48-hr</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6.07 ± 0.53</td>
<td>5.58 ± 0.22</td>
<td></td>
</tr>
<tr>
<td>5 x 10^{-6}M glyphosate</td>
<td>14.10 ± 0.60</td>
<td>16.8 ± 0.12</td>
<td></td>
</tr>
</tbody>
</table>
glyphosate of the specific activity of chorismate mutase would appear to be the first time that a change in the amount of extractable activity in response to such an external stimulus has been shown.

In contrast, several reports of such stimulation of SORase exist. The level of SORase along with that of dehydroquinate hydro-lyase in sweet potato increased after slicing (Minamikawa et al., 1966a, b) with a shorter lag phase than for PAL (Kojima et al., 1969) and also in white potato after slicing, together with prephenate:NADP oxidoreductase (Camm and Towers, 1973b) although increases were not as great as those exhibited by PAL. Red light has been reported to stimulate SORase in pea to the same extent as PAL (Ahmed and Swain, 1970) though this was not previously found with a crude enzyme preparation (Attridge and Smith, 1967). The significance of elevated levels of these enzymes is unclear since they may not necessarily reflect an increase in the rate of catalysis. It is also unclear as to how elevated levels are brought about; this could be by de novo synthesis, activation of latent enzyme or, since the level of soluble protein declined, a preferential protection from breakdown or inhibition of synthesis. Enhancement may be part of a more general effect on enzymes in this area of metabolism, since Muto et al (1969) showed that cut sweet potato exhibited increases in the levels of enzymes of the pentose phosphate pathway which generates erythrose-4-phosphate, one of the shikimic acid pathway precursors. It is unlikely that the magnitude of the increases in SORase and chorismate mutase activities are large enough to be of significance in the mode of action of
Polyphenol oxidases represent a terminal reaction of secondary compound metabolism, catalysing the hydroxylation ortho- to the existing hydroxy group of monophenols producing o-quinones. These are highly reactive compounds which readily condense with -SH and -NH₂ groups of protein, thus causing enzyme inactivation, or with -NH₂ groups of amino acids and primary amines producing red complexes (Butt, 1979). Alternatively, o-quinones can polymerise to form insoluble complexes. Wheat roots incubated in glyphosate developed a pink colouration as reported previously in roots of maize seedlings by Duke and Hoagland (1978) and could conceivably have represented increased o-quinone production, although colouration extended to the whole root, rather than being confined to the root tip. The increase in activity of PPO may thus have been responsible for an increased rate of phenolic compound oxidation in vivo. It is commonly observed that changes in PPO activity are not necessarily accompanied by corresponding changes in substrate levels probably because in normal plant cells enzyme and substrate are separated by compartmentation, hydroxyphenolic compounds mainly being found in the vacuole. In many cases the increases in phenolic oxidation observed in such instances as wounding or infection occur by breakdown of this compartmentation thus bringing enzyme and substrate together (Mayer and Harel, 1979). Increases in the level of extractable PPO activity have often been demonstrated however, for example in viral infection (Van Kammen and Brauwer, 1964; John and Weintraub, 1967; Nye and Hampton, 1966), fungal infection (Hyodo and Uritani,
Such increases have been ascribed to either new enzyme formation or activation of latent enzyme. Hyodo and Uritani (1966b) demonstrated that the stimulation of PPO activity by slicing in sweet potato could be repressed by antibiotics and also that new isoenzymes were formed (Hyodo and Uritani, 1967). PPO preparations from plant material can be increased by various chemical treatments (Kosuge, 1969) suggesting that rises in activity can sometimes be due to activation (Mayer and Harel, 1979). Again, the mechanism by which glyphosate brought about enhancement is unclear and it would be impossible to assess the contribution towards mode of action without determining the rate of hydroxyphenolic compound oxidation. The fact that active enzymes can be extracted from glyphosate treated material would seem to mitigate against any substantial quinone accumulation.

