LYSINE METABOLISM IN BARLEY LEAVES AND IN BARLEY POWDERY MILDEW

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ABBREVIATIONS USED IN THE TEXT

AAA	α-aminoadipic acid
ADC	Arginine decarboxylase
AdoDato	*S*-Adenosyl-1,8-diamino-3-thiooctane
APC	Aminopropylcadaverine
3APC	N,N-*bis*(3-aminopropyl)cadaverine
ARG	Arginine
ASA	Aspartic-β-semialdehyde
ATP	Adenosine triphosphate
BSA	Bovine serum albumin Fraction V
CAD	Cadaverine
CHA	Cyclohexylamine
DAI	Days after inoculation
DAP	Diminopimelic acid
DFMA	α-Difluoromethylarginine
DFMO	α-Difluoromethylornithine
DHDPS	Dihydrodipicolinic acid synthase
DMTA	5-Deoxy-5'-methylthioadenosine
DNA	Deoxyribose nucleic acid
DPM	Disintegrations per minute
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid, disodium salt
FDNB	1-Fluoro-2,4-dinitrobenzene
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
LDC	Lysine decarboxylase
M+C+W	Methanol+chloroform+water

MES-BTP	2-[N-Morpholino]ethanesulphonic acid/1,3-bis [tris(Hydroxymethyl)-methylamino]propane
MGBG	Methylglyoxyl-bis-guanylhydrazone
MTA	5'-methylthioadenosine
NADP	β-Nicotinamide adenine dinucleotide phosphate
NADPH	β-Nicotinamide adenine dinucleotide phosphate (reduced form)
NADH	β-Nicotinamide adenine dinucleotide (reduced form)
ODC	Ornithine decarboxylase
ORN	Ornithine
PAO	Polyamine oxidase
PAR	Photosynthetically active radiation
PEG	Polyethylene glycol
PLP	Pyridoxal-5'-phosphate
PUT	Putrescine
PVP	Polyvinylpyrrolidone
RNA	Ribose nucleic acid
RuBpCase	Ribulose bisphosphate decarboxylase
SAMdc	*s*-Adenosylmethionine decarboxylase
SPD	Spermidine
SPM	Spermine
TCA	Trichloroacetic acid
TLC	Thin layer chromatography
Tris-HCL	Tris(hydroxymethyl)aminoethane hydrochloride
V/V	Volume/volume

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ABSTRACT

LYSINE LEVELS AND ENZYME ACTIVITY

The activity of diaminopimelic acid decarboxylase, an enzyme of the diaminopimelic acid pathway of lysine biosynthesis, and usually associated with plants, bacteria and certain lower fungi, increased early in powdery mildew infection of barley leaves. The level of soluble lysine, the product of this enzyme, also increased early in the infection. Both soluble and bound lysine were reduced by 6 days after inoculation (DAI). At the same time, the activity of diaminopimelic acid decarboxylase was not significantly different in whole infected leaves when compared to healthy leaves, but was slightly reduced in isolated chloroplasts. Later in the infection (9 DAI), the activity of diaminopimelic acid decarboxylase was significantly greater in inter-pustule regions than in pustules.

Activity of dihydrodipicolinic acid synthase was reduced in infected leaves and in chloroplasts throughout the course of infection. This reduced activity was not due to increased levels of lysine, which has been shown to control the activity of this enzyme. It is suggested that changes in chloroplast integrity may have caused the observed reduction in activity of these two chloroplast localised enzymes. During sporulation the levels of both soluble and bound lysine were reduced in infected leaves.

Activity of lysine decarboxylase in whole infected barley leaves was reduced (3-9 DAI) compared to controls, and reductions were found to occur in both pustule and inter-pustule regions of the leaf. At the same time, activity was higher in chloroplasts isolated from whole infected leaves than in those isolated from healthy leaves, and a similar situation was found in chloroplasts isolated from pustules and inter-pustule regions 6 DAI. By 9 DAI, however, the activity of the enzyme in chloroplasts isolated from pustules was lower than in inter-pustule regions and healthy leaves. It is suggested that these changes in LDC activity are due to a reduction in activity of cytosolic LDC, while activity of the chloroplastic enzyme is stimulated in the inter-pustule regions of infected leaves.

The activities of two enzymes of the aminoadipic acid pathway of lysine biosynthesis were examined. This pathway is normally associated with higher fungi. Activity of saccharopine dehydrogenase was not significantly different in infected leaves until 9 DAI, when activity increased. In contrast to dihydrodipicolinic acid synthase and diaminopimelic acid decarboxylase, this increase was located in the pustules. It is likely that this increase was due to the presence of the mildew. Enzyme activity in isolated mildew mycelium was greater than in infected leaves. No aminoadipic acid reductase activity was found in healthy leaves. Activity of this enzyme and of

saccharopine dehydrogenase was similar in infected leaves, but the activity of aminoadipic acid reductase was higher in the isolated mycelium. It is suggested therefore that aminoadipic acid reductase is only found in the fungal mycelium.

^{14}C-ASPARTATE UPTAKE

Infection of barley with powdery mildew altered the uptake and metabolism of ^{14}C-aspartate. Uptake was greater in infected leaves 2 h after feeding, but was reduced thereafter. Aspartate was more rapidly metabolised in infected leaves than in healthy leaves, probably to homoserine and threonine. The fungus took up aspartate, homoserine and threonine from the plant, but these were apparently not further metabolised over the experimental period. This suggests that barley can metabolise aspartate, probably forming homoserine, threonine and lysine, while such metabolism does not occur in the powdery mildew fungus. This would confirm the presence of the diaminopimelic acid pathway for lysine biosynthesis in barley and its absence in *Erysiphe graminis*.

UPTAKE

Putrescine and ornithine both reduced the uptake of lysine by isolated powdery mildew mycelium, although these changes were not statistically significant. Also, a

slight, though again not statistically significant, increase in uptake in the presence of arginine was observed. The metabolic inhibitor, sodium azide, had no effect on lysine uptake.

Uptake of both aspartate and lysine increased during the first 30min of feeding but remained constant thereafter, until 120-240min, when the rate of uptake of lysine decreased. Lysine uptake was much greater than uptake of aspartate, and uptake of lysine was biphasic. Uptake of aspartate and lysine over the pH range 4-9 was not significantly different.

CADAVERINE AND DERIVATIVES

In leaves, inhibition of spermidine synthase/*S*-adenosylmethionine decarboxylase by pre-treatment with inhibitors caused a reduction in the formation of aminopropyl derivatives of cadaverine, suggesting the formation of these compounds by the normal route of polyamine biosynthesis, *via* aminopropyl transfer from *S*-adenosylmethionine, and not by Schiff base complex formation. This effect was only seen when the leaves were pre-treated with the inhibitors before addition of labelled lysine.

Inhibition of production of the cadaverine derivatives by the ornithine decarboxylase/arginine decarboxylase inhibitors difluoromethylornithine/difluoromethylarginine

suggests that cadaverine is formed by the activity of ornithine decarboxylase as well as by lysine decarboxylase activity. Labelling in infected leaves was always very much lower than in healthy leaves but the percentage inhibition was the same in both.

Unlike leaves, treatment of mycelium with difluoromethylornithine/difluoromethylarginine had no significant effect on labelling of cadaverine or its aminopropyl derivatives, even when LDC activity was significantly reduced. Also, methylglyoxyal bis(guanylhydrazone)/cyclohexyalmine had no significant effect on lysine decarboxylase activity. It is thus suggested that the Schiff base pathway for formation of cadaverine homologues may operate in the mycelium of powdery mildew.

1. INTRODUCTION

In the developed world the problem of food production would appear to be one of over-production. However, in view of the modern trend of growing crops in monocultural systems, uncontrolled outbreaks of pests and diseases could quickly change this over-production to a local or even country-wide shortage of the affected crop. It is, therefore, necessary to do everything possible to decrease the risk of infestation or infection.

1.1 METHODS OF PATHOGEN CONTROL

Russell (1978) lists three methods of controlling pests and diseases; avoidance, direct control and biological control. The first, avoidance, uses quarantine, good husbandry, crop rotation, clean seed, hygiene, nutrients, soil sterilisation, control of vectors and preventive chemicals (for example as seed dressings).

Direct control measures use chemicals which give control on contact (non-systemic) or which may be taken up by the crop (systemic). The former, which were the only ones available during the first half of this century, are mostly simple compounds containing sulphur, copper and mercury, as fungicides, and nicotine, derris and pyrethrum (all plant extracts) as insecticides.

Plant systemic fungicides are often fairly specific, for example, affecting nucleotide base synthesis,

polynucleotide or protein synthesis, steroid synthesis or components of lipoprotein membranes (Hassall, 1982). However cases of resistance to these fungicides have arisen. Whereas resistance to protective (non-systemic) fungicides is often short-lived, resistance to systemic compounds, where a single gene mutation is often involved, can be very long lasting (Hassall, 1982).

Many chemicals used as fungicides are very toxic to humans and may kill beneficial or non-pathogenic organisms, as well as controlling the target organism, and can prove phytotoxic under certain conditions. Their stability can cause environmental pollution and may lead to environmental problems in the future. A further disadvantage is that of induced resistance to or tolerance of a chemical previously toxic to the target organism, so that the chemical becomes less effective. The increased costs of development and registration of new synthetic chemicals may now make this method of control less attractive.

The third method of control cited by Russell (1978), biological control, shows promise for a more environmentally acceptable approach to disease control. Setting one organism, which is not ecologically damaging, to control another seems to be the perfect answer to a difficult problem and if it were so easy, chemical pesticides would soon become redundant. However, it seems that even where such systems exist (predatory mites can

control red spider mite in the glasshouse, parasitic wasps control white fly also in the glasshouse, ladybirds [*Coccinelid* sp.] versus aphids [*Aphis* sp.]), environmental factors control the effectiveness of these methods. More recently, chemical companies have taken up the challenge to produce 'ecologically friendly' pesticides.

Bacillus thuringiensis Berl. for example, first used against caterpillars in the thirties, was effective against Lepidoptera, Coleoptera and Diptera. Delta-endotoxins from this organism have now been inserted in killed *Pseudomonas* cells as an insecticide to give a flexible delivery system combined with greater persistence (Cannon, 1993). The addition of a *Pseudomonas* strain along with a non-pathogenic strain of *Fusarium* to pot plants and soil-less culture systems is said to give total control of *Fusarium* wilt (Alabouvette, Lemanceau & Steinberg, 1993). In Holland, growers of sweet pepper and tomato now use a system of integrated pest management which uses mainly biological control agents (van Schelt, 1993).

Other, commercially available, products include a biofungicide based on *Streptomyces griseoviridis* for the control of 'damping off' in cotton seedlings, bioherbicides based on *Colletotrichum gloeosporoides* Penz. used in rice, soya and citrus fruits, a *Fusarium lateritium* Desm. based product used in soya, and *Alternaria cassiae* for use in peanuts and soya (Powell & Jutsum,1993).

A further theoretical method of control has been suggested. Selective inhibition of fungi by, for example, changing host metabolism or the relationship of the plant with the fungus may be possible (Baldwin, 1984). Alternatively it may be possible to inactivate toxins or enzymes produced by the fungus during fungal attack (Baldwin, 1984). This approach relies on the specificity of the host/pathogen interaction (Baldwin, 1984).

1.2 FUNGAL DISEASES

Plant pathogenic fungi can be split into two broad groups according to their modes of nutrition and parasitism. The first contains fungi which can be grown axenically. This group of fungi, called necrotrophs, cause premature senescence or death of the host tissue, although this division is not absolute. They often produce large quantities of enzymes and phytotoxins. Directed translocation in the host does not play a large part in fungal nutrition. There is little stimulation of protein and nucleic acid biosynthesis in the host, and the host often produces phytoalexins in response to attack by this type of fungus.

The second class is difficult or impossible to grow axenically (powdery mildews and rusts). In these biotrophic fungi (obligate parasites), there is little death of host tissues (apart from, for example, cell death during the hypersensitive response), and a local

stimulation of host metabolism is seen along with stimulation of protein and nucleic acid synthesis.

1.2.1 Powdery Mildew

The barley powdery mildew fungus *Erysiphe graminis* f. sp. *hordei* Marchal is one of the most economically damaging diseases of cereals in Britain and can result in up to 50% losses in grain yield in susceptible late-sown varieties. The observed reduction in grain yield is due to the reduction in fertile tiller numbers, decrease in root system, decreases in the number of grains/tiller and in the size and weight of grains (Scott and Griffiths, 1980). It affects leaves (chlorosis) and roots (inhibition of mitosis in root tips).

The effects of disease are influenced by many independent factors, including genotypes of the host and pathogen as well as environmental factors. The presence of other diseases also influences the extent of damage and yield loss sustained.

Although chemical control of the disease is effective, fungicide residues have been found in grain as well as in other parts of the plant after harvest. Varieties carrying resistance genes are now the main means of control, but variation in the fungus can lead to breakdown of resistance. Since the sexual stage of the fungus (cleistothecium) does not play a large part in the spread

of the disease in temperate countries, the existence of variation within the fungus is thought to be due to somatic mutations and asexual recombinations (Russell, 1978). Breeders are constantly striving to overcome this problem by developing new, resistant varieties.

The powdery mildew fungus is a biotroph present on the leaf surface as a loose net of mycelium, which produces long strings of conidia. The dry spores are easily detached and dispersed by the wind. Low relative humidity and high wind speeds facilitate dispersal (Alexopoulos, 1962). Although both powdery mildew conidia and rust spores have relatively high fat content (which disappears during germination and is thought to be used as an energy source), only powdery mildew conidia germinate at very low relative humidity. Liquid water inhibits germination and may even cause death of the conidia. Infection usually occurs in relatively dry conditions. This is thought to be due to the relatively high water content of the spores (Cochrane, 1958; Somers and Horsfall, 1966). The white powdery coating on infected leaves, seen with the naked eye, is due to the large numbers of conidia produced by the conidiophores (Alexopoulos, 1962). A generative cell within each conidiophore produces the unicellular conidia. These are carried downwind to produce further infection. Asexual reproduction is diurnal (Alexopoulos, 1962) and the conidia show negative phototropism (Cochrane, 1958). This is also seen in sporidia and uredospores of *Puccinia*, and

the active wavelength is in the blue region of the visible spectrum (Cochrane, 1958). This type of fungus obtains metabolites from the host over long periods, since they do not disrupt host cells, even during sporulation (Gay & Manners, 1981).

Haustoria are formed in epidermal cells by invagination of the host plasmalemma (Fig. 1). This invagination is called the extra-haustorial membrane and between it and the haustorium is an amorphous matrix which is rich in polysaccharides. Although the haustorium is in fact outside of the cell plasmalemma, solutes and metabolites are unable to pass from the apoplast to the haustorium due to the close attachment of the two membranes at an impermeable ring (called a neck-band). Transfer of metabolites is, therefore, through the host cytoplasm and into the matrix *via* the invaginated portion of the plasmalemma, which is semi-permeable. Although this invaginated portion of the plasmalemma is part of the host cell, it differs from the remainder of the plasmalemma because it is more than twice as thick and highly convoluted (Gay & Manners, 1981).

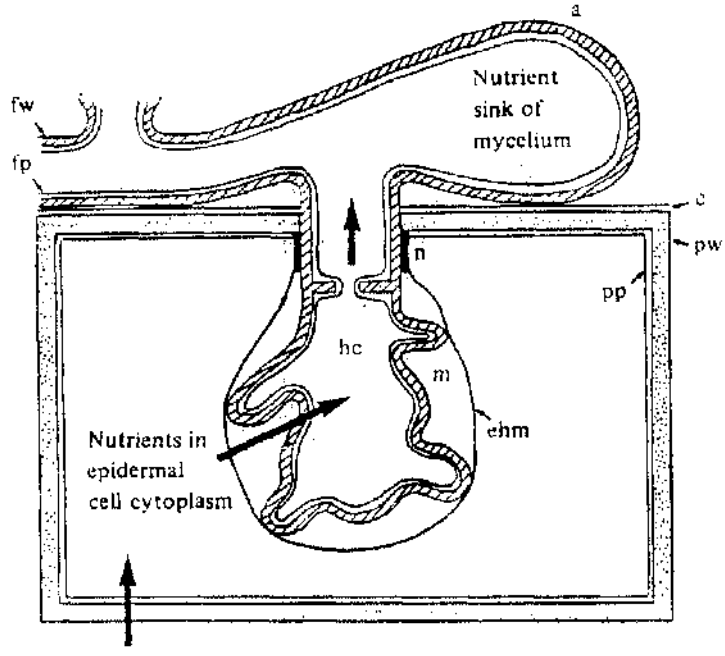
1.2.2 Invasion of Host Plants by Powdery Mildew.

Powdery mildews invade host tissues directly through the epidermis. Conidia are stimulated by plant exudates (Hancock and Huisman, 1981) on contact with a leaf, to

Fig 1: Mature fungal haustorium of *Erysiphe pisi*. Solid arrows indicate the postulated pathway of nutrient movement.

c = cuticle
pw = plant cell wall
pp = plasmalemma
ehm = extra haustorial membrane
m = matrix
hc = haustorial cytoplasm
n = neckband
a = appressorium
fw = fungal cell wall
fp = fungal plasmalemma

(Gay and Manners, 1981)


a
Nutrient
sink of
mycelium
fw
fp
c
pw
n
pp
hc
m
ehm
Nutrients in
epidermal
cell cytoplasm
Primary nutrient source
mesophyll and vascular tissue

produce proteins thought to be cutinases (Kunoh, Komura, Yamaoka and Kobayashi, 1988). In other species examined, these cutinases appear to be tissue specific (Trail and Koller, 1990 & 1993). Pascholati, Yoshioka, Kunoh & Nicholson (1992) showed that an exudate from powdery mildew conidia, released on contact with the surface of a barley leaf had cutinase activity. This was shown to be a serine esterase. This is the first time cutinase activity has been shown to occur in a powdery mildew fungus, but enzymes of this class are known to occur in other fungi (Pascholati *et al.*, 1992). Release of the enzyme into the liquid takes place in two stages. The second stage (10-15 min after contact) depends on temperature and protein synthesis (Kunoh, Nicholson, Yosioka, Yamaoka and Kobayashi, 1990). It is suggested that the enzyme is involved in erosion of the leaf surface which begins before the development of the appressorium and that this erosion may be necessary for recognition of the host surface or for adhesion (Pascholati *et al.*, 1992; Mendgen and Deising, 1993), and more efficient penetration of the leaf (Pascholati *et al.*, 1992). The exudate, which covers the entire conidial surface within ten minutes of contact, flows onto the leaf surface and is deposited around the ungerminated conidium. Release of the exudate is complete by thirty minutes after contact. However, appressorium formation does not begin until two hours after contact. Release of the exudate coincides with an apparent loss of structural integrity of the surface of the cuticle in contact with it (Pascholati

et al., 1992). Scott (1972) suggested that the germ tube elongates to form an appressorium which arises from a swelling of the tip of the germ tube. However, Carver and Ingerson (1987) appear to have shown that at least one primary germ tube is initiated before the appressorial germ tube forms and there is no further growth of the germ tube after it touches the leaf surface (Edwards, 1993). The appressorium is separated from the conidium by a septum (Kunoh, Ishizaki and Nakaya, 1977).

Two mechanisms combine to allow entry of the mildew into the epidermis. Firstly, enzymes formed following attachment of the appressorium to the outside of the epidermis, are released onto the surface of the leaves (Edwards and Allen, 1970). These cause enzymic digestion of the cuticle and cellulose of the epidermis. However, the infection hypha is capable of pushing through the epidermis without prior digestion by these digestive enzymes (Hardham, 1992). After attachment, a specialised layer containing melanin forms over the appressorium (McKeen and Rimmer, 1973). This layer is semi-permeable, and increased solute concentration, thought to be due to the breakdown of stored products, causes high internal hydrostatic pressure in the appressorium (Hardham, 1992). The penetration peg which forms then pushes through a layer of deposited secondary plant cell wall material (papilla: extracellular deposits of callose, proteins and phenolic compounds) on the under-side of the epidermal wall (Edwards

and Allen, 1970; Bushnell, 1972: Scott, 1972; McKeen and Rimmer, 1973).

The haustoria, involved in nutrient uptake from the host, form in an invagination of the host plasmalemma within the epidermal cells (Fig. 1) (Bushnell, 1972). They develop finger-like projections which effectively increase the surface area of these structures (Bushnell, 1972). Powdery mildews only invade epidermal cells, causing little disruption to the cell wall even during sporulation (Scott, 1972). There is no direct contact between the fungus and mesophyll cells and removal of photoassimilates occurs by production of nutrient gradients towards the fungus (Scott, 1972; Farrar, 1995).

1.2.3 Changes in Respiration During Infection.

As a general phenomenon, respiration is increased in infected plants (Farrar and Lewis, 1987). The energy generated is used for growth and maintenance of the fungus, as well as maintenance of the host and for host defence mechanisms (Kosuge and Kimpel, 1981; Farrar and Lewis, 1987). In addition, Smedegaard-Peterson & Stolen (1981) have shown that in incompatible host/pathogen combinations, there is an increased consumption of energy by the host.

Much of the work examining changes in respiration during biotrophic infection has used rust of wheat or barley. For example, in whole rusted barley leaves at

flecking, the rate of dark respiration was four times that of controls, while at sporulation and the green-island stage it was twice the control rate (Scholes and Farrar, 1986). In compatible host/parasite combinations, oxygen uptake in individual rust colonies on wheat leaves was greatly increased (10 - 15 times control), while tissues containing non-sporulating hyphae also showed increased oxygen uptake (2 - 4 times control), similar to the increase seen in pustules formed in non-compatible associations (Bushnell, 1970). However, respiration was also increased in wheat (Zulu, Farrar and Whitbread, 1991) and barley (Farrar and Rayns, 1987; McAinish, Ayres and Hetherington, 1991) infected with powdery mildew.

In rust, Scholes and Farrar (1986) thought that the observed increase was mainly due to respiration of the rapidly growing fungus, since growth and respiration are closely related (Farrar and Lewis, 1987). Although there was also an increase in regions between pustules, they speculate that this was due to the loss of chlorophyll and degeneration of chloroplasts, previously shown to occur in bluebell leaves infected with the rust *Uromyces muscari* (Scholes and Farrar, 1985).

In bluebell, increased respiration was only seen in rust pustules, which Farrar and Lewis (1987) cite as proof that the increase was due to the fungus. However, in barley leaves infected with powdery mildew, Millerd and

Scott (1963) and Bushnell and Allen (1962) showed, by removing the surface mycelium from infected leaves, that the increase was mainly in the leaves. In addition, respiration in mesophyll protoplasts isolated from powdery mildew infected barley leaves was higher than in healthy leaves (McAinish, Ayres and Hetherington, 1991).

Farrar and Rayns (1987) found that around half of the increased respiration in powdery mildew-infected barley was due to an increase in electron flow through the cytochrome chain and half through the alternative oxidase pathway. Although the latter pathway was increasingly engaged, the capacity was the same. Respiration was not limited by substrate or inorganic phosphate (Farrar and Rayns, 1987). They suggest that the observed increase in activity of the cytochrome pathway was due to adenylate regulation, but were not able to show why the alternative pathway was increasingly engaged. Resistant cultivars do not show alternative pathway activity (Farrar and Rayns, 1987). During infection, the pentose phosphate pathway (PPP), which is involved in the production of polyols and phytoalexins, becomes relatively more important than the glycolytic pathway (Farrar and Lewis, 1987). There is only a slight increase in the activity of some of the enzymes of glycolysis and these authors postulate that regulation of respiration is due to adenylate re-cycling, which is seen in healthy leaves, although it has been suggested that fungal toxins are involved (Farrar and Lewis, 1987).

1.2.4 Effects of Fungal Infection on Photosynthesis.

In powdery mildew-infected barley (at 1% CO_2) there was a slight increase in photosynthesis 2 DAI (Scott and Smillie, 1966). This increase was also observed at physiological CO_2 concentrations (Aist, Domes and Kranz, 1977). Thereafter photosynthesis decreased when compared to healthy leaves (Scott and Smillie, 1966; Aist *et al.*, 1977). In a further classical and often quoted piece of work on the effect of barley mildew on susceptible barley leaves (at 1% CO_2), Edwards (1970) found an initial stimulation of photosynthesis, although at physiological carbon dioxide concentrations there was biphasic inhibition of photosynthesis. The first phase of this inhibition was thought, by extrapolation, to take place 12h after inoculation (the time of initial penetration by primary haustoria) until about 3 DAI (when there was no visible chlorosis and chlorophyll content of healthy and inoculated leaves was still the same) (Edwards, 1970). The rate remained steady until about 6 DAI when the second phase of inhibition was seen. At this time sporulation was taking place rapidly and carbon was moving from the host to the fungus at a very rapid rate (Edwards and Allen, 1966). Similarly, in pea seedlings infected with powdery mildew, photosynthesis was reduced by 24h after inoculation (Ayres, 1976). Carbon dioxide fixation was not affected at this stage (Ayres, 1976). The early decrease in photosynthesis

in peas was due to an increase in photorespiration, while in the later stages reductions in stomatal opening were shown to be partially responsible for reduced net photosynthesis (Ayres, 1976). In barley the light reactions and photoreduction of NADP decreased and, at the same time, chlorophyll and activity of NADPH-diaphorase and aldolase, decreased (Scott and Smillie, 1966). From 3 DAI a greater proportion of photosynthate was used in respiration in peas infected with mildew and the carbon dioxide compensation point increased throughout the period of the experiment (7 DAI) (Ayres, 1976). However, during mildew infection, non-infected leaves may compensate for the reduced carbon dioxide fixation in infected leaves (Walters and Ayres, 1980). In powdery mildew-infected leaves, RuBPCase was reduced but in non-infected leaves on the same plant, the amount of this protein was increased (Walters and Ayres, 1980). In addition, the amount of photosynthate going to roots decreased (Walters and Ayres, 1983), export of photoassimilates from infected leaves decreased (Durbin, 1967; Goodman, Kiraly and Wood, 1986) and import of inorganic compounds (Durbin, 1967) and photosynthates into infected leaves increased (Kosuge, 1978). The reduction in activity and quantity of RuBPCase during infection may be due to the documented reduction in chloroplast ribosomes and rRNA as a consequence of the observed changes in the ultrastructure of chloroplasts (Walters, 1985). Alternatively, since RuBPCase can be used as a nitrogen source and since it is known that the uptake

of nitrate by roots of mildewed barley is inhibited, while at the same time nitrogen is taken up from the leaf by the fungus, it may be that nitrogen is released from RuBPCase protein and subsequently taken up by the fungus (Walters, 1985). It is also suggested that changes in pH, Mg^{2+} and/or P_i affects the activity of this enzyme. However, reductions in RuBPCase may not be solely responsible for the observed reductions in photosynthesis, since the activities of other enzymes (3-phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase [NAD^+ and $NADP^+$ activated forms]) were reduced after inoculation with powdery mildew (Walters, 1985).

Conflicting results have also been obtained when examining chloroplasts from infected leaves. For example, the number of chloroplasts in rust pustules on bluebell leaves was no different than in non-infected leaves (Scholes and Farrar, 1985). At the same time, oxygen evolution per unit area and per unit of chlorophyll was reduced in pustules when compared with areas between pustules and with uninfected leaves. Chloroplast volume, chlorophyll concentration and the ratio of chlorophyll a:b decreased, suggesting that there was a loss of chlorophyll from individual chloroplasts (Scholes and Farrar, 1985). Two of the major parameters of chlorophyll fluorescence kinetics (F_{var} and F_q) were reduced in pustules, whilst F_o increased before sporulation and decreased during sporulation. This suggests impairment of both non-cyclic

electron transport and the general integrity of the chloroplasts (Scholes and Farrar, 1985). It is further suggested that the increase in F_o may be due to disorientation of the chlorophyll a molecules, caused by the pathogen, increasing fluorescence between chlorophyll molecules and that the subsequent fall may be due to the observed loss of chlorophyll from pustules (Scholes and Farrar, 1985). However, in rust-infected barley leaves, there were fewer chloroplasts (per unit area). These remaining chloroplasts functioned at least as well as those in healthy leaves and *in vivo* kinetic data point to them maintaining the juvenile state for longer (Ahmad, Farrar and Whitbread, 1983). Although the rate of ferricyanide oxygen evolution was the same as controls, there was more starch and Pi and there was increased flux through the phosphate translocator on the chloroplast inner membrane (Ahmad *et al.*, 1983). The reduction in net photosynthesis in these leaves was due to the reduction in the number of functional chloroplasts rather than to reduced carbon dioxide fixation or changed water relations (transpiration was not reduced until two days after photosynthesis decreased) (Ahmad *et al.*, 1983). The decrease was approximately parallel to the decrease in leaf chlorophyll content (Ahmad *et al.*, 1983). In chloroplasts isolated from powdery mildew-infected sugar beet electron transport and ATP formation in non-cyclic photophosphorylation were reduced (water as electron donor, NADP as electron

acceptor) (Magyarosy, Schurmann and Buchanan, 1976). There was no change in ATP formation during cyclic photophosphorylation. The inhibition of non-cyclic photophosphorylation resulted in decreased photosynthetic carbon dioxide uptake (Magyarosy *et al.*, 1976). This inhibition may not be in the best interests of biotrophs since colony development was shown to stop when photosynthesis was blocked by adding inhibitors and the inhibition could be reversed by exogenous sucrose (Goodman, Kiraly and Wood, 1986). In powdery mildew-infected beet leaves, ultrastructural changes in chloroplasts were accompanied by decreased activity of the enzymes involved in organic acid synthesis (PEP carboxylase and malate dehydrogenase) (Magyarosy *et al.*, 1976). At the same time, labelling of 3-PGA and sugars was decreased and there was an increase in the levels of alanine, aspartate and glutamate (Magyarosy *et al.*, 1976). Montalbini and Buchanan (1974) found similar results with chloroplasts from rust infected *Vicia faba*.

