SUBSTRATES AND INHIBITORS OF DIAMINE OXIDASE

A thesis is presented in part fulfilment of the requirements for the Degree of Doctor of Philosophy

by

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Finally I would like to dedicate this thesis to my parents, Helen and Stephen, without whose daily support and guidance this and all other of my achievements would have been impossible.

ABBREVIATIONS

BCA	Bicinchoninic acid
BSA	Bovine serum albumin
BSOA	Bovine serum amine oxidase
DAO	Diamine oxidase
DAST	Diethylaminosulphur trifluoride
DFMO	a-Difluoromethylornithine
DMAB	3-(Dimethylamino)benzoic acid
DMAP	4-Dimethylaminopyridine
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
DNPH	2,4-Dinitrophenylhydrazine
DOPA	3,4-Dihydroxyphenylalanine
ee	Enantiomeric excess
EPR	Electron paramagnetic resonance
FAD	Flavine adenine dinucleotide
FPLC	Fast protein liquid chromatography
HPLC	High performance liquid chromatography
K_M	Michaelis Menten constant
LSIMS	Liquid secondary ion mass spectroscopy
MBTH	3-Methyl-2-benzothiazolinone hydrazone
NBT	Nitroblue tetrazolium
NMR	Nuclear magnetic resonance
ODC	Ornithine decarboxylase
PQQ	Pyrroloquinoline quinone
SDS	Sodium dodecyl sulphate
UV	Ultraviolet
V_{max}	Maximum rate

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SUMMARY

This thesis describes a study of the enzyme pea seedling diamine oxidase (ps DAO) and concentrates on four main areas :- (1) enzyme-catalysed oxidation of primary diamines by pea seedling diamine oxidase (2) oxidation of pyridine-amines by pea seedling diamine oxidase (3) inhibition of diamine oxidase and (4) further purification of pea seedling diamine oxidase.

(1) Oxidation of Primary Diamines by Pea Seedling Diamine Oxidase

Pea seedling diamine oxidase catalyses the oxidation of a range of diamines into the corresponding aminoaldehydes according to Scheme A.

$$H_2N(CH_2)_nNH_2 + H_2O + O_2 \xrightarrow{\text{Diamine Oxidase}} H_2N(CH_2)_{n-1}CHO + H_2O_2 + NH_3$$

Scheme A

Putrescine (i) and cadaverine (ii) are the natural substrates for the enzyme. Although much work has been carried out to determine the

relative effectiveness of these compounds and analogues thereof as substrates for pea seedling diamine oxidase, little data existed concerning the substrate potential of cyclic diamines.

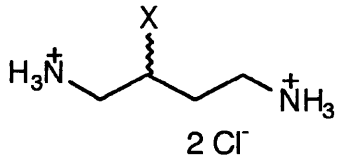
$H_2N(CH_2)_4NH_2$ (i)

$H_2N(CH_2)_5NH_2$ (ii)

A range of simple substituted aromatic and alicyclic diamines were synthesised in the form of the dihydrochloride salts and tested as substrates for the enzyme using an improved spectrophotometric assay system. The assay is dependent on the accurate measurement of hydrogen peroxide, produced as a product during the enzymatic oxidation. From the assay K_M and V_{max} values were obtained directly, giving an indication of the relative binding affinities and rate of oxidation of the various substrates. Analysis of these results suggested that although the cyclic diamines were poorer substrates than the natural substrates they did in general show a greater binding affinity for the enzyme than either putrescine (i) or cadaverine (ii). Comparison of the kinetic parameters obtained for a range of substituted aromatic diamines provided information on the effect of the position and nature of the substituents in determining the binding affinity and rate of oxidation of these compounds.

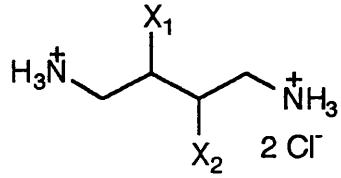
Comparison between the kinetic parameters found for analogues of the natural substrate with those of the natural substrate itself often provides useful clues into the nature of the active site. A range of putrescine analogues were tested as substrates for pea seedling diamine oxidase. All attempts to synthesise optically active 2-haloputrescines (iii) met with failure. A range of 2,3-

dihaloputrescines (iv) were however synthesised as the dihydrochlorides and kinetic data was collected for these substrates.



X = Br, Cl, F

(iii)

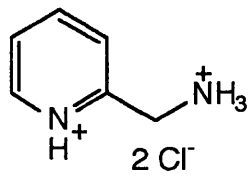


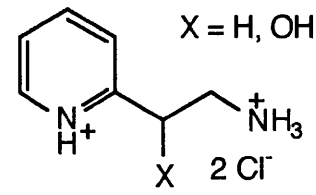
X1 = X2 = Br,Cl

(iv)

(2) Oxidation of Pyridine-Amines by Pea Seedling Diamine Oxidase

Pea seedling diamine oxidase is capable of catalysing the oxidation of some primary monoamines, albeit at reduced rates compared to the corresponding diamines. In order to examine more fully the role played by the second amine group during catalysis a range of (aminoalkyl)pyridine derivatives (v) and (vi) were synthesised as the corresponding dihydrochloride salts and assayed as substrates for the enzyme. The results obtained suggest that the presence of a second primary amine group is not required for efficient substrate binding however it does have a more definite role to play in promoting the deamination.





(v) (vi)

(3) Inhibition of Pea Seedling Diamine Oxidase

The oxidative deamination of diamines by diamine oxidase is a key step in polyamine metabolism. Polyamines are known to be essential for cell growth and replication. Inhibitors of the reaction catalysed by diamine oxidase may have a detrimental effect on polyamine metabolism and therefore on cell growth.

A range of compounds shown by our initial kinetic studies to be poor substrates but efficient binders for the enzyme were tested as inhibitors of pea seedling diamine oxidase. The tests were carried out using a modified version of the spectrophotometric assay used previously. Many of the compounds were shown to inhibit competitively the diamine oxidase catalysed deamination of putrescine. K_i values are reported for all compounds tested.

(4) Further Purification of Pea Seedling Diamine Oxidase

Pea seedling diamine oxidase can readily be isolated in a partially purified form from peas by means of a series of precipitations. The enzyme prepared in this manner was suitable for our kinetic studies. In an attempt to produce a homogeneous enzyme preparation a method was developed which utilised, as the final purification step,

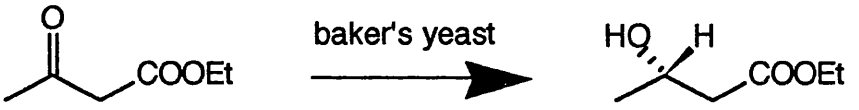
the technique of Fast Protein Liquid Chromatography. Although a homogeneous preparation was not obtained the 280-fold increase in specific activity observed compares well with previous purifications.

CHAPTER 1

INTRODUCTION

1.1 The Importance of Enzymes in Organic Synthesis

The use of enzymes in organic synthesis is of increasing importance with enzymatic reactions now being frequently employed to effect transformations that would be difficult to carry out by chemical means. In many cases enzyme catalysed processes offer significant advantages over chemical methods including those of efficiency, regioselectivity and stereoselectivity. The ability of enzymes to carry out biotransformations in extremely mild conditions, compatible with the most fragile organic molecules, also make their use an attractive option. Use of enzymes can provide methods for the preparation of optically active compounds. For instance, the reduction of ethylacetoacetate (1) to ethyl β-hydroxybutyrate (2) by baker's yeast,[1] gives an enantiomeric excess (ee) of 95-97% (Scheme 1.1).



(1) (2)

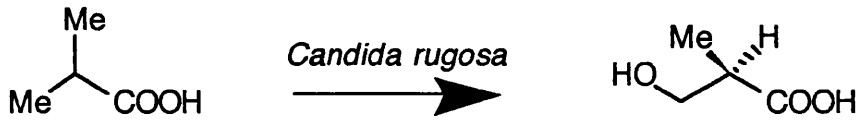
3 *S* : 95-97 % ee

Scheme 1.1

The ability to create essentially homochiral materials from prochiral starting materials is of particular interest in the area of drug manufacture. The vast majority of pharmaceutical drugs

available today, containing a centre of chirality, are marketed as racemates. In general, only one of the enantiomers of a racemic mixture is responsible for the desired activity towards the target organism. At best, the other enantiomer is "inert" and the mixture exhibits half the potency of the active enantiomer. However, in more extreme cases the unwanted enantiomer inhibits the desired effect of the active isomer and/or exhibits toxic side-effects. With the ever increasing demand for more selective drugs which show less toxic side-effects there is a greater emphasis being put on the synthesis of pure optically active compounds. With the increase in the number of commercially available biocatalysts, capable of catalysing a wide range of biotransformations, it would seem that enzymes have an important role to play in future developments in this area.

Although use of isolated enzymes is generally favoured, whole organisms have been successfully employed as biocatalysts. This is demonstrated in the synthesis of β-hydroxyisobutyric acid (Scheme 1.2). Here functionalisation of a methyl group of isobutyric acid (3) by *Candida rugosa*,[2] gives rise to the formation of (2*S*)-β-hydroxyisobutyric acid (4) in high enantiomeric excess. Functionalisation of this unactivated carbon centre by chemical means is not trivial.



(3) (4)

Scheme 1.2

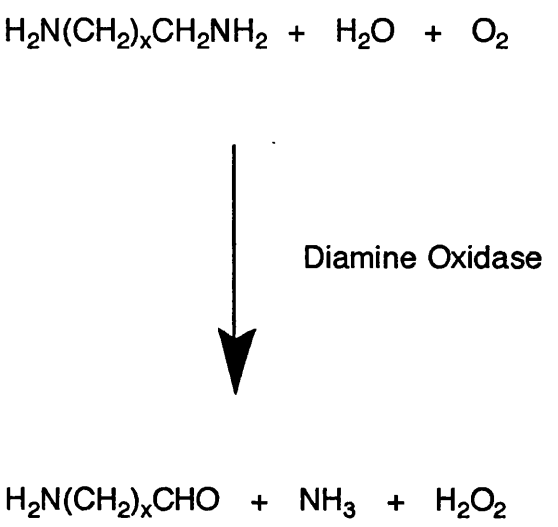
1.2 Immobilisation of Enzymes

Although, as discussed in 1.1, there may be advantages in the use of enzymes in organic synthesis, there are also some possible disadvantages in their use as organic catalysts, particularly in industrial processing. Many enzymes are inactivated under extreme conditions, for example, at high temperatures or in highly acidic or basic solutions. Indeed organic reagents themselves may be responsible for the loss of activity in some enzymes. Also, enzymes generally function optimally in aqueous solutions, rather than the organic solvents usually required to solubilise organic reagents. Moreover, enzymes are often inhibited by their substrates and their products at concentrations below those considered necessary for an economical process.

In recent years it has been shown[3] that the stability of an enzyme can often be improved by making the molecule more rigid through multipoint attachment to a solid carrier. This immobilisation leads to an increase in ionic strength in the microenvironment of the bound enzyme which is thought to decrease the amount of dissolved oxygen.[4] Various immobilisation techniques are now available to the organic chemist. This advancement in technology has opened the way for more efficient use of enzymes in industrial scale processing. Not only can the enzymes be made stable to the conditions of the industrial process but a batch reaction can more easily be terminated by the removal of an insoluble biocatalyst. Also, contamination of the organic product by the enzyme protein is normally significantly reduced.

1.3 Diamine Oxidase

Diamine oxidases (DAO, EC 1.4.3.6) catalyse the oxidative deamination of a range of primary diamines to the corresponding aminoaldehydes (Scheme 1.3).



Scheme 1.3

The mechanism by which the deamination takes place is not fully understood, but literature evidence currently available will be discussed in Chapter 2.

Enzymes which are capable of carrying out the above transformation are widespread in nature although two sources are particularly convenient. Pig kidney diamine oxidase is commercially available in a crude form and pea seedling diamine oxidase can be readily extracted from 10 day old pea seedlings.[5]

Diamine oxidases are copper containing proteins. It has been shown[6] that removal of the copper by chelating agents leads to

deactivation of the enzyme and that the activity is restored on the subsequent addition of Cu^{2+}.

The fact that diamine oxidases were shown to be inhibited by reagents capable of forming carbonyl derivatives[7] was strongly suggestive that the prosthetic group of the enzyme contained a carbonyl functionality. This was initially believed to be due to pyridoxal phosphate. However with the development of the hydrazine method,[8] the organic cofactor was claimed to be pyrroloquinoline quinone (PQQ). Recently further evidence has been published which suggests that PQQ may not be the cofactor and that topaquinone is the organic cofactor in a number of amine oxidases. The available evidence relating to the nature of the cofactor in diamine oxidases will be reviewed in Chapter 2.

1.4 Polyamines and Cell Growth

Diamine oxidase plays an important role in the regulation of cellular levels of natural polyamines.

Although Antoni van Leeuwenhoek was credited with the first observation of a polyamine crystal over 300 years ago, it is only in the last few years that major advances have been made into determining the biological importance of these compounds.[9]

All plants and animals are thought to contain at least one polyamine, with putrescine (5), spermine (6) and spermidine (7) being the most abundant.

$H_2N(CH_2)_4NH_2$ (5)

$H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$ (6)

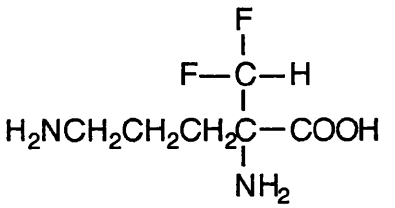
$H_2N(CH_2)_3NH(CH_2)_4NH_2$ (7)

Although polyamines are believed to have various physiological roles to play, it is their association with cell growth and replication that is the most important. This association stems from the ability of the protonated polyamine to interact strongly with the phosphate anions of the nucleic acids. This has a stabilising effect on both RNA and DNA and also speeds up every step in the transcription/translation sequence, that is the process where information coded by genes is used in protein synthesis.[9]

1.5 Inhibitors of Polyamine Biosynthesis

Final confirmation of the importance of polyamines in cell growth and replication came in the late 1970s with the development of specific inhibitors of polyamine biosynthesis. These inhibitors allowed biochemists to examine what happens to a system when the concentration of polyamines is significantly reduced. α-Difluoromethylornithine (DFMO) (8), synthesised by Merrell-Dow Pharmaceuticals, was found to bind specifically and irreversibly to ornithine decarboxylase (ODC), the enzyme involved in the first stage of polyamine biosynthesis. Inhibition of this enzyme led to a significant reduction in the formation of polyamines and this was shown to have a marked inhibitory effect on the proliferation of cells

in various cultures.[9] This showed that polyamines are indeed involved in cell growth and replication but also proved to have other benefits.



(8)

DFMO has been shown to possess interesting anti-tumour activity. Tumour cells proliferate rapidly and have a greater demand for polyamines, thus inhibition of polyamine biosynthesis has a more detrimental effect on tumour cells.

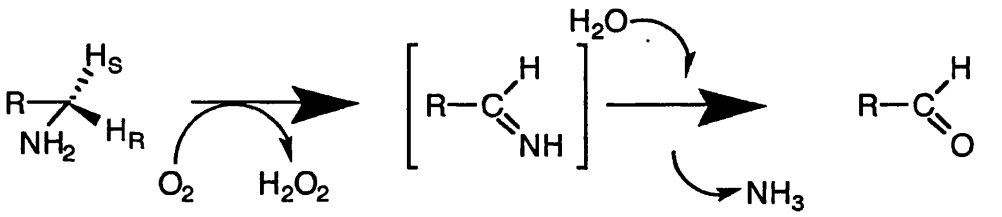
Experiments involving plant cells also produced interesting results. Plants possess two routes for polyamine biosynthesis. As well as the ODC pathway, polyamines can be produced from arginine via the enzyme arginine decarboxylase. So, if a particular fungus which infects a plant (or crop) possesses only the ODC pathway, then treatment with DFMO should lead to selective inhibition of polyamine biosynthesis via this route and hence the death of the fungus. The plant (or crop) will be unaffected. In 1985 Venkat Rajam and Galston showed that DFMO had an inhibitory effect on the growth of several fungi on artificial media.