3.5. Hydrolytic Enzymes

Symptoms of herbicide activity have often been compared to those of senescence and biochemical similarities may underlie these. The activation of degradatory hydrolytic activity is a general phenomenon of senescence and also occurs in response to a variety of external stimuli. The possibility that glyphosate may promote such activity was investigated with 3 such types of activity: protease, ribonuclease and acid phosphatase.

3.5.1. Results: The effect of glyphosate on proteolytic activity was of particular interest since an elevation of
this may have been responsible for the rapid decline in the level of soluble protein brought about by glyphosate. Proteolytic activity was investigated using azocasein substrate (Charney and Tomarelli, 1947). Azoproteins having been used to determine endoproteolytic activity as assayed by the release of TCA-soluble azopeptides although azo-amino acid complexes released by exoprotease activity may also contribute. Both acid (pH 5.0) and neutral (pH 7.0) activities were present in root tips, the pH 7.0 enzyme being slightly more active (Fig. 23). In wheat root tips, control activity at both pH values stayed relatively static for 3-days after seedling transfer to fresh CaSO₄, but after transfer to glyphosate at $5 \times 10^{-4}M$, activation occurred in both cases. At pH 5.0 specific activity was enhanced in excess of the control value after 1-day, with no significant change thereafter. At pH 7.0, specific activity was enhanced by about 50% after 1-day increasing to 100% after 3-days.

Ribonuclease (RNase) activity was affected in a similar manner. Assayed at pH 5.0, RNase increased by approximately 50% after exposure to glyphosate for 1-day and by nearly 200% after 2-days although control rates were rather variable (Fig. 24). Soluble acid phosphatase specific activity (pH 5.0) in controls remained constant for 3-days; but with glyphosate treatment increased by approximately 100% after 1-day, a level maintained after 2-days but which began to drop after 3-days (Fig. 25). Lysosomal acid phosphatase (Triton X-100 soluble) remained at a similar level per root tip (15nmol p-nitrophenol/hr) throughout the 3-days both in control and glyphosate treated material. Calculation of specific activity in this case was not possible since
Figure 23: Proteolytic activity against azocasein in wheat root tips
Figure 24: RNase in wheat root tips

- **control**
- **5 x 10^-4 M glyphosate**
Figure 25: Soluble acid phosphatase in wheat root tips
Triton X-100 interfered with the Lowry protein assay. With both proteolytic and RNase activity, rates per root tip did not change significantly with glyphosate treatment but did for soluble acid phosphatase. This was mainly because in the acid phosphatase experiment, soluble protein decline was greater than in those for protease and RNase.

3.5.2. Discussion: The biochemistry of senescence has been studied mainly in leaf tissue. A rapid loss of protein has been a known characteristic of detached and darkened leaves for some considerable time (Yemm, 1937; Wood and Cruikshank, 1944; Chibrale, 1954), a process associated with an increase in proteolytic activity (Anderson and Rowan, 1965). The enhancement of proteolytic activity has been shown to represent new enzyme formation since it was repressed by cyclohexamide (Martin and Thimann, 1972; Peterson and Huffaker, 1975) resulting in retardation of senescence. An increase in the activity of RNase has also been associated with senescence. Activity was higher in older than younger leaves and elevation occurred in response to dehydration, wounding, acute temperature change and infection (Dove, 1973). The increase of RNase activity was prevented by cyclohexamide (Udvardy et al., 1969; Thomas, 1974) and inhibitors of RNA synthesis (De Leo and Sacher, 1970) in senescing leaves and also in wounded turnip tissue (Sacher et al., 1975) although the increase observed in wounded potato was not due to de novo synthesis (Dove). Acid phosphatase has also been associated with leaf senescence, the appearance of which was prevented by inhibitors of RNA and protein synthesis (De Leo and Sacher, 1970).