Although the concentration of electron carriers and all photochemical activities decreased more rapidly in barley leaves infected with powdery mildew when compared on a fresh weight basis (Holloway, MacLean and Scott, 1992), these did not differ when compared on a chlorophyll basis (Holloway *et al.*, 1992). This suggests that the rate-limiting step of non-cyclic electron transport is the same in infected and control leaves (Holloway *et al.*, 1992)

and the differences are in the chlorophyll content of healthy and infected leaves (Goodman *et al.*, 1986). In contrast, Magyarosy and Malkin (1978) found a reduction in cytochrome levels in the electron transport chain in chloroplasts from powdery mildew-infected sugar beet plants and suggest that the reduction may be due to the reduction in ribosomal RNA in these organelles which occurred as early as one day after inoculation (Dyer and Scott, 1972).

1.2.5 Changes in Invertase Activity during Infection.

Increased acid invertase activity in barley leaves infected with powdery mildew (Hwang and Heitefuss, 1986; Scholes, 1992), caused a decrease in the activity and/or the quantity of the photosynthetic enzymes of the Calvin cycle (Scholes, 1992; 1995). Scholes (1992) suggests that this was due to end-product inhibition or to a direct effect of the carbohydrates on the genes which encode for these enzymes. However auxin, which may be produced by fungi, or host stimulated by the presence of the fungus, is known to regulate invertase activity (Scholes, 1992). It is not known if the increased enzyme activity is of host or fungal origin, although some increase in host invertase activity has been shown to occur. The cause of the increase in acid invertase activity does not appear to be known, but cannot be due to an increased requirement for its products since these accumulated. It is interesting to note that the observed changes have been shown to occur in

Arabidopsis plants which over-express yeast invertase in their cell walls and in leaves when fed with glucose (Scholes, 1995).

Brown rust of barley reduced the levels of sucrose and glucose, but not of fructose (Tetlow and Farrar, 1993). These authors suggest that this means increased invertase activity and uptake of glucose by the fungus. Total carbohydrates were reduced by 50% at flecking and sporulation (Tetlow and Farrar, 1993). They showed very low contamination by intracellular enzymes of the apoplast (using malate dehydrogenase activity). Both results, according to the authors, show that membranes are intact in the rusted leaves. Once in the epidermal cells, the metabolites enter the haustoria directly from the cytoplasm, due to the impermeable nature of the haustorial neckband (Bushnell and Gay, 1978). It has been shown that sucrose taken up from barley by mildew is quickly metabolised (Edwards and Allen, 1966). The main product is mannitol, with trehalose, arabitol, aspartic and glutamic acids amongst the other products (Edwards and Allen, 1966). Soluble fungal materials such as mannitol, are synthesised within the haustorium before being transported through the septal pore in the haustorial neck to hyphal cells (Bushnell and Gay, 1978). Hwang and Heitefuss (1986) on the other hand, have suggested that the increase in glucose, fructose and sucrose seen in barley infected with powdery mildew may be due to accumulation around infection

sites, reduced export from infected leaves and stimulation of host metabolic activity.

1.3 PATHWAYS OF LYSINE BIOSYNTHESIS.

Two pathways for the biosynthesis of lysine are known. The α-aminoadipic acid pathway (AAA) (Fig. 2a) of lysine biosynthesis and the diaminopimelic acid (DAP) pathway (Fig. 2b) (present only in organisms with cellulose in their cell walls) are examples of dichotomous evolution, where a single metabolic pathway has branched at some point in time to form two mutually exclusive pathways. These pathways are unrelated, but are responsible for the metabolism of lysine in different classes of organism (Bhattacharjee, 1992). The catabolism of lysine takes place by a reversal of the AAA pathway.

1.3.1 The α-Aminoadipic Acid Pathway of Lysine Biosynthesis.

In the α-aminoadipic acid (AAA) pathway of lysine biosynthesis (Fig. 2a), α-ketoglutarate (C5) and acetyl CoA (C2) are condensed to form homocitric acid (C7). After dehydration and re-hydration, an isomer of homocitric acid, homoisocitric acid (C7), is produced. Reduction of this compound gives oxaloglutaric acid (C7), which is decarboxylated to α-ketoadipic acid (C6). Following transamination, AAA (C6) is formed. Reduction of the AAA yields α-aminoadipate semialdehyde (C6), which is

Fig 2a: Enzymes of the α-Amino Adipic Acid Pathway of Lysine Biosynthesis

[1] Homocitrate synthase (EC 4.1.3.21)

[2] Homocitrate dehydratase

[3] Homoaconitate hydratase (EC 4.2.1.36)

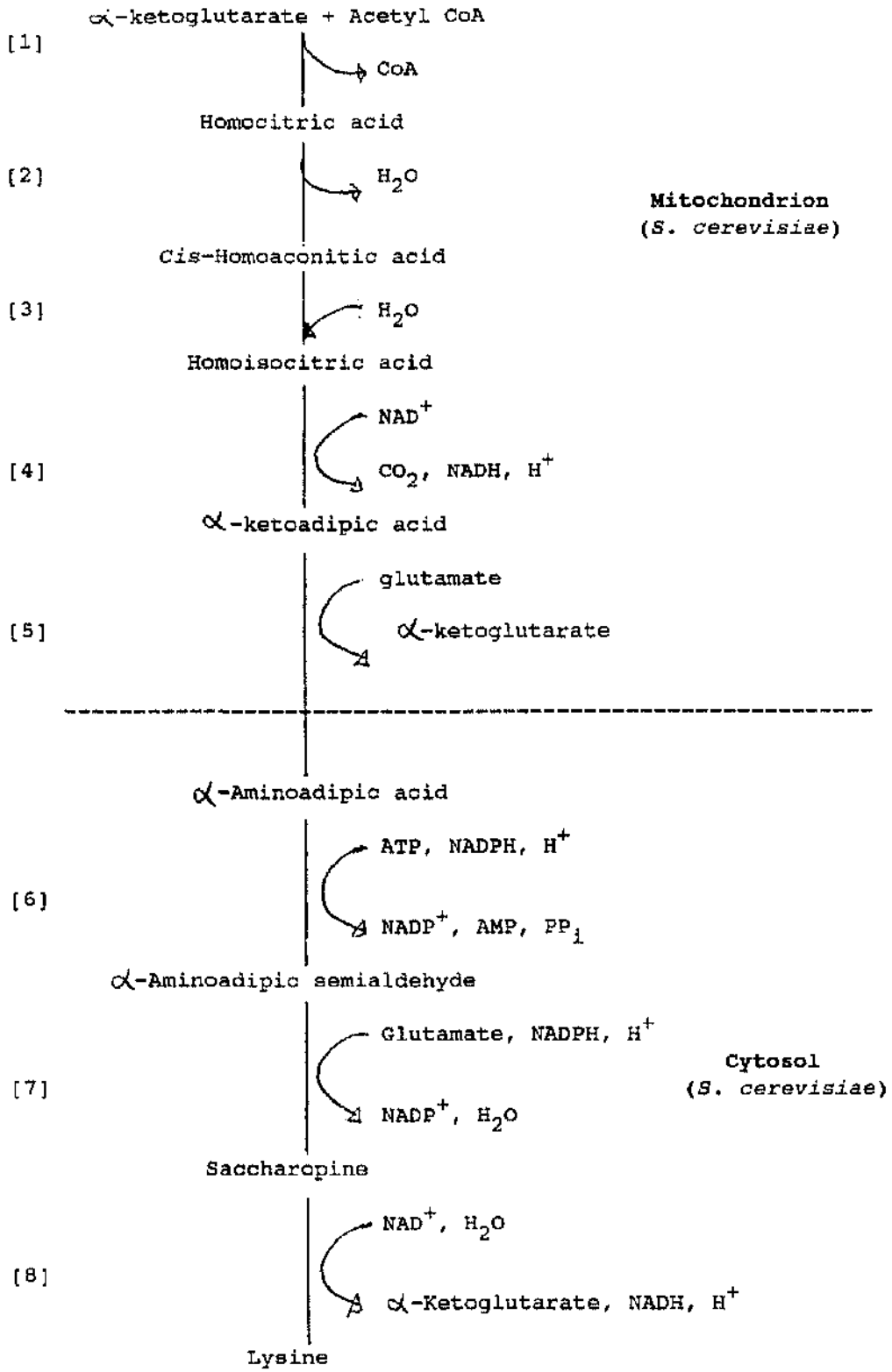
[4] 2-Hydroxy-3-carboxyadipate dehydrogenase (homoisocitric dehydrogenase) (EC 1.1.1.87)

[5] 2-Aminoadipate aminotransferase (α-ketoadipate glutamate aminotransferase) (EC 2.6.1.39)

[6] Aminoadipate-semialdehyde dehydrogenase (aminoadipic acid reductase) (EC 1.2.1.31)

[7] Saccharopine dehydrogenase (reductase) ($NADP^+$, L-glutamate forming) (EC 1.5.1.10)

[8] Saccharopine dehydrogenase (NAD^+, lysine forming) (EC 1.5.1.7)


α-ketoglutarate + Acetyl CoA
[1]
CoA
Homocitric acid
[2]
H_2O
Mitochondrion
(S. cerevisiae)
Cis-Homoaconitic acid
[3]
H_2O
Homoisocitric acid
[4]
NAD^+
CO_2, NADH, H^+
α-ketoadipic acid
[5]
glutamate
α-ketoglutarate
α-Aminoadipic acid
[6]
ATP, NADPH, H^+
$NADP^+$, AMP, PP_i
α-Aminoadipic semialdehyde
[7]
Glutamate, NADPH, H^+
$NADP^+$, H_2O
Cytosol
(S. cerevisiae)
Saccharopine
[8]
NAD^+, H_2O
α-Ketoglutarate, NADH, H^+
Lysine

condensed with glutamic acid (C5) to form saccharopine (C11), the immediate precursor of lysine (C6). This compound was first identified as an intermediate on the AAA pathway of lysine biosynthesis in the yeast *S. cerevisae* when it accumulated, briefly, from AAA and was subsequently converted to lysine (Kuo, Saunders and Broquist, 1964). The glutamate acts as an amino donor in the synthesis of the α-amino group of lysine (Kuo *et al.*, 1964).

This pathway for lysine biosynthesis has been shown to be present in the yeasts *Torulopsis utilis* (Sagisaki & Shimura, 1962a; Jones & Broquist, 1966), *S. cerevisae* (Kuo *et al.*, 1964; Kurtz and Bhattacharjee, 1975; Bhattacharjee, 1992) and *Rhodotorula glutinis* (Kurtz and Bhattacharjee, 1975) and in *Neurospora crassa* (Abelson and Vogel, 1955; Trupin & Broquist, 1965), *Pyricularia oryzae* (Wade, Thomson & Miflin, 1980), *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* (opportunistic fungal pathogens) (Garrard & Bhattacharjee, 1992).

1.3.1.1 Intra-cellular Localisation of the α-Aminoadipic Acid Pathway Enzymes.

From the evidence available, it may be said that most, if not all, of the metabolic enzymes involved in the α-aminoadipic acid (AAA) pathway are to be found in the mitochondria of some fungi.

A quarter of the activity of homocitrate synthase, the first enzyme of the AAA pathway was found in mitochondria in an isolate of *Penicillium chrysogenum*, while the remainder was cytosolic (Affenzeller, Jaklitsh, Honlinger and Kubicek, 1989). In *S. cerevisiae* the enzymes of this pathway, prior to the production of AAA, are localised in the mitochondria while the remaining steps are catalysed in the cytosol (Bhattacharjee, 1992).

In ox liver, both synthesis and degradation of lysine *via* saccharopine takes place in the mitochondria (Fellows, 1973). These reactions are catalysed by separate enzymes. Conversion of lysine to saccharopine and then to AAA takes place irreversibly in rat liver mitochondria (Rothstein & Miller, 1954) and in preparations of human liver in the presence of NADPH and ∝-ketoglutarate (Hutzler & Dancis, 1968; Higashino, Fujioka & Yamamura, 1971; Fellows & Lewis, 1973; Fjellstedt & Robinson, 1975). Fellows and Lewis (1973) went further to show that this conversion occurs in a range of mammals (ox, dog, cat, rat and pig). The same reaction was found to occur in mouse liver, but here the activity was not confined to the mitochondria. Catabolism of lysine has been shown to occur in the mitochondria in some mammals and at least some of the steps of lysine catabolism in fungi take place in these organelles (Betterton, Fjellstedt, Matsuda, Ogur & Tate, 1968). In barley, lysine catabolism occurred *via* a reversal of some

of the steps in the AAA pathway and this may take place in the mitochondria (Moller, 1976a).

1.3.1.2 Regulation of the ∝-Aminoadipic Acid Pathway Enzymes.

Unlike the DAP pathway of lysine biosynthesis, which is only one part of a highly branched biosynthetic pathway, the ∝-aminoadipic acid (AAA) pathway only produces lysine as a final product. Lysine metabolism by this pathway is regulated *in vivo* by, amongst other mechanisms, feedback inhibition (Bhattacharjee, 1985), repression by lysine (Schmidt, Bode & Birnbaum, 1990), general amino acid regulation and repression by specific genes.

Homocitrate synthase shows feedback inhibition by lysine in yeast (Bhattacharjee, 1985; Schmidt *et al.*, 1990) and in numerous species of fungi (Bhattacharjee, 1992).

AAA reductase in *Trichosporon adeninovorans*, which catalyses the formation of -aminoadipate semialdehyde (ASA) from AAA, is regulated by lysine and requires ATP, Mg^{2+} and NADPH (Schmidt *et al.*, 1990). AAA plays an important role in penicillin biosynthesis (Schmidt *et al.*, 1990). However, AAA reductase is also regulated by lysine in this species (Schmidt *et al.*, 1990). Although this enzyme in yeast has the same co-factor requirements for activity (Sagisaka and Shimura, 1962b), it is regulated by

glutamate rather than by lysine (Schmidt *et al.*, 1990). The optimum pH for the reaction in *T. utilis* was 7.6 - 8.0 and the enzyme was specific for AAA (Sagisaka and Shimura, 1962b).

Saccharopine oxidoreductase in ox liver mitochondria was not inhibited by lysine (Fellows and Lewis, 1973). The activity of this enzyme in the ox, which relies on a low lysine diet, is greater than in humans and other mammals studied (Fellows and Lewis, 1973).

1.3.2 Lysine Catabolism

The enzyme involved in the formation of saccharopine from lysine and α-ketoglutarate (lysine-α-ketoglutarate reductase) was found, in humans, mainly in the liver. The pH optimum for this reaction was 7.0 and there was a requirement for NADPH (NADH was no use) (Hutzler and Dancis, 1968). Saccharopine is a key intermediate in the degradation of lysine in mammals (Fellows and Lewis, 1973). However, very little saccharopine is found in human tissues and it is suggested that it is rapidly converted to α-aminoadipate semialdehyde and glutamate (Fellows and Lewis, 1973).

Saccharopine dehydrogenase (reductase), the enzyme which catalyses the degradation of saccharopine to α-aminoadipate semialdehyde and glutamate, was found in human

placenta (Fjellstedt and Robinson, 1975) and in human and rat liver (Fujioka and Nakatani, 1972). This enzyme was not able to be separated from lysine- α -ketoglutarate reductase. However, these were shown to be two distinct enzymes by the lack of inhibition of saccharopine dehydrogenase by an inhibitor of lysine- α -ketoglutarate (Fjellstedt and Robinson, 1975).

The optimum pH for the activity of saccharopine dehydrogenase was found to be from 8.5 - 8.9 (lower than the optimum pH for the yeast enzyme - pH 9.5) and there was a requirement for NAD or NADPH (Fjellstedt and Robinson, 1975). Glutamate was moderately inhibitory but no inhibition was observed with NAD or saccharopine (Fjellstedt and Robinson, 1975).

It was shown that these two reactions took place *in vitro* in the livers of rats, pigs, dogs, cats, oxen and sheep, as well as in the human liver (Fellows and Lewis, 1973) and in mouse liver (Fujioka and Nakatani, 1972).

The degradation of lysine in mammals is similar to biosynthesis in yeast and fungi, although there are differences in the enzyme and co-factor requirements (Fjellstedt and Robinson, 1975). For example, the saccharopine reductase enzyme from human placenta has a molecular weight of 480000 (Fjellstedt and Robinson, 1975), whereas that from yeast is 73000 (Jones and Broquist,

1966).

In *Achromobacter liquidum* (Soda and Misono, 1968) and *Flavobacterium fuscum* (Soda and Misono, 1968), the transfer of the terminal amino group of lysine to α-ketoglutarate was catalysed by the enzyme L-lysine- α -ketoglutarate aminotransferase. The optimum pH for the reaction was 8.3 - 8.5. This enzyme is also able to transfer the terminal amino group of L-ornithine to α -ketoglutarate, but the reaction takes place more slowly. The molecular weight of the enzyme is 116000 and PLP is required as a co-factor (Soda and Misono, 1968).

1.3.3 The Diaminopimelic Acid Pathway of Lysine Biosynthesis.

In this pathway (Fig. 2b), the formation of lysine begins when aspartate (C4) is converted to β-aspartyl phosphate in the presence of aspartate kinase and ATP. This is reduced to β-aspartic semialdehyde (C4) by β-aspartic semialdehyde dehydrogenase (Fig. 2b). β-aspartic semialdehyde and pyruvate (C3) are condensed to form dihydrodipicolinic acid (DHDP) (C7). This is catalysed by dihydrodipicolinic acid synthase (DHDPS). DHDP reductase, which requires NADPH as co-factor, reduces DHDP to Δ^1-piperideine-2,6-dicarboxylate (C7), which is further reduced in the presence of NADPH and ammonia by *meso*-diaminopimelate dehydrogenase, to *meso*-diaminopimelate

Fig 2b: Enzymes Of The Diaminopimelic Acid Pathway of Lysin Biosynthesis

[1] Aspartate kinase (EC 2.7.2.4)

[2] Aspartate- β -semialdehyde dehydrogenase (EC 1.2.1.11)

[3] Dihydrodipicolinic acid synthase (EC 4.2.1.52)

[4] Dihydrodipicolinic acid reductase (EC 1.3.1.26)

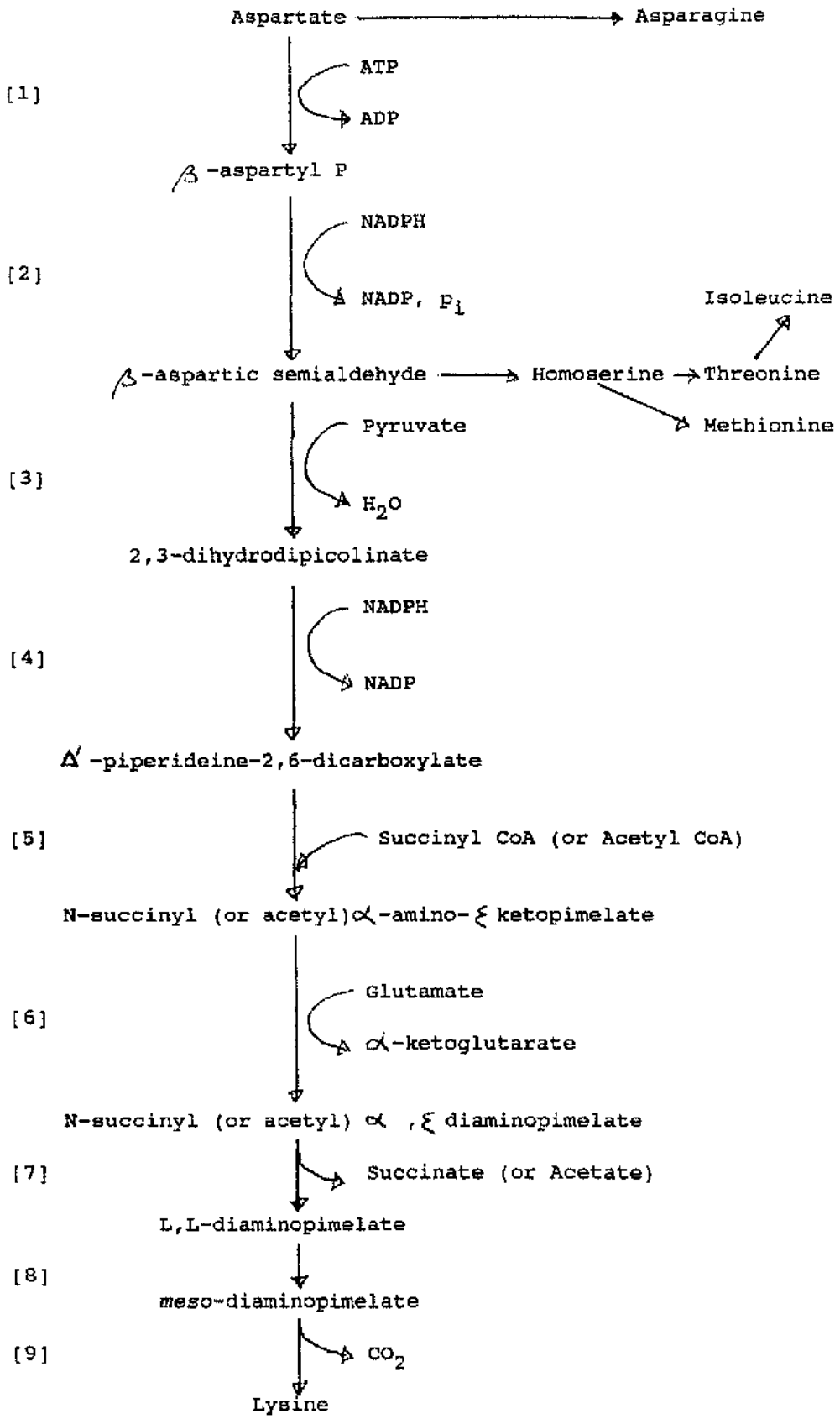
[5] Tetrahydropicolinate succinylase

[6] Succinyl-diaminopimelate (DAP) aminotransferase (EC 2.6.1.17)

[7] Succinyl-DAP desuccinylase (EC 3.5.1.18)

[8] DAP epimerase (EC 5.1.1.7)

[9] DAP decarboxylase (EC 4.1.1.20)


Aspartate
Asparagine
[1]
ATP
ADP
β-aspartyl P
[2]
NADPH
NADP, P_i
Isoleucine
β-aspartic semialdehyde
Homoserine
Threonine
Methionine
[3]
Pyruvate
H_2O
2,3-dihydrodipicolinate
[4]
NADPH
NADP
Δ′-piperideine-2,6-dicarboxylate
[5]
Succinyl CoA (or Acetyl CoA)
N-succinyl (or acetyl)α-amino-ξ ketopimelate
[6]
Glutamate
α-ketoglutarate
N-succinyl (or acetyl) α ,ξ diaminopimelate
[7]
Succinate (or Acetate)
L,L-diaminopimelate
[8]
meso-diaminopimelate
[9]
CO_2
Lysine

(DAP) (C7). Decarboxylation of this compound by *meso*-DAP decarboxylase gives lysine (C6). In *E. coli* however, *meso*-DAP dehydrogenase is not present and *meso*-DAP, the immediate precursor of lysine, is produced from tetraHDP by a series of four steps involving tetraHDP succinylase, succinyl DAP aminotransferase, succinyl DAP desuccinylase and DAP epimerase.

Although the DAP pathway was first investigated in *E. coli* (Gilvarg, 1958), many of the enzymes have been shown to occur in plants. Vogel (1959) showed that lysine synthesis in higher plants occurred *via* the DAP pathway (one part of a larger much-branched pathway).

1.3.3.1 Aspartate Kinase

Aspartate kinase (the first enzyme of the aspartate pathway) was found in the photosynthetic bacterium *Rhodopseudomonas sphaeroides* (Datta and Prakash, 1966). Activity of this enzyme has also been shown in maize (Cheshire and Miflin, 1975), barley seedlings (Shewry and Miflin, 1977), carrot root (Matthews and Widholm, 1978), soybean cotyledons and callus (Matthews and Widholm, 1979), *Nicotiana sylvestris* (Negruitiu, Cattoir-Reynearts, Verbruggen & Jacobs, 1984) and *Nicotiana* species (Shaul and Galili, 1992).

Aspartate kinase of *E. coli* is composed of four

identical sub-units (Umbarger, 1978). The enzyme from *Rhodopseudomonas sphaeroides* requires ATP and Mg^{2+} or Mn^{2+} ions and inorganic phosphate increases V_{max} (Datta and Prakash, 1966). In yeast, the enzyme required Mg^{2+}, Mn^{2+} or Fe^{2+} and was equally active from pH 5 - 9 (Black and Wright, 1954). In *B. polymyxa* the enzyme was inhibited by L-lysine and L-threonine at concentrations below 1mM (Paulus and Gray, 1967). At higher concentrations inhibition was seen with these amino acids individually (Paulus and Gray, 1967).

1.3.3.2 Dihydrodipicolinic Acid Synthase

Dihydrodipicolinic acid synthase (DHDPS), the first enzyme exclusive to the lysine biosynthetic pathway, has been isolated from *E. coli* and its physical properties determined by Laber, Gomis-Ruth, Romao and Huber (1992). This enzyme was also found in maize cotyledons (Cheshire and Miflin, 1975; Mazelis, Whatley and Whatley, 1977), carrot root (Matthews and Widholm, 1978), soybean cotyledons and callus (Matthews and Widholm, 1979) and *N. sylvestris* (Negruitiu *et al.*, 1984; Ghislain, Frankard & Jacobs, 1990) as well as in spinach (Kirk and Leech, 1972; Mazelis *et al.*, 1977), red kidney bean seedlings, cabbage, potato tubers, squash fruit and wheatgerm (Mazelis, *et al.* 1977) and wheat (Kumpaisal, Hashimoto and Yamada, 1987).

The DHDPS enzyme was first purified from *E. coli* by

Shedlarski and Gilvarg (1970). The molecular weight of the enzyme, which was made up of four sub-units, was 134000. The tetrameric form of the enzyme in the same species was confirmed by Laber *et al.* (1992) who calculated the molecular weight to be 112000. The four identical sub-units had molecular weights of 32000 (Laber *et al.*, 1992).

This enzyme in *N. sylvestris* has a molecular weight of 164000 and also has four identical sub-units of 38500 (Ghislain *et al.*, 1990). The tetrameric structure is stabilised by pyruvate. In wheat where the molecular weight was 123000 (Kumpaisal *et al.*, 1987) and *Bacillus licheniformis* the enzyme was again stabilised by pyruvate.

In *B. licheniformis*, the molecular weight of the enzyme was 108000 - 117500 and was again composed of four identical subunits each of 28000 (Halling and Stahly, 1976). In *B. subtilis*, the enzyme from sporulating and non-sporulating cells was identical, with a molecular weight of 124000 (Yamakura, Ikeda, Kimura & Sasakawa, 1974). The activity of the enzyme was four to six times greater during sporulation in *B. cereus* (Hoganson and Stahly, 1975).

Thus, the molecular weight of the enzyme from different species and from the same species when calculated by different workers is of the same order of magnitude. However, the pH for optimum activity of the enzyme was

somewhat different in wheat (8.0) (Kumpaisal *et al.*, 1987), *E. coli* (8.4) (Yugari and Gilvarg, 1965) and *B. subtilis* (9.5) (Yamakura *et al.*, 1974).

Regulation of this enzyme can control this branch of the pathway (Negruitiu *et al.*, 1984). The activity of the enzyme in *N. sylvestris* is strongly inhibited by lysine (Ghislain *et al.*, 1990) and, while two lysine analogues were poor inhibitors, a pyruvate analogue caused inhibition by binding at the pyruvate-binding site (Ghislain *et al.*, 1990). In wheat, the enzyme was inhibited by lysine and its analogues, by some metal ions (Zn^{2+}, Cd^{2+}, Hg^{2+}) and certain sulphydryl inhibitors (Kumpaisal *et al.*, 1987).

The only substrate for this enzyme in *B. licheniformis* was L-ASA, but both D- and L-ASA inhibited its activity (Stahly, 1969). The inhibition arose as pyruvate was not able to saturate the enzyme in the presence of these compounds (Stahly, 1969). These workers were unable to find any other inhibitors of the enzyme. The enzyme in maize (Cheshire and Miflin, 1975), soybean seedlings, callus and suspension cultures (Matthews and Widholm, 1979) was inhibited 95% by 1mM lysine. In carrot root suspension cultures and whole carrot root, inhibition was greater than 80% in the presence of 0.5mM lysine (Matthews and Widholm, 1978).

1.3.3.3 DAP Decarboxylase

This enzyme has been isolated from *E. coli* (Work, 1962; White and Kelly, 1965). It is specific for *meso*-diaminopimelate (DAP) and has an optimum pH of 7.2 (Work, 1962), although White and Kelly (1965), also using a phosphate buffer, found the optimum pH in this species to be around 6.7 - 6.8. There was a requirement for a thiol compound and pyridoxal-5'-phosphate (PLP) (White and Kelly, 1965). Work (1962) did not show a requirement for additional PLP, but suggested that this compound is tightly bound to the enzyme and acts as a co-enzyme. There was no evidence that the reaction was reversible (Work, 1962). The molecular weight of the enzyme in this species was 200000 (White and Kelly, 1965).

The enzyme in *Vicia faba* has been shown to be present exclusively in the chloroplast (Mazelis, Miflin and Pratt, 1976). In this species, the optimum pH was 7.0, which lies between the pH optima calculated for the two *E. coli* isolates examined by Work (1962) and White and Kelly (1965).