Since diamine oxidase is involved in the determination of cellular levels of polyamines it would seem reasonable to expect, that inhibition of this enzyme may lead to useful anti-tumour and/or anti-fungal activity.

The kinetics of enzyme inhibition is discussed in Chapter 3, and the testing of some derivatives as inhibitors of pea seedling DAO is discussed in Chapter 6.

1.6 Stereochemistry and Regiochemistry in Reactions of Diamine Oxidase

During the enzymatic deamination of a diamine by DAO, a proton from the prochiral methylene group adjacent to the nitrogen is lost (Scheme 1.4). The absolute stereochemistry associated with this loss has been determined by various methods.



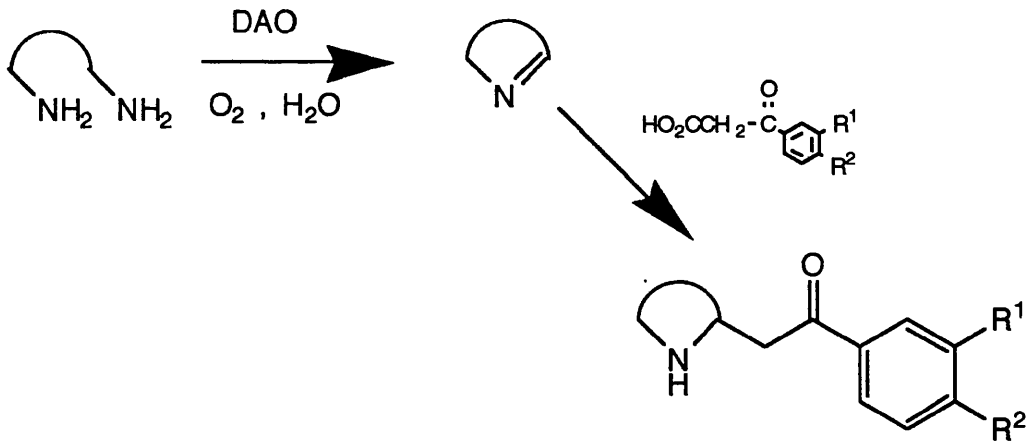
Scheme 1.4

The consistent result obtained on examination of a wide range of substrates,[10-12] all of which had been enantiomerically labelled with either deuterium or tritium, suggested that it is the *pro-S* hydrogen that is lost during the enzymic process.

An insight into the regioselectivity of DAO was given by Santaniello *et al.*[13] It was shown that using pea seedling DAO, both (*R*)- and (*S*)-2-methylputrescine were regioselectively oxidised at the C-4 position, whereas with pig kidney DAO the regiochemistry differed depending on the stereochemistry of the substrate.

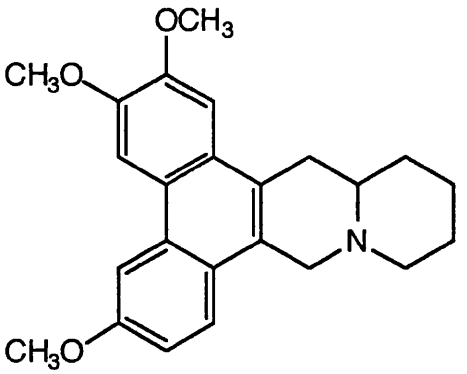
1.7 Synthetic Applications of Diamine Oxidase

Cragg and Herbert have used diamine oxidase as an efficient catalyst in the synthesis of a wide range of phenacyl derivatives.[14] The enzyme catalysed oxidation of a suitable diamine, followed by the *in situ* condensation of the resultant imine with a benzoylacetic acid derivative, gave access to a variety of novel phenacyl compounds (Scheme 1.5).



Scheme 1.5

These derivatives are important intermediates in the synthesis of biologically active alkaloids. Cragg and Herbert[15] have produced the alkaloids cryptopleurine (9), tylophorine (10) and analogues by further transformations of phenacyl derivatives synthesised via a DAO catalysed oxidation.



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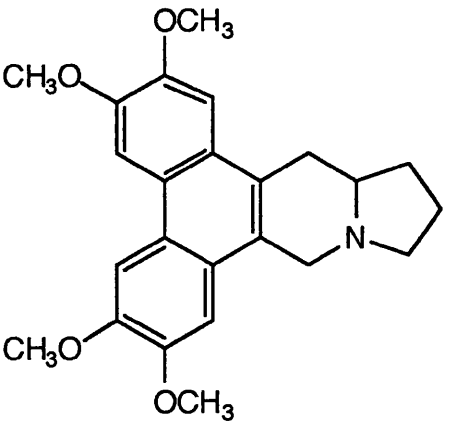
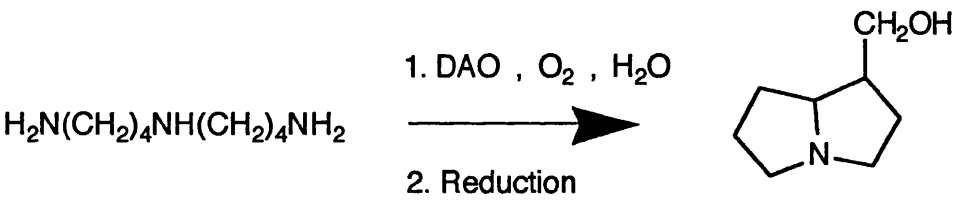


(10)

The pyrrolizidine alkaloid trachelanthamidine (12) can be conveniently formed[16] through the incubation of homospermidine (11) with DAO followed by chemical or enzymic reduction of the likely product, 1-formylpyrrolizidine (Scheme 1.6). Homospermidine is an intermediate in the biosynthetic pathway to pyrrolizidine alkaloids.



(11) (12)

Scheme 1.6

This use of isolated enzymes could allow the synthesis of a wide range of new alkaloid analogues.

1.8 Substrate Specificity of Diamine Oxidase

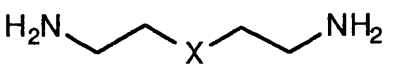
The substrate specificity of diamine oxidase has been investigated by many authors. In 1955, Mann[17] showed that a series of diamines which included histamine and the amino acids lysine and ornithine were substrates for partially purified pea seedling diamine oxidase. Confirmation that the above oxidations were due to one particular enzyme came in 1961 when Werle *et al.*[18] reproduced the results using highly purified diamine oxidase.

Since then a wide range of diamines have been examined as substrates for the enzyme.

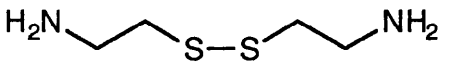
Frydman *et al.*[19] showed that a range of N- and C-alkylputrescines acted as substrates for both animal and plant diamine oxidase.

Equi *et al.*[20] have shown that a series of a,v-diamines with chain lengths varying from 2 to 12 are oxidatively deaminated by pea seedling diamine oxidase, with the natural substrates putrescine and cadaverine being most efficiently oxidised.

Systems containing heteroatoms have also been examined as substrates for diamine oxidase. Lanthionamine (13) and the oxygen analogue (14) have been shown to be oxidised by pea seedling diamine oxidase. Other thiodiamines like cystamine (15) are oxidatively deaminated by pig kidney diamine oxidase[21] as are the corresponding seleno-analogues.[22]



X = S (13)
X = O (14)



(15)

Although some monoamines (e.g. benzylamine) are substrates for diamine oxidase, their poor rate of oxidation suggests that the presence of a second amino group is essential for an efficient process.

Even though the catalytic functions of pea and pig kidney diamine oxidase are similar, it is believed that there do exist some differences in structure between the two enzymes. Macholan[21] in his experiments using hydroxydiamines showed that the affinities of these substrates towards the two forms of diamine oxidase were significantly different. Frydman *et al.*[19] has shown that the rate of enzymatic deamination of the substrates 1-propylputrescine and 2-methylputrescine vary considerably for plant and mammalian oxidases. These results, together with those reported by Santaniello *et al.* (Section 1.6) suggest that certain differences exist at the active sites of the enzymes.

This summary of diamine oxidase is developed in detail in the following Chapter, and enzyme kinetics are discussed in Chapter 3.

1.9 Work Described in this Thesis

Diamines containing ring systems (alicyclic or aromatic) have been studied as substrates for pea seedling DAO. The synthesis of a number of cyclic diamines and their behaviour with DAO are discussed in Chapter 4.

The range of putrescine derivatives which have been studied as substrates for DAO has been extended by incorporating a number of halogen-containing compounds. This work is described in Chapter 5.

Some pyridine derivatives containing an amino group in the side chain have been synthesised and tested as substrates or inhibitors of pea seedling DAO. The results are discussed in Chapter 6.

An attempted purification of pea seedling DAO using the technique of Fast Protein Liquid Chromatography is discussed in Chapter 7.

CHAPTER 2

DIAMINE OXIDASE - A REVIEW

2.1 Isolation and Purification of Diamine Oxidase (DAO).

Diamine oxidases are widespread in nature; however two sources are particularly convenient for their isolation and purification. Pig kidney diamine oxidase is commercially available, whereas pea seedling diamine oxidase can be extracted and purified from young pea seedlings.[22] It has been shown[23] that maximum diamine oxidase activity can be found in the peas between 7 and 16 days after germination.

Hill[5] has developed a convenient method for the purification of pea seedling diamine oxidase. Much of the unwanted material is removed from the crude extract via precipitation with an ethanol/chloroform (2:1) mixture. The enzyme is then isolated through a series of ammonium sulphate precipitations followed by a pH dependent precipitation according to the method of Tabor.[24] Finally, chromatography on hydroxyapatite DEAE-cellulose columns produces the purified enzyme. The final chromatography step is only necessary when a highly purified sample is required.

Diamine oxidase has been isolated from pea epicotyls.[25] Treatment of the crude extract with a 5% protamine solution followed by saturation with ammonium sulphate (65%) yielded the enzyme which was purified to homogenity on phosphocellulose and MGBG-Sepharose columns. The above procedure resulted in a 32-fold increase in specific activity.

The molecular weight of the homogeneous enzyme was found to be 180 000 daltons. This agreed with reported results.[26,27] Sodium dodecylsulphate (SDS) gel electrophoresis produced a single band at mass 85 000 daltons. These results are strongly suggestive that the enzyme consists of two identical subunits.

More recently high performance liquid chromatography (HPLC) has been employed in the purification of DAO.[28]

2.2 The Role of Copper in DAO

A study of the inhibition of diamine oxidase by chelating agents[29] suggested that the enzyme is a metalloprotein. Mann[22] has shown by spectrographic analysis that highly purified preparations of pea seedling diamine oxidase contain 0.08-0.09 % of copper as well as trace amounts of other metals.

It has been shown[29] that when copper is removed through complexation with sodium diethyldithiocarbamate, the enzyme loses its activity. The activity was restored with subsequent addition of Cu(II) ions. No reactivation of the enzyme was observed on addition of other metal ions e.g. Mg^{2+}, Fe^{3+}, Ni^{2+} or Zn^{2+}. Other chelating ligands which cause inhibition of diamine oxidase include 8-hydroxyquinoline and 1,10-phenanthroline. The inhibition caused by these ligands could be reversed by the addition of a wide range of metal ions. Reversal of the inhibition appears to depend on the ability of the metal ion to displace the chelating ligand combined with the enzyme bound copper, leaving the active, copper containing protein. These results suggest that copper may form part of the prosthetic group of the enzyme.

The precise role of copper in the catalytic process has long been a source of debate. The failure of electron paramagnetic resonance (EPR) experiments[30] to detect copper oxidation state changes during the enzymic process has led to the suggestions that Cu(II) may act as a Lewis acid;[31] that it has an indirect role in catalysis;[32] or that it merely serves a structural role.[33]

Using the biquinolyl estimation method,[34] it was reported that no copper in the Cu(I) form was present in diamine oxidase. Recently, however, new evidence has been produced, suggesting that a Cu(I)-state may be the reactive catalytic intermediate.[35] These findings will be discussed later (Section 2.13).

2.3 Initially Proposed Cofactors for Diamine Oxidase

In the last 30 years considerable research has been undertaken in an attempt to establish the nature of the prosthetic groups in diamine oxidase.

It has been shown[36] that diamine oxidase is inhibited by various reagents capable of forming derivatives with carbonyl groups. This result is strongly suggestive that the active site of the enzyme contains a functional aldehyde or ketone grouping. Mann[22] suggested that this carbonyl group formed a complex with copper and that this complex made up the prosthetic group of the enzyme. Since then there has been much debate over the identity of the carbonyl cofactor.

Evidence was produced suggesting that both animal diamine oxidase[37] and pea seedling diamine oxidase[38] contain pyridoxal phosphate. However, since hydrogen peroxide is produced during the catalytic deamination, it was thought that the enzymes were

flavoproteins. Indeed, work carried out by Kapeller-Adler[39] and Gorychenkova[40] concluded that the prosthetic group in both plant and animal diamine oxidase contained flavine adenine dinucleotide (FAD). Although Yamada and Yasunobu[41] have shown that absorption spectra of spermine oxidase possess bands consistent with the presence of pyridoxyl phosphate and FAD, no such evidence is available in relation to plant or animal diamine oxidase. Mann[22] concluded that the absorption spectrum of highly purified pea seedling diamine oxidase was not typical of an enzyme containing either pyridoxal phosphate or FAD.

The debate surrounding the cofactor took on a new dimension when, in 1984, two independent groups reported that bovine plasma amine oxidase contained pyrroloquinoline quinone (PQQ) as the cofactor.[8,42]

2.4 The Isolation and Characterisation of Pyrroloquinoline Quinone (PQQ)

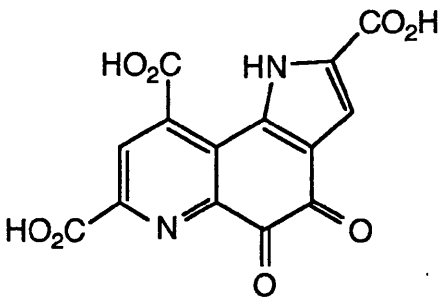
Although, upon surveying the literature, it is possible to find early indications of the existence of PQQ (16), it was not until 1964 that substantial evidence became available.

Hauge,[43] whilst working on a bacterial glucose dehydrogenase, discovered a dissociable cofactor which had different characteristics to those of well established cofactors. Spectral studies carried out by this group indicated that the unknown cofactor contained a naphthoquinone grouping.

Duine and co-workers,[44] during experiments on methanol dehydrogenase, used ESR techniques to indicate the presence of an organic free radical which had properties compatible with those of an

o-quinone. Examination of the hyperfine structure of the ESR spectrum revealed the presence of three hydrogens and two nitrogens. The subsequent discovery that methylotrophic bacteria excrete large amounts of the cofactor into their media,[45] led to suitable quantities being isolated for structure elucidation.

An X-ray characterisation of the acetone adduct of the cofactor,[46] provided the final confirmation that the structure was that of PQQ (16).



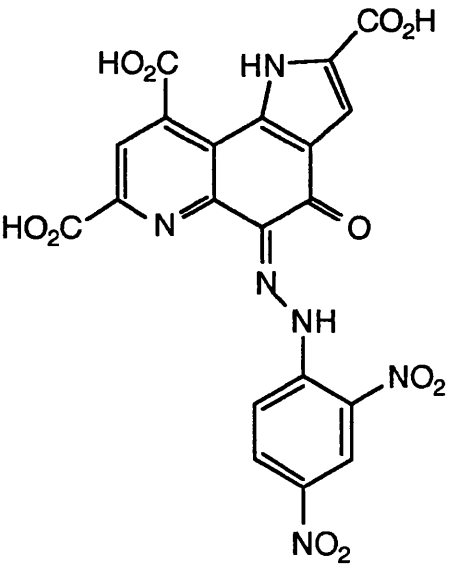
(16)

2.5 Pyrroloquinoline Quinone (PQQ) as a Cofactor in Copper Amine Oxidases

The first indication that PQQ exists in a covalently bound form resulted from work carried out on methylamine dehydrogenase.[47] The remarkable similarity between this enzyme and copper-containing amine oxidases led to the suggestion that PQQ may be a cofactor for this class of enzymes.