The contribution of these enhanced activities to mode
of action are debatable. Proteolytic activity against azocasein was very low in control root tips in relation to other enzymes assayed and the increase upon this observed with glyphosate would not be likely to account for the rapid loss in soluble protein, but could be a contributory factor. If the hydrolytic degradation rates of RNase and acid phosphatase reflect in vivo rates these would also be contributory, albeit secondary factors in toxicity. The lack of specific activity values for lysosomal acid phosphatase renders unclear whether the increase in soluble activity was due to breakage of lysosomes. The elevated levels of these enzymes cannot have metabolic significance after 1-day since respiratory activity was negligible after this time and it is probable that the increases observed are merely general stress reactions.

3.6. Ethane Evolution and Membrane Bound Enzymes

The cell membrane is a known site of action for some herbicides. Brecke showed that in isolated bean leaf cells glyphosate did not promote the leakage of electrolytes or pre-loaded $^{86}$Rb with the conclusion that gross changes in membrane conformation were not caused which would have resulted in increased permeability. However, in the same tissue, the uptake of both $^{86}$Rb and $^{32}$Pi were rapidly inhibited, a phenomenon ascribed to an inhibition of ATPase linked monovalent ion pumps of the cell membrane. It thus appeared that glyphosate could cause subtle effects at the cell membrane without changing the gross properties of the lipid barrier. Membrane effects of glyphosate have therefore been re-examined here using different techniques, firstly by investigating the effect of glyphosate on the evolution of ethane, a known indicator of membrane breakdown, and secondly by investigating the effect on selected membrane bound enzymes. Any interference with the structure of membranes would
result in the decreased stability of enzymes situated therein and accordingly the activity of such enzymes may be expected to be inhibited. Enzymes chosen, in accordance with Hodges and Leonard (1973) as membrane markers in roots were: IDPase (Golgi apparatus), NADPH-dependent cytochrome c reductase (endoplasmic reticulum) and NADH-dependent cytochrome c reductase (tonoplast). ATPases, both non K⁺- and K⁺-stimulatable were included to prove or disprove Brecke's hypothesis as well as β-glucan synthetase, thought to be located in the Golgi apparatus and/or plasma membrane (Villemez et al., 1968; Ray et al., 1969; Van der Woude et al., 1972, 1974) since ¹⁴C-glucose incorporation in Agropyron single node buds was sensitive to glyphosate.

3.6.1. Results: Ethane has been established as a marker of membrane lipid oxidation in both animals and plants (Rieley et al., 1974; Konze and Elstner, 1978 respectively). In the experiments in which the evolution of ethylene by wheat seedlings in response to glyphosate was determined by GLC, the production of ethane was also recorded. Results are presented in Fig.26. Negligible ethane was given off by control material over the 6-day period but seedlings incubated with glyphosate showed an approximately linear increase in ethane evolution with a doubling in rate after 1-day at 5 x 10⁻⁴M glyphosate and a 10-fold increase at 10⁻³M increasing to 7-fold after 6-days at 5 x 10⁻⁴M and 13-fold at 10⁻³M. Glyphosate thus considerably promoted the production of ethane by wheat seedlings indicating the probability of membrane breakdown at some point in the seedling.

The effect of glyphosate on the activity of membrane bound enzymes in root tips was determined after incubation
Figure 26: EVOLUTION OF ETHANE BY WHEAT SEEDLINGS.

(●) control, (○) 5 x 10⁻⁴M glyphosate, (▲) 10⁻³M glyphosate.

For experimental details, see Fig. 21.
in glyphosate for 1-day in 2 concentrations, 2.5 x 10^{-4}M and 5 x 10^{-4}M. These were chosen because 5 x 10^{-4}M glyphosate gave substantial inhibition of formazan reduction and 2.5 x 10^{-4}M an intermediate value. The ATPase activity of mitochondria was determined by assaying non K^{+}- and K^{+}-activated ATPase at pH 9.0. The protein content of the mitochondrial pellet was not significantly affected by glyphosate at either concentration (Fig.27a) but both non K^{+}- and K^{+}-activated ATPase activity were marginally depressed at 2.5 x 10^{-4}M and slightly more so at 5 x 10^{-4}M. Inhibition was less than the more drastic effects on formazan staining of whole root tips and thus in spite of the decreased rate of respiratory metabolism, isolated mitochondria retained most of their ATPase activity. Negligible activity at pH 6.0 was found in controls, in agreement with Hodges and Leonard (1973).