1.3.3.4 Other Enzymes of the DAP Pathway

Dihydrodipicolinic acid reductase (DHDPR) (Tyagi, Henke and Farkas, 1985b), an enzyme first identified in *E. coli* by Farkas and Gilvarg (1965), and diaminopimelic acid

epimerase (DAP epimerase) (Tyagi, Henke and Farkas, 1985a), were also present in maize. The optimum pH for the activity of DHDPR in *E. coli* was around 7.0 and the enzyme was strongly inhibited by dipicolinic acid (Tyagi *et al.*, 1985b). In the same species, the pH optimum for DAP epimerase was 7.7 - 8.3 (Antia, Hoare and Work, 1957). There was no requirement for PLP, but the enzyme was inhibited by carbonyl-binding reagents. This inhibition was overcome by thiols (Antia *et al.*, 1957).

Succinyl-DAP aminotransferase had an optimum pH of 8.0 and was specific for glutamate. α-Ketoglutarate inhibited both the forward and reverse reactions and there was inhibition by hydroxylamine (typical of a PLP dependent enzyme) (Peterofsky and Gilvarg, 1961). Succinyl-DAP succinylase in *E. coli* also had an optimum pH of 8.0 and had a requirement for Co^{2+} as a co-factor (Kindler and Gilvarg, 1960). This enzyme was also identified in *Corynebacterium diptheriae* and *B. cereus* (Kindler and Gilvarg, 1960).

1.3.3.5 Intra-cellular Localisation of the DAP Pathway Enzymes.

In contrast to the AAA pathway of lysine biosynthesis in fungi, where the enzymes appear to be localised within the mitochondria, the enzymes of the DAP pathway are thought to be present in the chloroplasts of green plants.

In fact, most of the enzymes in amino acid biosynthesis from aspartate are also present in these organelles.

DAP decarboxylase activity in *Vicia faba* (Mazelis *et al.*, 1976) and DHDPS activity in spinach were localised within the chloroplast (Wallsgrove & Mazelis, 1981). In *Nicotiana sylvestris* (Ghislain *et al.*, 1990), the activity of the latter enzyme was more specifically associated with the stroma of the chloroplast. In spinach, aspartate aminotransferase was also shown to be associated with the stroma of the chloroplast (Miflin, 1974). Lysine and the other amino acids of the aspartate pathway (threonine, methionine and isoleucine) were synthesised in chloroplasts isolated from pea leaves (Mills, Lea & Miflin, 1980). Chloroplasts of *V. faba* synthesised 19 amino acids, 17 of which were formed from alanine or aspartate (Kirk & Leech, 1972). Oxaloacetate was only found in the cytoplasm and these workers suggest that the supply of this compound from the cytoplasm is an important factor in controlling amino acid synthesis by chloroplasts. However, Wallsgrove, Lea & Miflin (1983) suggest that lysine and threonine are synthesised in the chloroplast in green leaves, but the final step in methionine biosynthesis takes place in the cytoplasm.

It may be possible that the origin of chloroplasts in green plants derives from an endosymbiotic relationship (Carr and Craig, 1970). Examples of this, in algae, show

the possibility of such symbioses. For example, a blue-green alga, *Glaucocystis nostochinearum*, functions in the role of a chloroplast within an unnamed colourless alga. Diaminopimelic acid was not detected in the photosynthetic alga, although β-carotene and allophycocyanin, characteristic of free living blue-green algae, were present. Photosynthetic processes in the blue-green algae and in some photosynthetic bacteria are essentially the same as those found in chloroplasts. In the blue-green algae, the photosynthetic lamellae are situated around the periphery of the organism and in contact with the cytoplasm. In the chloroplast, however, the lamellae are separated from the cytoplasm by being enclosed within the chloroplast membrane (Carr and Craig, 1970).

The components of RNA in chloroplasts, bacteria and algae are similar, and different from those of the cytoplasm (Carr and Craig, 1970).

1.3.3.6 Regulation of the DAP Pathway Enzymes.

Aspartate kinase and DHDPS from maize (Cheshire and Miflin, 1975) and DHDPS from *Nicotiana sylvestris* (similar in function to the bacterial enzyme) (Ghislain, Frankard and Jacobs, 1990) and from *E. coli* (Laber *et al.*, 1992) were inhibited by lysine. In maize, lysine also protects the aspartate kinase enzyme from heat inactivation

(Cheshire and Miflin, 1975). Both aspartate kinase and DHDPS in *N. sylvestris* are regulated by quite small amounts of lysine (Negruitiu *et al.*, 1984). Aspartate kinase is inhibited, *in vivo*, by lysine in the same species (Shaul and Galili, 1992). In soybean cotyledons, aspartate kinase and homoserine dehydrogenase were inhibited by lysine or threonine, but were inhibited synergistically in the presence of both of these compounds (Matthews and Widholm, 1979). In barley seedlings, aspartate kinase was regulated by cooperative feedback regulation in the presence of lysine and low concentrations of methionine (Shewry and Miflin, 1977).

Unlike most bacterial and plant enzymes, aspartate kinase in the photosynthetic bacterium *Rhodopseudomonas sphaeroides* was not inhibited by any of the end-product amino acids of this pathway (Datta and Prakash, 1966). A key intermediate in the synthesis of all of the aspartate pathway amino acids, aspartate-β-semialdehyde was, however, strongly inhibitory. This inhibition was competitive with aspartate and ATP and is thought to provide an effective method of controlling the synthesis of lysine, threonine, methionine and isoleucine (Datta and Prakash, 1966).

Synergistic inhibition of growth in *Mimulus cardinalis* seedlings (Henke, Wilson, McClure and Treik, 1974) and in wheat and barley embryos (Bright, Shewry and Miflin, 1978) occurred in the presence of lysine (1mM) and threonine

(1mM). This inhibition was relieved by methionine, homocysteine and homoserine, suggesting feed-back regulation of aspartate kinase by lysine and homoserine dehydrogenase by threonine (Henke *et al.*, 1974; Bright, Wood and Miflin, 1978). Similarly, inhibition of protein synthesis was seen in *Mimulus cardinalis* seedlings (Henke and Wilson, 1974).

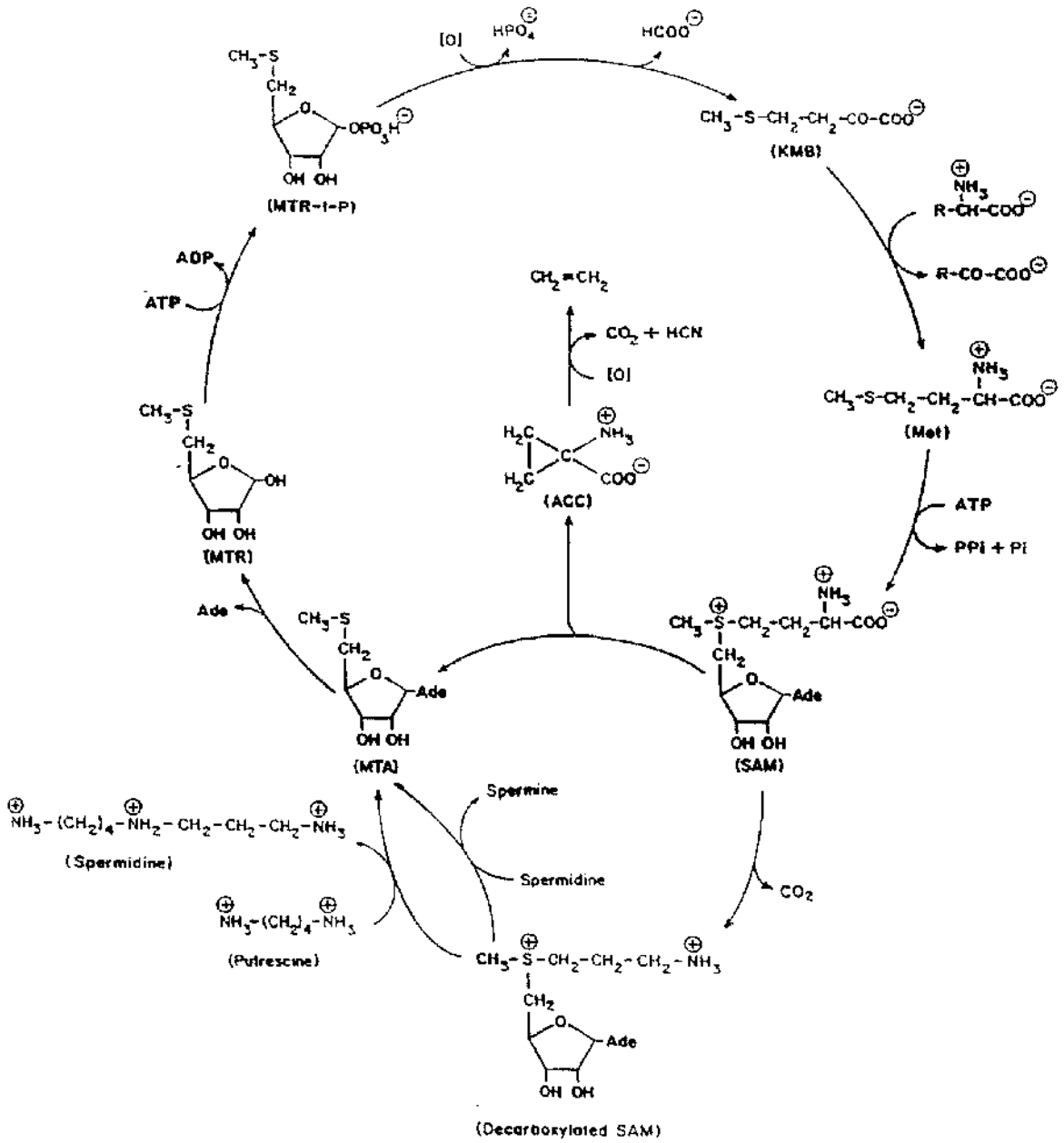
Inhibition of the growth of gemmalings of *Marchantia polymorpha* in the presence of lysine and threonine was due to inhibition of protein synthesis rather than to changes in amino acid uptake (Dunham and Bryan, 1971). These workers suggest that there is concerted feedback inhibition of aspartate kinase activity in the presence of lysine plus threonine, since there is a reduction in aspartate derivatives. The addition of methionine with these two amino acids relieved the synergistic effect on protein synthesis (Dunham and Bryan, 1971).

1.4 Polyamines.

The common polyamines putrescine (PUT), spermidine (SPD) and spermine (SPM) are ubiquitous in nature (Porter and Sufrin, 1986). The non-conjugated forms of these low molecular weight aliphatic amines at cellular pH occur as organic polycations and may act as metabolic buffers (Hamana and Matsuzaki, 1987; Galston and Kaur-Sawhney,

Fig. 3: The methionine cycle and the relationship between synthesis of polyamines and ethylene (Myazaki and Yang, 1987).

Abbreviations:
ACC = 1-aminocyclopropane-1-carboxylic acid
Ade = adenine
KMB = 2-keto-4-methylthiobutyric acid
Met = methionine
MTA = 5'-methylthioadenosine
MTR = 5-methylthioribose
MTR-1-P = 5-methylthioribose-1-phosphate
SAM = *S*-adenosylmethionine


[O]
HPO4
HCOO
(MTR-1-P)
(KMB)
(Met)
ATP
PPi + Pi
ADP
ATP
CH2=CH2
CO2 + HCN
[O]
(ACC)
(MTR)
Ade
(MTA)
(SAM)
Spermine
Spermidine
(Spermidine)
(Putrescine)
CO2
(Decarboxylated SAM)

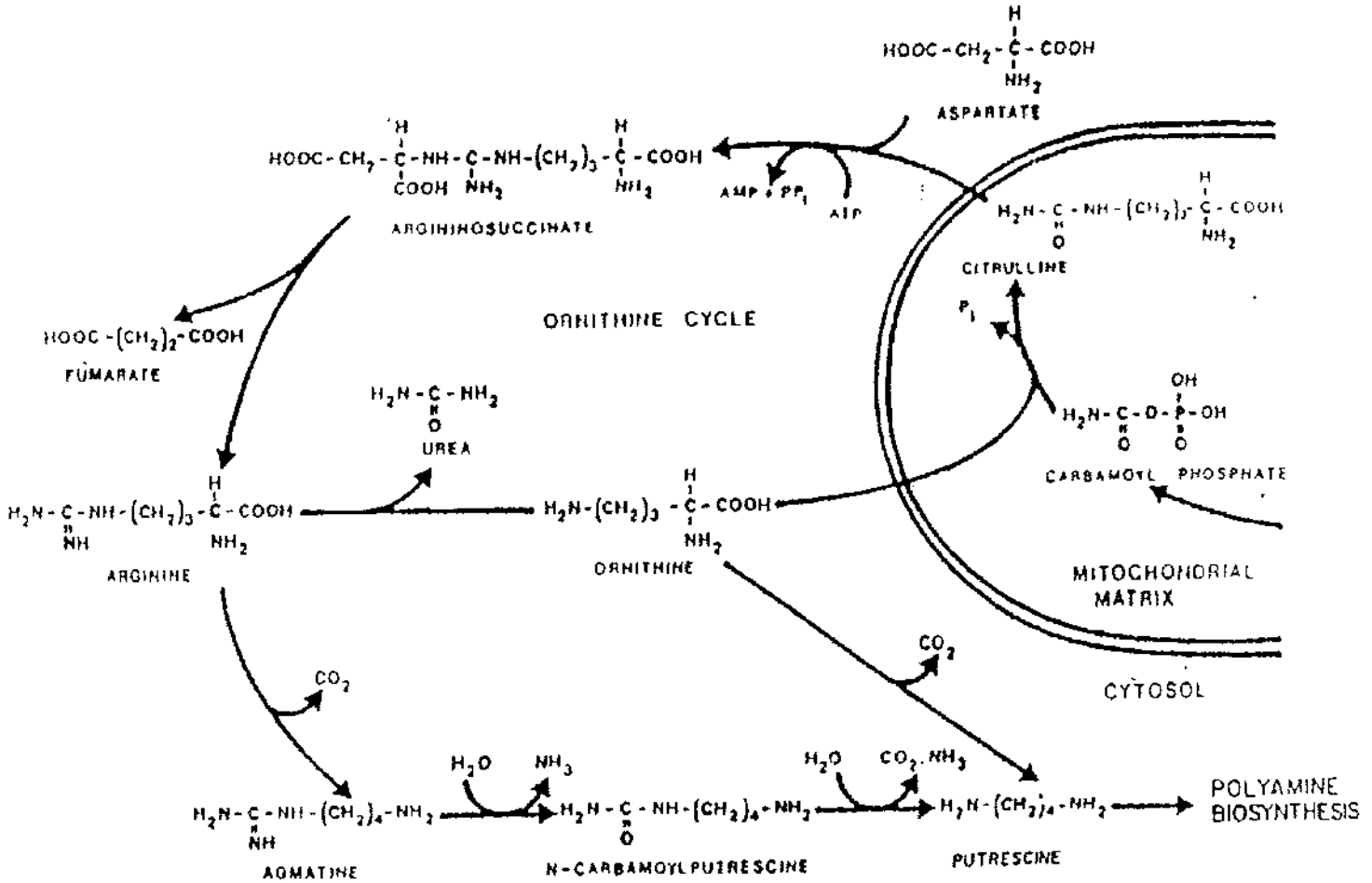
1990). However, they are often conjugated to, for example, small molecules (phenolic acids) or macromolecules (DNA, RNA, ribosomes, proteins, cell wall components) (Janne, Alhonen-Hongisto, Seppanen and Holtta, 1981; Hamana and Matsuzaki, 1987; Galston and Kaur-Sawhney, 1990). Variations in the concentrations within the cell (μM - mM) may occur in response to environmental stress (light, temperature, chemical and physical stress) (Galston and Kaur-Sawhney, 1990) and, in addition, spermidine protects nucleic acids from enzyme degradation and chemical and physical stresses (Porter and Sufrin, 1986).

Polyamines are required during cell division (Janne *et al.*, 1981; Evans and Malmberg, 1989) and are thought to be involved in DNA replication. Indeed, they are essential to the development of tobacco and tomato fruit and ovaries, and may also be required during differentiation of floral meristems (Evans and Malmberg, 1989). In addition, polyamines can delay the start of senescence (Evans and Malmberg, 1989; Galston and Kaur-Sawhney, 1990), probably by inhibiting the production of ethylene (Galston and Kaur-Sawhney, 1990), which may compete with polyamine biosynthesis for S-adenosylmethionine (Miyazaki and Yang, 1987) (Fig. 3). For example, in powdery mildew-infected barley leaves, free polyamine levels were higher in green-islands than in surrounding tissues and ethylene production was reduced (Coghlan and Walters, 1990).

Although only one pathway for polyamine biosynthesis

has been shown to occur in mammals and fungi, the ornithine decarboxylase (ODC) pathway, there are known to be two pathways in plants (Slocum, Kaur-Sawhney and Galston, 1984) (Fig. 4). Ornithine may be converted to the diamine, PUT, directly by decarboxylation, or may first be converted, *via* the ornithine cycle, to arginine, which is subsequently decarboxylated to give agmatine. On further reaction agmatine also yields PUT (Fig. 4). In higher plants arginine may also be converted to ornithine in the presence of the enzyme arginase. SPD and SPM are formed by the addition of aminopropyl moieties from decarboxylated *S*-adenosylmethionine to PUT and SPD respectively. These reactions are catalysed by the action of aminopropyltransferases with quite narrow substrate specificity. The enzymes which convert ornithine or arginine to putrescine, ODC and arginine decarboxylase (ADC), are thought to be compartmentalised within the cell. Both ODC and SamDC are rate limiting enzymes in polyamine biosynthesis (Holm, Persson, Pegg & Heby, 1989). Although the subcellular distribution, intracellular binding sites and bound/unbound pool sizes are not known with certaintly (Porter and Sufrin, 1986), ODC has been associated with organelles which contain DNA, while ADC is thought to be cytosolic (Galston and Kaur-Sawhney, 1990). In the fungus, *N. crassa*, however, ODC was found only in the cytosol (Tabor and Tabor, 1985). Polyamines produced by the activity of ODC are seen as essential for DNA replication

Fig 4: Putrescine biosynthesis from arginine or ornithine. Conversion of ornithine to arginine takes place *via* the ornithine cycle (Slocum, Kaur-Sawhney and Galston, 1984).


ASPARTATE
ARGININOSUCCINATE
AMP + PP$_i$
ATP
CITRULLINE
ORNITHINE CYCLE
P_i
FUMARATE
UREA
CARBAMOYL PHOSPHATE
ARGININE
ORNITHINE
MITOCHONDRIAL MATRIX
CYTOSOL
CO_2
H_2O
NH_3
CO_2, NH_3
AGMATINE
N-CARBAMOYLPUTRESCINE
PUTRESCINE
POLYAMINE BIOSYNTHESIS

and cell division, whereas activation of ADC and accumulation of PUT tends to be associated with plant responses to stress (Evans and Malmberg, 1989; Galston and Kaur-Sawhney, 1990). An exception to this occurs in *Nicotiana tabacum* cv *xanthi*, where ADC activity was found to coincide with the production of free amines (agmatine and PUT), while ODC was involved in the biosynthesis of PUT conjugates (hydroxycinnamoyl putrescines) (Burtin, Matin-Tanguy, Paynot and Rossin, 1989). The inhibitor of ADC activity, difluoromethylarginine (DFMA), caused a reduction in fresh and dry weight of *N. tabacum* cv. *xanthi*, while difluoromethylornithine (DFMO), which inhibits ODC, increased fresh and dry weight (Burtin *et al.*, 1989). They thus suggest that ADC is involved in cell division and callus induction (see above).

A decrease in hydroxycinnamoyl putrescines in *N. tabacum* cv *xanthi* was associated with bud formation (Burtin *et al.*, 1989). However, Torrigiani, Altamura, Capitani, Serafini-Fracassini and Bagni (1989) showed that these compounds were associated with flowering and the development of reproductive organs in the same species. Large amounts of these conjugates were also found in vegetative organs, callus and roots (Torrigiani *et al.*, 1989).

1.4.1 Unusual Polyamines

Polyamines other than PUT, SPD and SPM have been found

Table A: Unusual polyamines identified from natural sources.

1,3-Diaminopropane	$H_2N(CH_2)_3NH_2$
Putrescine	$H_2N(CH_2)_4NH_2$
Cadaverine	$H_2N(CH_2)_5NH_2$
Norspermidine	$H_2N(CH_2)_3NH(CH_2)_3NH_2$
Spermidine	$H_2N(CH_2)_3NH(CH_2)_4NH_2$
Aminopropyl-cadaverine	$H_2N(CH_2)_3NH(CH_2)_5NH_2$
Homospermidine	$H_2N(CH_2)_4NH(CH_2)_4NH_2$
Norspermine (Thermine)	$H_2N(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH_2$
Thermospermine	$H_2N(CH_2)_3NH(CH_2)_3NH(CH_2)_4NH_2$
Aminopentenyl-norspermidine	$H_2N(CH_2)_3NH(CH_2)_3NH(CH_2)_5NH_2$
Spermine	$H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$
Aminopropyl-homospermidine	$H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_4NH_2$
Aminobutyl-homospermidine	$H_2N(CH_2)_4NH(CH_2)_4NH(CH_2)_4NH_2$
N,N'-*bis*(3-amino-propyl)cadaverine	$H_2N(CH_2)_3NH(CH_2)_5NH(CH_2)_3NH_2$
Caldopentamine	$H_2N(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH_2$
Homocaldopentamine	$H_2N(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH(CH_2)_4NH_2$

in various organisms. Because of similarities in chromatographic properties these unusual compounds were not able to be separated from the common polyamines (Tait, 1985). In recent years, however, many have been separated and identified using HPLC and infra red analysis. Diamines and polyamines found in bacteria are listed in the table below (Table A) (Tait, 1985; Tabor and Tabor, 1985; Galston and Kaur-Sawhney, 1990; Hamana, Matsuzaki, Niitsu and Samejima, 1990).

It has been shown that these unusual polyamines may be related to: stabilising macromolecules under abnormal conditions (Tait, 1985), protecting bacterial enzymes against heat denaturation (*Thermus thermophilus*) (Tabor and Tabor, 1985), increased growth rate (*Rhizobium*) (Galston and Sawhney, 1990). Differences in the occurrence of these compounds within groups of bacteria may assist in their taxonomic classification (Hamana *et al.*, 1990). No polyamines were found in a number of halophytic Eubacteria. It is not known at present if this is characteristic of all halophytic species (Hamana *et al.*, 1990).

Unusual polyamines have rarely been identified in mammalian tissues. However Matsuzaki and co-workers (Matsuzaki, Xiao, Suzuki, Hamana, Niitsu and Samejima, 1987) were able to show the presence of several of these compounds (*sym*-homospermidine, N'-acetylspermidine, N'-acetylspermine, aminopropylhomospermidine and canavalmine)

in hamster epididymis and suggest that aminopropylhomospermidine is formed from homospermidine by the action of spermine synthase.

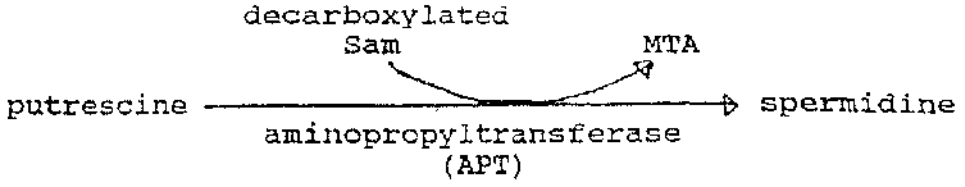
1.4.2 Biosynthesis of Uncommon Polyamines

Synthesis of spermidine from putrescine, and spermine from spermidine occurs in most organisms by the addition of an aminopropyl group from decarboxylated *S*-adenosylmethionine, catalysed by an aminopropyltransferase (Fig. 5a) (Tait, 1985). However, an alternative pathway for the synthesis of spermidine operates in some bacteria. Here the aminopropyl group is donated by L-aspartic-β-semialdehyde (Tait, 1976), a metabolite of aspartic acid and a precursor of the aspartate pathway amino acids (Fig. 5b). This alternative pathway would still be competitive with ethylene since L-aspartate-β-semialdehyde is a precursor of methionine and, therefore, SAMdc. Putrescine and L-aspartic-β-semialdehyde condense to form a Schiff base complex. Reduction of this complex forms carboxyspermidine, which is decarboxylated to give spermidine (Fig. 5b). Both of these pathways are present in *Lathyrus sativus* seedlings (Srivenugopal and Adiga, 1980). In two *Vibrio* species the action of this alternative pathway gave rise to norspermidine (Yamamoto, Hamanaka, Suemoto, Ono & Shinoda, 1986). These workers found no aminopropyltransferase capable of using SAMdc.

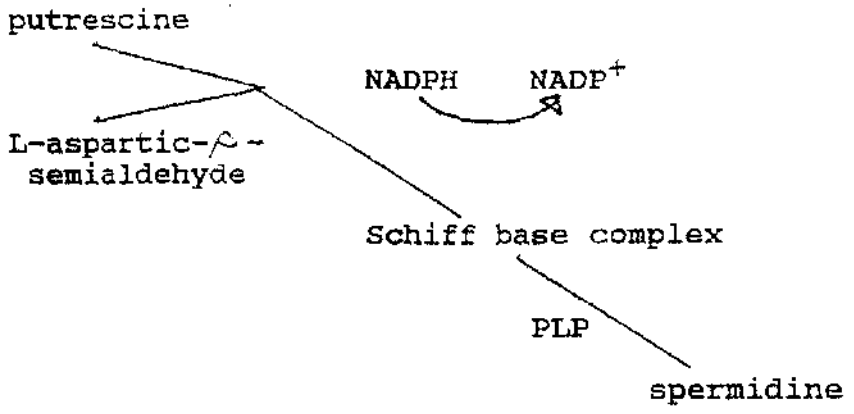
In polyamines which have only aminopropyl groups

Fig. 5: Pathways for the biosynthesis of spermidine (a & b) norspermidine (c) and Homospermidine (d) (Tait, 1985).

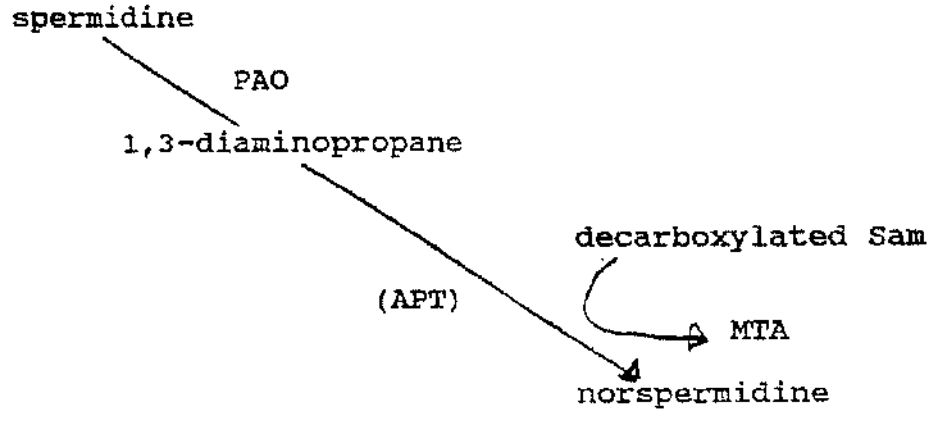
(a)


decarboxylated
Sam
MTA
putrescine
spermidine
aminopropyltransferase
(APT)

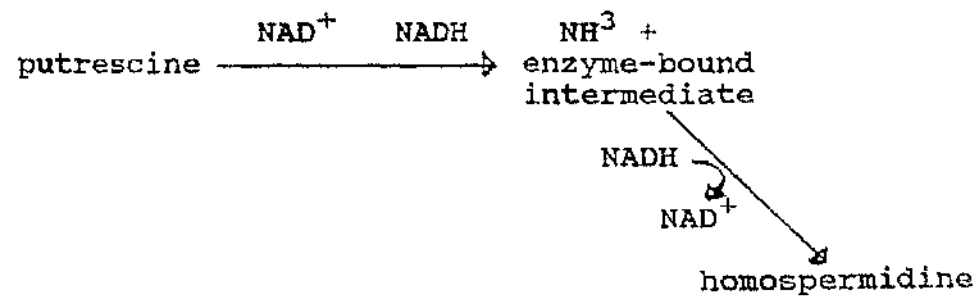
(b)


putrescine
NADPH
NADP+
L-aspartic-β-
semialdehyde
Schiff base complex
PLP
spermidine

(c)


spermidine
PAO
1,3-diaminopropane
decarboxylated Sam
(APT)
MTA
norspermidine

(d)


NAD+
NADH
NH3 +
putrescine
enzyme-bound
intermediate
NADH
NAD+
homospermidine

(norspermidine, norspermine, caldopentamine) or aminobutyl groups (homospermidine, aminobutylhomospermidine) synthesis probably occurs *via* reactions c) and d) respectively (Fig. 5) (Tait, 1985).