In 1984 this feeling gained strength when Amenyama *et al.*[42] indicated spectral similarities between PQQ and chromophores isolated from bovine plasma amine oxidase.

Thusfar, any attempts to detach the covalent cofactor from the protein had met with problems. Due to the reactive nature of PQQ, staightforward hydrolysis had resulted in the formation of a variety of unidentifiable compounds. Lobenstein-Verbeek *et al.* overcame this problem by initially derivatising the cofactor by treatment of bovine serum amine oxidase with dinitrophenylhydrazine.[8] This adduct proved to be stable enough to survive subsequent enzyme proteolysis and comparisons could then be made between it and the hydrazone formed when authentic PQQ was treated with DNPH. The homogeneity of the derivatised product was shown by HPLC and it was found to have an identical retention time and absorption spectrum to that of the model compound. Extensive NMR studies on the derivatised compound indicated the structure was that of the monohydrazone (17).



(17)

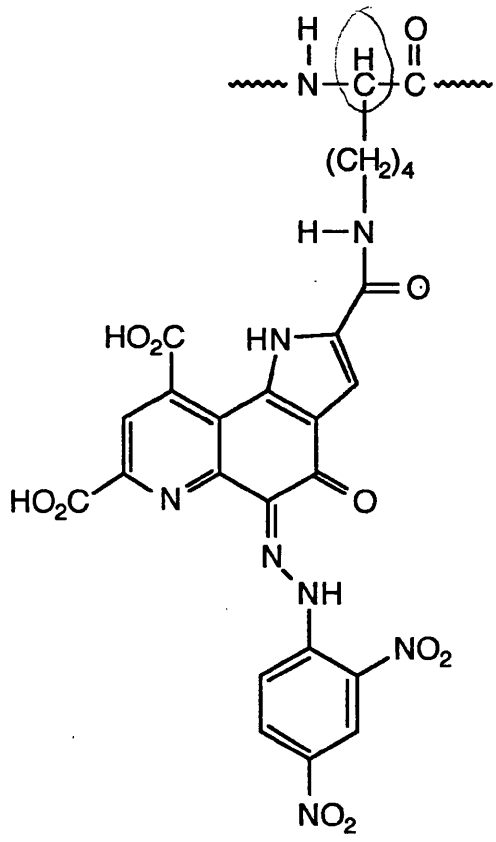
Subsequent degradation of this compound in alkaline media produced a fluorescing compound which had identical chromatographic properties to those of the compound obtained directly from PQQ. These results were strongly indicative that bovine serum amine oxidase contains PQQ which can be detached on proteolysis. Since these initial findings, applications of the "hydrazine method" have suggested that covalently bound PQQ is the second cofactor in a number of amine oxidases.

Duine and co-workers[48] extended the original approach to determine the nature of the cofactor in pig kidney diamine oxidase. Derivatisation with dinitrophenylhydrazine followed by proteolytic degradation produced a compound possessing the same chromatographic properties, absorption spectrum and ^{1}H NMR spectrum as authentic derivatised PQQ. It was also noted that the yield for derivatisation of the enzyme-bound cofactor could be significantly enhanced by carrying out the reaction in the presence of molecular oxygen.

Further studies by the same group,[49] resulted in the determination of the primary structure of a peptide containing PQQ which was isolated from pig kidney DAO. Derivatisation was effected as above; however proteolysis was carried out using the specific protease trypsin. The resultant hydrolysate contained a peptide in which the derivatised cofactor (PQQ) was covalently bound. The peptide was purified to homogeneity and the amino acid sequence was determined. On analysis the sequence was found to be :-

```
1                5            8              11
His-Ser-Asp-Ala-Val-Phe-Thr-Lys-Asn-Tyr-Arg
                             |
                            PQQ
```

Evidence collected suggested that the cofactor was attached to a lysyl residue probably through the interaction of an NH_2 group with a COOH (probably the most acidic group at C-2) of PQQ. Comparisons between synthetically prepared lysine-PQQ-DNPH (18) and the product obtained from further digestion of the peptide supported this proposal. Identical retention times and absorption spectra were obtained suggesting that the derivatised residue was indeed lysine-PQQ-DNPH (18).



(18)

Although use of the "hydrazine method" has provided much information on the nature of the cofactor of pig kidney DAO, no such strategy has been employed in relation to pea seedling DAO.

Glatz *et al.* [50] noted the ability of pea seedling DAO to form derivatives with a wide variety of reagents known to interact with PQQ. Also, examination of the optical and electrochemical properties of the native enzyme was strongly suggestive that the enzyme contained PQQ as a tightly bound cofactor. The fluorescence spectrum of purified DAO showed emissions consistent with that of a PQQ-containing enzyme. The existence of an *o*-quinone was demonstrated by the addition of dimethoxyaniline to the enzyme. The resultant Schiff base gave a characteristic absorbance (>500nm) which was consistent with the presence of an *o*-quinone whose carbonyl groups are located on the rings of an aromatic compound.[51] On the basis of the above observations it was concluded that the carbonyl cofactor in pea seedling DAO was firmly bound PQQ.

2.6 Improvements in the "Hydrazine Method"

Although significant advances had been made in the area of cofactor chemistry using the hydrazine method, concern existed over the low yield in which the adduct could be isolated from the enzyme. It was even suggested that the hydrazone which could be isolated might have resulted from the action of DNPH on a PQQ impurity rather than on the cofactor as reported.

During the initial studies,[8] a yield of only 6% of the DNPH derivative (17) was reported. Although absorbance measurements suggested that all the adduct was present after derivatisation, it was

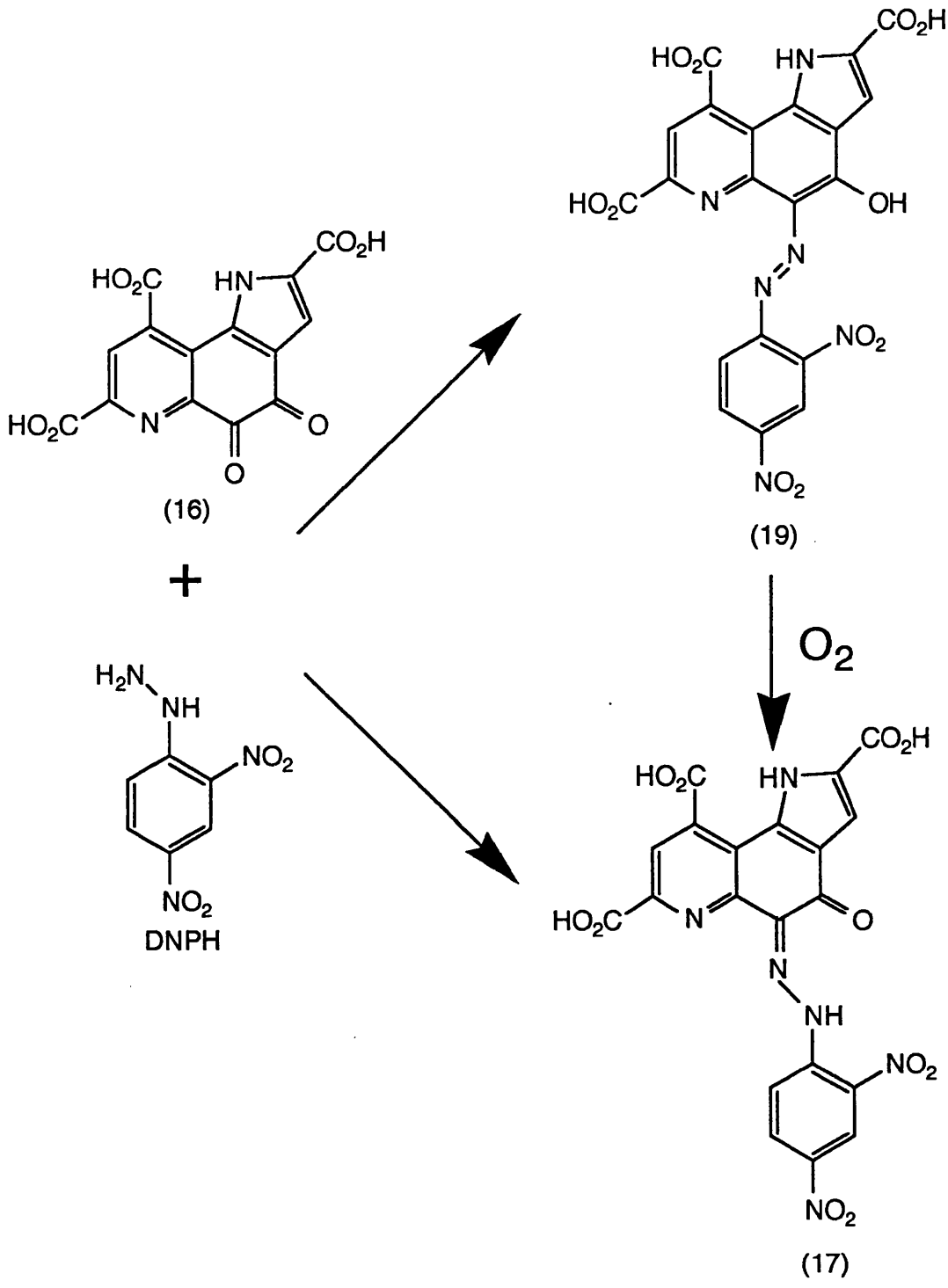
believed that the destructive action of the pronase during proteolysis was responsible for the low yield.

Duine and co-workers,[49] during a reinvestigation of the problem, discovered that reaction of DNPH with the enzyme produced the desired hydrazone as the minor product and that the major product was a coloured compound possessing significantly different properties. The identity of this compound was not established, but it was discovered that, by carrying out the derivatisation under an oxygen atmosphere, a ten times higher yield of the desired hydrazone could be obtained. After greater insight was obtained into the conditions for hydrazone formation it was realised that treatment of the enzyme with DNPH initially resulted in almost complete conversion into the azo compound (19) and not the hydrazone (17). This azo compound, however, was converted into the hydrazone at high oxygen concentrations[52](Scheme 2.1). Thus by ensuring that the initial derivatisation was carried out in an oxygen atmosphere the overall method could be significantly improved.

This improved method has been used to provide evidence that covalently bound PQQ is the cofactor in a wide range of enzymes.[53,54]

2.7 Alternative Methods for the Detection of PQQ

Although the "hydrazine method" offers many advantages, particularly if a quantitative analysis is required, a variety of other techniques have been used for the detection of PQQ in copper amine oxidases.



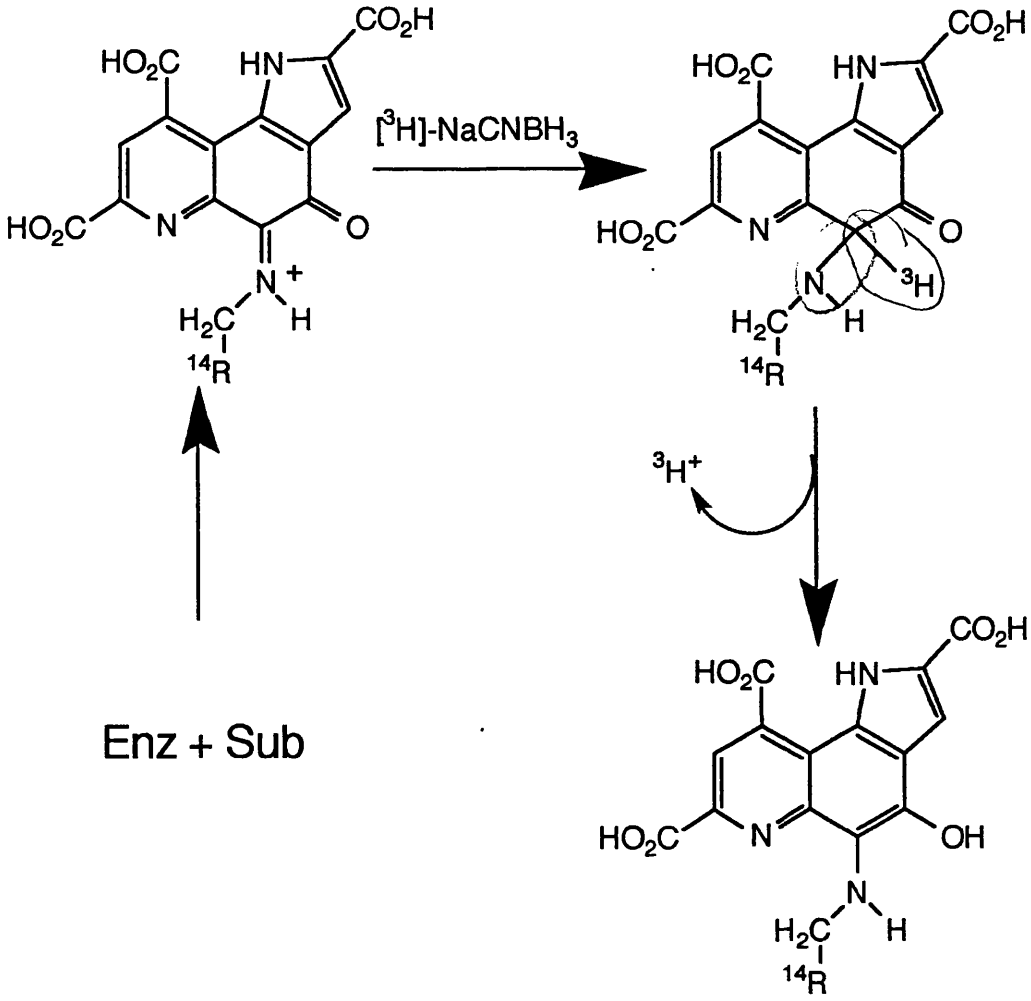
Scheme 2.1

Using the technique of resonance Raman spectroscopy, Dooley and co-workers[55] have produced a detailed structural characterisation of various derivatives of mammalian amine oxidases. Comparisons have been made between derivatives prepared directly from either PQQ or pyridoxal phosphate and those obtained from bovine or porcine plasma amine oxidase. In each case the observed frequencies and relative intensities obtained for the derivatised proteins were almost identical to those of the PQQ derivatives. Overall, the results ruled out the possibility of pyridoxal phosphate being the cofactor for the enzymes studied and provided strong evidence that PQQ, or a derivative thereof, is indeed present.

Other groups have used resonance Raman spectroscopy for the purpose of cofactor structure elucidaton.[56,57,58] Problems have arisen, however, due to the lack of understanding over the conditions required for hydrazone formation (See 2.6), and it appears that the absorption spectrum for the azo compound formed *in vivo* (and not the hydrazone) has often been compared to that of the model hydrazone. The obvious differences which exist were attributed, wrongly, to active site residues (in the case of the protein bound adduct). Cofactor determinations made by this method have therefore been the subject of much criticism.

The studies of Hartmann and Klinmann[59] have involved the use of labelled isotopes to examine the properties of the active site cofactor in bovine plasma amine oxidase. Experiments involved incubation of the enzyme with a high concentration of a ^{14}C-labelled amine substrate followed by reduction of the Schiff base intermediate with [^{3}H]-sodium cyanoborohydride. It was discovered that the above treatment resulted in a stoichiometric incorporation of the ^{14}C-labelled substrate into the enzyme. However further

investigation failed to detect the incorporation of any tritium. It was suggested that these observations were consistent with the presence of a PQQ cofactor. Reduction of the substrate-cofactor complex would be expected to label the enzyme initially. However the unique α-dicarbonyl structure of PQQ possesses a highly acidic proton at C-5 which promotes enolisation with subsequent loss of tritium (Scheme 2.2).