The protein content of the microsomal fraction was much more sensitive to glyphosate, being reduced by 31% and 74% at 2.5 x 10^{-4}M and 5 x 10^{-4}M glyphosate respectively (Fig.27b). This pattern was reflected in the activity of microsomal ATPase pH 6.0, both non K^{+}- and K^{+}-activated. In controls, less ATPase pH 9.0 activity was found, but the same pattern of inhibition was found for non K^{+}-activated enzyme. Discrepancies amongst replicates produced values for K^{+}-activation somewhat different from this pattern both in comparison with each other end as a proportion of the total activity. All other enzymes showed a similar inhibition to that of microsomal protein.

In order to ascertain whether glyphosate had a direct effect on ATPases as suggested by Brecke (1976), a microsomal fraction was isolated from wheat roots and glyphosate
Figure 27a: Mitochondrial ATPase activity and protein from wheat root tips

i) ATPase pH 9.0 including monovalent ion-activated component, assayed from 13000 x g fraction of root tips excised after 24hrs incubation.

ii) Protein content of 13000 x g fraction.
Figure 27b: Microsomal enzymes and protein from wheat root tips
Assayed from 80000 x g fraction of root tips excised after 24hrs incubation.
added in vitro to the reaction media. At $10^{-3}$M, glyphosate did not inhibit either non $K^+$- or $K^+$-activated ATPase at pH 6.0 or 9.0 (Table 15).

3.6.2. Discussion: The promotion by glyphosate of ethane evolution in wheat seedlings suggested the desirability of a more detailed study of membrane properties. The principle effect of glyphosate was a substantial decline in the level of microsomal protein which correlated with those of soluble protein (Fig.19c) and formazan reduction (Fig.20). All microsomal enzymes declined to a similar degree, i.e. specific activities were unaffected. There was no additional inhibition which could have been ascribed to a direct interaction of glyphosate with these enzymes or with the membranes in which they were situated.

Although ATPases were assayed in a complete microsomal fraction it has been shown that a high proportion of microsomal ATPases are associated with plasma membrane. Density gradient centrifugation of oat root microsomes revealed that ATPases found at the density of plasma membrane particles had an alkaline pH optimum whereas ATPases with higher pH optima were associated with other organelles (Leonard et al., 1973; Hodges and Leonard, 1973). In this work, wheat root microsomes contained predominantly ATPase activity at pH 6.0 which may have substantially been accounted for by plasma membrane. The addition of glyphosate in vitro did not inhibit this activity nor that at pH 9.0 either with or without $K^+$. It has been established by Fisher et al (1970) that a correlation exists in cereal roots between monovalent ion influx and microsomal monovalent ion-stimulated ATPase activity (assayed at pH 7.2),
TABLE 15

MICROSOMAL ATPases FROM WHEAT ROOTS; ADDITION OF $10^{-3}$M GLYPHOSATE IN VITRO.

<table>
<thead>
<tr>
<th>ATPase</th>
<th>μmoles Pi/mg protein/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>pH 6.0 -K⁺</td>
<td>8.45 ± 0.99</td>
</tr>
<tr>
<td>+K⁺</td>
<td>2.58 ± 0.65</td>
</tr>
<tr>
<td>pH 9.0 -K⁺</td>
<td>1.23 ± 0.32</td>
</tr>
<tr>
<td>+K⁺</td>
<td>3.19 ± 0.27</td>
</tr>
</tbody>
</table>

An 80,000 x g preparation was obtained from whole roots of seedlings germinated for 3 days at 27°C. Reaction mixtures contained 34.1 μg protein and enzymes were assayed at pH 6.0 for 30 mins and at pH 9.0 for 60 mins. Glyphosate did not cause pH change of the reaction mixture in either case.
which suggests an obligatory role for ATP hydrolysis in ion transport, particularly since zero monovalent ion stimulated ATPase activity correlated with zero ion uptake. It would therefore seem unlikely that the inhibition of ion transport observed by Brecke (1976) was accounted for by a direct interaction of glyphosate with ATPases.