1.4.3 Cadaverine and its Aminopropyl Derivatives

In tumour cells (Janne *et al.*, 1981) and Chinese hamster ovary cells (Holtta and Pohjanpelto, 1983) depleted of SPD and SPM by treatment with the ODC inhibitor DFMO, the formation of cadaverine (CAD: a diamine formed by the decarboxylation of lysine) is induced. A similar effect has been observed in the mycorrhizal fungus *Paxillus involutus* (Zarb and Walters, 1994). Removal of ORN from the growth medium had the same effect on the ovary cells (Holtta and Pohjanpelto, 1983). The cadaverine is converted to aminopropylcadaverine (APC) and N,N-*bis*(3-aminopropyl)cadaverine (3APC) by the addition of aminopropyl moieties in a manner analogous to the formation of SPD from PUT and SPM from SPD (Fig. 5a) (Janne *et al.*, 1981). These authors suggest that cadaverine is formed from lysine by the action of ODC. ODC from rat liver has been shown to catalyse this reaction. However, the affinity of the enzyme for ornithine is around 100 times greater than for lysine (Pegg and McGill, 1979). It is thought that these compounds can substitute for PUT, SPD and SPM in some cellular functions (Janne *et al.*, 1981). In tumour cells treated with DFMO, and in which PUT and SPD

levels were reduced, conversion of lysine to CAD and APC was high. However, cell growth was much slower and they behaved like polyamine depleted cells (Alhonen-Hongisto and Janne, 1980). Although the role of CAD and its aminopropyl derivatives in animal cells is not known, they suggest that this alternative pathway may allow cells to remain alive during depletion of the common polyamines (Holtta and Pohjanpelto, 1983). In mutant forms of *N. crassa*, which were unable to synthesise PUT, de-repression of an ODC with weak lysine decarboxylase (LDC) activity allowed the synthesis of CAD and APC. This allowed a reduced rate of growth to continue (Tabor and Tabor, 1985). Blocking PUT biosynthesis in *E. coli* allowed synthesis of CAD and its aminopropyl derivatives by an inducible LDC, which was specific for lysine (Tabor and Tabor, 1985).

1.4.4 Inhibitors of Polyamine Biosynthesis

To assist studies on the functioning of the pathways of polyamine biosynthesis, a number of inhibitors of the enzymes of these pathways have been developed. Difluoromethylornithine (DFMO) and difluoromethylarginine (DFMA), which inhibit ODC and ADC respectively, are enzyme-activated irreversible inhibitors. The α-carbon of ornithine and arginine are substituted by a difluoromethyl group (Galston & Kaur-Sawhney, 1990). It is known that DFMO is decarboxylated by ODC.

MGBG, an inhibitor of *S*-adenosylmethionine decarboxylase (SAMdc) may inhibit by competition for an uptake system and is a reversible, non-specific inhibitor of spermidine synthase (Porter & Bergeron, 1988). This compound is also a potent inhibitor of diamine oxidase and, in addition, induces SPD/SPM N'-acetyltransferase. MGBG has been shown to accumulate in mammalian cells and cause damage to mitochondria. There may also be less direct effects on the synthesis of macromolecules such as DNA (Pegg, 1988). Pegg (1988) suggests that an increase in the level of SAMdc on treatment with MGBG is due to enzyme stabilisation, preventing proteolytic degradation.

AdoDato is a specific and potent inhibitor of spermidine synthase (Porter & Bergeron, 1988). This compound is a 'transition state analogue'. Treatment of cancer cells with AdoDato decreased spermidine but increased spermine concentrations and caused derepression of SAMdc (Pegg, 1988).

DMTA, an analogue of methylthioadenisine (MTA) (Fig. 3), is a potent inhibitor of spermine synthase and also inhibits SAMdc (Porter & Sufrin, 1986). MTA is produced by the aminopropyl transferases during polyamine biosynthesis. It does not accumulate in the cell due to rapid breakdown by MTA phosphorylase, to adenine and 5-methylthioribose 1-phosphate. DMTA may act by mimicking the observed feed-back regulation of spermidine and spermine synthase by MTA. This compound also inhibits

spermidine synthase at high concentrations (Pegg, 1988).

AdoDato and DMTA are analogues of *S*-adenosyl methionine (AdoMet). Holm *et al.* (1989) have suggested that there may be competition between AdoMet and AdoDato for the active site on spermidine synthase.

1.5 Aims and Objectives.

When alternative pathways for synthesis of a compound exist in plants and fungi, inhibition of the fungal pathway may be fungitoxic without harming the plant. For example, two pathways for the synthesis of polyamines are known to exist: the arginine decarboxylase (ADC) pathway and the ornithine decarboxylase pathway (ODC). Both pathways are present in plants, but only ODC is present in most phytopathogenic fungi. Inhibition of the enzymes of this latter pathway *in vivo* has been shown to be fungicidal in many plant/pathogen systems (e.g. West and Walters, 1988 a & b; Foster and Walters, 1990; Walters and Kingham, 1990; Walters, Havis, Foster and Robins, 1992; Walters, 1995).

Since two pathways are known to exist for lysine biosynthesis, and work on *N. Crassa* and *S. cerevisiae* suggests that all higher fungi might utilise the AAA pathway which is absent from plants, the possibility exists that specific inhibition of the fungal pathway might be fungicidal.

Although lysine biosynthesis and catabolism in barley have been examined by Moller (1974 and 1976 a & b), little work has been done on lysine metabolism in powdery mildew-infected leaves or in the mildew fungus. This project aims to examine lysine biosynthesis and catabolism in barley, the powdery mildew fungus and in the barley:powdery mildew interaction. The objective is to determine whether barley and the powdery mildew fungus do indeed utilise different pathways for lysine biosynthesis, and also to show how mildew infection alters the metabolism of lysine in barley. Such information is important in helping to determine the suitability of lysine biosynthesis as a novel target for future fungicide development.

More specifically, this project will examine changes in lysine levels in healthy and mildewed leaves as mildew infection progresses. A study of lysine biosynthesis and catabolism in infected leaves, in pustules and in isolated mildew mycelium will also be carried out. This will include measurement of the activity of selected enzymes of both pathways, to establish if these are indeed present in the leaves, the fungus and/or the host/fungus combination. By feeding infected leaves with labelled precursors it should be possible to determine if the fungus takes these up from the leaf and if they are metabolised by the fungus. Lysine biosynthesis is thought to be carried out in the chloroplast and structural and functional changes are known to occur in these organelles during powdery mildew

infection (Walters, 1985). An examination of enzyme activity in isolated chloroplasts during the course of infection will therefore be carried out.

Since any changes seen in the synthesis of the diamine cadaverine (a metabolite of lysine) and its derivatives (APC and 3APC) during infection may affect the synthesis of lysine, an examination of this pathway both in the leaves and in the mycelium, will be carried out. Several inhibitors of polyamine biosynthesis, known to be effective in blocking key enzymes, will be employed in these studies.

2. MATERIALS AND METHODS

2.1 General Materials and Methods.

2.1.1 Growth and Maintenance of Plants before Inoculation.

Barley plants (*Hordeum vulgare* L. cv. Golden Promise) were grown and maintained in a ventilated glasshouse under a day/night temperature regime of 24/12^{o}C. Natural daylight was supplemented with 400W mercury vapour lamps to give a 16 hour photoperiod.

Seeds were sown (30) in Fisons Levington M3 compost, in half size seed trays (156 x 208mm). Plants emerged approximately 4 days after sowing and were inoculated 8 days later.

2.1.2 Maintenance of the Pathogen.

Golden Promise seeds were sown every week (3 x 25 seeds in 152mm pots) and when the plants were 7 days old they were inoculated with spores of the powdery mildew fungus *Erysiphe graminis* f.sp. *hordei* Marchal and maintained under the same conditions as described in Section 2.1.1. In this way there was a continuous supply of fresh spores available to be used for plant inoculation.

2.1.3 Inoculation of Plants.

Spores from a pot of freshly sporulating stock plants were shaken over the trays and allowed to settle for a few hours. The inoculated and uninoculated (control) plants were maintained under the conditions described in Section 2.1.1.

2.1.4 Harvesting and Storage of Leaves.

First leaves of barley plants were harvested (1, 3, 6, 9 and 15 days after inoculation (DAI)) and, if not required immediately, frozen (-18^{o}C) until required. Pustules and inter-pustules (the green areas between pustules) were obtained from fresh leaves (9 DAI) by cutting around the infected areas with a sharp scalpel and placing them immediately into a universal bottle surrounded with ice. These were frozen until required.

2.1.5 Protein Estimation.

Protein was estimated using the method described by Lowry, Rosebrough, Farr and Randall (1951). The standard curve used is shown in the Appendix, Fig. B.

2.1.6 Statistical Analysis.

All values shown (except those in Table 2 - Section 5 and DHDPS work) are the means of two to five replicates and most experiments were repeated two or three times. Results were analysed using Student's t-test on Minitab Data Analysis Software Release 6.1.1.

2.1.7 Chemicals.

Chemicals were obtained from Sigma Chemical Co. Ltd., Poole, Dorset and Aldrich Chemical Co. Ltd., Gillingham, Dorset. TLC plates were also supplied by Sigma Chemical Co. Ltd. White 'unwire' test tube racks were supplied by Merck Ltd. (BDH), Thornliebank, Glasgow.

Aspartic-β-semialdehyde was kindly supplied by Professor D. J. Robins, Chemistry Department, Glasgow University.

2.1.8 Radioisotopes.

L-[U-^{14}C]aspartic acid, (DL-*meso*)-2,6-diamino[1,7-^{14}C]pimelic acid hydrochloride and L-[U-^{14}C]lysine

monohydrochloride were supplied by Amersham International PLC, Aylesbury, Buckinghamshire and ICN Biomedicals Ltd., High Wycombe, Buckinghamshire.

2.2 Lysine Concentrations in Healthy Barley Leaves and in Leaves Inoculated with *Erysiphe graminis*.

2.2.1 Determination of Lysine.

2.2.1.1 Extraction of Soluble and Hydrolysed Lysine.

Leaves were prepared by the method of Wade *et al.* (1980).

Leaves were cut into small pieces (1g; 1.0 - 2.0 mm) and placed in a boiling tube with aqueous ethanol (10ml; 70% ethanol; 80°C for 3 min). The liquid was carefully decanted. This was repeated once more with ethanol and once with distilled water. All of the decanted liquid (the soluble fraction) was combined and dried down on a rotary evaporator (Buchi Rotavapor R110; 30 - 40°C) and taken up in sterile distilled water (5ml). Distilled water (3ml) was added to the plant material left after extraction. This was sonicated (10 min at half power; MSE Soniprep) and the same volume of hydrochloric acid (6M) added before flushing with nitrogen. The sealed tubes were maintained at 110°C for 20h. The resulting hydrolysate was then filtered through glass wool and dried on the rotary evaporator (60°C). The evaporator was allowed to run for 20 min after the samples became dry, to ensure complete removal of the

hydrochloric acid. These samples were reconstituted by adding sterile distilled water (5ml) (hydrolysed or bound fraction).

2.2.1.2 Preparation of Lysine Decarboxylase (EC 4.1.1.18).

The enzyme (Sigma Type 1 from *Bacterium cadaveris*) was prepared, according to the method of Hutzler (1968), immediately before use. This was done by dispersing it in maleate buffer (0.02M; 0.4ml g^{-1} enzyme) held on ice. The suspension was centrifuged (MSE Europa 24M; 1000g (3000 r.p.m.) for 10 min; 4°C). The supernatant was discarded and the enzyme resuspended in maleate buffer (0.2M; 0.2ml g^{-1} enzyme) containing pyridoxal 5-phosphate (Pyridoxal-5-phosphoric acid; Co-decarboxylase) and used immediately.

2.2.1.3 Preparation of 1-Fluoro-2,4-dinitrobenzene (FDNB).

FDNB (4mg ml^{-1}) was dissolved in absolute ethanol shortly before use and kept in the fridge until required. Health and safety information contained in the Hazard Data Sheets (BDH) was consulted and precautions observed during the preparation of this solution.

2.2.1.4 Lysine Assay.

The method of Hutzler, Odievre and Dancis (1967) was used to measure lysine levels. The standard curve used is shown in the Appendix, Fig. A.

Following the addition of the prepared lysine decarboxylase solution (0.5ml) to sample (0.5ml) and water (0.5ml), the tubes (16 x 125mm) were incubated (45 min at 37^{o}C). After incubation, zinc sulphate (2ml; 0.3M) and sodium hydroxide (2ml; 0.5M) were added: these were allowed to run slowly down the sides of the test tubes. The tubes were capped, inverted and centrifuged (1500g (2400 r.p.m.); 20 min).

Aliquots (2ml) from each supernatant were pipetted into clean (13 x 100mm) test tubes. Borate buffer (0.4ml; 1.0M) and FDNB (2ml) were added. Samples were mixed well and incubated in a water bath (30 min at 60^{o}C). Potassium hydroxide (1ml; 1M) and chloroform (4ml) were added to each sample and shaken well, centrifuged (5min; 500g (2000 r.p.m.)) and absorbance of the lower, chloroform, phase read (400nm; Gallenkamp Visi-spec). Reagent blanks (distilled water + 0.2M maleate buffer) and sample blanks (sample + 0.2M maleate buffer) were used in each experiment. Lysine standards were also run in each experiment.

2.3 Biosynthesis and Catabolism of Lysine in Barley Leaves and Chloroplasts.

2.3.1 The Diaminopimelic Acid Pathway.

2.3.1.1 Assay of Diaminopimelic Acid Decarboxylase (EC 4.1.1.20) Activity.

Whole leaves:

All sample preparation procedures were carried out between 0 - 5°C. Using a modification of the method of Sadler & Shaw (1980) whole leaves (1g) were cut (1.0 - 2.0mm) with scissors and ground in buffer (0.1M Tris-HCl [tris(hydroxymethyl)aminomethane hydrochloride]; pH 8.0; 10ml) containing 2-mercaptoethanol (10mM). After sonication (MSE Soniprep 150; 3 times for 10s on and 10s off) the sample was filtered and squeezed through muslin (4 layers) and centrifuged (20000g (15500 r.p.m.); 30 min). The supernatant was used as the enzyme source. Aliquots of supernatant (0.5ml) were added to (DL-*meso*)-2,6-diamino[1,7-^{14}C]pimelic acid (DAP) (0.0185 MBq; specific activity 4.33 GBq mmol^{-1}; 5 μl). The tubes were closed with tight-fitting rubber stoppers and incubated (37°C; 30 min). The stoppers were fitted with syringe needles holding filter paper squares (0.5 x 0.5cm; Whatmans No 1 qualitative) impregnated with potassium hydroxide (KOH; 10 μl; 2M). After incubation the tubes were removed to a tray of iced water until trichloroacetic acid (TCA) (0.2ml; 6%) was added. The stoppered tubes were then replaced in the water bath for a further 30 min. The filter papers were

removed and placed in scintillant (10ml; Packard Emulsifier Safe) and left overnight before reading on a liquid scintillation analyser (Canberra Packard Tri-carb 1900 TR). Each sample was replicated (5 times) and blanks (3; TCA added before incubation) were used in each experiment.

Chloroplasts:

Chloroplasts were isolated by the method of Ahmad, Farrar and Whitbread (1983).

Plants used for chloroplast isolation were obtained early in the light period and placed in the dark (1h) before harvesting. This removed any starch stored in the chloroplasts and lessened the risk of damage during the isolation procedures. Samples were prepared by cutting leaves (2g) into small pieces (1.0 - 2.0mm). These were homogenised in isolation medium (10ml) (Appendix) in a Waring blender at the 'Hi' setting (5 x 1s). The homogenate was filtered and squeezed through muslin (4 layers) and the filtrate centrifuged (1500g (4000 r.p.m.); 5min). The supernatant was discarded and the chloroplasts gently resuspended in buffer (Tris-HCl; pH 8.0; 4ml) containing 2-mercaptoethanol (10mM), using a brush made from fine nylon mesh (Henry Simon, London).

The chloroplast suspension was sonicated as described for whole leaves and DAP decarboxylase activity was measured by a modification of the method used for whole

leaves. The volumes of sample and substrate were halved in order to conserve ^{14}C-DAP. The remainder of the assay was carried out as described for whole leaves.

2.3.1.2 Assay of Dihydrodipicolinic Acid Synthase (EC 4.2.1.52) Activity.

Whole leaf and chloroplast samples were prepared as described for the DAP decarboxylase assay (Section 2.3.1.1), using a different grinding (whole leaves) and resuspension (chloroplasts) buffer (50mM potassium phosphate (pH 8.0), 10mM sodium pyruvate, 5mM ethylenediaminetetraacetic acid, disodium salt [EDTA] and 10mM 2-mercaptoethanol) (Wallsgrove and Mazelis, 1981). The supernatant (whole leaves) and disrupted chloroplast isolates were dialysed overnight against potassium phosphate buffer (20 volumes; 50mM; pH 8.0) containing sodium pyruvate (10mM). Dialysis was necessary to remove the 2-mercaptoethanol, an inhibitor of dihydrodipicolinic acid synthase (DHDPS) (Wallsgrove and Mazelis, 1981). Aliquots of the dialysed sample (0.25ml) were added to tubes along with the reaction medium (Tris-HCl; 100mM; pH 8.5; 100mM sodium pyruvate; 5mM aspartic-β-semialdehyde; 0.25ml) (Wallsgrove & Mazelis, 1980). Sample blanks without aspartic-β-semialdehyde (ASA) were also run. Reagent blanks, containing dialysis buffer (0.25ml) and reaction medium without ASA (0.25ml) were included. The tubes were incubated (30^{o}C; 30 or 60 min) and the reaction

stopped by adding sodium citrate/sodium phosphate buffer (0.22M/0.55M; pH 5.0; 2ml) containing *p*-dimethylaminobenzaldehyde (0.25mg ml^{-1}) which was previously dissolved in a minimum of cold absolute ethanol. The colour was allowed to develop (2h; at room temperature) before the samples were centrifuged (500g (2000 r.p.m.);10 min) and absorbance was read (520nm).

2.3.1.3 Assay of Lysine Decarboxylase (EC 4.1.1.18) Activity (Icekson, Bakhanashvili and Apelbaum, 1986).

Samples were prepared as described for the diaminopimelic acid (DAP) decarboxylase assay (Section 2.3.1.1) except for the extraction buffer (Tris-HCl; 50mM; pH 8.0; 0.5mM EDTA; 5mM dithiothreitol (DTT)). The filtrate was centrifuged (5000g (7500 r.p.m.); 15 min) and the supernatant used as the enzyme source.

Aliquots of the enzyme extract (50μl) were added to L-[U-^{14}C]lysine monohydrochloride (7.34×10^{-1} MBq; specific activity 11.86 GBq $mmol^{-1}$; 4 μl) and reaction medium (Tris-HCl; 10mM; pH 8.0; 1mM DTT; 0.1mM EDTA; 5mM L-lysine; 0.1mM pyridoxal 5-phosphate (added immediately before use); 0.2ml). The samples were incubated (45^{o}C; 1h) and subsequent treatment and recovery of the ^{14}C-CO_2 was that used for the DAP decarboxylase assay.

2.3.2 The Aminoadipic Acid Pathway.

2.3.2.1 Assay of Aminoadipate Reductase (EC 1.2.1.31) Activity.

Leaves (1g) were ground in buffer (0.1M Tris-HCl, 10mM 2-mercaptoethanol; pH 8.0), sonicated (10s on, 10s off; 3 times), immediately filtered through four layers of muslin and centrifuged (2500g (5500 r.p.m.); 30min; 4°C). All procedures were carried out using pre-chilled buffers and equipment, which was kept on ice during sample preparation. Mycelium (from 10 leaves) was collected using a soft camel hair brush and shaken into iced extraction buffer (see above). This was sonicated (10s on, 10s off; 6 times) and treated as for leaves, after filtration through four layers of muslin.

Supernatant (0.5ml) was added to reaction medium (6 μmoles α-aminoadipic acid (AAA), 2 μmoles adenosine 5'-triphosphate (ATP), 2 μmoles magnesium chloride ($MgCl_2$), 0.09 μmoles manganese chloride ($MnCl_2$), 0.024 μmoles - nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), 0.1 μmole glutathione (GSH); 0.5ml; pH 6.5) and incubated (2.5h at 37°C) (Sagisaka & Shimura, 1962b).

Estimation of α-aminoadipic acid-β-semialdehyde was carried out by the method of Sagisaka and Shimura (1962b). This involved addition of *p*-dimethylaminobenzaldehyde solution (0.5ml; 0.5g in 5ml 10M HCl added to 20ml acetone (Edwards, 1970) and potassium phosphate buffer (0.2ml of

0.25M; pH 7.0) to aliquots (0.5ml) of the incubated samples. This was heated (100°C for 20min), cooled to room temperature and the volume adjusted (3ml) with aqueous ethanol (ethanol:water, 60:40 v/v). The samples were centrifuged (2500g (5500 r.p.m.); 10min) and absorbance of the supernatant read at 480 nm. Water was added to blanks in place of AAA.

2.3.2.2 Assay of Saccharopine Dehydrogenase (EC 1.5.1.7) Activity.

Samples were prepared as for the ∝-aminoadipic acid reductase assay (Section 2.3.2.1), using phosphate extraction buffer (5mM dipotassium phosphate (K_2HPO_4), 10mM 2-mercaptoethanol, 1mM EDTA).

Supernatant (0.5ml) was added to reaction medium (100 μmoles L-lysine.HCl, 20 μmoles μ-ketoglutarate, 250 μmoles potassium phosphate buffer (pH 7.0), 0.25 μmoles β-nicotinamide adenine dinucleotide (NADH); 1.5ml) (Saunders and Broquist, 1966). The tube was inverted to mix the contents and the change in absorbance was read (340nm; 30s intervals for 5min; room temperature). A blank containing ∝-ketoglutarate (20 moles in 2ml) was used.

2.4 Uptake and Metabolism of ^{14}C-Aspartate.

2.4.1 Feeding with ^{14}C-Aspartate.

Plants were removed from the trays (6 DAI) and the

first leaves excised under water. These were immediately transferred to Pyrex test tubes (12 x 75mm; 1 leaf per tube) containing distilled water (50μl; pH 7.5) and L-[U-^{14}C]aspartic acid (0.085MBq; specific activity 7.96GBq mmol^{-1}; 10μl) and allowed to take up solution for 2, 4 or 8h. Test tubes were placed vertically in racks and incubated (22^{o}C; 70% RH; 237 μmol m^{-2} s^{-1} PAR* [natural daylight]) during uptake. Healthy leaves from healthy plants, infected leaves with mycelium attached and infected leaves with mycelium removed before feeding were used in these uptake studies. The mycelium was removed from some leaves after feeding (using small pieces of damp cotton wool) and, in all cases, tissues were extracted as described below. After uptake, the cut ends were washed in distilled water (0.2ml x 3), dried on tissue paper and the leaves weighed. These were then immediately frozen in liquid nitrogen and stored until required.

2.4.2 Extraction.

The frozen leaves were finely chopped and added to methanol + chloroform + distilled water (12 + 5 + 3 by volume; M+C+W; 2ml) and kept at -18^{o}C until required. Extraction was carried out using a modification of the method of Bielski and Turner (1966). The leaves were

* Photosynthetically active radiation.

homogenized, using a Potter Elvehjem homogeniser, at room temperature and decanted into centrifuge tubes. The homogeniser was washed out with M+C+W (1ml), the washings added to the centrifuge tube for centrifugation (5000g for 5 min) and the supernatant decanted into a glass vial (10ml). The pellet was re-suspended in M+C+W (2ml) and shaken for 5 min, re-centrifuged as above and the supernatant added to the glass vial. Chloroform (1ml) and water (1.5ml) were added to the M+C+W extract, which was then shaken and the chloroform layer, separated by centrifuging as above, was removed and discarded. The pellet remaining after the second M+C+W extraction was re-extracted in hot ethanol + water (80 + 20 by volume; 80^{o}C; 2ml). The sample was shaken for 4 min and centrifuged as above. The supernatant was added to the M+C+W extract. This was repeated a further three times and the combined extracts (soluble label/pool) dried down under vacuum at 35^{o}C and taken up in propan-2-ol + water (10 + 90 by volume; 2ml).

Fungal samples were extracted by the method described for leaves.

2.4.3 Oxidation.

The pellet remaining after the final extraction step (insoluble label/pool) was removed from the tube with tissue (3cm^{2}), added to two paper oxidising thimbles and the whole oxidised (1 min) in a Canberra Packard Oxidiser

(model 306 M).

2.4.4 Thin Layer Chromatography (TLC).

Samples (50μl for plant or 100μl for fungal extracts) were spotted onto TLC plates (K2F Whatman, 20 x 20cm cellulose) and run sequentially, in the same direction, in the solvent systems: butan-2-one + acetone + pyridine + water + formic acid (35 + 35 + 15 + 15 + 2 by volume) and propan-2-ol + water + formic acid (20 + 5 + 1 by volume). The plates were dried and then scanned for radioactivity using a Stratec Raytest Rita-3200 Radio-TLC-Analyser. Plant samples were scanned for 1000s and fungal samples for 2000s and peak areas expressed in terms of total counts over the time period.

2.4.5 Pre-feeding.

Leaves for pre-feeding were placed in distilled water (200μl) containing lysine (10mM) or threonine (10mM) for 2h before feeding for 4h, as described above. The leaves were then processed as described above.

2.5 Studies on Uptake Kinetics using Isolated *Erysiphe graminis* Mycelium.

2.5.1 Introduction.

The isolation method used here was first used by

Gregory (1992) to isolate *E. graminis* mycelium from barley leaves for the study of carbohydrate uptake.

2.5.2 Preparation of Isolated Mycelium.

The mycelium was removed from approximately 100 first leaves of barley, using a wetted spatula. This was placed in homogenising medium (20ml; Appendix) and stirred to produce a fine suspension. Aliquots (2ml) of the prepared mycelium were pipetted into centrifuge tubes, centrifuged (5800g (8500 r.p.m.); 10min) and the supernatant discarded.

2.5.3 Measurement of Optimum pH for the Uptake of Aspartate and Lysine.

Mycelium was prepared as described in Section 2.5.2. The mycelium in each tube was resuspended in homogenising medium (0.5ml; containing aspartate or lysine; 1mM). The pH of each sample was adjusted (pH 4.0, 5.0, 6.0, 7.0, 8.0 or 9.0) with hydrochloric acid or potassium hydroxide. Aliquots (0.1ml) were added to labelled aspartate or labelled lysine (1μl) and incubated (40 min; room temperature). Ice-cold homogenising medium (2ml) was added before centrifuging (5800g; 10 min). This step was repeated once more. The mycelium was resuspended in water (0.5ml), by rotamixing briefly, and decanted into a scintillation vial. The tubes were further washed (2 x 0.5ml Soluene (Packard)) before incubation (3h; 60^{o}C). The samples were neutralized (25% (v/v) acetic acid); 0.2ml) and left to stand in Hionic Fluor (10ml) for 24h.

Disintegrations per minute (DPM) were measured using a Packard 1900 TR 'Tri-Carb' scintillation counter.

2.5.4 Time-Course of Aspartate or Lysine Uptake.

Mycelium was prepared for this experiment as described in Section 2.5.2. After resuspension in homogenising medium (pH 7.0; 7ml; containing aspartate or lysine; 1mM) aliquots (0.1ml) of the uniformly dispersed mycelial suspension were incubated with labelled aspartate or labelled lysine (1μl) for 10, 20, 30, 60, 90, 120 and 240 min. At the end of each time period the reaction was stopped by adding ice cold homogenising medium (2ml). All samples were then treated as in Section 2.5.3.

2.5.5 Measurement of Lysine Uptake at different Concentrations.

Mycelium was prepared as in Section 2.5.2. The mycelium in each tube was resuspended in the same buffer (pH 7.0; 1.5ml) containing lysine at different concentrations (0.005, 0.01, 0.1, 1.0, 10, 25, 50 & 100mM). Aliquots (0.4ml) were added to ^{14}C-lysine (1μl) and incubated (40 min; 25^{o}C). All samples were then treated as in Section 2.5.3.

2.5.6 The Effects of Amines and a Metabolic Inhibitor on the Uptake of Lysine by Isolated *E. graminis* Mycelium.

Mycelium was prepared as in Section 2.5.2. The mycelium in each tube was resuspended in homogenising medium. Both lysine stock solution and the amine (ornithine, arginine, putrescine) or inhibitor under test (sodium azide) were added to give the same molarity (1mM). Labelled lysine (2.5μl) was added at the same time. Samples were incubated (40 min; 25^{0}C) and treated thereafter as in Section 2.5.3.

2.6 Formation of Cadaverine Derivatives in Barley Leaves and Powdery Mildew Mycelium.

2.6.1 Leaves.

2.6.1.1 Leaves not pre-treated with Inhibitors.

Leaves were prepared and the lysine decarboxylase assay carried out as described in Section 2.3.1.3 with the exception that the inhibitor solutions (3mM; MGBG/CHA) were added at the start of the assay. A similar volume of buffer was added to the control samples. At the end of the assay period, perchloric acid (10%) was added to terminate the reaction in place of TCA, used in the original protocol. The reaction medium was retained for labelled polyamine determination (Section 2.6.3).

2.6.1.2 Leaves Pre-treated with Inhibitors.

This work was carried out in order to determine the

effect of pre-treatment with the inhibitors used in Section 2.6.1.1.

Leaves were cut up ($\simeq$ 1mm) and placed in buffer (10mM Tris-acetic acid, pH 6.0; 0.5mM calcium chloride; 5 ml g^{-1}) containing the inhibitors (3mM; MGBG/CHA). These were incubated in the light on a reciprocal shaker (120 strokes/min; 2h; 25^{o}C). The molarity of the buffer in each sample was adjusted to 1mM by the addition of unlabelled lysine from a stock solution and at the same time, labelled lysine was added (5 μl ml^{-1}) and the samples incubated as above (2h). Uptake was stopped by adding ice cold distilled water (10ml). This was removed by pipette and the same step repeated (x2) after which the leaves were stored until required (-18^{o}C). Samples were prepared and the LDC assay carried out as described in Section 2.3.1.3.