Scheme 2.2

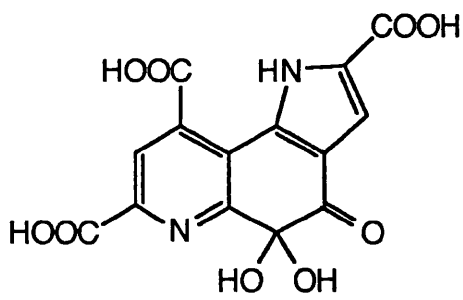
Due to the apparent widespread nature of PQQ as a cofactor there was a need for a new fast analytical method allowing detection and quantitative analysis of PQQ in biological samples. Citro *et al.*[60] developed a system where antibodies were used to react with both free and protein-bound PQQ, producing specific antibodies which allowed the detection of PQQ in certain amine oxidases.

Gallop[61] questioned the validity of direct methods of PQQ determination and produced a highly selective colourimetric assay which allowed detection in cell extracts. The assay involved the reduction of PQQ by glycine followed by reduction of the $PQQH_2$ intermediate by nitroblue tetrazolium (NBT). Gallop used this technique to indicate the presence of PQQ in a variety of enzymes and foodstuffs. However the validity of the method has been questioned.[62] The reactivity of certain amino acids towards PQQ leads to the formation of unreactive oxazole condensation products. Therefore the assay is not quantitative in the case of quinoproteins. Secondly, it has been shown[62] that many other substances can behave like PQQ in the assay. Ascorbic acid and riboflavin, present in foods shown by the assay to contain high levels of PQQ, have a similar activity towards the assay as PQQ.

2.8 Structures of Naturally Occurring Forms of PQQ

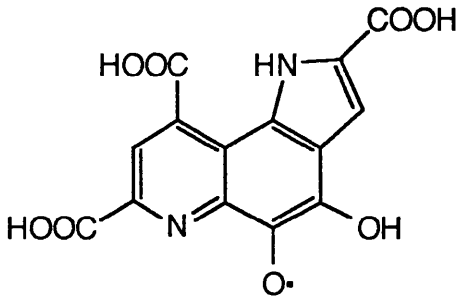
All mammalian quinoproteins, so far investigated, have been shown to contain PQQ in a covalently bound form. It has been suggested that the cofactor is anchored to the protein by an amide or ester bond via a carboxylic acid grouping.

Spectroscopic investigations, carried out on aqueous solutions of PQQ, have indicated the presence of two interconverting species. Due to the unusually high reactivity of the C-5 carbonyl group towards nucleophiles it is believed that, under these conditions, an equilibrium state exists between unhydrated and hydrated PQQ (PQQ-H_2O) (20). Other adducts can also be formed in a similar manner.

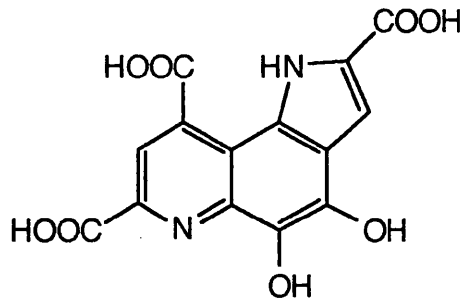


(20)

Being a redox cofactor it is no surprise that PQQ has been found in the reduced form. Two natural forms, PQQH· (21) and $PQQH_2$ (22) have so far been discovered. However, since reoxidation of these compounds occurs in aerobic conditions at pH>4, it is unlikely that either will be present under physiological conditions in the free form.[63] However, they are able to exist in a protein bound form due to the increased stability associated with the protein environment and the shielding effect which prevents oxygen attack.



(21)

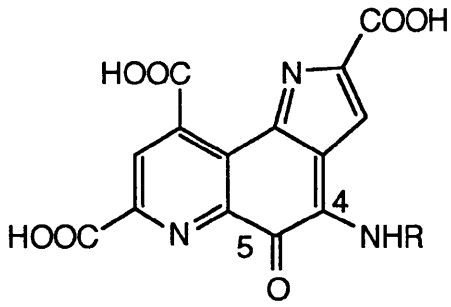


(22)

2.9 The Mechanistic Involvement of PQQ

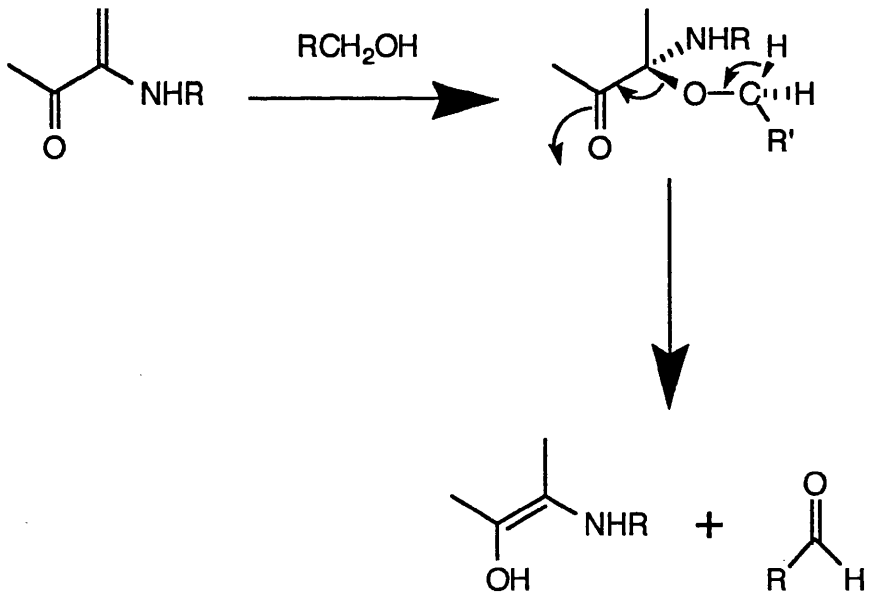
Attempts to investigate the reaction mechanism in a PQQ containing enzyme have largely revolved around studies on methanol dehydrogenase. Work by Mincey *et al.* [64] indicated a radical signal by EPR which accounted for only 13% of the cofactor. This result was consistent with the observation that inactivation of the enzyme with cyclopropanol was complete at 14% incorporation of the labelled substrate. On the basis of these results it was suggested that the radical species was the active form of the enzyme. However, work by Parkes and Abeles[65] cast doubt on the importance of a radical intermediate for activity.

Several investigators[66,67] have noted the activation of PQQ-dependent enzymes by ammonia or primary amines. It has therefore been proposed that compound (23), formed via the interaction of an amine with PQQ, is the reactive intermediate in the mechanistic process.[68]



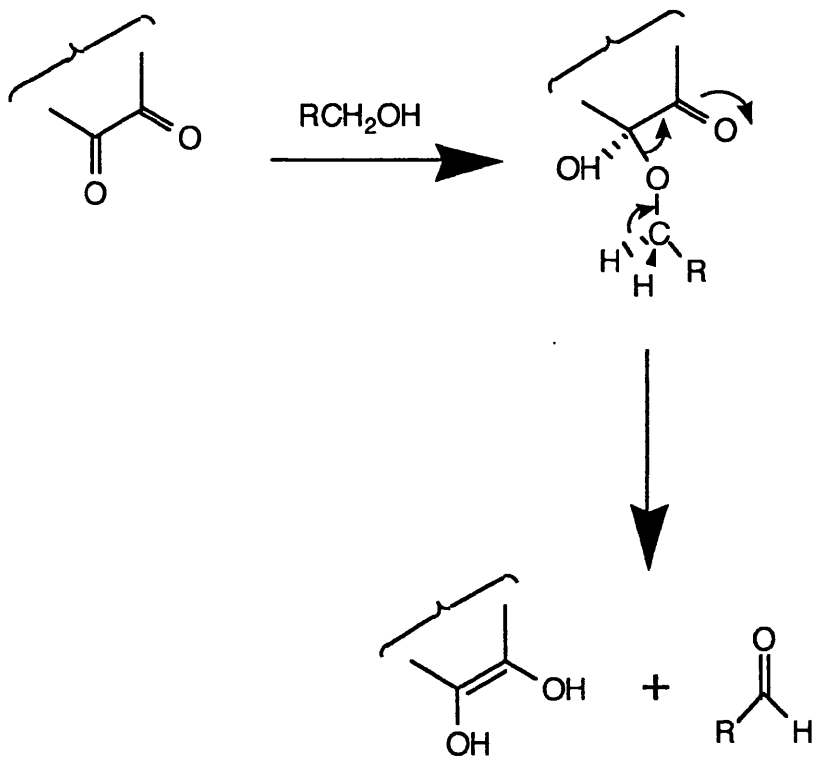
(23)

Forrest *et al.*[68] suggested a reaction scheme (Scheme 2.3) which involves attack of an alcohol at C-4 of the amine intermediate (23) producing a carbinolamine derivative. Subsequent oxidation of this derivative produces an amino quinol, containing nitrogen at the C-4 position, as the reduced form of the cofactor.



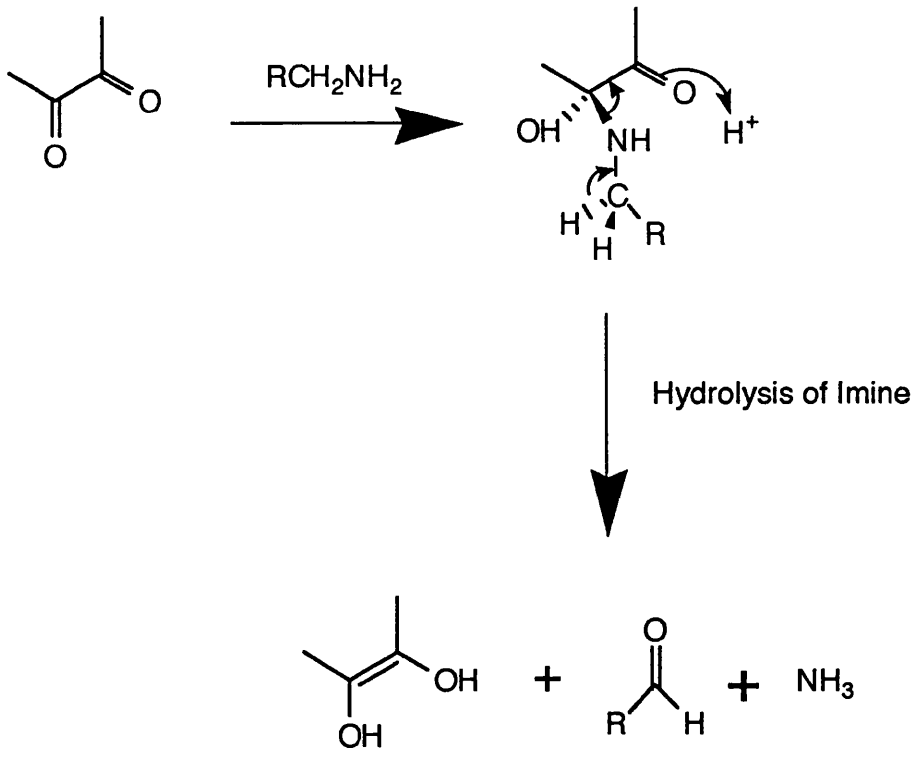
Scheme 2.3

The major difficulty with the above scheme is the suggestion that the reactive intermediate is formed via nucleophilic attack at the C-4 centre, rather than at the highly reactive C-5 centre.[69] An alternative mechanism which takes this fact into account is given (Scheme 2.4). Here, initial formation of the hemiacetal at C-5 is followed by substrate oxidation, leaving PQQ in the reduced quinol form.



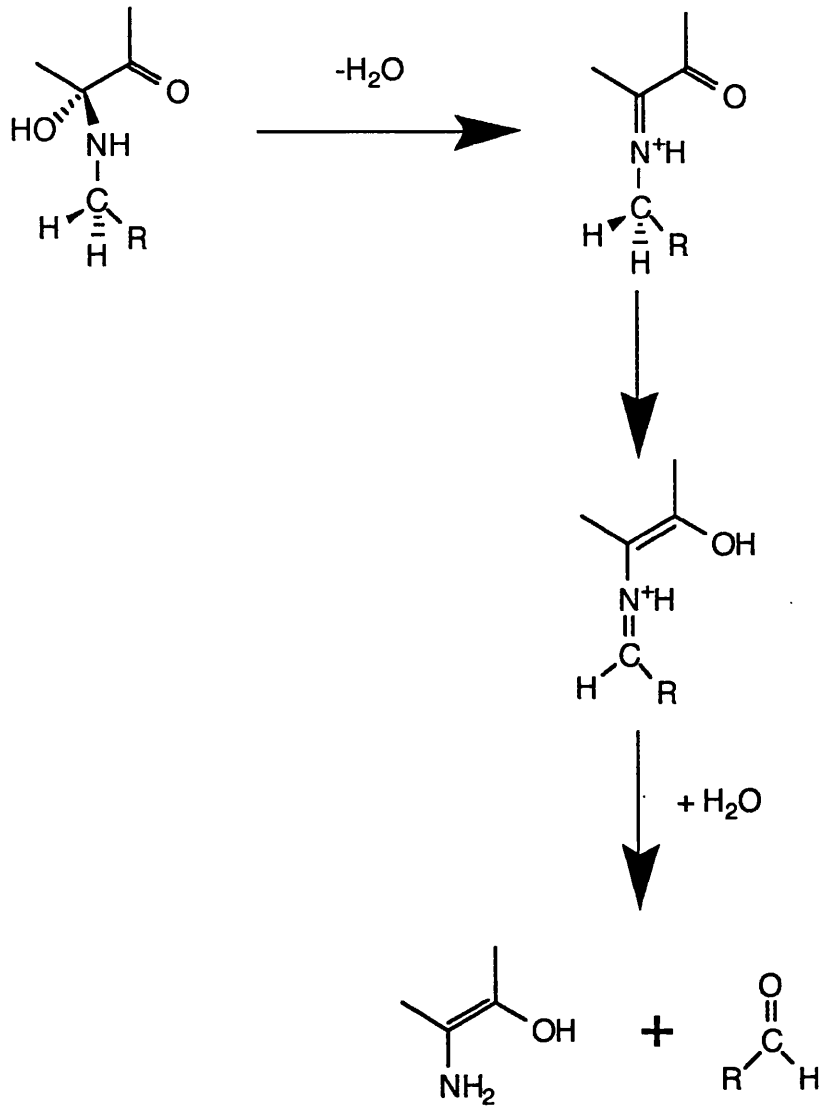
Scheme 2.4

This idea has been extended into the area of amine oxidation. An analogous mechanism has been proposed (Scheme 2.5) which supports the observation that the quinol form of PQQ is the major product of the enzymic reaction.



Scheme 2.5

At this point it was thought that these similar mechanisms were an adequate representation for the role of PQQ in both classes of enzyme. However, a fundamental difference exists between amines and alcohols, namely the ability of amines to form stable Schiff base intermediates with PQQ. Taking account of this feature, Bruice and co-workers[70] proposed a minor pathway for PQQ catalysed amine oxidation (Scheme 2.6). In order to establish which of the two mechanisms is relevant for amine oxidases, an examination of the nitrogen transfer from substrate to cofactor during cofactor reduction would be required.