The plasma membrane has been shown to be a target of the herbicide dinitramine, thought to act primarily by inhibition of cell division. Travis and Woods (1977) demonstrated a rapid inhibition of $K^+$-activated ATPase and $\beta$-glucan synthetase of plasma membrane particles from soybean roots, being manifested after 15-minutes. This was apparently due to disruption of membrane integrity since membrane permeability was increased. A report also exists of inhibition of monovalent ion stimulated ATPase of rice roots by the commercial formulations of thiolbencarb and propanil (Toth and Zsoldos, 1976). These effects could have been due to the presence of surfactants.

The lack of effect of glyphosate on the specific activity of microsomal enzymes would seem to indicate that mode of action does not involve an attack on membrane integrity. Since the measurement of ethane evolution involved the use of whole seedlings, production may have been from some part of the seedling other than the root tip.
Concluding Discussion
CONCLUDING DISCUSSION

Glyphosate has been observed to have many effects on biochemical and physiological processes, but in relatively few cases can a connection with a primary mode of action be surmised.

Chlorophyll formation and photosynthesis were particularly sensitive to glyphosate although these observations were obviously unconnected with mode of action within meristems. The biochemical interaction underlying the inhibition of chlorophyll formation was not identified and whilst this phenomenon was almost certainly not due to an inhibition of carotenoid synthesis with a resultant photooxidation of chlorophyll, various studies did not suggest chloroplastic protein or RNA synthesis as a site of action. This was despite the reduction in levels of chloroplast rRNA observed. The enhancement of nitrate reductase activity by glyphosate was essentially a transient phenomenon likely to have limited biochemical consequences. Induction of nitrate reductase would be a useful tool with which to investigate the effect on protein synthesis in meristems since this enzyme has a very short half-life with a high rate of turnover.

The failure of metal ions to inactivate glyphosate demonstrated the unlikelihood of chelation being an important factor in mode of action, a contention supported by the inactivity of glyphosate towards metal-requiring enzymes. Glyphosate did not appear to act an an analogue of glycine but this should not deter work with other endogenous compounds.
The syntheses of macromolecules are obviously processes which will be affected by any herbicide at some point in the sequence of events leading to cell death. Also, any process dependent upon the production of ATP will be inhibited by a reduction in its availability and many herbicides which affect RNA and protein synthesis (Moreland et al., 1970) do indeed reduce levels of ATP (Gruenhagen and Moreland, 1971; Ashton and Crafts, 1973). The object of examining macromolecule synthesis in single node buds of *Agropyron* was to discern any differences in sensitivity between DNA, RNA, protein and cellulose synthesis. DNA synthesis was considered of special importance with regard to a herbicide acting by prevention of meristematic growth, but appeared to be less sensitive than other processes. Rates of protein synthesis were determined using 3 labelled precursors, i.e. $^{14}$C-phe, $^{14}$C-leu and $^{14}$C-protein hydrolysate. The incorporation of $^{14}$C-protein hydrolysate was inhibited to similar extents as those of RNA and cellulose synthesis but the incorporation of $^{14}$C-leu was more sensitive (Table 11), a difference which could be ascribed to the inclusion of $^{14}$C-phe in the hydrolysate since the incorporation of $^{14}$C-phe was only inhibited to a limited extent. The incorporation of $^{14}$C-leu was thus the most sensitive biosynthetic function observed in single node buds.