2.6.2 Isolated Mycelium.

2.6.2.1 Isolated Mycelium not Pre-treated with Inhibitors.

Mycelium was isolated by the method used previously (Section 2.5.2) using the buffer described in Section 2.3.1.3 (lysine decarboxylation medium) and stirred to ensure a uniform suspension. This was held on ice during sonication (10s on and 20s off 10 times, MSE Soniprep) and centrifuged (5000g (7500 rpm); 15 min). The supernatant was adjusted to 1mM with a stock lysine solution. Aliquots (250μl) were added to ^{14}C-lysine (4μl) and incubated as for

the lysine decarboxylase assay (Section 4.2.3). The reaction medium was retained for analysis of labelled polyamines (Section 2.6.3).

2.6.2.2 Isolated Mycelium Pre-treated with Inhibitors.

Again, this work was carried out in order to assess the effects of pre-treatment with the inhibitors used. Mycelium was isolated by the method used previously (Section 2.5.2) using the buffer described in Section 2.3.1.3 (Tris-HCl reaction medium) and stirred to ensure a uniform suspension. Aliquots (1ml) of the mycelial suspension were added to each tube and inhibitor solution (1ml) (made up at double the required molarity) added to bring the solution to the required molarity. A similar volume of buffer was added to control samples before incubation (25; 2h). After the incubation period the samples were sonicated as above and all subsequent treatments were the same (Section 2.6.2.1).

2.6.3 Determination of Labelled Polyamines by Dansylation (Zarb & Walters, 1993).

Aliquots (100 μl) of the reaction medium remaining after lysine decarboxylase assay were added to saturated sodium carbonate (200μl) and dansyl chloride (10mg ml^{-1} acetone; 400μl). These were incubated in the dark (60^{o}C; 30 min). Proline (100 μg ml^{-1} water; 100μl) was added and the samples incubated for a further 10 min at room

temperature. Toluene (500μl) was then added and the samples shaken (20s). Aliquots (25μl) of the toluene layer were spotted onto silica gel TLC plates, activated by heating at 110^{o}C for 90min (Whatman LK6D). After drying the spots, the plates were run in chloroform:triethylamine (12:1 v/v; approximately 40 min). The plates were then allowed to dry in a fume hood (15 min) and cadaverine, aminopropylcadaverine (APC) and *N,N'-bis*(3-aminopropyl)cadaverine (3APC) standards, run on the same plate as markers, were traced under UV light. Areas with the same Rf value as the standards were scraped from the plates and placed in scintillant (10ml) and DPM calculated as before (Section 2.3.1.1).

3. RESULTS

3.1 Lysine Levels and the Activities of some Enzymes of the DAP and AAA Pathways of Lysine Biosynthesis

3.1.1 Lysine in Healthy and Powdery Mildew Infected Barley Leaves

The levels of soluble and bound lysine were examined in various tissues from both healthy first leaves of barley and leaves inoculated with *E. graminis* (powdery mildew), at various times after inoculation.

Concentrations of soluble lysine were lower than concentrations of bound lysine, both in healthy leaves and in those inoculated with powdery mildew (Figs 6.1 & 6.2).

This was also true in pustules and inter-pustule regions isolated from infected leaves 9 days after inoculation (9 DAI) (Fig. 6.3). A comparison of healthy and infected leaves showed that soluble lysine levels were significantly lower in infected leaves, except at 3 DAI when the opposite was true (Fig. 6.1). Bound lysine levels were significantly lower in infected leaves from 6 - 15 DAI (Fig. 6.2). Both soluble and bound lysine levels were significantly lower in pustules than in inter-pustules at 9 DAI (Fig. 6.3).

3.1.2 Lysine Biosynthesis in Healthy and Powdery Mildew-Infected Barley Leaves: Activities of some of the Enzymes of the Diaminopimelic Acid Pathway

The activities of the first (dihydrodipicolinic acid synthase) and the last (diaminopimelic acid decarboxylase) enzymes in the diaminopimelic acid pathway of lysine biosynthesis were examined in healthy and mildew infected first leaves of barley, at different times after inoculation. The activities of these enzymes were also measured in pustules and inter-pustule regions taken from infected leaves, and in chloroplasts isolated from both healthy and infected leaves. The activity of the enzyme which catalyses the synthesis of cadaverine from lysine (lysine decarboxylase) was also measured in these tissues.

3.1.2.1 Diaminopimelic Acid (DAP) Decarboxylase Activity

The activity of this enzyme was consistently greater

in infected leaves (Fig. 7.1), though this difference was only significant at one day after inoculation (1 DAI) and 12 DAI. Enzyme activity was significantly greater in inter-pustule regions than in pustules at 9 DAI (Fig. 7.2).

However, DAP decarboxylase activity was greater in chloroplasts isolated from infected leaves at 3 DAI, although it was reduced thereafter (Fig. 7.3).

3.1.2.2 Dihydrodipicolinic Acid Synthase (DHDPS) Activity

One day after inoculation (1 DAI) the activity of this enzyme was similar in healthy and infected leaves (Fig. 7.4). By 3 DAI however, activity was reduced in infected leaves. DHDPS activity in the pustule regions was similar to that observed in controls (Fig. 7.5) whereas activity in inter-pustule regions was somewhat greater than in healthy leaves.

In isolated chloroplasts from infected leaves, DHDPS activity was consistently less than in those isolated from healthy leaves (Fig. 7.6). This was especially evident at 6 and 9 DAI.

3.1.2.3 Lysine Decarboxylase Activity

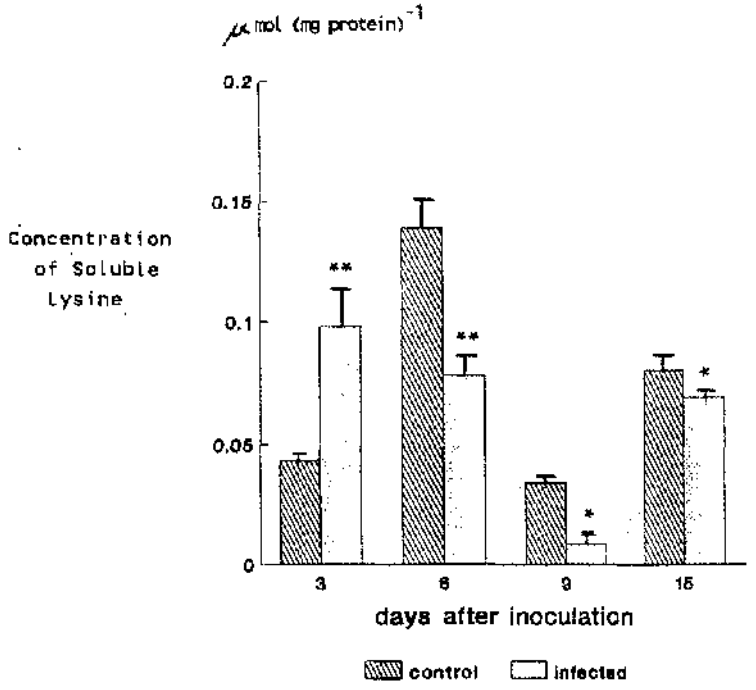
Lysine decarboxylase activity (LDC) was significantly greater in infected leaves at one day after inoculation (1 DAI), but was significantly reduced at 3 - 9 DAI (Fig. 7.7). Enzyme activity was significantly reduced in both

pustules and inter-pustule regions compared to healthy leaves at 9 DAI (Fig 7.8).

Although enzyme activity was reduced in chloroplasts from mildewed leaves 1 DAI, it was increased in chloroplasts from mildewed leaves thereafter (Fig. 7.9). LDC activity in chloroplasts isolated from pustules and inter-pustule regions (6 DAI) and inter-pustules regions (9 DAI) was greater than in control leaves. However LDC activity in chloroplasts isolated from pustules at 9 DAI was less than in healthy leaves (Fig. 7.10).

Fig. 6.1: Soluble lysine in healthy (control) and mildew infected first leaves of barley at different times after inoculation. Significant differences shown as p = 0.1 (*); p = 0.05 (**).

Fig. 6.2: Bound (hydrolysed) lysine in healthy (control) and mildew infected first leaves of barley at different times after inoculation. Significant differences shown as p = 0.1 (*); p = 0.05 (**).


μ mol (mg protein)$^{-1}$
Concentration of Soluble Lysine
0.2
0.15
0.1
0.05
0
**
**
*
*
3
6
9
15
days after inoculation
control
infected

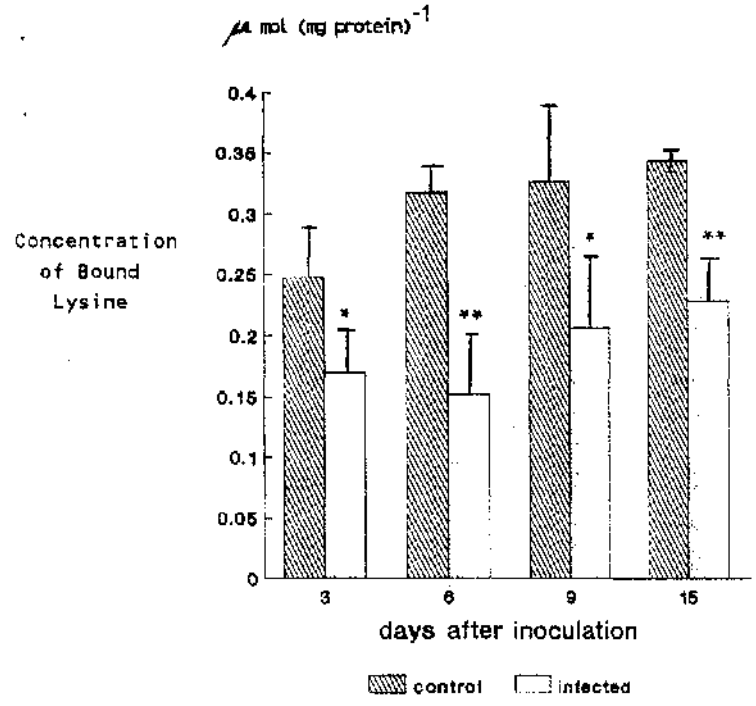

μ mol (mg protein)$^{-1}$
Concentration of Bound Lysine
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
*
**
*
**
3
6
9
15
days after inoculation
control
infected

Fig. 6.3: Soluble and bound (hydrolysed) lysine in pustules and inter-pustule regions isolated from mildew infected first leaves of barley 9 days after inoculation. Significant differences shown as p = 0.05 (**); p = 0.01 (***).

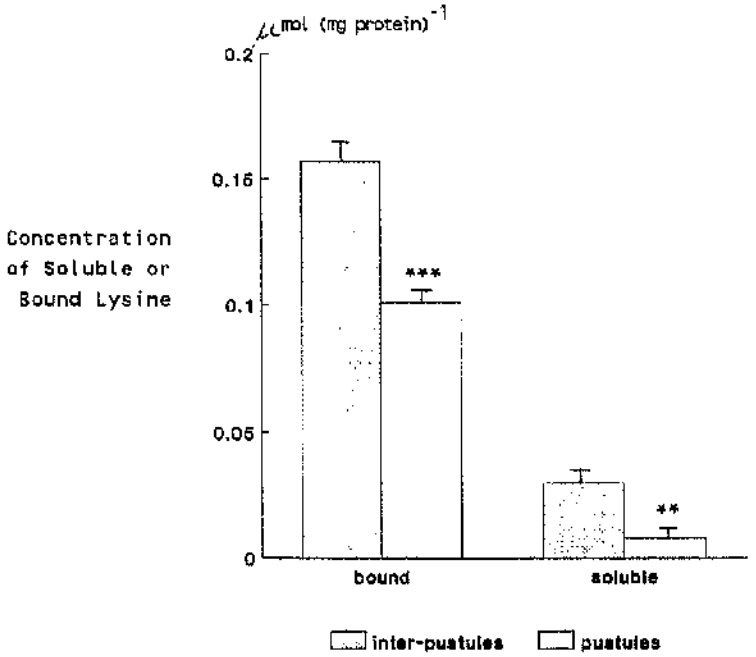
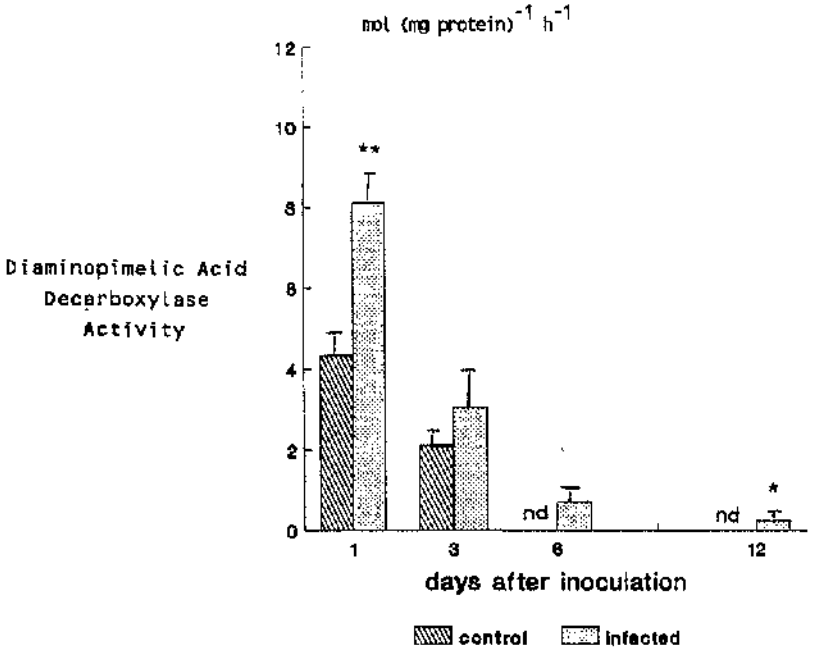

Concentration of Soluble or Bound Lysine
μmol (mg protein)$^{-1}$
0.2
0.16
0.1
0.05
0
***
**
bound
soluble
inter-pustules
pustules

Fig. 7.1: Diaminopimelic acid decarboxylase activity in healthy (control) and mildew infected first leaves of barley at different times after inoculation. Significant differences shown as p = 0.1 (*); p = 0.05 (**). nd = not detected.

Fig. 7.2: Diaminopimelic acid decarboxylase activity in pustules and inter-pustule regions of mildew infected first leaves of barley 9 days after inoculation. Significant differences shown as p = 0.05 (**).


mol (mg protein)$^{-1}$ h^{-1}
Diaminopimelic Acid Decarboxylase Activity
12
10
8
6
4
2
0
**
nd
nd
*
1
3
6
12
days after inoculation
control
infected

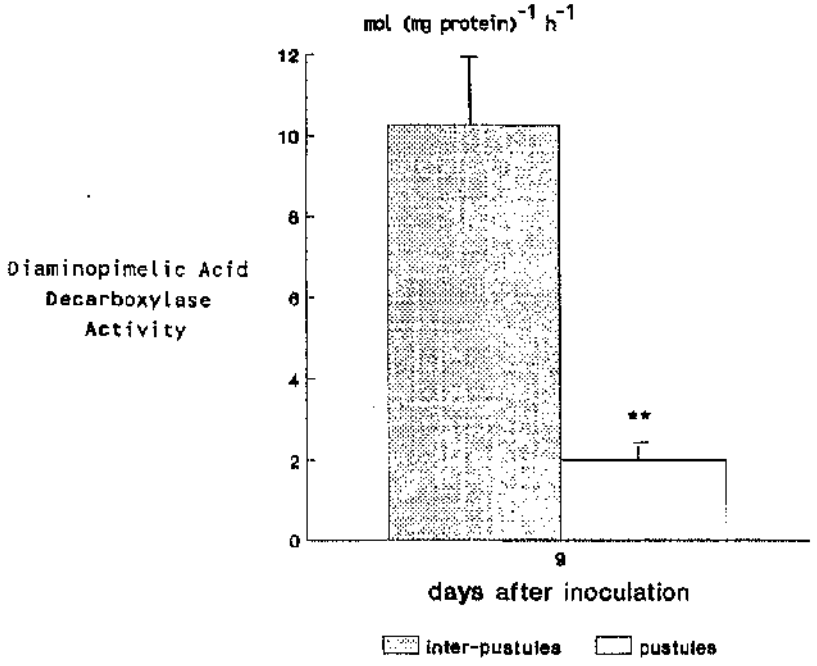

mol (mg protein)$^{-1}$ h^{-1}
Diaminopimelic Acid Decarboxylase Activity
12
10
8
6
4
2
0
**
9
days after inoculation
inter-pustules
pustules

Fig. 7.3: Diaminopimelic acid decarboxylase activity in chloroplasts isolated from healthy (control) and mildew infected first leaves of barley at different times after inoculation. Significant differences shown as p = 0.1 (*); p = 0.05 (**). nd = not determined.

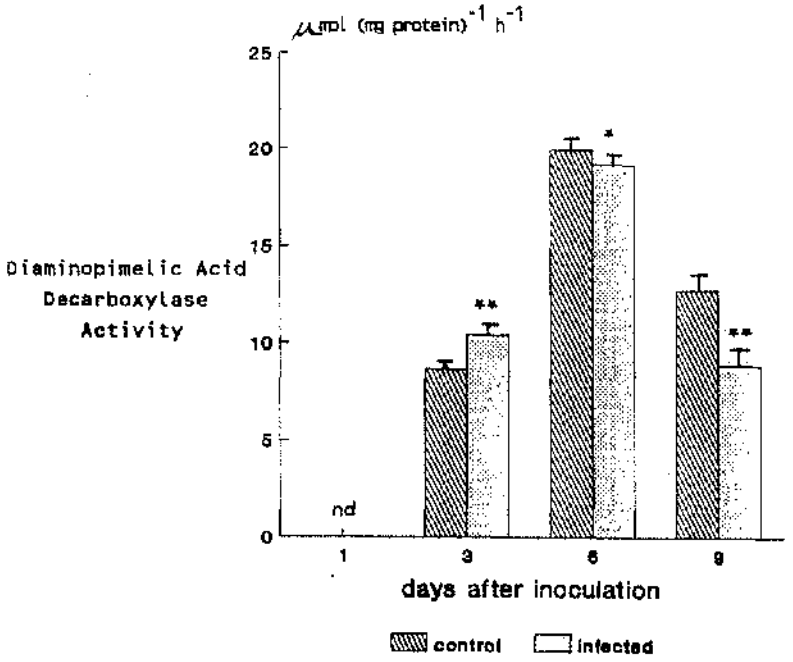
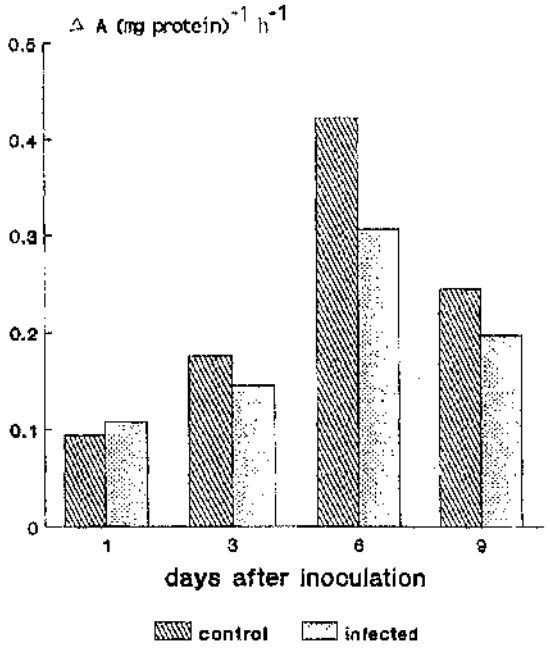

μmol (mg protein)$^{-1}$ h^{-1}
Diaminopimelic Acid Decarboxylase Activity
25
20
15
10
5
0
nd
**
*
**
1
3
6
9
days after inoculation
control
infected

Fig. 7.4: Dihydrodipicolinic acid synthase activity in healthy (control) and mildew infected first leaves of barley at different times after inoculation.

Fig. 7.5: Dihydrodipicolinic acid synthase activity in healthy (control) first leaves and in pustules and inter-pustules taken from mildew infected first leaves of barley 6 & 9 days after inoculation.

Dihydrodipicolinic Acid Synthase Activity



Dihydrodipicolinic Acid Synthase Activity

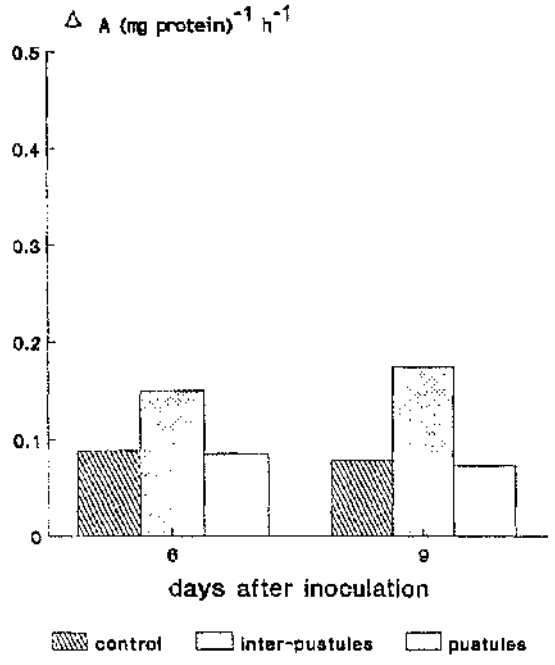


Fig. 7.6: Dihydrodipicolinic acid synthase activity in chloroplasts isolated from healthy (control) and mildew infected first leaves of barley at different times after inoculation.

Dihydrodipicolinic Acid Synthase Activity

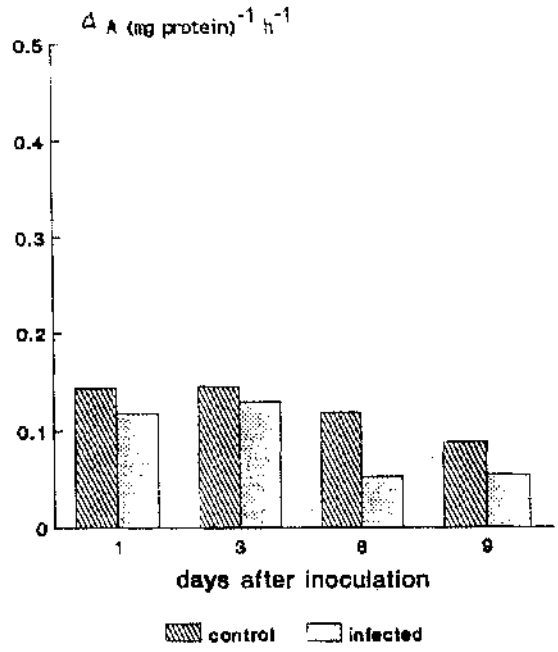
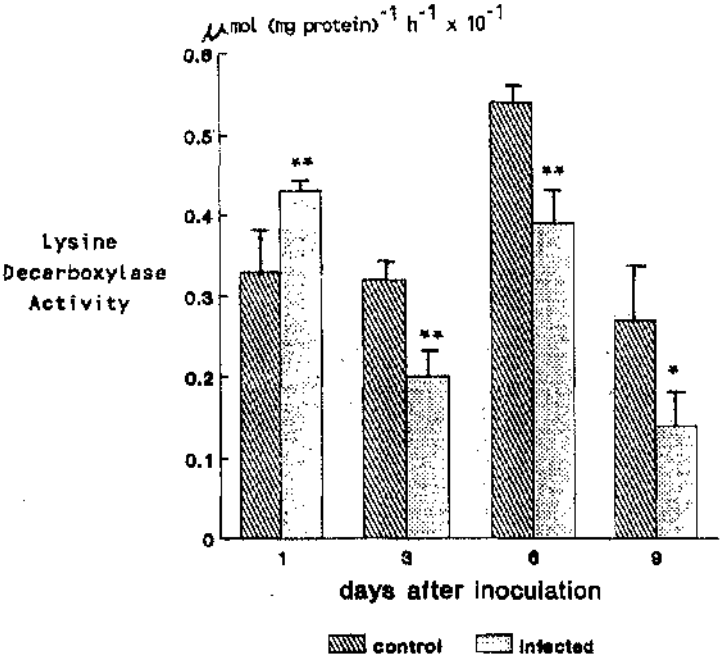


Fig. 7.7: Lysine decarboxylase activity in healthy (control) and mildew infected first leaves of barley at different times after inoculation. Significant differences shown as p = 0.1 (*); p = 0.05 (**).

Fig. 7.8 Lysine decarboxylase activity in healthy leaves (control) and in pustule and inter-pustule regions from mildew infected first leaves of barley 9 days after inoculation. Significant differences shown as p = 0.1 (*).


μmol (mg protein)$^{-1}$ h^{-1} x 10^{-1}
Lysine Decarboxylase Activity
0.6
0.5
0.4
0.3
0.2
0.1
0
**
**
**
*
1
3
6
9
days after inoculation
control
infected

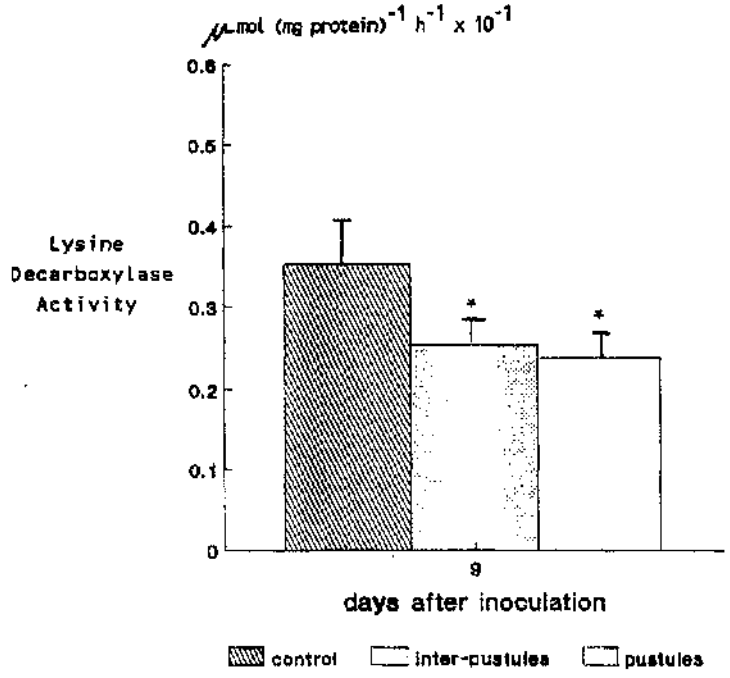
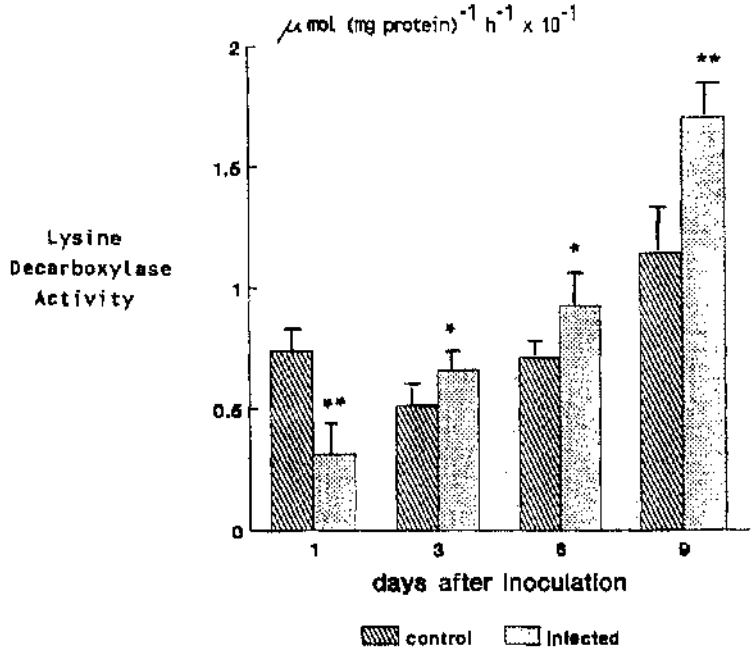
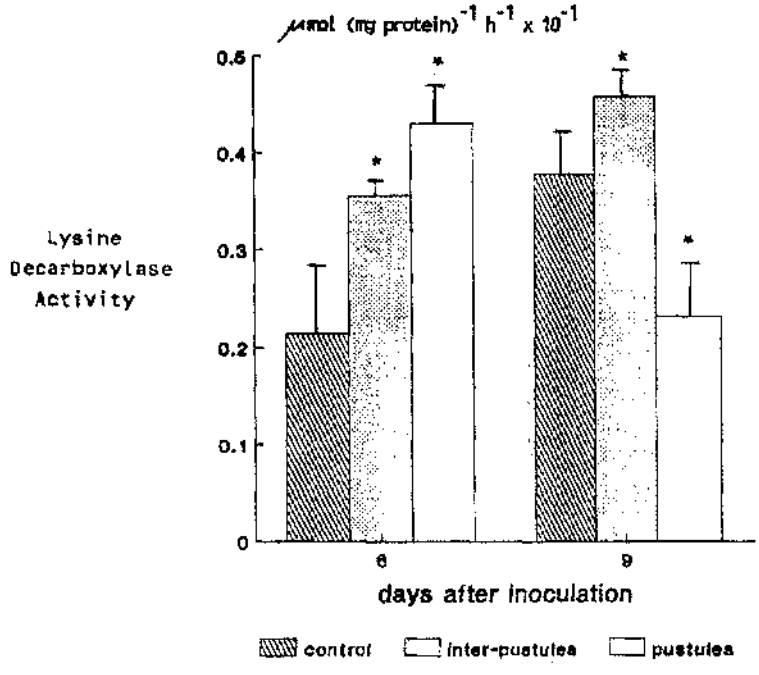

μmol (mg protein)$^{-1}$ h^{-1} x 10^{-1}
Lysine Decarboxylase Activity
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
9
days after inoculation
control
inter-pustules
pustules

Fig. 7.9: Lysine decarboxylase activity in chloroplasts isolated from healthy (control) and mildew infected first leaves of barley at different times after inoculation. Significant differences shown as p = 0.1 (*); p = 0.05 (**).