Scheme 2.6

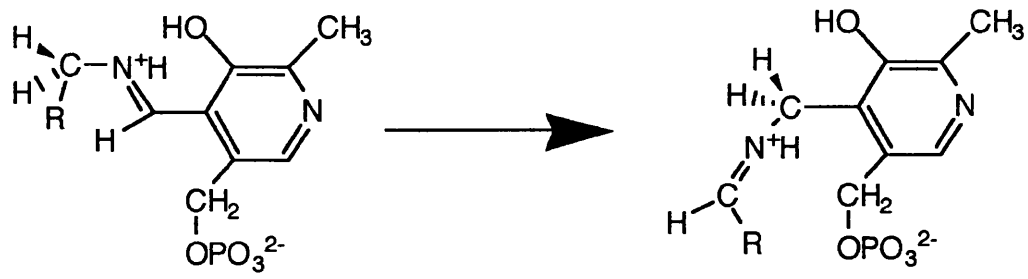
It has been reported that amine oxidations involving porcine plasma amine oxidase produce a burst of ammonia. This observation appeared to rule out the aminotransferase mechanism (Scheme 2.6), since in this pathway ammonia is not produced as a byproduct. It was later discovered, however, that comparison studies between native enzyme and inhibited enzyme during the oxidation process showed no significant difference in ammonia release.[71] This suggests

that it is non-specifically bound ammonia that is being released during the process. Using a quench flow technique and a sensitive assay for ammonia detection, Ruis *et al.*[72] clearly showed that the ammonia release, during the enzymic oxidation of benzylamine, correlated with the enzymic re-oxidation and not benzyldehyde formation. Overall, on the basis of these results, it is felt that the aminotransferase mechanism (Scheme 2.6) provides a more accurate description of the involvement of PQQ in enzyme catalysed amine oxidations.

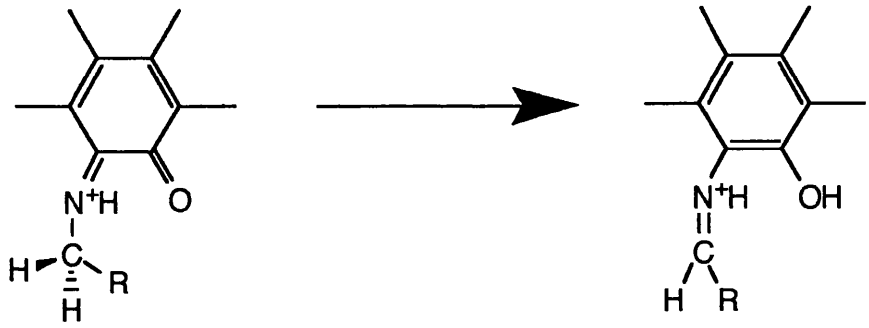
2.10 Further Insight into the Mechanistic Involvement of PQQ

So far, the description of the interaction of amine substrates with PQQ appears to be very similar to the known interaction of amines with pyridoxal phosphate. Both involve Schiff base formation followed by proton abstraction from C-1. A proposed difference in the mechanistic involvement of the two cofactors is in the fate of the α-hydrogen during substrate oxidation (Scheme 2.7). The pyridoxal phosphate-substrate complex undergoes a 1,3-prototropic shift whereas the unique α-dicarbonyl structure of PQQ may promote proton transfer to the C-4 carbonyl oxygen.

PYRIDOXAL PHOSPHATE :

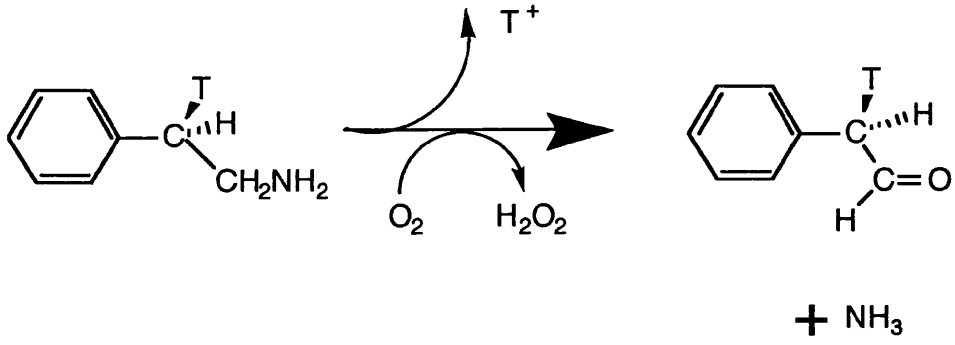


PQQ:



Scheme 2.7

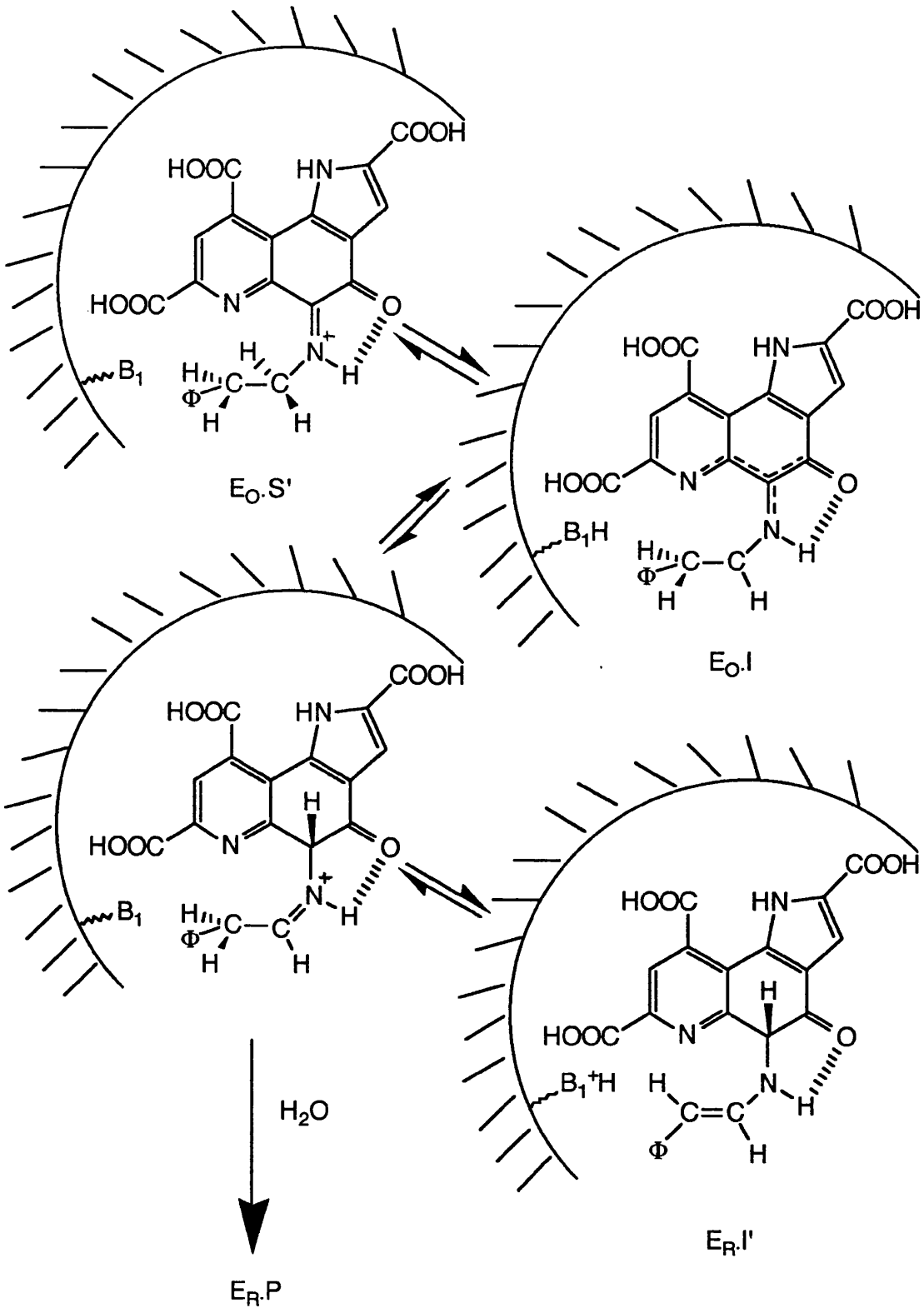
Further insight into this concept was gained through studies carried out on plasma amine oxidase.[73] It was noted that enzymic oxidation of phenethylamines gave rise to a product which had undergone β-hydrogen exchange (Scheme 2.8). It was found that this exchange was a kinetically rapid one compared to the rate of cofactor reduction. Further kinetic studies on this process showed that the transfer of reducing equivalents from substrate to cofactor required to be reversible and that a subsequent step to cofactor reduction is partially rate limiting. As a consequence of the kinetic inequality, it was proposed that bovine plasma amine oxidase functioned via a two base mechanism.



Scheme 2.8

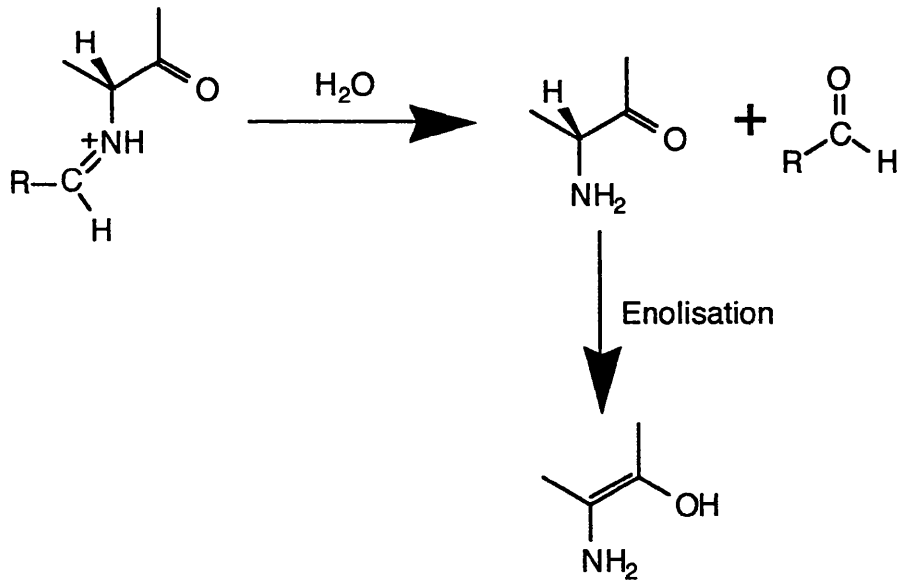
Subsequent pH dependency studies[74] led to the observation that identical pK_a values existed for both the exchange process and the substrate oxidation process. This result was strongly suggestive that an identical residue was involved in both steps. This theory has recently gained support through a series of stereochemical probes.[75] If the possibility of a two base mechanism is dismissed then another explanation for the kinetic inequality must be found.

Current understanding of the mechanism has returned to the involvement of a 1,3-prototropic shift analogous to that observed with pyridoxal phosphate. This proposal has been incorporated into Scheme 2.9. This mechanism illustrates (1) formation of a Schiff base complex; (2) oxidation of the substrate via a proton abstraction mechanism; (3) the transfer of both hydrogen and nitrogen from C-1 of substrate to cofactor in the reductive half equation.



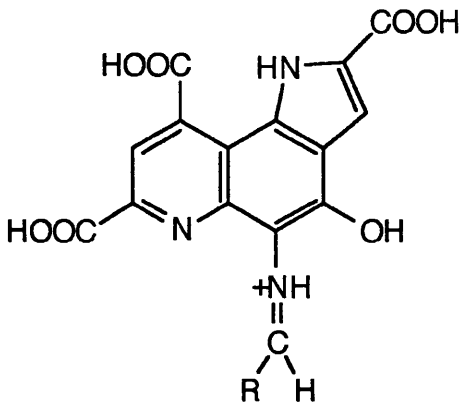
Scheme 2.9

Scheme 2.9 shows the proposed mechanism for bovine plasma amine oxidase, in which a single active site residue, EB1, catalyses both substrate oxidation and exchange. $E_O.S'$ is the Schiff base complex between amine and C-5 of the cofactor; $E_O.I$ is the transiently formed carbanionic intermediate; $E_R.P$ is the product Schiff base, involving 1,3-prototropic shift from substrate to cofactor; $E_R.I'$ is the enamine formed in the course of hydrogen exchange. Although this mechanism seemed best equipped to explain reported observations, certain aspects of it had to be justified. Namely, the postulated mechanism seems at first to contradict the results of Hartmann and Klinmann[59] (Section 2.7), who noted the loss of a tritium label from C-5 through a proposed enolisation process. It has been suggested however, that although enolisation does occur, it is a slow process within the product-imine complex and only occurs after hydrolysis of this intermediate (Scheme 2.10). This is in accordance with the proposed mechanism.



Scheme 2.10

A second feature of the mechanism which had to be justified was the the suggestion that protonation occurs at C-5. This process would appear to be unfavourable since it prevents the formation of a stable aromatic product (24).



(24)

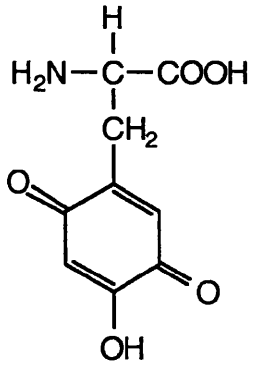
It was argued however, that formation of such a product would be expected to reduce significantly the rate of hydrolysis. This is contrary to the observed kinetics which show that the rate of hydrolysis is fast relative to other steps.

2.11 Arguments Against PQQ as a Cofactor

Although much evidence has been collected in support of PQQ as the organic cofactor in a variety of enzymes, the lack of direct evidence for the presence of PQQ at a reactive site of a mammalian protein was a concern. As discussed earlier (Section 2.5), an active site cofactor-containing peptide has been isolated from pig kidney diamine oxidase;[49] however the extremely poor yield obtained (0.1%) limited the characterisation to amino acid sequencing.

Recent work by Hol and co-workers has cast doubt over the nature of some cofactors previously reported to be PQQ. X-Ray studies on methylamine dehydrogenase from *Thiobacillus versutus*[75a] have produced diffraction patterns which indicate the presence of an active site dicarbonyl, lacking the pyridine ring found in PQQ. This is a direct contradiction of earlier work by Duine and co-workers.[52] In 1992 Ito *et al.*[76] reported the crystal structure of galactose oxidase, previously claimed to contain PQQ. The observed electron density distribution could be accounted for by the known primary structure of the protein and the solvent ions in solution. No density corresponding to PQQ was observed.

In 1990 direct evidence was obtained which supported the argument that PQQ was not the cofactor in mammalian protein. Janes *et al.*,[77] isolated the active site cofactor-containing protein from bovine serum amine oxidase (BSAO). By optimising the conditions for proteolysis the peptide could be isolated in a 40% yield. This high yield, combined with the relatively small size of the protein, allowed a complete structural analysis to be undertaken. The result of this analysis suggested that the organic cofactor for this protein was not PQQ but topaquinone (TOPA) (25).



(25)

(1) Amino acid composition

The cofactor-containing peptide was stabilised, as before, through derivatisation with phenylhydrazine. Proteolytic digestion of the derivatised BSAO in the presence of urea produced the desired peptide in 40% yield. Purification was carried out using HPLC.

The sequence was found to be:-

- Leu - Asn - X - Asp - Tyr -

Since only one amino acid was detected at each round of peptide sequencing, it was concluded that the cofactor had a single point of attachment to the protein. This sequence was verified for every sample used for spectroscopic characterisation.