The enhancement of extractable PAL activity, initially demonstrated by Duke and Hoagland (1978) was shown in both single node buds and wheat root tips to be a very sensitive process. Several other phenomena which could have been related to this were observed. The differential effects of glyphosate on the incorporation of $^{14}$C-leu and $^{14}$C-phe indicated that the protein precursor pool of phe was probably
markedly reduced and that this was the major reason for the inhibition of protein synthesis. The levels of soluble and microsomal protein in root tips declined rapidly at a rate similar to the inhibition of respiratory activity as measured by the formazan reduction assay. Both phenomena showed an inverse correlation with the enhancement of PAL activity, but this did not prove a causal relationship, PAL enhancement may merely have been a symptom of toxicity, a possibility supported by the fact that PAL inhibitors did not alleviate toxicity. Since the addition of L-phe or mixed aromatic amino acids did not alleviate toxicity, a mode of action purely through a deficit of free phe is questioned.

No evidence presented suggested that the enhancement of PAL activity was responsible for the probable decline in the level of free phe. Experiments by Tymonko (1978) with shikimic acid pathway intermediates strongly suggested an in vivo inhibition of chorismate mutase. The consequent reduction in the free phe pool which would be resultant could have caused an elevation in PAL activity by the decrease in PAL products thereby relieving end product inhibition and repression, particularly since elevation did not appear to be due to the production of ethylene. The differences between workers however seem to be irreconcilable at the moment. There is no evidence to suggest that glyphosate does not inhibit chorismate mutase. Jaworski's work (1972) showing that chorismic acid partially repressed growth inhibition would indicate an inhibition point prior to chorismate mutase. Both Roisch and Lingens (1974) and Gresshoff (1979) have reported that chorismate
mutase from \textit{E. Coli}, an organism susceptible to glyphosate was not inhibited \textit{in vitro} and Gresshoff further reported that glyphosate did not alter the extractable chorismate mutase activity in \textit{E. Coli}. In this work, extractable chorismate mutase actually increased by a considerable amount. An \textit{in vivo} inhibition of this enzyme might be expected to produce a diversion of chorismic acid carbon to tryp via anthranilate. It is possible that diversion of carbon is achieved through activation of anthranilate synthetase without a direct effect on chorismate mutase. The effect of glyphosate on levels of tryp has not been recorded. The report by Duke and Hoagland (1979) that the PAL inhibitor L-AOPP abolished the free phe deficit brought about by glyphosate would initially suggest that \textit{in vivo} PAL activity was responsible for free phe depletion. However, as an analogue of phe, L-AOPP may also have caused allosteric inhibition of chorismate mutase.

The response of glyphosate treated tissues to exogenous aromatic amino acids presents the most perplexing aspect of mode of action. Response falls into 3 categories, either reversal of inhibition (Jaworski, 1972; Roisch and Lingens, 1974; Haderlie, 1975; Gresshoff, 1979), increase in inhibition (Duke and Hoagland, 1978) or no effect (Brecke, 1976 and this study). It is obviously difficult to reconcile these differences on the basis of a common mode of action. Further work should include more time-course experiments in which the various phenomena shown to be involved can be related more fully. The usefulness of this has been demonstrated by Tymonko (1978) who showed that the decrease in free phe was preceded by the onset of protein decline and
accumulation of total free amino acids. However, the amount of any amino acid available for protein synthesis may constitute only a part of the total free pool (Bidwell et al., 1964; Oaks, 1965; Holleman and Key, 1966; Berlin and Widholm, 1978) and could be modified independently of other pools of the amino acid. Exogenous amino acids may increase total free pools without influencing protein precursor pools, which may have explained the lack of toxicity reversal with such agents reported here, although Holleman and Key (1966) found in soybean that supplemental feeding enhanced the protein precursor pools much more so than the total soluble pools.