Fig. 7.10: Lysine decarboxylase activity in chloroplasts isolated from healthy leaves (control) and from pustules and inter-pustule regions of mildew infected first leaves of barley 6 & 9 days after inoculation. Significant differences shown as p = 0.1 (*).


μmol (mg protein)$^{-1}$ h^{-1} x 10^{-1}
Lysine Decarboxylase Activity
2
1.5
1
0.5
0
**
*
*
**
1
3
6
9
days after inoculation
control
infected


μmol (mg protein)$^{-1}$ h^{-1} x 10^{-1}
Lysine Decarboxylase Activity
0.5
0.4
0.3
0.2
0.1
0
*
*
*
*
6
9
days after inoculation
control
inter-pustules
pustules

3.1.3 Activities of Enzymes of the α-Aminoadipic Acid Pathway in Healthy and Powdery Mildew-Infected Barley Leaves

3.1.3.1 α-Aminoadipic Acid Reductase Activity

The activity of this enzyme was examined in healthy and powdery mildew infected leaves and in mycelium removed from infected leaves (9 DAI). Although there was no activity detected in healthy leaves, some activity was seen in infected leaves. Activity was, however, much greater in the mycelium (Table 1).

3.1.3.2 Saccharopine Dehydrogenase Activity

The activity of saccharopine dehydrogenase was examined in healthy and powdery mildew infected leaves at various times after inoculation and in pustules, inter-pustule regions and mycelium removed from infected leaves (9 DAI). In healthy and infected leaves differences were not significant at 3 and 6 DAI, but by 9 DAI the activity in healthy leaves had decreased significantly compared to the infected leaves (Table 2). By 12 DAI, however, activity in the infected leaves was significantly lower than in healthy leaves. Activity of the enzyme in pustules was significantly greater than in inter-pustules. In the mycelium, activity was significantly greater than in all other tissues examined (Table 2).

Table 1: Activity of Aminoadipic Acid Reductase in healthy (control) and powdery mildew inoculated (infected) 1st leaves of barley and in isolated powdery mildew 9 days after inoculation. ±SEM. Significant differences at: p = 0.01 (***).

Sample	Δ A min^{-1} mg $protein^{-1}$
control leaf	nd
infected leaf	0.027±0.003
mycelium	0.213±0.026***

nd = not detected.

Table 2: Activity of Saccharopine Dehydrogenase in healthy (control) and powdery mildew inoculated (infected) first leaves of barley at various times after inoculation, and in pustules, inter-pustule regions and mycelium removed from inoculated leaves 9 days after inoculation. Enzyme activity expressed as $\Delta A\ min^{-1}$ mg $protein^{-1}$. ± SEM. Significant differences at: p = 0.05 (**); p = 0.01 (***).

Sample	days after inoculation			
	3	6	9	12
control leaf	0.020±0.005	0.021±0.001	0.012±0.001	0.017±0.001
infected leaf	0.017±0.002	0.023±0.001	0.021±0.001**	0.009±0***
inter-pustules			0.015±0.002	
pustules			0.024±0.001**	
mycelium			0.047±0.005***	

3.2 Feeding With ^{14}C-Aspartate

3.2.1 Uptake of ^{14}C-aspartate

3.2.1.1 SOLUBLE LABEL

The uptake of ^{14}C-aspartate by both healthy and infected leaves (6 DAI) increased with time (Table 3). At 2h uptake by infected leaves was greater than in control leaves. When mycelium was removed from the infected leaves before feeding uptake was less than in infected leaves with the mycelium still present.

By 4h after the start of feeding, uptake was greater in control leaves than in infected leaves with mycelium present or in infected leaves from which the mycelium had been removed either before or after the feeding began. This trend continued at 8h except in leaves with the mycelium removed before feeding. In these leaves uptake was greater than in healthy leaves.

3.2.1.2 BOUND LABEL

Again, as seen with the soluble fraction, labelling in the bound (hydrolysed) fraction increased with time in all samples (Table 4).

Although there was less bound label in infected leaves than in healthy leaves at 2h after feeding began there was little difference thereafter. When the mycelium was removed (either before or after feeding), however, there

was considerably less bound label present in these leaves.

The ratio of bound label to soluble label changed with time. At 2h there was less bound label than soluble label and at 4h the amounts were similar. By 8h, however, there was greater labelling in the bound fraction. This trend was also seen in the mycelial extracts (Tables 3 & 4).

3.2.2 Metabolism of ^{14}C-aspartate

3.2.2.1 LEAVES

Metabolism of aspartate appeared to take place faster in infected leaves. 2h after the start of feeding only 22% of the label was present as aspartate in contrast to 45% in control leaves (Table 5). In the infected leaves, much of the label had moved to peak 3 (tentatively identified as homoserine and threonine) (Table 6) and peak 5 as yet unidentified but possibly methionine.

By 4h, only 18.5% of the label in infected leaves was in aspartate but there was still 35% in control leaves. There also appeared to be movement of label from peak 5 to peak 3 (Table 5).

Leaves from which the mycelium was removed before feeding contained 26% of ^{14}C-aspartate at 4h (Table 5), greater than in infected leaves with the mycelium still attached, but lower than in control leaves.

The profiles of extracts from infected leaves from which the mycelium was removed before (Fig. 8.2a) and after feeding (Fig. 8.2b) were quite different. Where the mycelium was removed before feeding the profile resembled that of control leaves (Fig. 8.1a) but that for leaves where fungal mycelium was removed after feeding resembled the profile of infected leaves with mycelium still attached (Fig. 8.1b). This was true for all samples taken over the 4-8h period.

3.2.2.2 MYCELIUM

The distribution of label in infected leaves and in mycelium detached after feeding showed large differences as indicated by the two main peaks - aspartate and peak 3 (Fig. 8.3). There was much more aspartate (as % of soluble label) in the mycelium and, while the percentage of label in aspartate in infected leaves decreased with time, it remained relatively constant in the mycelium.

Peaks 4 and 5 were lower and higher respectively in the mycelial extracts (Fig. 8.3).

3.2.3 Pre-Feeding with Unlabelled 10mM Lysine or Threonine

Both in control and infected leaves, pre-feeding with 10mM lysine was more inhibitory to aspartate uptake than pre-feeding with 10mM threonine (Table 7). Uptake by infected leaves was inhibited less than that in control

leaves (Table 7).

Labelling appeared greater in mycelium removed from the lysine treated leaves than in controls or those treated with threonine, although this increase was not significant (Table 8).

Table 3: Total radioactivity present in the soluble fraction of healthy and infected barley leaves, and in powdery mildew mycelium, at different times after feeding with ^{14}C-aspartate (see text for details). Results are expressed as dps g^{-1} f wt (leaves) and dps per sample (mycelium). Values are the means of 4 replicates ± SEM. Significant differences at: p = 0.1 (*)

	hours after treatment		
	2	4	8
control	4460 ± 270	9720 ± 1020	15960 ± 160
infected	5640 ± 300*	8540 ± 150	13270 ± 190*
infected (w) [a]	4570 ± 380	8790 ± 180	17190 ± 280
infected (wa) [a]	nd	8280 ± 210	12070 ± 300*
mycelium	nd	40 ± 2	50 ± 6

[a] Mycelium was removed from leaf surfaces before (w) or after (wa) feeding. nd = not determined.

Table 4: Total radioactivity present in the insoluble fraction of healthy and infected barley leaves, and in powdery mildew mycelium, at different times after feeding with ^{14}C-aspartate. Results are expressed as dps g^{-1} f wt (leaves) and dps per sample (mycelium).

	hours after treatment		
	2	4	8
control	2820	11320	25950
infected	2580	11980	26100
infected (w) [a]	nd	8060	nd
infected (wa) [a]	nd	8390	17830
mycelium	nd	40	110

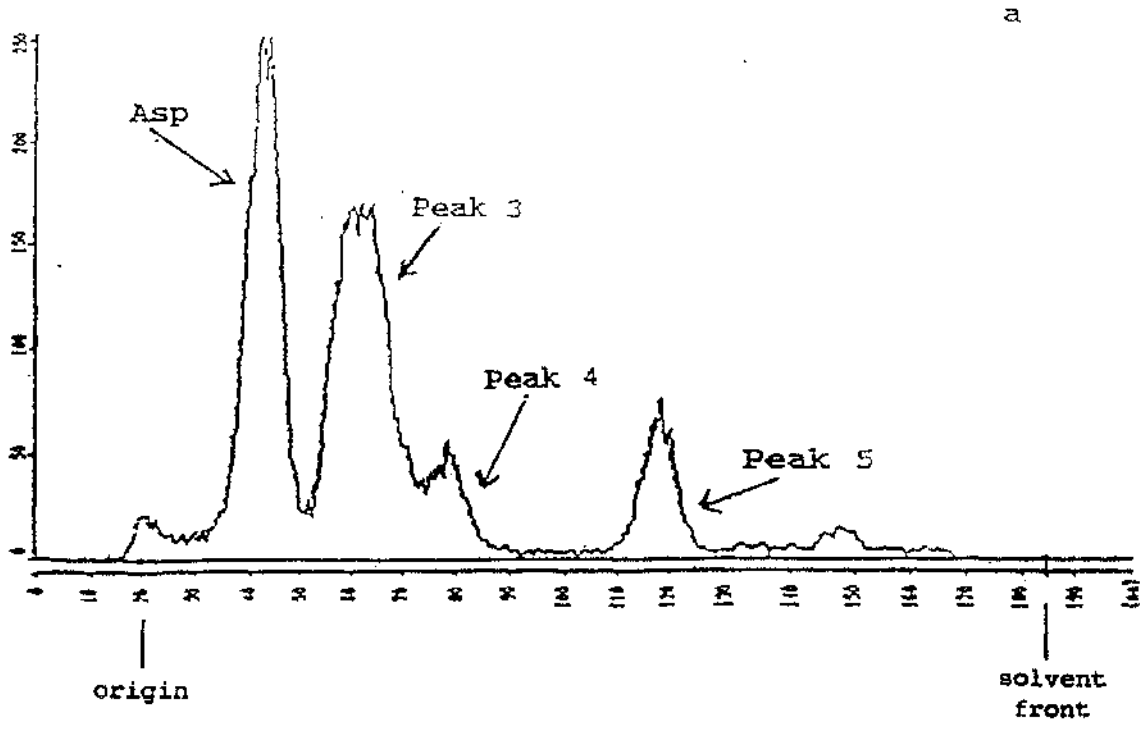
[a] Mycelium was removed from leaf surfaces before (w) or after (wa) feeding. nd = not determined.

Table 5: Labelled aspartate and metabolites as % of total soluble radioactivity following feeding of first leaves with ^{14}C-aspartate for 2, 4 or 8h. Values are the means of 2 replicates ± SEM. Significant differences shown at: p = 0.1 (*); p = 0.05 (**).

	hours after treatment		
	2	4	8
Aspartate			
control	44.85 ± 1.8	34.90 ± 1.5	25.10 ± 0.4
infected	21.65 ± 1.9*	18.40 ± 0.5*	10.40 ± 0.1 **
infected (a)[a]	32.10 ± 2.9	26.30 ± 0.7	17.05 ± 0.35**
infected (b)[a]	nd	18.20 ± 0.4*	16.50 ± 0.1 **
mycelium (c)[a]	nd	29.45 ± 3.3	33.30 ± 1.4
Peak 3			
control	22.75 ± 0.15	35.55 ± 2.4	43.25 ± 0.75
infected	33.35 ± 2.4	50.30 ± 1.8	58.15 ± 2.9
infected (a)[a]	26.65 ± 0.75	44.20 ± 1.5	44.55 ± 5.3
infected (b)[a]	nd	52.45 ± 0.65*	58.60 ± 1.5 *
mycelium (c)[a]	nd	18.95 ± 0.05*	17.40 ± 0.3 **
Peak 4			
control	7.70 ± 0.9	6.55 ± 1.1	8.65 ± 3.2
infected	8.40 ± 1.9	9.20 ± 0.9	10.35 ± 3.2
infected (a)[a]	7.40 ± 0.9	7.40 ± 1.8	12.60 ± 5.3
infected (b)[a]	nd	7.95 ± 0.15	5.70 ± 1.1
mycelium (c)[a]	nd	5.35 ± 0.15	4.25 ± 0.75
Peak 5			
control	11.55 ± 0.25	9.45 ± 0.15	9.35 ± 0.15
infected	26.25 ± 0.15**	12.10 ± 0.3 *	9.00 ± 0.1
infected (a)[a]	20.25 ± 0.05**	11.15 ± 0.35	13.25 ± 0.35*
infected (b)[a]	nd	10.80 ± 0.4	5.75 ± 0.35*
mycelium (c)[a]	nd	11.00 ± 1.2	12.50 ± 1.7

[a] (a) = inoculated - mycelium removed before feeding; (b) inoculated - mycelium removed after feeding; (c) mycelium removed from inf (b) leaves. nd = not determined.

Fig. 8.1: Radio-TLC-analyser traces of extracts after one dimensional TLC in 2 solvent systems. a = control leaves 4h after feeding; b = infected leaves 4h after feeding [x axis = mm; y axis = dps].


a
Asp
Peak 3
Peak 4
Peak 5
origin
solvent
front

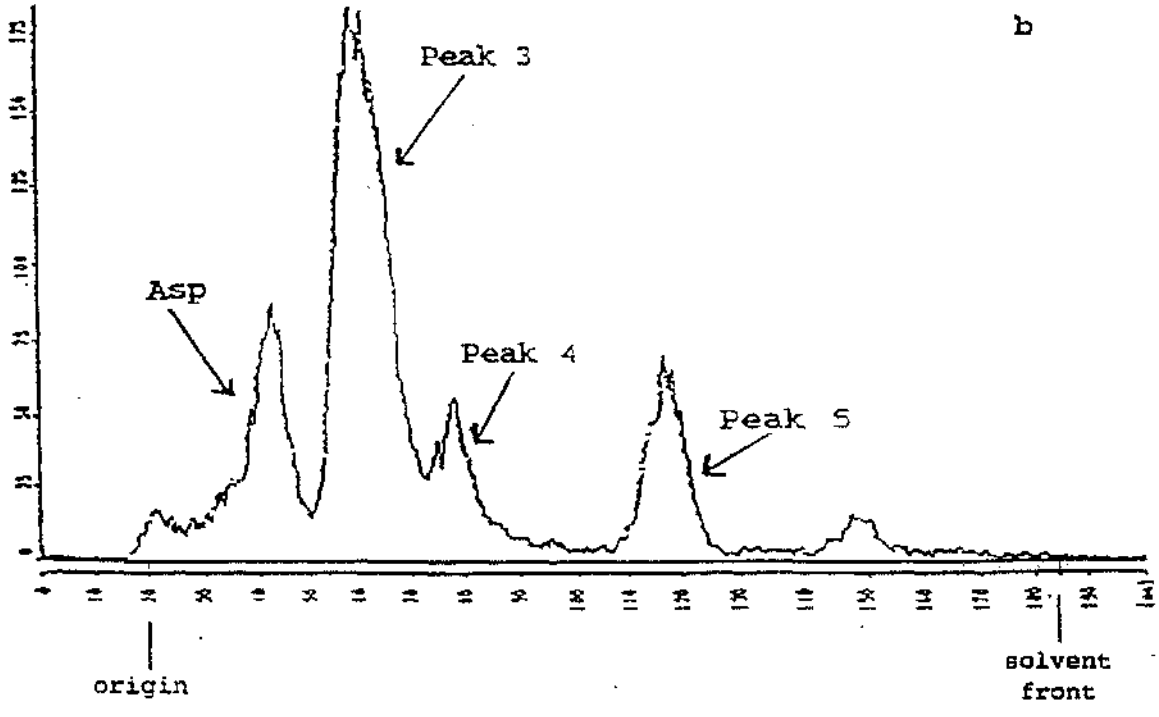
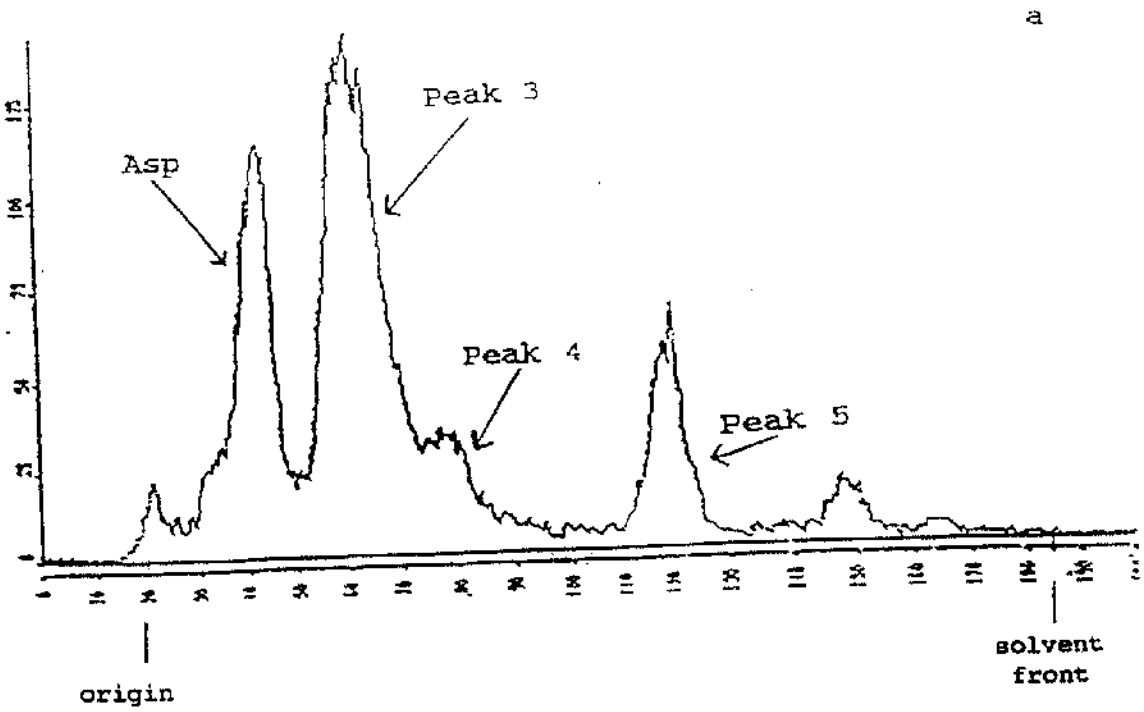

b
Peak 3
Asp
Peak 4
Peak 5
origin
solvent
front

Fig. 8.2: Radio-TLC-analyser traces of extracts after one dimensional TLC in 2 solvent systems. a = control leaves 4h after feeding (mycelium removed before feeding); b = infected leaves 4h after feeding (mycelium removed after feeding).


a
Peak 3
Asp
Peak 4
Peak 5
origin
solvent
front

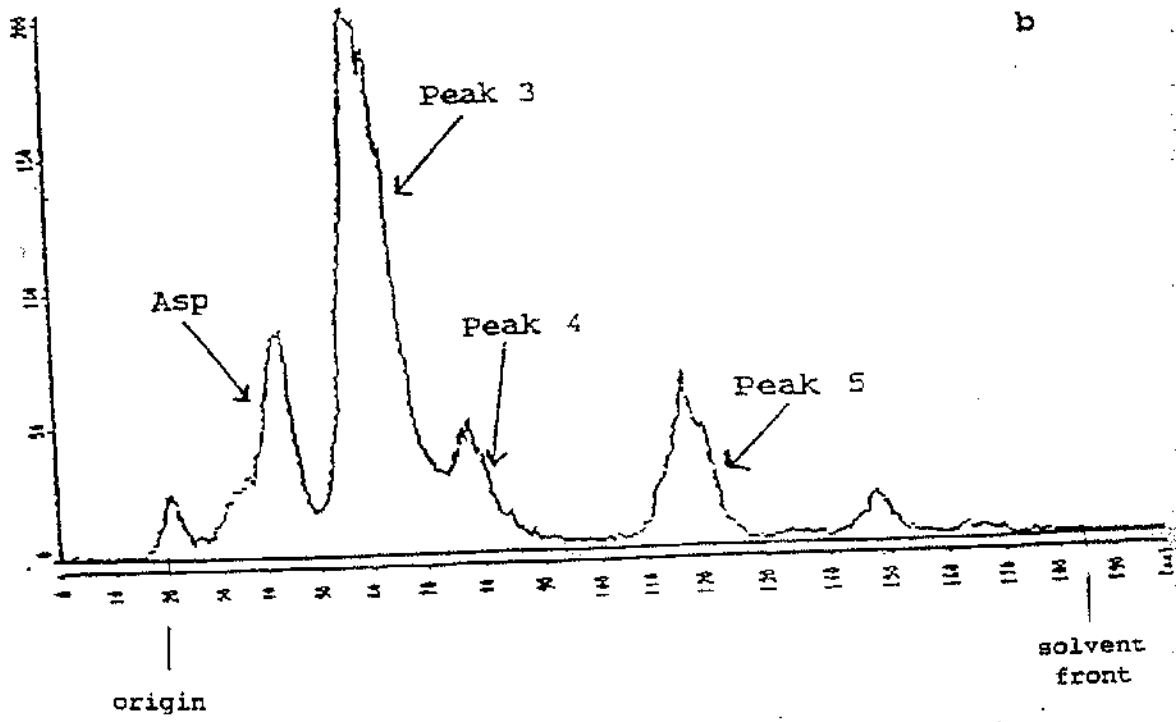

b
Peak 3
Asp
Peak 4
Peak 5
origin
solvent
front

Fig. 8.3: A comparison of the radioactivity in aspartate and metabolites in leaves infected with *E. graminis*, and in *E. graminis* mycelium removed from infected leaves after feeding with labelled aspartate for 4 or 8h. Significant differences shown as p = 0.1 (*); p = 0.05 (**); p = 0.01 (***).

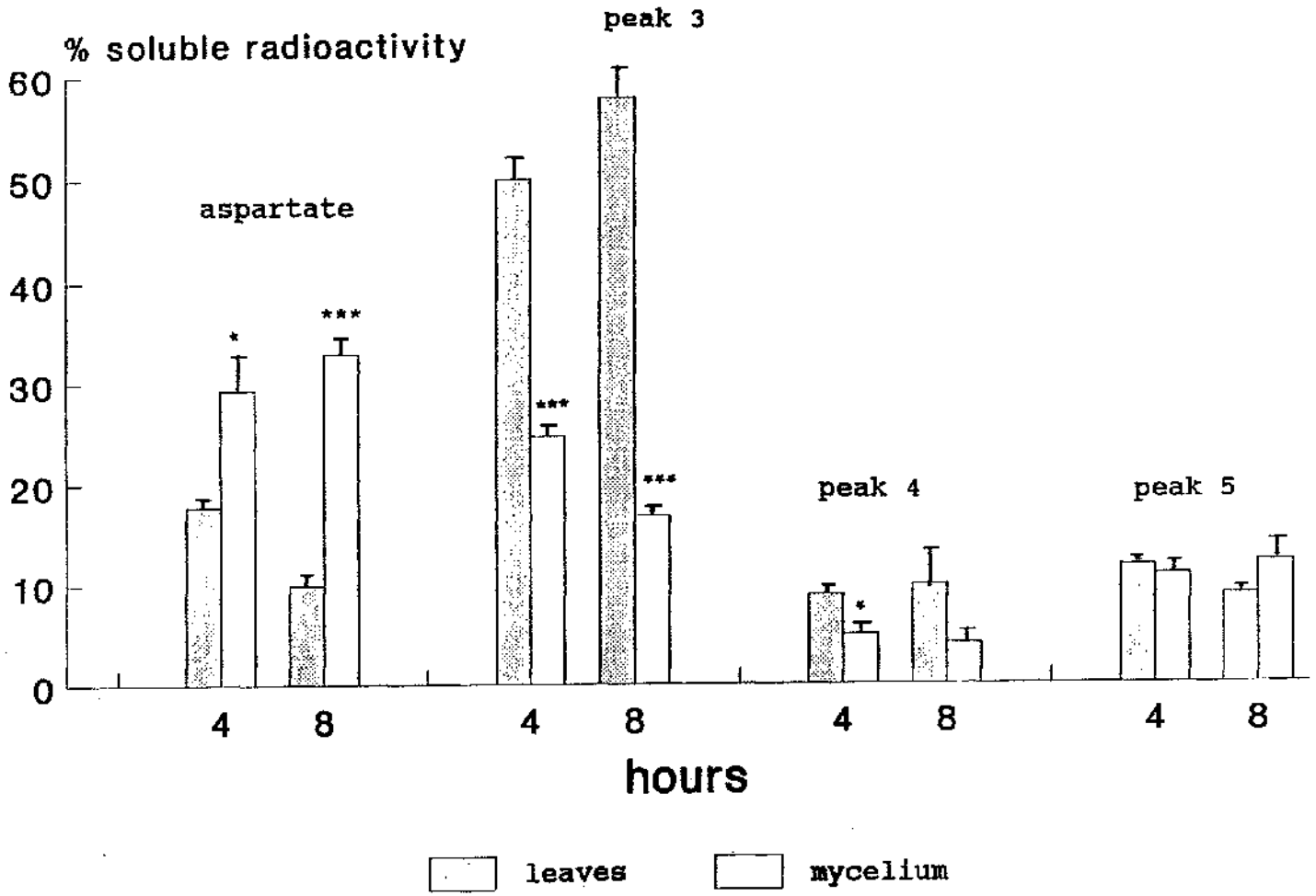

% soluble radioactivity
peak 3
60
50
40
30
20
10
0
aspartate
*
***
***
***
*
peak 4
peak 5
4
8
4
8
4
8
4
8
hours
leaves
mycelium

Table 6: Rf values of the main peaks of radioactivity in leaf and mycelial samples and of standards run in parallel.

peak	Rf	standard	Rf
		cystathionine	0.13
		asparagine	0.20
		lysine	0.20
2	0.23	aspartate	0.22
3	0.32	homoserine	0.33
		threonine	0.34
4	0.43		
5	0.63	methionine	0.65
		isoleucine	0.76

Table 7: Total soluble radioactivity in barley leaves pre-fed with water (control), 10 mM lysine or 10 mM threonine, for 2 hrs, and then fed with ^{14}C-aspartate for 4 hrs. Values are the mean of 4 replicates ± SEM. Significant differences shown as: p = 0.1 (*); p = 0.05 (**); p = 0.01 (***).

	Radioactivity dps g^{-1} f wt	
pre-treatment	control	infected
water	11070 ± 140	8120 ± 800
lysine	6060 ± 410*	5460 ± 180***
threonine	7560 ± 100**	6520 ± 520**

Table 8: Total soluble radioactivity in mycelium, removed from infected leaves pre-fed with water (control), 10 mM lysine or 10 mM threonine, for 2 hrs, and then fed with ^{14}C-aspartate for 4 hrs. Values are the mean of 4 replicates ± SEM. Differences not statistically significant.

pre-treatment	radioactivity (dps per sample)
water	129.9 ± 37
lysine	142.3 ± 20
threonine	129.9 ± 19

3.3 Studies On Uptake Kinetics Using Isolated *E. graminis* Mycelium

3.3.1 Measurement of Optimum pH for the Uptake of Aspartate and Lysine

Uptake of aspartate and lysine by isolated powdery mildew mycelium was examined as described in 2.5.

Uptake of aspartate was greater at pH 4 and pH 7 although differences in uptake over the pH range employed were not significant (Fig. 9.1). Uptake of lysine appeared to be somewhat greater at pH 4 and pH 9. These differences were not, however, significant. Homogenising medium adjusted to pH 7.0 was used for subsequent work.

It is interesting to note that uptake of lysine was greater than uptake of aspartate at all values of pH examined.

3.3.2 Time Course of Aspartate or Lysine Uptake

The rate of uptake of both aspartate and lysine increased initially to a constant level, at 30 minutes after the start of the experiment (Fig. 9.2).

The rate of uptake of lysine during the first 30 minutes was, however, much greater and appeared to decrease between 120 and 240 minutes.

The level of uptake of lysine was much greater than that for aspartate.

3.3.3 Measurement of Lysine Uptake at Different Concentrations

Lysine uptake was linear over the whole concentration range (0.005 - 100 mM) (Fig. 9.3). Re-calculation of the data to produce an Eadie-Hofstee plot showed that uptake was biphasic (Fig. 9.4). Values for K_m and V_{max}, determined by linear regression, were obtained for both systems (Table 9). K_m and V_{max} for system 1 were much smaller than for system 2.

3.3.4 The Effects of Amines and a Metabolic Inhibitor on the Uptake of Lysine by Isolated *E. graminis* Mycelium

Lysine uptake decreased in the presence of putrescine and, especially, ornithine but was increased in the presence of arginine (Fig. 9.4). These differences were not significant.

Uptake was unaffected by the presence of 1mM sodium azide.

Fig. 9.1: Uptake of aspartate or lysine, by isolated *E. graminis* mycelium, at different pH values.