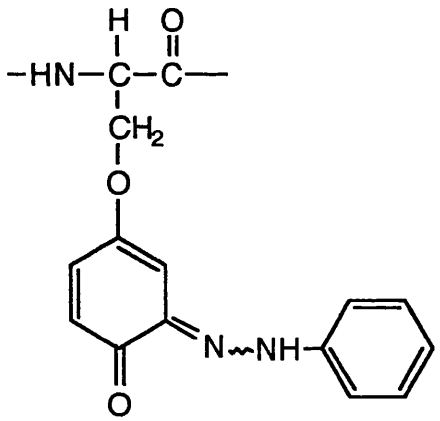
(2) Peptide characterisation by mass spectrometry (MS)

Initial studies involving the use of liquid secondary ion mass spectrometry (LSIMS) gave a molecular ion (MH^+) of 807.5 daltons for the phenylhydrazone pentapeptide isolated from BSAO. If the cofactor was initially derivatised with [1-^{15}N]phenylhydrazine, then a molecular ion at 808.5 daltons was obtained. This result indicates that the phenylhydrazone moiety is preserved in the isolated peptide. Derivatisation of the carboxylic acid groups with acidic hexanol produced a new MH^+ at 975.5 daltons. Since derivatisation of each acid group should give rise to an increase of 84.1 daltons, the observed difference of 168.2 daltons corresponds exactly to the formation of two ester linkages. Therefore, it would appear that two carboxylic acid groups are present in the isolated peptide. As both of these groups are present in the pentapeptide backbone (at Asp and at the carboxyl terminus) it would appear that no more free carboxyl

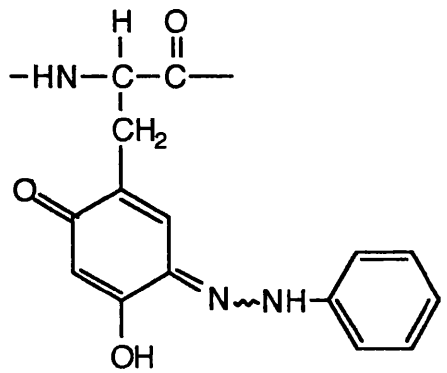
groups are present in the cofactor. Even although one of the carboxylic acid groups could conceivably be involved in an amide linkage with the peptide itself, the absence of the remaining two groups would seem to rule out the presence of PQQ as the cofactor in this enzyme.

Subsequent studies provided an accurate mass for the dihexyl derivative of 974.5123 daltons which on subtraction of the two hexyl derivatives, left a figure of 806.3245 daltons for the peptide. On further subtraction of the accurate masses for the known amino acid components a value of 283.0967 daltons was obtained for X. Five empirical formulae were found which were within ±5ppm of this value. However, only one, $C_{15}H_{13}N_3O_3$, was compatible with both the UV/Vis absorbance properties of the active site cofactor and the presence of a phenylhydrazone in X.

Two possible structures (26) and (27) compatible with this empirical formula are shown.



(26)



(27)

Structure (26) is a serine residue attached to the phenylhydrazone of a catechol ring through an ether linkage.

Structure (27) is the phenylhydrazone of a trihydroxyphenylalanine derivative.

The collision-induced dissociation (CID) mass spectrum of the derivatised pentapeptide was examined in an attempt to distinguish between the above structures. The results obtained therefrom allowed a tentative assignment of the active site cofactor in BSAO to be TOPA (25).

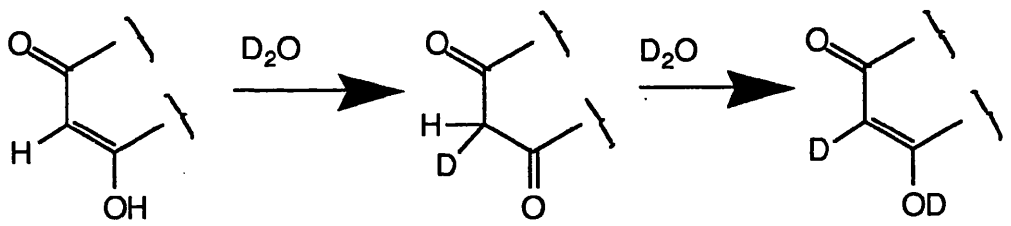
(3) Peptide characterisation by UV/Vis spectroscopy

Consistent with earlier work, Janes *et al.* observed λ_{max} at 448 nm for the phenylhydrazone-derivatised bovine serum amine oxidase. This value is in agreement with the expected λ_{max} for the phenylhydrazone derivative of PQQ, and has in the past been cited as evidence for the presence of PQQ. However, it was shown that the λ_{max} for the derivative of the isolated peptide was shifted to 434 nm and this new value was in agreement with the observed λ_{max} for the phenylhydrazone derivative of topaquinone. It was suggested that the effect of the protein side chain is to shift the λ_{max} to a value which, by coincidence, is in agreement with the absorbance data obtained for PQQ.

(4) Peptide characterisation by NMR spectroscopy

Proton NMR studies, initially undertaken in D_2O, produced a spectrum consistent with a pentapeptide backbone and tyrosine side chain. In addition to these were three further resonances at δ 7.5, 7.2 and 6.9 which integrated in the ratio 4-5:1:1 respectively. It was initially believed that the equivalent protons were due to the

phenylhydrazone ring and that the remaining two resonances were due to protons associated with the cofactor. However, when the nitrophenylhydrazone derivative was studied in place of the phenylhydrazone derivative, the signal at δ 7.2 disappeared, indicating that it could be attributed to the phenylhydrazone ring. Thus only one proton resonance was observed for the cofactor when the sample was run in D_2O. This is not surprising since in TOPA the C-5 proton lies between an enol and a ketone and in the presence of D_2O would be expected to undergo an enol-keto tautomerism forming a deuteriated cofactor with a single proton resonance (Scheme 2.11).



Scheme 2.11

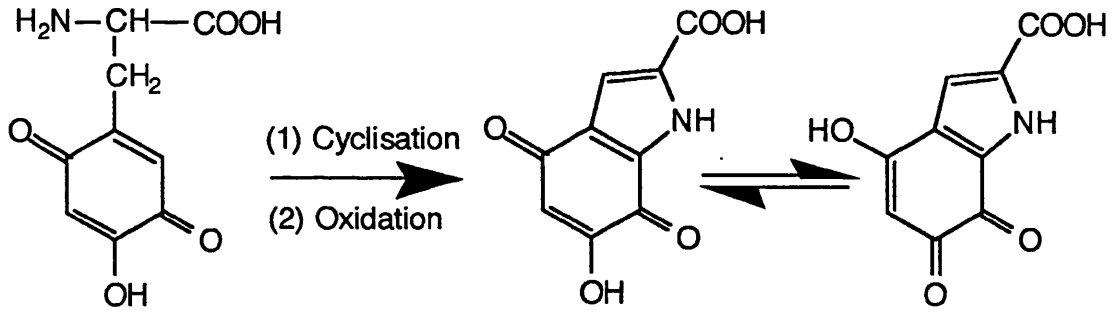
This was confirmed by running the sample in H_2O. This provided a spectrum which produced an unambiguous characterisation of the cofactor structure.

Since it is likely that all copper amine oxidases have a common cofactor, these findings had far reaching consequences in relation to other enzymes.

If TOPA is to replace PQQ as the common cofactor, how could it be that two apparently different structures could have been confused?

The vast majority of PQQ structure elucidations have relied on the observation that isolated phenylhydrazone derivatives obtained from the studied enzyme have coeluted on HPLC with authentic samples of PQQ phenylhydrazones.

In view of the above findings Janes *et al.* suggested that a derivative of TOPA phenylhydrazone, formed from BSAO during proteolysis, may fortuitously coelute with the phenylhydrazone of PQQ. This at first may seem unlikely. However, topa quinones readily undergo rapid intramolecular cyclisation giving rise to the products shown (Scheme 2.12).



(25) (28) (29)

Scheme 2.12

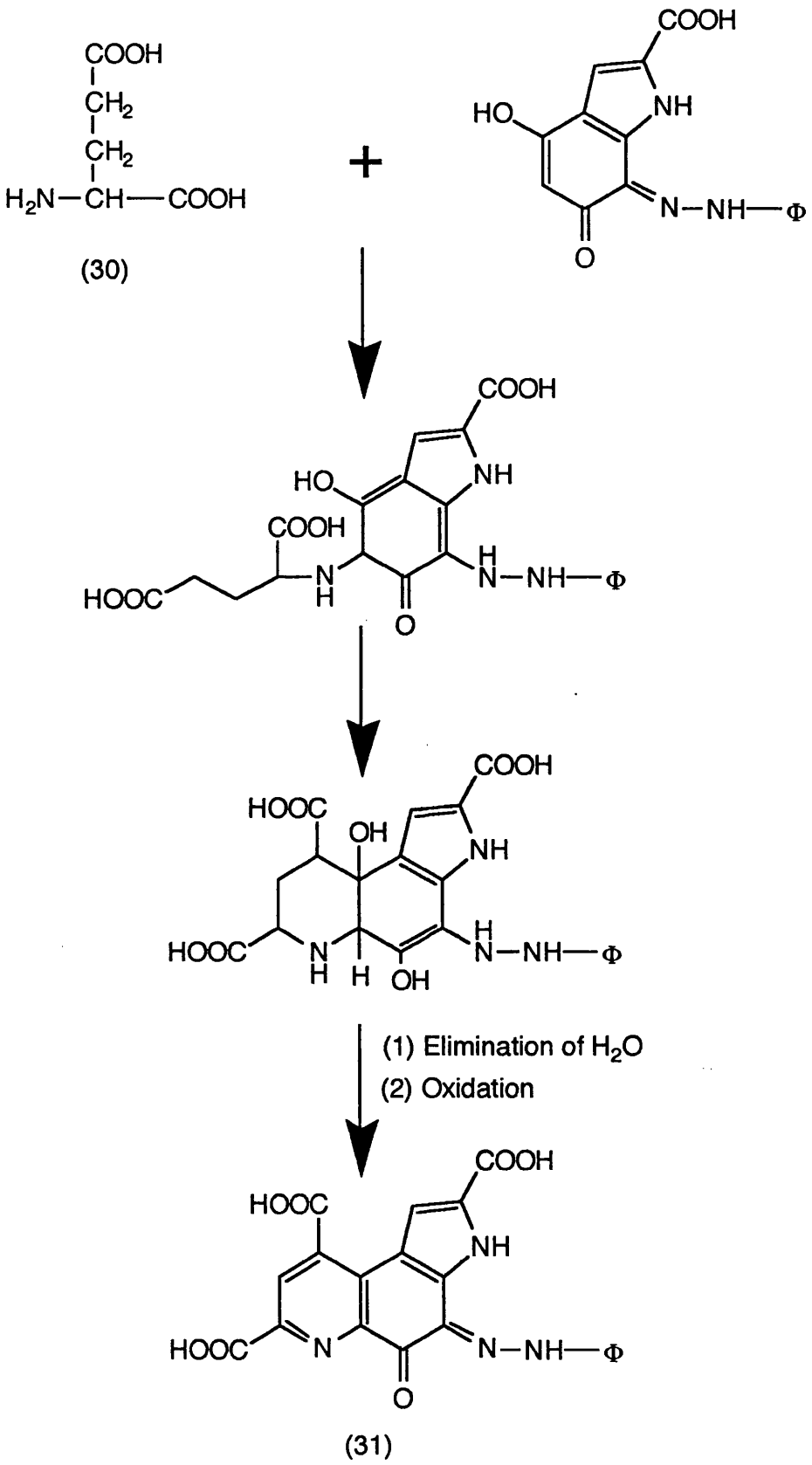
Although these compounds closely resemble two of the rings of PQQ, it is highly unlikely that their phenylhydrazone derivatives would coelute with the phenylhydrazone of PQQ. However, we would expect these compounds to be susceptible to Michael addition with a range of nucleophilic compounds. Since proteolysis of oxidatively damaged proteins results in the formation of a high concentration of glutamate, it would seem feasible that after such a step a sufficient amount of glutamate would be available to form a Michael adduct

with compound (29). Furthermore, the availability of a second nucleophilic site in glutamate would promote the formation of a six membered ring via a second Michael addition. This compound, after subsequent elimination of H_2O and oxidation produces a compound which would be expected to possess similar properties to PQQ (Scheme 2.13).

Janes suggested that proteolysis of phenylhydrazone derivatives of copper amine oxidases produce the phenylhydrazone of topaquinone which, after cyclisation, reacts via a double Michael condensation with glutamate to form compound (31). This will have a similar retention time on HPLC to the phenylhydrazone of PQQ. This provides a rational explanation for the confusion surrounding the nature of the organic cofactor in this class of enzymes.

In a subsequent study, Janes *et al.*[77a] investigated the identity of the active site cofactor in porcine plasma, porcine kidney and pea seedling amine oxidases. Proteolytic degradation on the radiolabelled p-nitrophenylhydrazine derivatised enzymes led to the isolation of relatively small, easily sequenced, active site peptides. In the case of the pea enzyme, the radiolabel was spread over three main peaks with the major peak responsible for 26% of the total activity. Resonance Raman studies revealed that the intensity of the signals and the relative peak positions on the spectrum of the isolated peptide were superimposable on those of a model derivatised topaquinone. Similar results were obtained for both porcine plasma and porcine kidney amine oxidases.

On the basis of these findings and earlier reported results regarding bovine serum amine oxidase[77] Janes has concluded that topaquinone is a ubiquitous cofactor in nature and likely to be the carbonyl containing cofactor in all copper amine oxidases.


COOH
CH2
CH2
H2N—CH—COOH
(30)
+
COOH
HO
NH
N—NH—Φ
O
COOH
HO
NH
COOH
HOOC
N
H
H
N—NH—Φ
O
COOH
HOOC
OH
NH
HOOC
N
H
H
OH
H
N—NH—Φ
(1) Elimination of H2O
(2) Oxidation
COOH
HOOC
NH
HOOC
N
N—NH—Φ
O
(31)

Scheme 2.13

2.12 Current Understanding of the Nature of the Cofactor

The work by Janes *et al.*[77, 77a] has significantly shifted the bias towards topa as the cofactor in copper amine oxidases. There are however a number of questionmarks still existing over the presence of this compound at the active site.

Firstly, topa is neurotoxic[78] and since this toxicity has been traced to the redox properties of the compound, it would seem unusual that the same properties would enable it to perform as an active site cofactor.

Secondly, how could topa arise in the enzyme? It could conceivably be incorporated via the oxidation of an active site tyrosine, or since dopa is a naturally occurring amino acid, via direct incorporation into the growing chain. If tyrosine oxidation is the mechanism for topa formation, is it performed by a second enzyme or alternatively, do the active site metal atoms in the copper containing amine oxidases play a catalytic role in the event?

In short, it would appear that some questions remain unanswered in relation to the nature of the cofactor. X-ray studies carried out on the crystallised enzymes are apparently required before a final confirmation of the identity of the cofactor can be given.