The enhancement of PAL induction by glyphosate raises the possibility that related enzymes may behave similarly, since enzymes following PAL in the pathway of phenolic compound synthesis are also inducable. Co-ordinate light induction of cinnamate-4-hydroxylase with PAL (Amrhein and Zenk, 1968, 1970) and p-coumarate-ligase with PAL (Hahlbrock et al., 1971a) have been shown and a study with parsley cell culture has shown that 2 sets of consecutive enzymes in the flavonoid synthesis pathway initiated by PAL are induced by light at different intervals (Hahlbrock et al., 1971b; Grisebach and Hahlbrock, 1974). It has been suggested that these 2 sets are under the control of 2 corresponding operons and if a similar phenomenon was brought about by glyphosate, this could indicate action at the genetic level.

Jangaard (1974a) has discussed PAL as a target for rationally designed herbicides since as a sensitive metabolic control point, inhibitory compounds could have high phytotoxicity with negligible action towards animals. Some
inhibition was observed with several herbicides but no example of stimulation was seen (Jangaard, 1974b). Davies (1972) has reported that the formation of PAL was delayed by 2,4-D. Enhancement of PAL activity was reported by Pal'chenko and Volnyets (1977) for MCPA, TCA and dalapon and Hoagland and Duke (1979) reported that in soybean seedlings both amitrole and paraquat enhanced activity, in the latter case by as much as glyphosate. This would suggest that PAL enhancement by glyphosate might be due to a more general stress reaction, but the failure of glyphosate to elicit a stimulation in the production of ethylene indicates that a typical wound reaction did not occur. Inhibition of enzymes of the shikimic acid pathway has also been proposed as a mechanism of action of future herbicides for similar reasons as PAL inhibition, but analogues of dehydroshikimic acid which were inhibitory to SORase were found not to possess herbicidal properties (Baille et al., 1972).

The increases in the specific activities of SORase, chorismate mutase, PPO and hydrolytic enzymes are all considered to be secondary effects and the main question here is how these are brought about. If the increases represent de novo protein synthesis they would have to concur with that of PAL, before protein synthesis was inhibited. Alternatives would be activation of previously latent enzyme or a rate of turnover reduced to below average. In the discussions of these results, comparisons were made with characteristics of wound and senescence metabolism and although some of the effects here, e.g. the enhancement of SORase are characteristic of wounding, the analogy between herbicide treatment and wounding is perhaps superficial.
Wound metabolism represents the adaption of the cell to a new environment and in the case of storage tissues is characterised by a rapid synthesis of protein and increase in respiration with increases in glycolytic enzyme activities and ultimately new cell division (Kahl, 1973; Van Steveninck, 1975). This contrasts with the mainly catabolic changes of senescence.

The effects of glyphosate on membranes are equivocal. The intimation of oxidative membrane damage as shown by the activation of ethane generation was not borne out by studies on the activity of membrane bound enzymes. The retention of control specific activity values indicated that the various membranes of the cell maintained an integrity essential to the operation of enzymes so located. Membrane integrity requires a constant turnover of components, dependent upon ATP supply (Rivera and Penner, 1979) and the vast reduction in respiratory competence observed at $5 \times 10^{-6}$M glyphosate must have drastically curtailed this. The main effect was that of a decline in the level of microsomal protein, again supporting a mode of action through protein depletion. The fact that the specific activity of NADH-cytochrome c reductase (thought to be a tonoplast marker) did not change meant that the integrity of the tonoplast was presumably maintained and thus breakdown of compartmentation between polyphenol oxidase activity and a possible build-up of its vacuolar substrates with increased oxidation was unlikely.

In conclusion, it is difficult at present to suggest at which site the herbicide glyphosate exerts a primary effect. The involvement of glyphosate with the metabolism of aromatic amino acids is unequivocal, but the point of action
is unclear. The consequences are a probable depletion in the protein precursor pool of phe with an inhibition of protein synthesis and a consequent rapid decline in protein levels. The level of secondary product would be likely to decrease rather than increase, a phenomenon which could explain the enhancement of PAL activity. The failure in this study of exogenous aromatic amino acids to reverse the toxicity of glyphosate suggests that additional factors may be involved in its mode of action and although other processes have been studied, these factor(s) have not yet been identified.
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