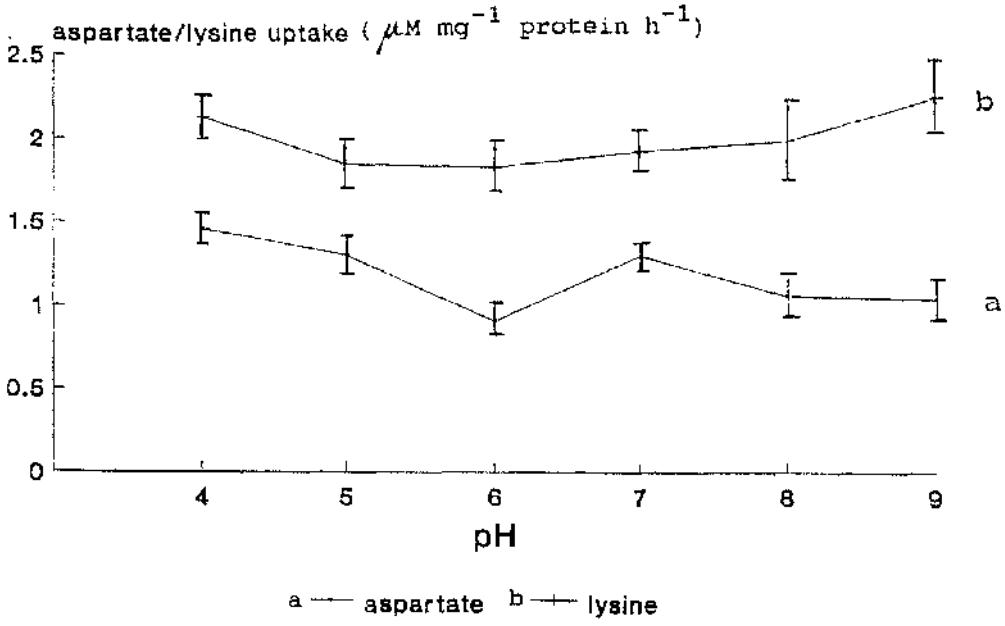

aspartate/lysine uptake (μM mg-1 protein h-1)
2.5
2
1.5
1
0.5
0
4
5
6
7
8
9
pH
b
a
a aspartate b lysine

Fig. 9.2: Time course of uptake of aspartate or lysine, by isolated *E. graminis* mycelium.

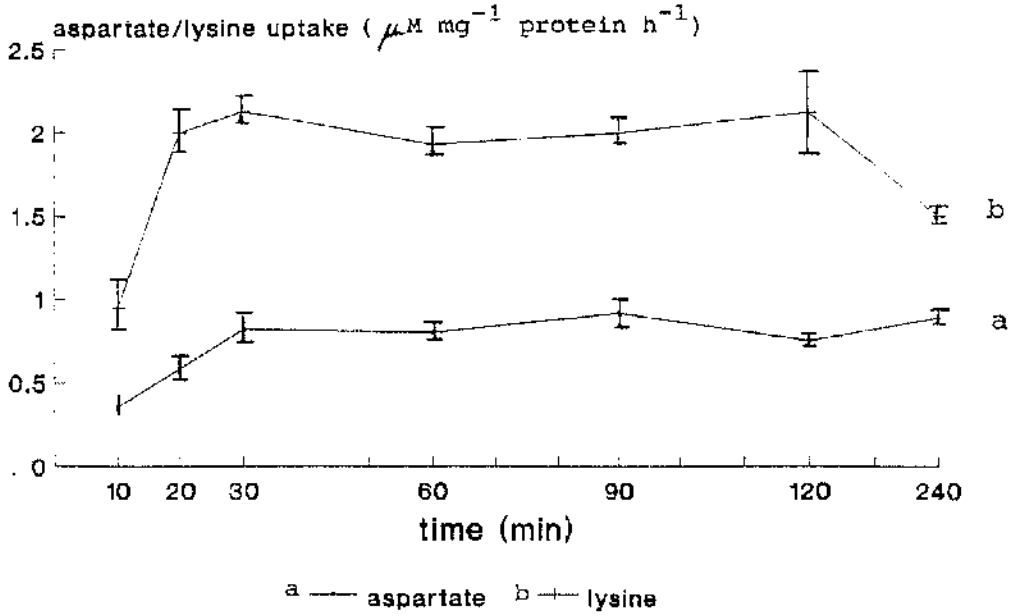
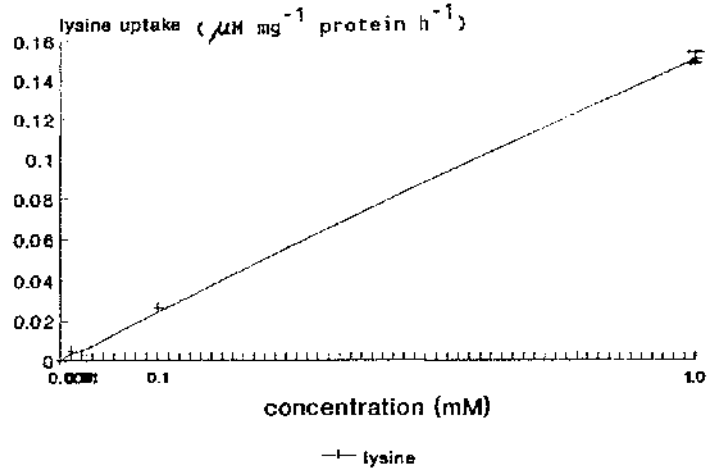

aspartate/lysine uptake (μM mg⁻¹ protein h⁻¹)
2.5
2
1.5
1
0.5
0
10
20
30
60
90
120
240
time (min)
b
a
a aspartate
b lysine

Fig. 9.3: Effect of concentration on uptake of lysine by isolated *E. graminis* mycelium.


lysine uptake (μM mg-1 protein h-1)
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0.1
1.0
concentration (mM)
lysine

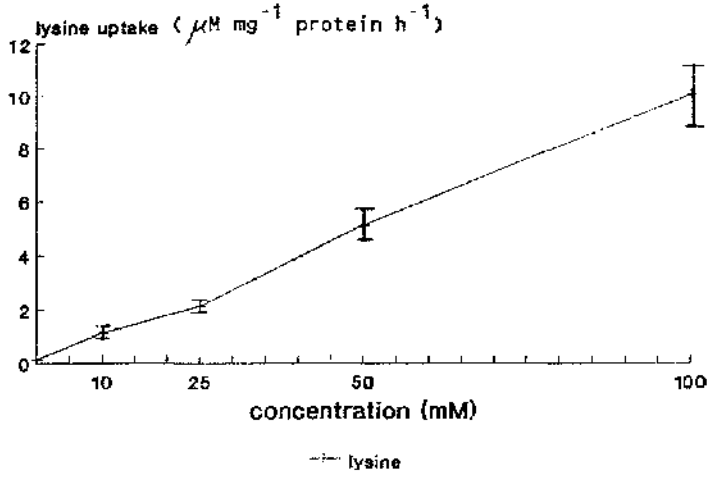

lysine uptake (μM mg-1 protein h-1)
12
10
8
6
4
2
0
10
25
50
100
concentration (mM)
lysine

Fig. 9.4: Eadie-Hofstee plots for lysine uptake by *E. graminis*: showing lysine uptake versus lysine uptake/lysine concentration, over the concentration range 0.005 - 100mM.

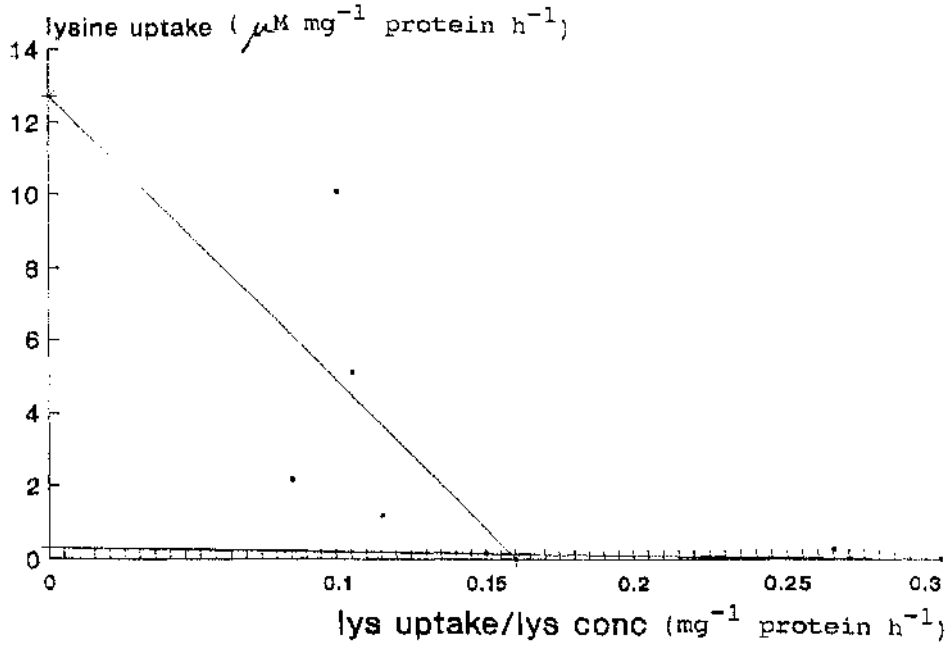
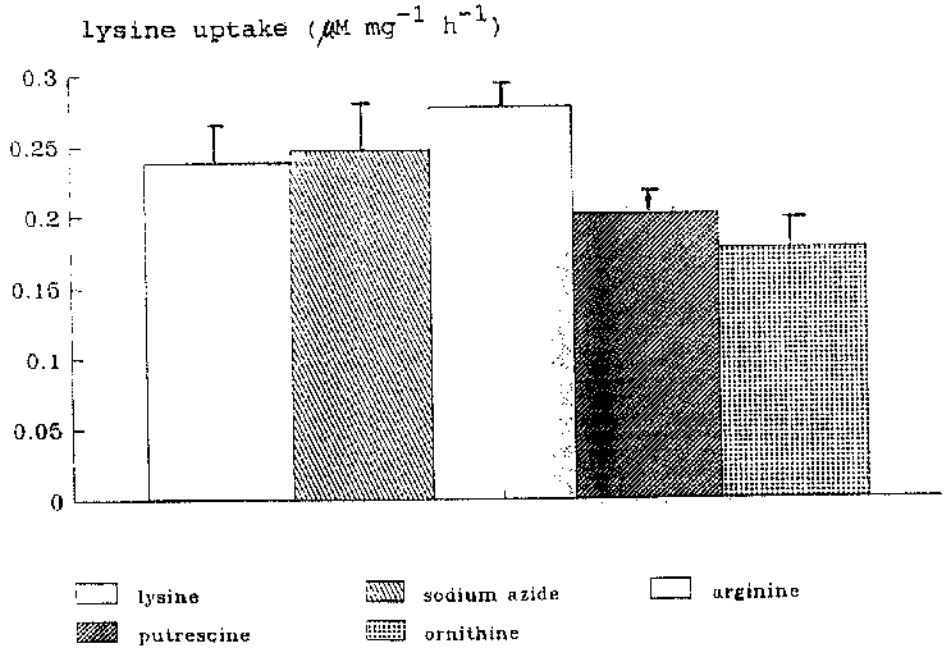

lysine uptake (μM mg-1 protein h-1)
14
12
10
8
6
4
2
0
0
0.1
0.15
0.2
0.25
0.3
lys uptake/lys conc (mg-1 protein h-1)

Table 9: K_m and V_{max} values for lysine uptake by *E. graminis*. Values calculated by linear regression from Eadie-Hofstee plots.

	System 1		System 2	
	K_m (mM)	V_{max} (m mol g^{-1} h^{-1})	K_m (mM)	V_{max} (m mol g^{-1} h^{-1})
Lysine	1.09	0.31	80.0	12.7

Fig. 9.5: Uptake of lysine by isolated *E. graminis* mycelium in the presence of 1mM sodium azide, arginine, putrescine or ornithine. Differences not significant at p = 0.1.


lysine uptake (μM mg^{-1} h^{-1})
0.3
0.25
0.2
0.15
0.1
0.05
0
lysine
putrescine
sodium azide
ornithine
arginine

3.4 Formation of Cadaverine Derivatives

3.4.1 Incorporation of ^{14}C-Lysine into Cadaverine Derivatives in Healthy and Mildewed Barley Leaves

The amount of radioactivity appearing in cadaverine and N,N'-*bis*(3-aminopropyl)cadaverine (3APC), a higher homologue of cadaverine, was significantly reduced in leaves infected with *E. graminis* compared to healthy leaves of the same age (Table 10). In contrast, labelling of aminopropylcadaverine (APC) was significantly increased in mildewed leaves. Addition of 3mM MGBG (a SAMdc inhibitor) and 3mM CHA (a spermidine synthase inhibitor) had no significant effect on labelling in either healthy or mildew infected leaves (Table 10). However, treatment with the same inhibitors for 2h before addition of labelled lysine significantly reduced labelling of 3APC (to 10% and 11% of that found in the non-inhibitor treated healthy and mildew infected leaves respectively) (Table 11). APC, detected in the non-inhibitor treated healthy leaves was absent from those treated with inhibitor and from both treated and non-treated infected leaves. No radioactivity appeared in cadaverine (Table 11).

Pre-treatment of healthy and infected leaves with 5mM each of DFMO/DFMA reduced labelling of 3APC to 53% and 50% of the non-inhibitor treated leaves respectively (Table 12). However, although label appeared in APC in the healthy leaves treated with DFMO/DFMA, this compound was absent from non-inhibitor treated healthy and infected

leaves and from all other inhibitor treated samples (Table 12).

When 5mM MGBG/5mM CHA was combined with 5mM DFMO/5mM DFMA, there was no labelling of cadaverine or either of its higher homologues. A similar result was obtained when leaves were pre-treated with 0.2mM AdoDato (a spermidine synthase inhibitor) + 5mM CHA (Table 12).

In preliminary work it was shown that labelling of 3APC was much greater in inter-pustules than in pustule regions and labelling was significantly reduced in both inter-pustules and pustules when treated with 0.2mM AdoDato + 5mM CHA (data not shown).

3.4.2 Incorporation of ^{14}C-Lysine into Cadaverine Derivatives in Isolated Mycelium of *E. graminis*

When isolated powdery mildew mycelium was fed with ^{14}C-lysine, some radioactivity appeared in APC but most of the label appeared in its higher homologue, 3APC (Table 13). A similar situation was observed *in vitro*, when extracts of the mycelium were incubated with ^{14}C-lysine (Table 14). Following pre-treatment with 2mM DFMO/2mM DFMA, labelling of 3APC was not significantly altered (Tables 13 & 14). Pre-treatment with these inhibitors at 5mM also had no significant effect on the labelling of 3APC, although labelling of APC was significantly reduced (Table 15). Treatment with a combination of 5mM MGBG/5mM CHA and 5mM

DFMO/5mM DFMA caused a reduction in labelling of 3APC. Labelling of cadaverine and APC were not significantly altered. Exposure to 5mM MGBG/5mM CHA alone, as with 5mM DFMO/5mM DFMA alone, produced no significant effect on labelling of 3APC (Table 15).

Interestingly, DFMO/DFMA used at 2mM or 5mM *in vitro* reduced LDC activity, although only the latter treatment was significant (Table 16). A similar result was obtained *in vivo* when mildew mycelium was incubated with 2mM DFMO/2MmM DFMA prior to the LDC assay (Table 17). Treatment with 5mM MGBG/5mM CHA produced no significant effect on LDC activity, although a combination of MGBG/CHA/DFMO/DFMA reduced enzyme activity (Table 18).

Labelling of 3APC in isolated mycelium increased with increasing molarity of DMTA (a spermine synthase inhibitor) (Table 19). This increase was only significant at the highest molarity (10mM) of the inhibitor used. LDC activity was not significantly altered over the range of inhibitor concentrations used (Table 20).

Table 10: *In vitro* incorporation of ^{14}C-lysine into cadaverine derivatives in extracts from healthy and mildewed first leaves of barley, ± MGBG and CHA. Values are the means of 5 replicates ±SEM. Significant differences at: p = 0.1 (*); p = 0.01 (***); p = 0.001 (****).

Treatment	Radioactivity in polyamine (dpm μg^{-1} protein)		
	CAD	APC	3APC
control	0.68±0.20	2.84±0.79	32.50±1.00
3mM MGBG + 3mM + CHA	0.32±0.08	3.43±0.92	30.56±3.70
infected	0.13±0.06*	7.02±0.49***	12.76±1.20****
3mM MGBG + 3mM + CHA	0.16±0.04*	9.81±1.40***	9.93±1.00****

Table 11: *In vitro* incorporation of ^{14}C-lysine into cadaverine derivatives in healthy and mildewed first leaves pre-fed with MGBG and CHA. Values are the means of 5 replicates ±SEM. Significant differences at: p = 0.001 (****).

Treatment	Radioactivity in polyamine (dpm g^{-1} protein)		
	CAD	APC	3APC
control	nd	14.28±2.20	56.62±2.80
3mM MGBG + 3mM+ CHA	nd	nd	5.43±0.74****
infected	nd	nd	12.40±1.60****
3mM MGBG + 3mM+ CHA	nd	nd	1.32±0.37****

nd = not detected

Table 12: *In vitro* incorporation of ^{14}C-lysine into cadaverine derivatives in extracts of healthy and mildewed barley leaves pre-treated with inhibitors for 2h. Values are the means of 5 replicates ± SEM. Significant differences at: p = 0.01 (***); p = 0.001 (****).

Treatment	Radioactivity in polyamine (dpm g^{-1} protein)		
	CAD	APC	3APC
control	nd	nd	81.68±6.11
+ 5mM DFMO/5mM DFMA	nd	26.60±0.97	43.25±2.41***
+ 5mM DFMO/5mM DFMA & 5mM MGBG/5mM CHA	nd	nd	nd
+ 0.2mM AdoDato/ 5mM CHA	nd	nd	nd
infected	nd	nd	20.24±1.75****
+ 5mM DFMO/5mM DFMA	nd	nd	10.17±1.84****
+ 5mM DFMO/5mM DFMA & 5mM MGBG/5mM CHA	nd	nd	nd
+ 0.2mM AdoDato/ 5mM CHA	nd	nd	nd

nd = not detected

Table 13: *In vivo* incorporation of ^{14}C-lysine into cadaverine derivatives in isolated mycelium of *E. graminis*, pre-fed with 2 mM DFMO and 2mM DFMA. Values are the means of 5 replicates ± SEM. Differences not significant.

Treatment	Radioactivity in polyamine (dpm g^{-1} protein)		
	CAD	APC	3APC
control	nd	7.34±3.63	106.47±17.91
2mM DFMO + 2mM DFMA	nd	11.96±5.16	94.19±12.86

nd = not detected

Table 14: *In vitro* incorporation of ^{14}C-lysine into cadaverine derivatives in extracts of *E. graminis* mycelium ± DFMO and DFMA. Values are the means of 5 replicates ± SEM. Differences not significant.

Treatment	Radioactivity in polyamine (dpm g^{-1} protein)		
	CAD	APC	3APC
control	nd	19.9±5.3	176.8±32.4
2mM DFMO + 2mM DFMA	nd	10.9±2.0	211.0±19.9

nd = not detected

Table 15: *In vitro* incorporation of ^{14}C-lysine into cadaverine derivatives in extracts of *E. graminis* mycelium in the presence of inhibitors. Values are the means of 5 replicates ± SEM. Significant differences at: p = 0.1 (*); p = 0.05 (**).

Treatment	Radioactivity in polyamine (dpm/µg^{-1} protein)		
	CAD	APC	3APC
control	0.38±0.04	1.66±0.18	46.17±3.32
5mM MGBG/5mM CHA + 5mM DFMO/5mM DFMA	0.27±0.09	1.35±0.32	33.87±2.45**
5mM MGBG/5mM CHA	0.36±0.05	1.36±0.27	41.21±5.30
5mM DFMO/5mM DFMA	0.19±0.11	0.86±0.21*	42.37±3.90

Table 16: *In vitro* lysine decarboxylase activity in extracts of isolated *E. graminis* mycelium in the presence of DFMO/DFMA. Values are the means of 5 replicates ± SEM. Significant differences at: p=0.1 (*).

Treatment	enzyme activity (μmol CO_2 mg protein^{-1} h^{-1})
control	0.73±0.23
2mM DFMO + 2mM DFMA	0.52±0.08
5mM DFMO + 2mM DFMA	0.45±0.07*

Table 17: *In vivo* lysine decarboxylase activity in extracts from isolated *E. graminis* mycelium pre-treated with DFMO/DFMA. Values are the means of 5 replicates ± SEM. Significant differences at: p = 0.05 (**).

Treatment	enzyme activity (μmol CO_2 mg protein^{-1} h^{-1})
control	1.19 ± 0.26
2mM DFMO + 2mM DFMA	0.81 ± 0.25**

Table 18: *In vivo* lysine decarboxylase activity in isolated *E. graminis* mycelium in the presence of various inhibitor combinations. Values are the means of 5 replicates ± SEM. Differences not significant.

Treatment	enzyme activity (μmol CO_2 mg protein^{-1} h^{-1})
control	0.60±0.13
5mM MGBG/5mM CHA + 5mM DFMO/5mM DFMA	0.32±0.08
5mM MGBG/5mM CHA	0.57±0.02

Table 19: *In vitro* incorporation of ^{14}C-lysine into cadaverine derivatives in extracts of *E. graminis* mycelium + DMTA. Values are the means of 5 replicates ± SEM. Significant differences at: p=0.1 (*); p=0.05 (**); p=0.001 (****).

Treatment	Radioactivity in polyamine (dpm μg^{-1} protein)		
	CAD	APC	3APC
control	0.47±0.55	17.68±8.26	124.69±22.23
2mM DMTA	1.34±0.35**	17.31±8.73	133.99±22.63
5mM DMTA	2.15±0.12****	13.60±8.80	146.73±17.52
10mM DMTA	1.46±0.00**	8.00±2.24*	160.60±30.10*

Table 20: Lysine decarboxylase activity in extracts of isolated *E. graminis* mycelium in the presence of DMTA. Values are the means of 5 replicates ± SEM. Differences not significant.

Treatment	enzyme activity (μmol CO_2 mg protein^{-1} h^{-1})
control	0.63±0.25
2mM DMTA	0.64±0.21
5mM DMTA	0.68±0.14
10mM DMTA	0.59±0.14

4. DISCUSSION

4.1 Lysine And Enzymes Of Lysine Biosynthesis.

The enhanced activity of DAP decarboxylase in whole infected barley leaves early in the infection process (Fig. 7.1), confirmed by an examination of isolated chloroplasts (Fig. 7.3), may be due to an increased requirement for lysine either by the leaf or by the developing fungus. Indeed, the level of soluble lysine in these leaves was increased at 3 DAI (Fig. 6.1). This is in agreement with the work of Sadler and Shaw (1980) who found increased lysine levels in the mycelium of the rust fungus *Melampsora lini*, on flax cotyledons and Jaeger and Reisener (1969) who found that lysine accumulated in pustules of *Puccinia graminis* on wheat leaves. Also, labelled lysine or its metabolites were transferred from *Phaseolus vulgaris* to haustoria and then to intercellular hyphae of *Uromyces phaseoli* (Mendgen, 1979). In rust-infected bean however, there were no major fluctuations in free and bound lysine during the early stages of infection with rust (Raggi, 1974), although there were increases in free lysine during primary and secondary sporulation on rusted pinto bean leaves and, at flecking, free lysine was decreased in the areas immediately around pustules (Raggi, 1974). In the present study, the levels of both soluble and bound lysine were decreased in whole infected leaves by 6 DAI (Fig. 6.1, 6.2). DAP activity in whole infected leaves was not significantly different from controls at this time,

although enzyme activity was decreased in chloroplasts. Subsequently, DAP decarboxylase activity was significantly greater within the inter-pustule regions than in pustules (Fig. 7.2). The lower DAP decarboxylase activity in pustules may be due to the breakdown in chloroplast function within these regions. Indeed, it is well known that there is a loss of chloroplast polysomes as early as 24h after inoculation of leaves with powdery mildew and that chloroplast disintegration occurs later in infection (e.g. Dyer & Scott, 1972; Callow, 1983; Magyarosy *et al.*, 1976). This is in contrast to brown rust of barley, where chloroplast integrity was not greatly affected within pustules (Ahmad *et al.*, 1983; Scholes and Farrar, 1986). Mills *et al.* (1980) have shown that the enzymes of lysine biosynthesis are located within the chloroplast in green plants and evidence of loss of chlorophyll from individual chloroplasts, decrease in chloroplast volume and decrease in the ratio of chlorophyll a:b have been shown to occur in rust pustules on bluebell leaves (Scholes and Farrar, 1985). These differences were evident by the pre-sporulation stage, when chlorophyll in the pustule areas was 54% of the control value and changes in chlorophyll fluorescence, suggesting impairment of non-cyclic electron transport and chloroplast integrity, were also observed. Although it can be speculated that these changes may be due to, for example, a diffusible toxin as suggested for powdery mildew infection of peas (Aked and Hall, 1993b),

there may be a more straightforward explanation. Thus, Scholes (1992) suggests that, in powdery mildew infected barley, the decrease in the rate of photosynthesis occurs primarily as a result of an accumulation of carbohydrates, following an increase in invertase activity. The subsequent reduction in activity of the Calvin cycle enzymes down-regulates electron transfer. Thus, the mildew does not affect photosynthesis by producing a toxin, but by altering source/sink relations in the infected leaf.

The increased respiration seen during infection requires a greater proportion of photosynthate (Ayres, 1976). This may mean a diversion of precursors away from the lysine pathway. It may also be that more of the precursors of this pathway are diverted to the other branches to form threonine, methionine or isoleucine, since lysine can inhibit the formation of these amino acids (for example: Henke and Wilson, 1974; Henke *et al.*, 1974; Bright *et al.*, 1978; Matthews and Widholm, 1979). Such changes may contribute to the reduction in lysine concentration in mildewed leaves.

The reduced activity of DHDPS, the first enzyme unique to lysine biosynthesis, in both whole infected leaves (Fig. 7.4) and in isolated chloroplasts (Fig. 7.6), throughout the course of the infection, suggests differential effects of infection on these two chloroplast localised enzymes (i.e. DHDPS & DAP decarboxylase). DHDPS is regulated by lysine (e.g. Cheshire and Miflin, 1975; Negruitiu *et al.*,

1984; Ghislain *et al.*, 1990) and although the level of soluble lysine in these leaves was greater at 3 DAI, both soluble and bound lysine were reduced thereafter, as previously stated. Thus, regulation by lysine is not responsible for this effect. In fact, increased activity of this enzyme might be expected because of the reduction in lysine levels. As with DAP decarboxylase, this reduced activity may be due to changes in the integrity of the chloroplasts (although it should be noted that activity was greater in inter-pustule regions than in pustules or healthy leaves at 6 & 9 DAI). For example, in powdery mildew of beet, activity of some of the enzymes involved in organic acid synthesis (which occurs in chloroplasts) was reduced while, at the same time, the relative amounts of amino acids increased (Magyarosy *et al.*, 1976). It is most likely that lysine is taken up from the leaf by fungi (Jaeger and Reisener, 1969; Mendgen, 1979) and Sadler and Shaw (1980) have suggested that the increased production of lysine seen in rust infected flax may provide this amino acid for fungal uptake. This lysine might become bound within the fungus (eg. in proteins) or be dispersed (eg. spores). The levels of both soluble and bound lysine in pustules was lower than in inter-pustule regions at 9 DAI (Fig. 6.3), coincident with sporulation.

The observed reduction in lysine decarboxylase (LDC) activity, seen in whole infected leaves (3-9 DAI) (Fig. 7.7), which is consistent both with the reduced labelling

of cadaverine and its aminopropyl derivatives seen at 9 DAI (Section 3.4) and the reduction in free and bound lysine already discussed, is not due to reduced activity of the enzyme in chloroplasts since activity in these organelles was increased at this time (Fig. 7.9). Activity in chloroplasts isolated from pustules at 9 DAI was reduced (Fig. 7.10), probably due to the previously observed reduction in chloroplast integrity within powdery mildew pustules. However, activity in chloroplasts from inter-pustules at this time remained higher than in healthy leaves. Since LDC is not thought to be found only in chloroplasts, it may be that this anomalous situation is due to reduced activity of the enzyme outwith the chloroplast. This could perhaps cause a stimulation of activity in chloroplasts and may point to some association between the separate locations of this enyzme.

It is also seen from Section 3.4 that the assay used is not specific for LDC and may also measure ODC activity. ODC can catalyse the conversion of lysine to cadaverine, albeit very weakly (Janne *et al.*, 1981). Nevertheless, since ODC is thought to be associated with DNA (Galston & Kaur-Sawhney, 1990), it is possible that the reduction seen in whole leaves is due more to reduced activity of this enzyme. If the increase in LDC seen in chloroplasts is not sufficient to compensate for this reduction then, overall, a reduction in activity would be seen (Fig. 7.8).

DHDPS and DAP decarboxylase are enzymes of lysine biosynthesis in healthy plants. However, the powdery mildew fungus, like most fungi except *Oomycetes*, is thought to synthesise lysine using enzymes of the AAA pathway. The activity of saccharopine dehydrogenase, one of the enzymes of this pathway, in powdery mildew-infected barley leaves (Table 2) was no different from healthy leaves until 9 DAI, when activity increased. In direct contrast to DHDPS (Fig. 7.5) and DAP decarboxylase (Fig. 7.2), this effective increase in activity was located in the pustules. This was almost certainly due to the presence of the fungal mycelium, since activity in isolated mycelium was greater than in mildewed leaves. The increased activity was not due to an increase in the level of lysine, the substrate, but may have been due to a reduction in the levels of saccharopine or AAA, causing release of feedback inhibition. Also, if there is a requirement for either of these compounds by the fungus, especially during sporulation, then the activity of these enzymes might be expected to remain high, or to increase at this time. It is possible that lysine taken up from the leaves is used in the production of proteins and the excess degraded to saccharopine or AAA, which may be required by the fungus or may be used in some other metabolic process. The reduced activity of saccharopine dehydrogenase in infected leaves 12 DAI may reflect both damage to the leaf and to a loss of activity of the fungal enzymes. This latter effect may be due to reduced availability of the substrate (lysine) or to

a reduced requirement for these compounds at this stage in the infection. Thus the level of lysine in these leaves would be reduced and since the DAP pathway enzymes may be damaged by the breakdown of chloroplasts at this stage of infection, then less or no new lysine would be synthesised.