2.13 Interaction of the Prosthetic Groups in Diamine Oxidase

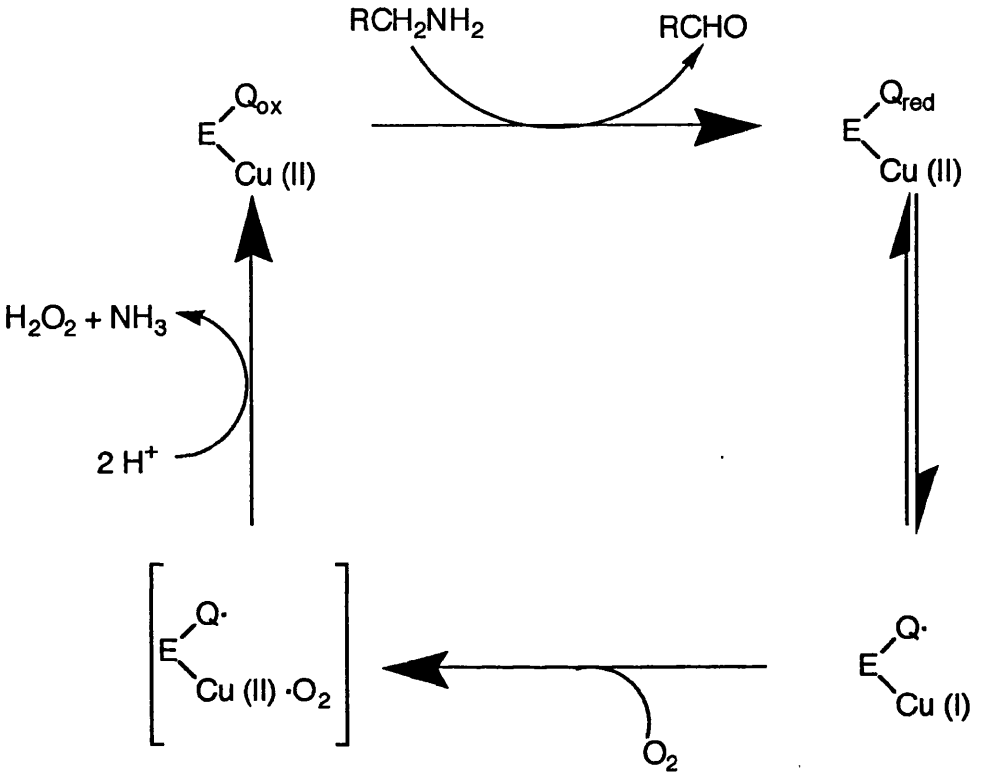
Although its structure has not been confirmed beyond doubt, it would appear that the active site of diamine oxidase contains a redox

cofactor. The presence of copper has already been discussed. A possible function for copper in these enzymes is in the reoxidation of the substrate-reduced enzyme. The problem with this proposal is that it is unlikely that a single copper centre, capable of undergoing a one electron change, can catalyse a two-electron transfer from substrate to O_2.

Recent studies using electron paramagnetic resonance techniques[35] have detected the presence of a Cu(I)-semiquinone which has been proposed as the catalytic intermediate capable of undergoing a two-electron oxidation. EPR spectral changes accompanying the addition of appropriate amines to several amine oxidases (including pea and pig diamine oxidase) were observed. It was noted that a common signal existed in each case and since the spectrum is independent of substrate and enzyme used, the radical must be associated with a moiety that is conserved among the enzymes examined. Addition of cyanide to the reduced form of the enzyme produced a significant enhancement of this signal. This observation is in accordance with the presence of the proposed intermediate since cyanide would have a stabilising effect on the Cu(I)-semiquinone through its interaction with Cu(I). These results have led to the development of a plausible reaction mechanism which presents well-precedented roles for both copper and the quinone (Scheme 2.13).

Apparently the failure of previous investigations to detect the Cu(I)-semiquinone has been due to the fact that this state is in thermal equilibrium with the Cu(II)-reduced quinone. Internal electron transfer from copper to the semiquinone is favoured by low temperatures and since earlier work has been carried out using low temperature EPR, the position of the equilibrium would favour the

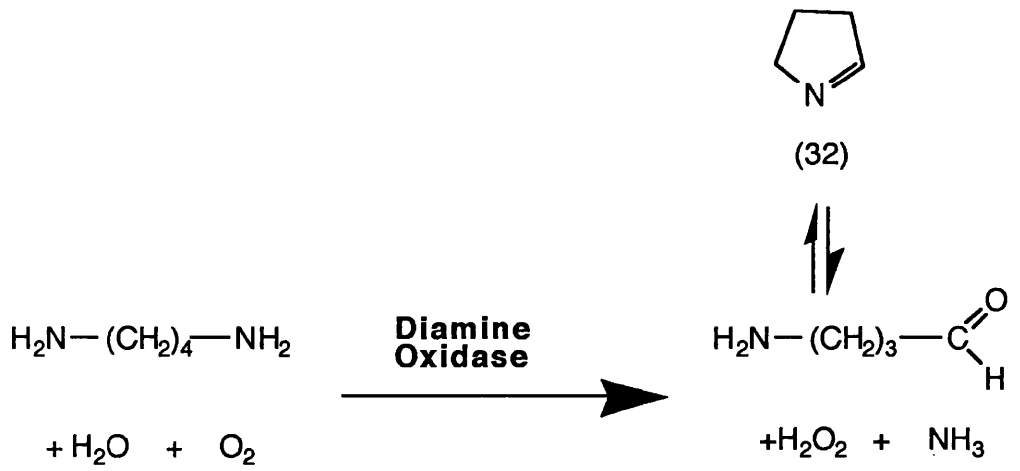
Cu(II)-reduced quinone state. The semiquinone was therefore missed. Further study should provide an insight into whether the quinone is actually bonded to copper or whether the substrates interact at the metal site.



Scheme 2.13

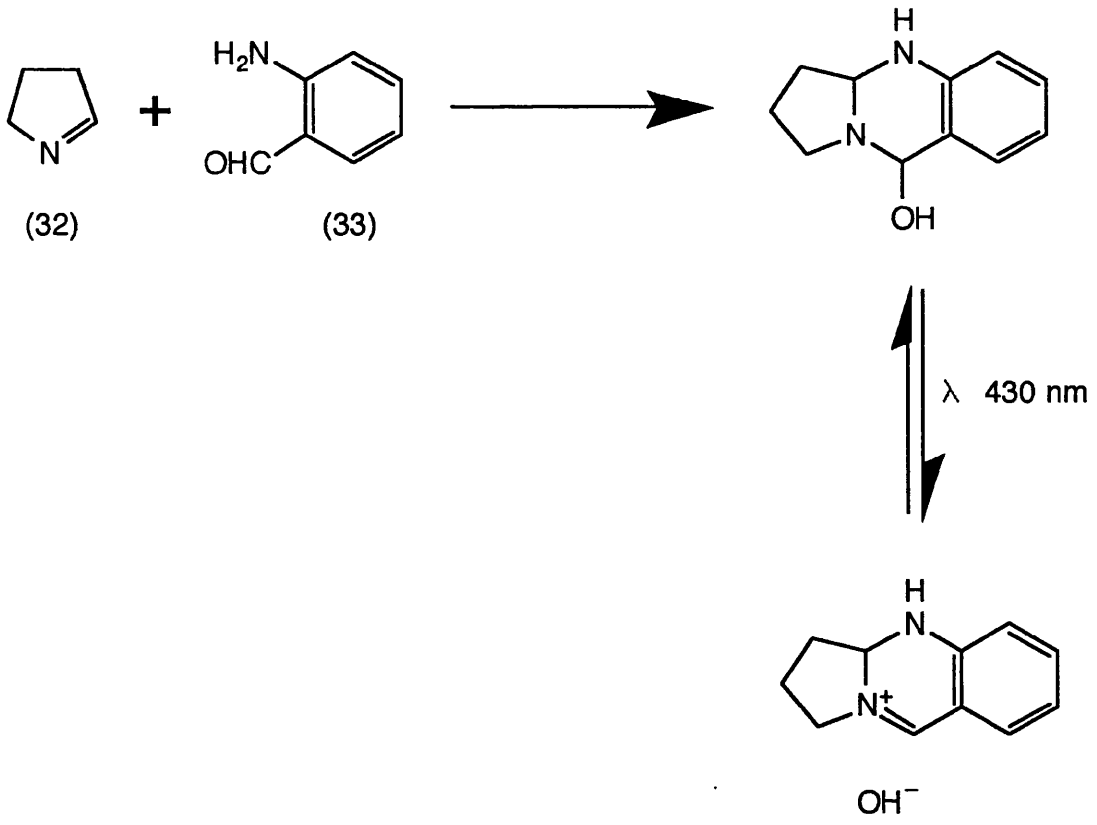
2.14 Determination of Diamine Oxidase Activity - Assay Systems Used

As mentioned previously, diamine oxidase catalyses the oxidation of a range of primary diamines into the corresponding aminoaldehydes (Scheme 2.14).



Scheme 2.14

The rate of this conversion provides a direct measure of the activity of the enzyme. Over the years a variety of assay systems have been developed for this purpose.[79-82] Early studies focussed on the requirement for oxygen consumption during enzymatic catalysis. Systems were developed whereby diamine oxidase activity was determined by the manometric measurement of oxygen uptake.[83] Other methods have relied on the trapping of the organic product from the enzymatic reaction. Holmstedt and Tham[84] used the production of 1-pyrroline (32) from the reaction mixture of putrescine and diamine oxidase to determine the activity of the enzyme. 1-Pyrroline, in the presence of *o*-aminobenzaldehyde (33), forms a yellow compound, the rate of formation of which can be determined spectrophotometrically (Scheme 2.15).



Scheme 2.15

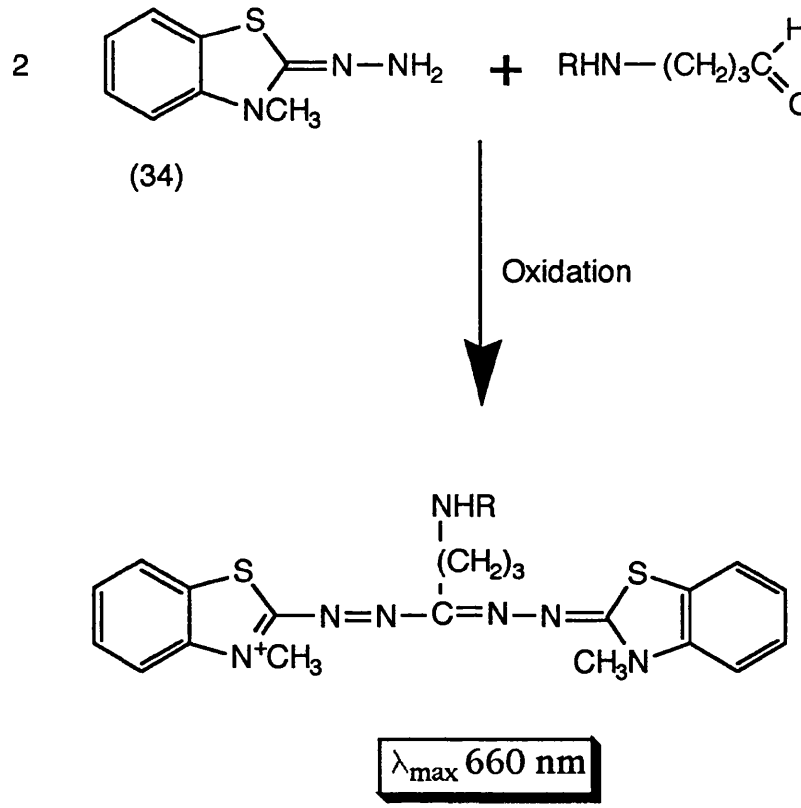
This assay was subsequently calibrated[85] using known concentrations of γ-aminobutanal (in the form of the more stable acetal). This procedure allowed activity measurements to be calculated in units of micromoles per mg of enzyme per hour. This allowed comparisons to be made between this method and those dependent on oxygen uptake.

A rapid and sensitive colourimetric assay for diamine oxidase was described by Naik *et al.*[86] This involved the enzymatic oxidation of putrescine to 1-pyrroline followed by treatment of the cyclic product with ninhydrin reagent in a strongly acidic medium. The coloured product thus formed had a characteristic absorbance maximum at 510 nm. The coloured compound was shown to be

stable for approximately 24 hours compared to the one hour reported in the method of Holmstedt and Tham. This work was later extended by Pec *et al.*[87] allowing the determination of diamine oxidase activity using cadaverine as substrate.

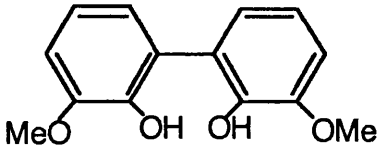
The problem with methods using the production of 1-pyrroline (32) (or in the case of cadaverine, 1-piperideine) for the determination of enzymatic activity is that they are restricted to use with putrescine (or cadaverine) as substrate.

Frydman *et al.* [19] developed an assay which was used for the determination of the rates of oxidation of *N*-alkylputrescine derivatives by diamine oxidase. This method involved condensation of the oxidation product of the enzymic reaction (i.e. the aminoaldehyde) with 3-methyl-2-benzothiazolinone hydrazone (MBTH) (34) to give a bis-hydrazone cation having an absorbance maximum at 660 nm. (Scheme 2.16).



Scheme 2.16

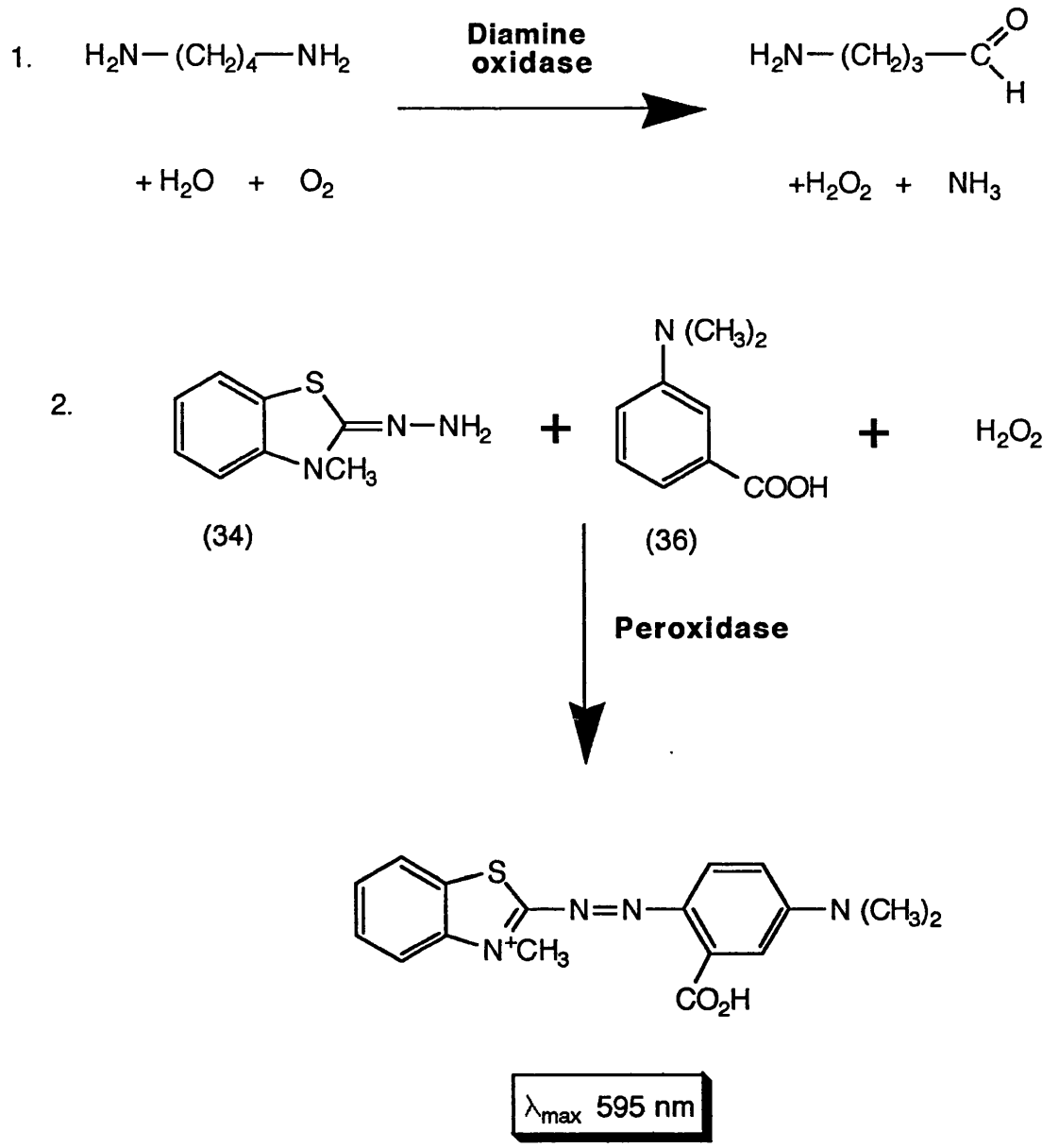
Procedures have been developed for the measurement of hydrogen peroxide production during enzymatic catalysis. Booth and Saunders[88] developed a peroxidase coupling reaction in which hydrogen peroxide, in the presence of peroxidase, rapidly oxidises guaiacol leading to the production of a brown-red solid from which 2,2'-dihydroxy-3,3'-dimethoxybiphenyl (35) can be isolated.