It is interesting to note that, although AAA reductase (Table 1) and saccharopine dehydrogenase (Table 2) activities in infected leaves were similar, the activity of AAA reductase in isolated mycelium (Table 1) was much greater than the activity of saccharopine dehydrogenase in this tissue. The lack of activity of AAA reductase in non-infected leaves thus suggests that this enzyme is only found in the fungal mycelium. In contrast, saccharopine dehydrogenase activity was detected in healthy barley leaves. Interestingly, although biodegradative saccharopine dehydrogenase activity is known to occur in barley, some evidence has been published for the biosynthetic activity of this enzyme in green tissue (Moller, 1976b).

Light is involved as a catalyst in some enzyme reactions (Buchanan, Hutcheson, Magyarosy and Montalbini, 1981). Thus, light regulates the reductive pentose phosphate pathway cycle at a number of key points which may be rate limiting. Changes seen are in the concentration of H^{+} and Mg^{+2} ions, the redox state of, for example, thioredoxins, and increases in the concentration of

metabolites which change enzyme activity (ATP and NADPH). These changes caused by light are reversed in the dark, so that photosynthetic and biosynthetic enzymes are active during the day and degradative enzymes are active at night. It may be, therefore, that some of the changes seen in mildew and rust pustules are due directly to changes in light levels at the surface of the leaf. Fungal mycelia are hyaline but presumably absorb or reflect some light (pustules look white to the naked eye, suggesting that all of the visible light striking these areas is reflected). Light is also involved in chlorophyll production (quality as well as quantity). Shading by the mycelium may cause the switching off of the biosynthetic functions of these key enzymes and the switching on of their biodegradative functions. This would depend on the maintenance of the integrity of chloroplast, plasmalemma and tonoplast membranes. Accelerated senescence would be observed at the original site of infection, where primary sporulation would take place. At the same time, the fungus might grow into outlying regions and the same process could begin again. Reduced photosynthesis and biosynthesis in pustule regions might stimulate these processes in non-infected areas. The development of green-islands, which only takes place in detached leaves or late in infection when natural senescence is advanced, may be induced. The production of cytokinins either by the host or the fungus is thought to be involved in this phenomenon (Kiraly, El Hammady and Pozsar, 1967), although firm evidence is lacking.

Similarly, greatly elevated polyamine concentrations are associated with green-islands on mildewed barley leaves (Coghlan and Walters, 1990). Green-island formation may allow the complete development of spores already initiated.

4.2 Uptake of ^{14}C-aspartate.

The uptake of ^{14}C-aspartate by both healthy and infected leaves increased with time (Table 3). The increased uptake of aspartate by infected leaves at 2h was probably the result of the active metabolic sink created by the presence of the rapidly growing fungus (Bushnell & Gay, 1978). Indeed, removal of the fungal mycelium before feeding reduced uptake at this stage (Table 3), although uptake increased in these leaves from 4h. Other workers have shown that powdery mildew haustoria continue to take up label even after the mycelium has been removed (Shaw & Samborski, 1956). At 4h and subsequently, aspartate uptake by infected leaves bearing mycelium was reduced (Table 3). Since there was no difference in insoluble label (see Section 2.4) between control and infected leaves at 2, 4 or 8h (Table 4) and all of the label had been taken up from the feed water by 8h (results not shown), the lower soluble counts (see Section 2.3) in infected leaves was not due to movement away from the soluble pool to the insoluble pool. This makes the reduction in soluble label in infected leaves at 8h (Table 3) difficult to explain. Nevertheless, there may have been a loss of label as ^{14}C-carbon dioxide

(CO_2), due to spore release, fungal metabolism of aspartate or to altered leaf metabolism as a result of more general effects of the fungus. However, the increased label remaining in the infected leaves from which mycelium was removed before feeding seems to point to loss of label due to fungal metabolism rather than to the latter (Table 3).

4.2.1 Metabolism of ^{14}C-aspartate.

Metabolism of labelled aspartate appeared to take place faster in the infected leaves in that only 22% of the label found in these leaves after 2h was still present as aspartate in contrast to 45% in control leaves (Table 5). In the infected leaves, much of the label had moved to peak 3 (tentatively identified as homoserine and threonine [Table 6]) and peak 5 (as yet unidentified, but possibly a precursor of homoserine i.e. 2-aspartyl phosphate, or of threonine i.e. o-phospho-homoserine; or perhaps methionine [Table 6]). Mills *et al.* (1980) showed that homoserine was the main product formed after feeding pea chloroplasts with ^{14}C-aspartate. Lysine, threonine and isoleucine, as well as alanine, asparagine and glutamate were formed in much smaller quantities. By 4h, only 18.5% of the label in infected leaves was in aspartate but there was still 35% in control leaves. There also appeared to be movement of label from peak 5 to peak 3 (Table 5). Leaves from which the mycelium was removed before feeding contained 26% ^{14}C-aspartate at 4h (Table 5), greater than in infected leaves

with the mycelium still attached, but lower than in control leaves. This suggests that although the surface mycelium played some part in the increased metabolism of the labelled aspartate, there was still a residual effect on metabolism when this was removed.

The radio TLC profiles of extracts from infected leaves from which the mycelium was removed before (Fig. 8.2a) and after feeding (Fig. 8.2b) were quite different. Where the mycelium was removed before feeding the profile resembled that of control leaves (Fig. 8.1a) but that for leaves where fungal mycelium was removed after feeding resembled that for infected leaves with mycelium still attached (Fig. 8.1b). This was true for all samples taken over the 4 - 8h period.

The distribution of label in infected leaves and in mycelium detached after feeding showed large differences as indicated by the two main peaks - aspartate (peak 2) and peak 3 in Fig. 8.3. There was much more ^{14}C-aspartate (as % of soluble label) in the mycelium and, while the % label in aspartate in the infected leaves decreased with time, it remained relatively constant in the mycelium. The decreased aspartate in the infected leaves probably reflects both enhanced metabolism and fungal uptake. The aspartate in the fungus cannot be metabolised to lysine (Vogel, 1959) and probably becomes incorporated into protein only slowly. The amount of label in peak 3 (which

may be homoserine and/or threonine) was lower in the mycelium, and again remained constant while it increased in the leaves. Since the fungus probably cannot make homoserine or threonine (Vogel, 1959), the presence of these amino acids in the mycelium relects uptake from the plant. Peaks 4 and 5 were lower and higher respectively in the mycelial extracts (Fig. 8.3). This selective uptake of amino acids has been shown to occur in rust on coltsfoot (*Tussilago farfara* L.) (Burrell & Lewis, 1977).

4.2.2 Pre-feeding With Unlabelled 10 mM Lysine Or Threonine.

Pre-feeding with lysine or threonine has been shown to inhibit enzymes of the aspartate pathway (Mills *et al.*, 1980). It seemed that, both in control and infected leaves, pre-feeding with 10 mM lysine was more inhibitory to aspartate uptake than pre-feeding with 10 mM threonine (Table 7). However, enzyme inhibition was lower in the infected leaves, possibly due to metabolites being removed from the leaves by the fungus, as suggested above, and would probably lower the enzyme inhibition to some extent. Although uptake of label in the infected leaves pre-fed with lysine was significantly lower than in those pre-fed with threonine, there was the opposite trend in the mycelial extracts (Table 8).

4.3 Uptake Kinetics.

The K_m values for lysine uptake in *E. graminis* (Table 9) were found to be similar to those observed for the uptake of DFMO into barley seedlings (Walters and Kingham, 1990) and putrescine and spermidine uptake into Saintpaulia petals (Bagni and Pistocchi, 1985) but were much higher than uptake of putrescine and spermidine by the plant pathogenic fungus *F. culmorum* (West and Walters, 1991). Further, the decreased uptake of lysine by isolated powdery mildew mycelium in the presence of putrescine and ornithine (Fig. 9.5) suggests that these compounds share an uptake system (or systems). In cultured human epithelial cell lines from kidney the inhibition of lysine uptake by arginine and ornithine was suggested to show the presence of a shared di-basic amino acid transport system (States, Foreman, Lee, Harris and Segal, 1987). There was a high affinity (low concentration) system and a low affinity (high concentration) system for uptake (States *et al.*, 1987). Similarly, in sugarcane cells there are at least two transport sites for arginine and lysine (Maretzki and Thom, 1970). One has a high affinity for arginine and a low extracellular concentration but a second carrier system may function at higher arginine concentrations. In barley roots, where there are also two systems for the uptake of basic amino acids (Bright, Kueh and Rognes, 1983), uptake of lysine, arginine and ornithine was reduced in the roots of mutant plants which did not have the low concentration-

high affinity system. At the same time lysine uptake by leaves was unaffected in these mutants (Bright *et al.*, 1983). Again, in *Chromatium vinosum* (a purple photosynthetic bacterium) it is suggested that there are two transport systems. One can transport either lysine or arginine while the second is specific for arginine (Young-Ae and Knaff, 1988). There was no co-transport of protons (H^+) or sodium ions (Na^+) and it is thought that transport may take place by an electrogenic uniport (Young-Ae and Knaff, 1988).

In contrast, in Saintpaulia petals, the stimulation of spermidine uptake by potassium ions (K^+) may be due to a co-transport mechanism (Pistocchi, Bagni and Creus, 1986). Lysine transport in *Corynebacterium glutamicum* was highly specific, energy dependent and active, and there was exchange of intra- and extra-cellular lysine. Pores, which were thought to be used for lysine excretion and whose permeability depend on membrane structure, appeared to open when intra-cellular lysine concentrations increased (Luntz, Zhdanova and Bourd, 1986).

There was no reduction in uptake of lysine in the presence of arginine in powdery mildew mycelium (Fig. 9.5). In fact, there was a slight though not significant increase. In higher plants, Kinraide (1981) suggests, there are two amino acid transport channels. Channel two transports basic amino acids, with greatest affinity for arginine followed by lysine and then histidine. At the

same time affinities for other amino acids in this channel are low. Channel one, on the other hand, is a general transport system (Kinraide, 1981). In oat coleoptiles, however, there are different mechanisms for the uptake of basic, neutral and acidic amino acids (Kinraide and Etherton, 1980). Here neutral amino acids are thought to be co-transported with a proton and accumulation appears to depend on both a change in pH and the electrical potential difference in protons. With acidic amino acids each molecule is associated with a cation and a proton. In this case accumulation only depends on the electrical potential difference. Since basic amino acids (e.g. ornithine, arginine, lysine) are permanently protonated they do not seem to be co-transported with an additional proton but 'depend on the membrane potential component of the proton motive force' (Kinraide and Etherton, 1980).

In cultured tobacco cells lysine accumulated against a concentration gradient (Harrington and Henke, 1981), and uptake was biphasic. Interestingly, transport was stimulated by low pH and both systems were inhibited by respiratory inhibitors, unlike the uptake of both aspartate and lysine in powdery mildew mycelium (Table 9.1). Uptake of the basic amino acids, arginine and ornithine, was also pH dependent in *Fusarium culmorum* and took place most rapidly at acidic pH (West & Walters, 1991). In cultured tobacco cells, System I was only inhibited by basic amino acids and, to a lesser extent, sulphur amino acids while

system II was inhibited by all of the amino acids tested (Harrington and Henke, 1981). Uptake of lysine does not appear to be an active process in powdery mildew since the metabolic inhibitor sodium azide did not affect uptake (Fig. 9.5). However, the rapid response of arginine and lysine transport in sugar cane cells to metabolic inhibitors shows that, in these cells, uptake requires energy (Maretzki and Thom, 1970), as shown for *Corynebacterium glutamicum* (Luntz *et al.*, 1986). Phosphorylation uncouplers were specially fast in inhibiting uptake in sugarcane cells, suggesting that ATP storage was limited and ATP unavailable unless it was being continuously reformed. In these cells both lysine and canavanine were poor inhibitors of arginine uptake. The lower inhibition of arginine uptake, compared with lysine, on pre-incubation with a metabolic inhibitor is further evidence of two separate transport sites for these amino acids in sugarcane cells (Maretzki and Thom, 1970).

4.4 Formation of Cadaverine Derivatives.

4.4.1 In Barley Leaves.

The accepted route for the formation of polyamines is *via* aminopropyl transfer from decarboxylated *s*-adenosylmethionine (SamDC) (Fig. 5a). However, the existence of a second route which involves the formation of a Schiff base complex has been shown to occur in some

bacteria and in the legume *Lathyrus sativus* (Srivenugopal and Adiga, 1980; in Tait, 1985) (Fig. 5b).

Reduced formation of aminopropyl derivatives of cadaverine following pre-treatment of barley leaves with CHA/MGBG (Table 11) and AdoDato/CHA (both inhibitors of spermidine synthase) (Table 12) suggests that the formation of these compounds takes place by the action of the aminopropyl transferases, although partial operation of the Schiff base complex route proposed by Tait (1985) cannot be ruled out. A similar situation was observed in a number of fungi, which appeared to form CAD and its higher derivatives *via* both routes (Zarb and Walters, 1993). This is unlike the results obtained by Hamana, Matsuzaki, Niitsu and Samejima (1989), where inhibition of the aminopropyl transferases had little effect on the formation of tri- and tetra-amines from putrescine in *Agrobacterium* species. It is suggested that these enzymes have a very narrow specificity (Goodman, Kiraly & Wood, 1986). However, Matsuzaki *et al.* (1987) postulate that homospermine (aminopropylhomospermidine) may be formed from homospermidine by spermine synthase (Matsuzaki *et al.*, 1987). Also, aminopropyltransferases other than spermidine and spermine synthase are involved in the production of norspermidine and norspermine from 1,3-diaminopropane in some thermophilic Eubacteria and Archaebacteria (Hamana *et al.*, 1989).

Pre-treatment of leaves with DFMO/DFMA (Table 12)

substantially reduced (50%) the labelling of 3APC in both healthy and powdery mildew-infected leaves, although APC was also present in the healthy inhibitor treated leaves (Table 12). This suggests that the precursor, cadaverine, is formed by the action of ODC/ADC as well as by lysine decarboxylase, since these inhibitors should inhibit ODC and ADC activity. It should be noted however, that both DFMO and DFMA can be metabolised in plant tissue and this may result in incomplete inhibition of ODC and ADC.

Inhibition of ODC also inhibited production of cadaverine and its aminopropyl derivatives in Chinese hamster ovary cells (Holtta and Pohjanpelto, 1983). These authors suggested that cadaverine is formed from lysine by the action of ODC, which has been shown to catalyse this reaction in rat liver cells, although the affinity of the enzyme for ornithine is around one hundred times greater (Pegg and McGill, 1979; In Holtta and Pohjanpelto, 1983). Again, in the filamentous fungus *N. crassa*, ODC activity was greatly enhanced (de-repressed) when ornithine was depleted (Paulus, Kiyono & Davis, 1982). Cadaverine and APC are produced from lysine, following decarboxylation by ODC, since mutants of this species without ODC activity cannot decarboxylate lysine (Paulus *et al.*, 1982). ODC catalysed the formation of cadaverine (*in vitro*) in polyamine depleted cells of *E. coli* and APC was synthesised from cadaverine, although (*in vivo*) the formation of APC was inhibited by ODC (Igarishi, Kashiwagi, Hamasaki, Miura,

Kakegawa, Hirose and Matsuzaki, 1986). In contrast, the activity of ODC (*in vivo*) in tumour cells exposed to DFMO was greatly increased and DFMO appeared to act as a competitive inhibitor rather than as an irreversible inhibitor (Alhonen-Hongisto and Janne, 1980). The increased activity of the enzyme in these cells was thought to be due to stabilisation of the enzyme by DFMO, preventing proteolysis. Alhonen-Hongisto and Janne (1980) suggest that it may also be due to the depletion of putrescine and spermidine which may act as feedback inhibitors (Alhonen-Hongisto and Janne, 1980). Inhibition of ODC led to enhanced SAMdc activity in this case (Janne *et al.*, 1981). When the phytopathological fungus, *P. avenae*, was grown in media amended with DFMO or DFMO/MGBG the concentrations of CAD, PUT and SPD were reduced significantly (Foster and Walters, 1990). Here, DFMO reduced ODC activity greatly and the activity of SAMdc was greatly enhanced. In addition, MGBG reduced ODC activity, which is in contrast to results seen with tumour cells, where activity of this enzyme was greatly increased when treated with this compound (Porter and Sufrin, 1986). This latter result may be due to stabilisation of ODC protein in these inhibitor treated cells (Janne, Alhonen-Hongisto, Nikula and Elo, 1985) as previously suggested with DFMO (Alhonen Hongisto and Janne, 1980). However, the inhibition of ADC and SAMdc, by ethylene, in pea seedlings led to an increase in lysine decarboxylase activity and

allowed accumulation of CAD (Icekson *et al.*, 1986). These authors postulate that the observed increases compensate for the inhibition of the 'normal' route of polyamine biosynthesis.

4.4.2 In Powdery Mildew Mycelium.

Unlike the results obtained with leaves, treatment of mycelium with DFMO/DFMA had no significant effect on labelling of CAD and its aminopropyl derivatives (Tables 13, 14 & 15). This was in spite of the fact that the activity of LDC (or ODC) was reduced significantly in the presence of these inhibitors (Tables 16 & 17). Without the addition of DFMO/DFMA, MGBG/CHA had no effect on LDC activity (Table 18). Since MGBG/CHA also had no effect on labelling, it may be that the alternative Schiff base pathway is present in this fungus, unlike the situation in barley leaves. This is also unlike the results obtained by Zarb and Walters (1993; 1994) with a number of phytopathological and mycorrhizal fungi. Here labelling was, in most species, reduced significantly by treatment with MGBG/CHA. Zarb and Walters (1993) suggest, however, that the remaining label may be due to the operation of the Schiff base route. The significant reduction in labelling observed in the presence of MGBG/CHA and DFMO/DFMA (Table 15), which was not observed with MGBG/CHA or DFMO/DFMA alone may be due to a non-specific effect of the combined inhibitors.

Total labelling was increased in the presence of increasing concentrations of the spermine synthase inhibitor DMTA (Table 19). The increased labelling of 3APC in the presence of this inhibitor points to the activity of an aminopropyltransferase other than SPM synthase. It is interesting to note that there was no significant difference in the activity of LDC in the presence of DMTA (Table 20).

5. CONCLUSIONS

Since plants and some pathogenic fungi have separate pathways for lysine biosynthesis, the specific inhibition of lysine biosynthesis in these fungi might provide a new approach to fungal control. However before this can be achieved, information on lysine metabolism in the host and fungus must be gained.

Results discussed in this thesis show that there are significant changes in the metabolism of aspartate and lysine in barley leaves infected with powdery mildew. Although there was greater uptake of labelled aspartate by infected leaves initially, uptake decreased thereafter. Metabolism of the labelled aspartate was greater in the infected leaves compared to controls, while in the fungus aspartate and homoserine were taken up from the leaves without further metabolism. Furthermore, the apparent inability to metabolise these compounds suggests that the

diaminopimelic acid pathway for lysine biosynthesis is not present in the fungus. The early increase in DAP decarboxylase activity and soluble lysine may provide lysine for the rapidly growing fungus. This growing sink for nutrients could cause an increase in metabolism by releasing feedback inhibition of enzymes. For example, inhibition of aspartate uptake by lysine and threonine was reduced in infected leaves. This increased metabolism is especially apparent in the work done using labelled aspartate. An examination of the uptake of lysine and aspartate by powdery mildew mycelium showed that the fungus is apparently quite able to take up both of these compounds, even when isolated from the leaves.

The reductions in soluble and bound lysine by 6 days after inoculation (DAI) may be due to loss in fungal spores or metabolism by the fungus, as suggested for the loss of label seen during uptake of labelled aspartate. The activities of three enzymes of the DAP pathway (DHDPS, DAP decarboxylase and LDC) were also decreased in pustules by 9 DAI and may be due to the documented loss of chloroplast integrity.

By contrast, the activity of two enzymes (saccharopine dehydrogenase and aminoadipic acid reductase), thought to be of fungal origin, were increased at 9 DAI. These enzymes may also be involved in lysine catabolism. Furthermore, it was demonstrated that the formation of CAD and its aminopropyl derivatives was very much lower in infected

leaves, showing that the reductions in lysine levels in infected leaves are not due to increased cadaverine formation. However, this reduction in cadaverine formation may be due to the reduced levels of lysine in the infected leaves or to a reduced requirement for these compounds. This alternative pathway for the formation of polyamines is thought to be used mainly when there are insufficient 'normal' polyamines (putrescine, spermidine, spermine) available. Since polyamine levels are known to increase in infected leaves (e.g. Walters, Wilson & Shuttleton, 1985; Walters & Wylie, 1986; Coghlan & Walters, 1990; Machatschke, Kamrowski, Moerschbacher & Reisener, 1990; Foster & Walters, 1990), use of this pathway may not be required. Interestingly, a further alternative pathway for the formation of cadaverine derivatives, *via* Schiff base formation, may be present in the fungus.

This work confirms that the DAP pathway of lysine biosynthesis is present in barley leaves, while the AAA pathway is present in the powdery mildew fungus. It has also shown that the fungus is capable of taking up both precursors and metabolites of lysine. Thus, although the fungus has the capacity to synthesise lysine, it may also require to take up lysine from the leaves for sustained growth and reproduction. At present, therefore, it is not clear whether specific inhibition of fungal lysine biosynthesis will be fungicidal. Before this can be determined, it will be necessary to ascertain whether the

mildew synthesises most of its own lysine requirement.

6. FUTURE WORK.

The usual method for specifying and quantifying compounds required for fungal growth is by growing the fungus on modified medium lacking the compound under test, or by adding the radiolabelled compound to the medium and examining the biosynthetic products. It is also possible in the same way to look at the activity of enzymes and at enzyme induction. While biodegradative enzymes are inducible and biosynthetic enzymes are constitutive, they can be distinguished by the co-factors and redox conditions required for optimum activity. This relatively simple method cannot, unfortunately, be used with powdery mildews since there is at present no way that these can be grown axenically. A different approach must, therefore be tried, perhaps by injecting the labelled substrate into the infected leaves or by allowing it to be taken up by the isolated leaves. Removal of the mycelium or the pustules after a measured time would allow the metabolic products of the substrate to be determined and measured.

The latter method was used to examine the uptake and metabolism of labelled aspartate. It was not possible to show the exact products of aspartate metabolism in the leaves or fungus during the work on uptake of ^{14}C-aspartate. This was due to the difficulty of finding a TLC

system which effectively separated the metabolic products. The main problem lay in the co-chromatography of aspartate with lysine or lysine with homoserine and/or threonine, in most of the solvent systems used. A further range of solvent systems tried since the completion of this work, shows that it may indeed be possible to separate these products. For example, ethanol:28% ammonium hydroxide (80:20) separated all four compounds although homoserine and threonine ran very closely together. Running the same plate in butanol:acetic acid:water (4:1:1) in the second dimension might give better separation of these latter compounds. Methionine should also be separated using this system.

As an extension of the work done using labelled aspartate, the uptake of ^{14}C-lysine from infected leaves into the mycelium might give a measure of the fungal requirement for this amino acid. This might be more easily measured than the previous work since the potential number of compounds produced from lysine should be smaller. This could also, perhaps, give an indication of how lysine is used within the area infected by the fungus. For example, it might show that lysine is, as has been suggested, removed from the leaves in spores or show if label is lost in the form of carbon dioxide. Preliminary unpublished work using labelled lysine has shown that there is a greater evolution of labelled carbon dioxide from infected leaves than from healthy leaves. It may also be that some

of the lysine is degraded to saccharopine. Transamination of the saccharopine could, theoretically, yield glutamate by a reversal of reaction 7 (Fig. 2a). Detection of labelled saccharopine and glutamate after feeding with labelled lysine would confirm this. Other amino acids might be produced by transamination of the glutamate thus formed. This may also help to explain the reduction in lysine levels in infected leaves.

The work done on cadaverine and its higher homologues has shown that, at 9 DAI, the synthesis of these compounds was very much reduced in infected leaves. At the same time lysine levels and the activities of two of the enzymes of the DAP pathway were reduced within powdery mildew pustules. It would be interesting to know if this reduction in the levels of APC and 3APC only occurs during sporulation or if this is also seen earlier in the course of infection. An examination of isolated pustules and inter-pustule regions, as has been carried out for DAP decarboxylase, DHDPS and LYS decarboxylase would be useful in determining whether these changes are generalised or localised within a specific area of the infected leaves. Preliminary work has already shown that labelling of 3APC was much greater in inter-pustule regions than in pustules. This is in spite of the fact that these reactions also occur in the mycelium of powdery mildew.

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APPENDIX

Chloroplast Isolation Medium (Ahmad *et al.*, 1983).

400 mM	sucrose
50 mM	Hepes (pH 8.0)
10 mM	sodium chloride
1 mM	magnesium chloride
1 mM	EDTA
4.1 mM	cysteine
0.25%	BSA
1%	PEG

Mycelium Homogenising Medium (Gregory, 1992).

50 mM	Mes-BTP
1.5 mM	magnesium chloride
2.0 mM	EDTA
5 mM	glucose
30 mM	glycine betaine
2%	PEG
0.5%	PVP
0.1%	BSA
0.3%	DTT

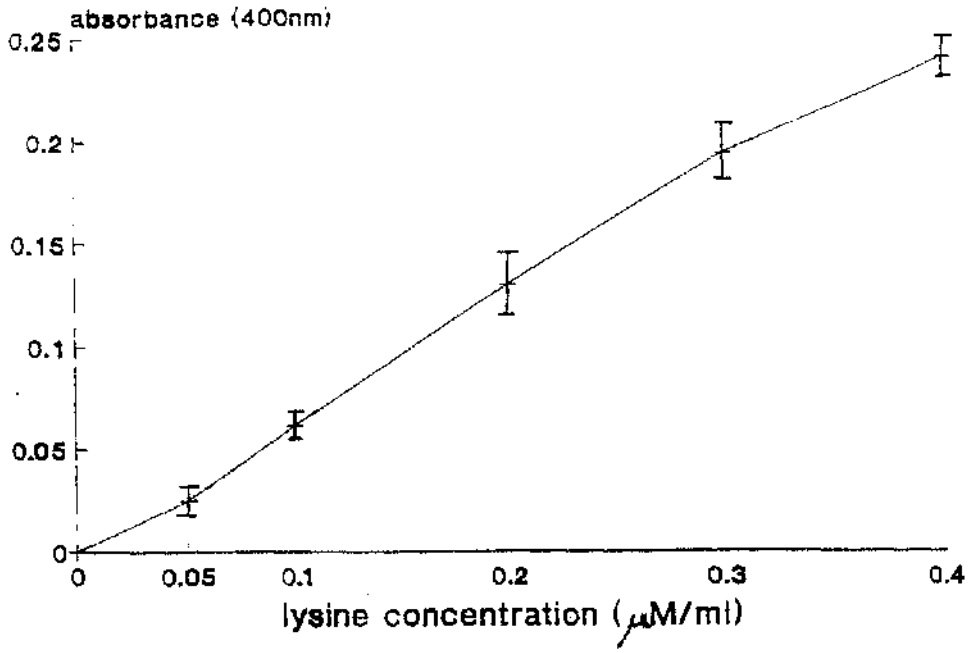

Fig A: Lysine standard curve constructed using data obtained after performing the Hutzler lysine assay described in Section 2.2.1.4. Six replicates were used and the standard errors are shown.

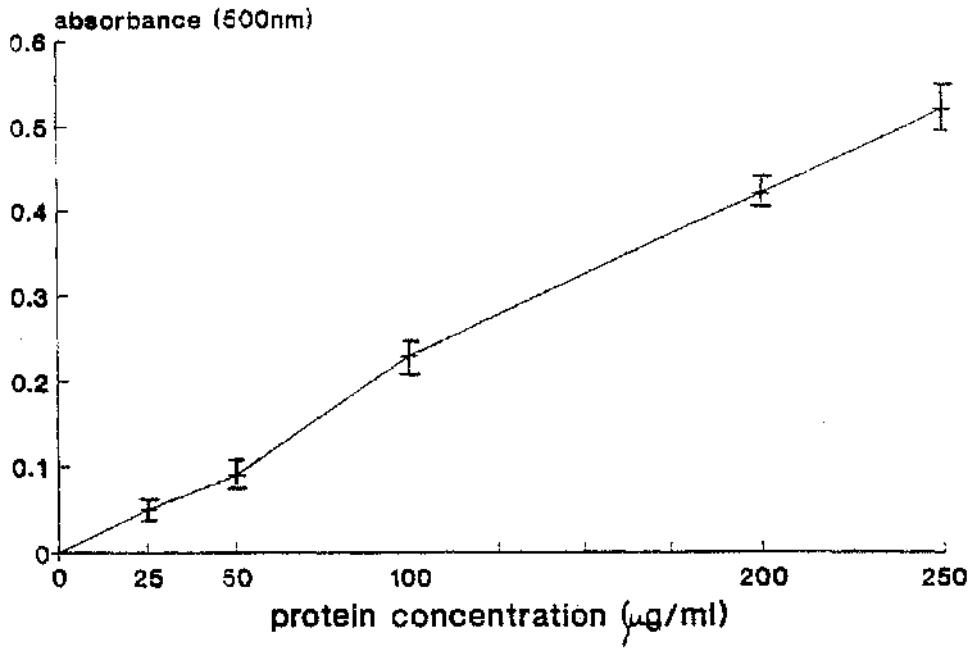

Fig B: Protein standard curve constructed following protein assay (Section 2.1.5.) of known concentrations of bovine serum albumin (fraction V). Two replicates were used and the standard errors are shown.