(35)

This reaction has been used to determine the activity of diamine oxidase. Smith[89] developed a colourimetric procedure utilising the peroxidase/guaiacol assay which had been adapted for use in amine oxidases. Despite the fact that the stoichiometry obtained with the varying substrates suggests that the method provides a good estimate of enzymatic activity, complications are associated with this assay. Products from the oxidation of guaiacol include quinones[88] which are highly reactive. Reaction of these quinones with other compounds in the reaction mixture could seriously inhibit the enzymatic process. It is also known that 1-pyrroline, in the presence of peroxidase, gives a product of further oxidation. This again could affect the accuracy of the assay.

In 1985, Stoner[90] reported an improved spectrophotometric assay for the measurement of diamine oxidase activity. This procedure involved a peroxidase-coupled assay to monitor continuously the hydrogen peroxide released during diamine oxidation. In the presence of hydrogen peroxide, 3-methyl-2-benzothiazolinone hydrazone (MBTH) (34) reacts with 3-(dimethylamino)benzoic acid (DMAB) (36) producing a purple indamine dye with a characteristic absorbance maximum at 595 nm (Scheme 2.17). Stoner has shown that this assay is efficient for the measurement of diamine oxidase activity with histamine as substrate. However it has since been shown to be effective in activity determinations using a wide range of substrates.[91,92]



Scheme 2.17

2.15 Substrate Specificity and the Active Site

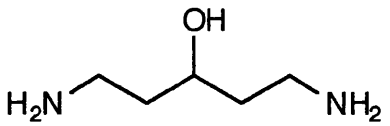
The substrate specificity of both pea seedling and pig kidney diamine oxidase has been investigated by a variety of authors using the assay systems previously discussed (Section 2.14). Although both enzymes

have shown a broad substrate tolerance, it would appear that some differences regarding the active site geometries in the two enzymes do exist.

Mann[17] has shown that partially purified preparations of pea seedling diamine oxidase catalyse the oxidation of a wide range of substrates including the amino acids lysine and ornithine. Costa *et al.*[93] in a series of experiments involving pig kidney diamine oxidase, failed to observe the oxidation of lysine by this enzyme. Frydman *et al.*[19] studied the oxidation of a range of *N*- and *C*-alkylated putrescine derivatives by both plant and animal oxidases. The results obtained showed that *N*-ethyl-, *N*-propyl- and *N*-butylputrescine were all efficiently oxidised by both plant and mammalian forms of the enzyme whereas *N*-methylputrescine showed a very low rate of oxidation in both cases. The *C*-alkylputrescines were generally poorer substrates than the corresponding *N*-alkyl-derivatives with 1,4-dimethylputrescine failing to be oxidised by either enzyme. The specificity of diamine oxidase towards 1-propylputrescine was significantly different for the two enzyme forms. It was shown to be an effective substrate for pea diamine oxidase but a poor substrate for the mammalian form. This result, combined with the fact that 1-methylputrescine and 2-methylputrescine are oxidised at very different rates by plant and mammalian DAO, appears to indicate that differences exist at the active sites of the two enzyme forms.

Macholan[21] attempted to highlight these apparent differences in a series of experiments involving hydroxydiamines. He showed that the binding affinities of 3-hydroxypentane-1,5-diamine (37), 2-hydroxybutane-1,4-diamine (38) and 2-hydroxypentane-1,5-

diamine (39) towards the two forms of diamine oxidase were significantly different.



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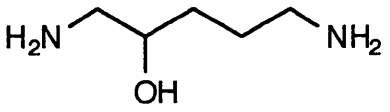
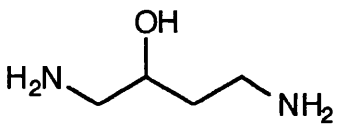


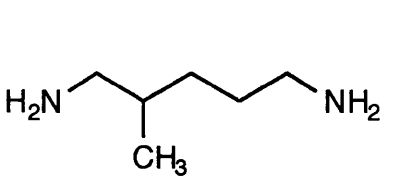
(39)



(38)

Robins and co-workers[91] have since repeated the work of Frydman using the improved spectrophotometric assay of Stoner.[90] Contrary to the report of Frydman[19] it was found that *N*-methylputrescine was a good substrate for both plant and animal diamine oxidase, being oxidised significantly faster than the *N*-ethyl and *N*-propyl derivatives. This work was extended into the area of cadaverine analogues.[92] Due to the poor specific activity of the commercial pig product, accurate kinetic data were only reported for experiments using partially purified pea seedling diamine oxidase. It was shown that 2-methylcadaverine (40), 3-methylcadaverine (41), 3,3-dimethylcadaverine (42), 3-hydroxy-3-methylcadaverine (43) and *meso*-2,4-dimethylcadaverine (44) are all substrates of the enzyme. There appears to be a progressive decrease in the rate of oxidation as the size of substituents increase. It was found that 3-hydroxy-3-methylcadaverine (43) was a better substrate than 3,3-

dimethylcadaverine (42). It was felt that the incorporation of a polar substituent into the backbone of the substrate led to a more efficient process.



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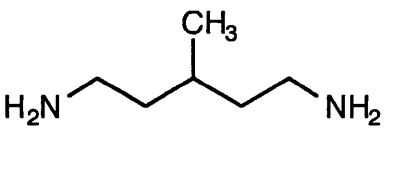

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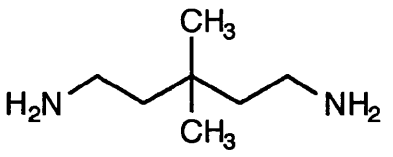

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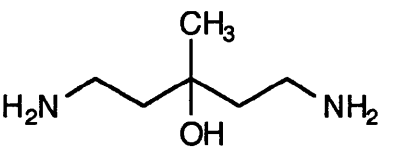

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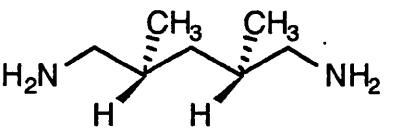


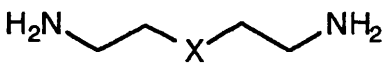
(44)

Equi *et al.*[20] have also shown that a series of α,ω-diamines with chain lengths varying from 2 to 12 are oxidatively deaminated by pea seedling diamine oxidase. An association drawn between chain length and binding affinity suggested that the most efficient binders possessed carbon chain lengths between 4 and 7, with the best binders being the natural substrates (putrescine C4 and cadaverine C5). The rate of oxidation was also shown to be strongly dependent on chain length, with the natural substrates being most efficiently oxidised.

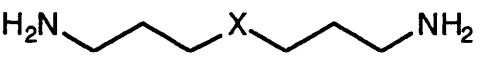
Cragg *et al.*[94] have shown that a range of thiodiamines and their oxygen analogues are oxidised by diamine oxidase.

Lanthionamine (13) is oxidatively deaminated by pea seedling DAO whereas homocystamine (45) and homolanthionamine (46) are substrates for pig kidney DAO.

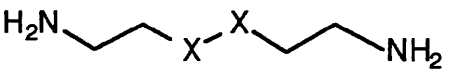
Corda *et al.*[95] examined the effect of pig kidney diamine oxidase on a range of selenium-containing substrates. Selenocystamine (47) and selenolanthionamine (48) were efficiently oxidised by pig kidney diamine oxidase. Seleno-homocystamine (49) and seleno-homolanthionamine (50) were also shown to be substrates for the mammalian enzyme. However the rates of oxidation of these compounds were significantly lower than that for selenolanthionamine (48).



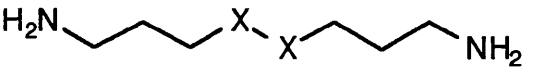
X = S (13)
X = Se (48)



X = S (46)
X = Se (50)



X = Se (47)



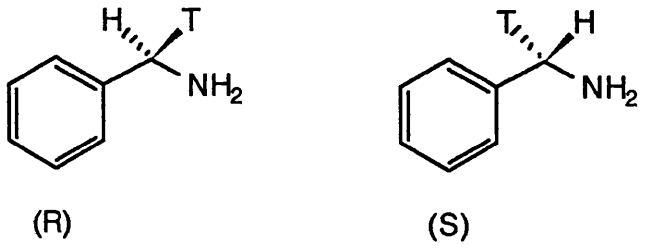
X = S (45)
X = Se (49)

Some monoamines (e.g. benzylamine) are substrates for diamine oxidase. However the rates of oxidation observed for these compounds are lower than for the corresponding diamines. It is believed that the presence of the second amine group is essential for an efficient process.

2.16 Stereochemical Control During DAO-Catalysed Reactions

The mechanism of the enzyme catalysed deamination of diamines has been discussed in much detail (Sections 2.9, 2.10). However the stereochemistry associated with hydrogen abstraction, from the methylene group adjacent to the nitrogen, has not yet been considered in detail. The absolute stereochemistry of the abstraction of a hydrogen atom from the prochiral methylene group has been determined in several instances by a variety of methods. A precondition of all these methods was the availability of substrates which were labelled at the methylene group with tritium or deuterium and whose absolute stereochemistry had been determined by correlation with compounds of known chirality.

Battersby *et al.*[11] investigated the problem by first synthesising *(R)*- and *(S)*-[methylene-$^3H_1$]benzylamine (51). Three experiments were then carried out involving the incubation of pea seedling diamine oxidase with (1) *(R)*-[methylene-$^3H_1$]benzylamine (2) *(S)*-[methylene-$^3H_1$]benzylamine and (3) a 50:50 mixture of the above isomers.

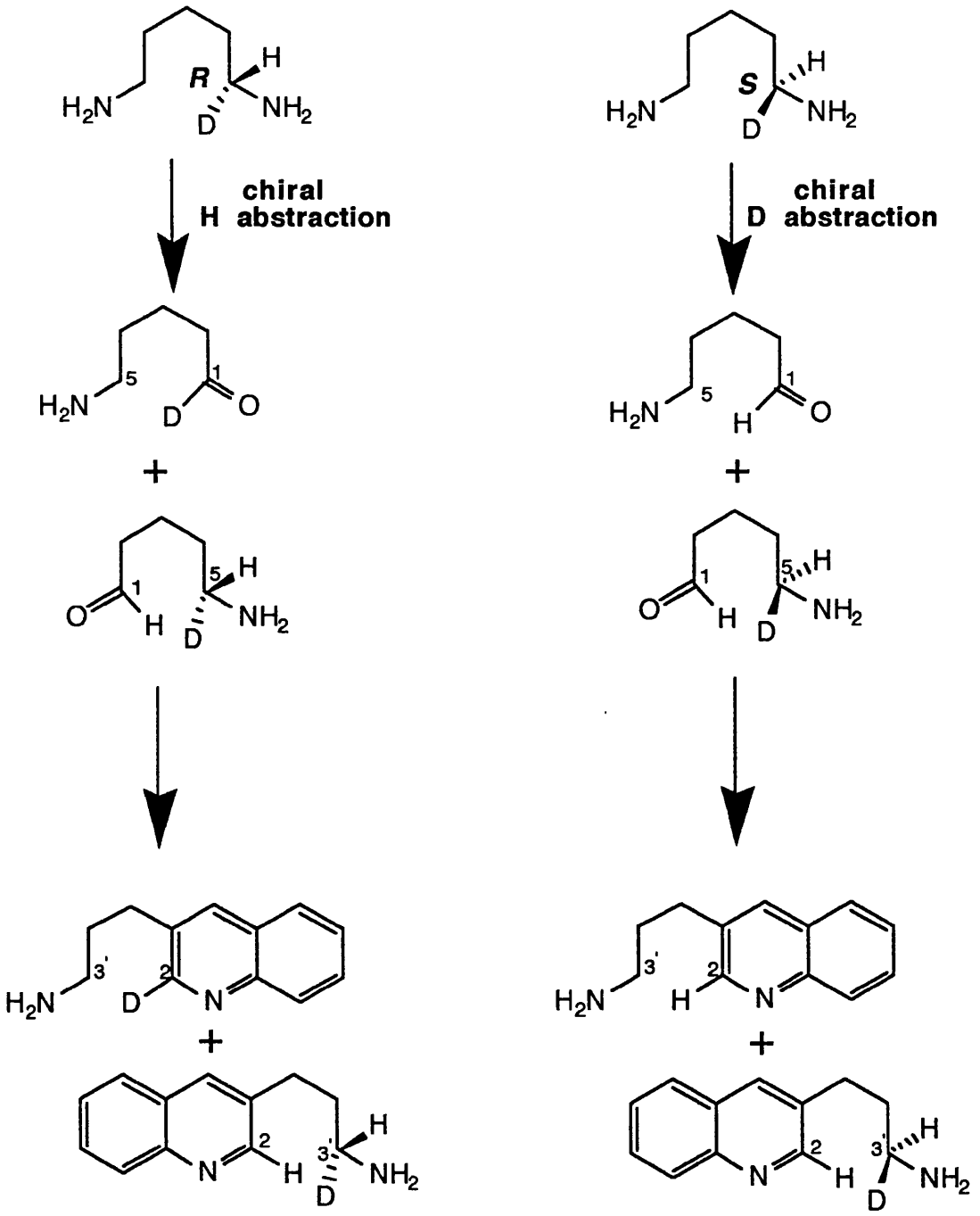


(R) (S)

(51)

In order to ease product isolation the resultant aminoaldehydes were treated with a mixture of liver alcohol dehydrogenase, NAD^+ and cyclopentanol giving rise to the corresponding alcohols which were then converted into their 2,4-dinitrobenzoates. The product isolated from experiment (1) showed almost complete retention of the tritium label whereas in experiment (2) only a fraction of the original activity remained. The result of experiment (3) was consistent with the previous two experiments, with approximately 50% of the label being retained. These results showed that it is the *pro-S* hydrogen that is abstracted during the pea seedling diamine oxidase catalysed oxidation of benzylamine.

Richards and Spenser[10] used ^2H NMR spectroscopy to investigate the stereochemistry of the deamination of cadaverine by pig kidney diamine oxidase. *(S)*-[1-$^2H_1$]cadaverine, *(R)*-[1-$^2H_1$] cadaverine and [1,1-$^2H_2$]cadaverine were prepared with high configurational purity. The products from the enzyme catalysed oxidation of these compounds by pig kidney DAO were trapped with *o*-aminobenzaldehyde leading to the formation of 3-(3'-aminopropyl)quinoline (52) (Scheme 2.18). The products obtained from the incubation of diamine oxidase with *(R)*-[1-$^2H_1$]cadaverine and [1,1-$^2H_2$]cadaverine both showed two signals in their ^2H NMR spectra whereas with *(S)*-[1-$^2H_1$]cadaverine only one signal was observed. This clearly indicates that the *pro-S* hydrogen is again lost during the catalytic deamination process.



(52)

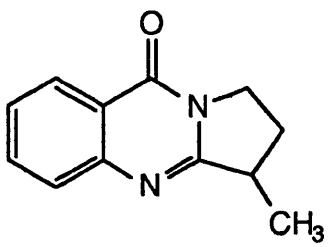
Scheme 2.18

Battersby *et al.*[96] continued work in this area by examining the stereo-control involved in the catalytic oxidation of cadaverine with pea diamine oxidase. Incubation of [1-^{3}H]-labelled cadaverine with pea DAO yielded the corresponding aminoaldehyde which in the presence of a suitable dehydrogenase was converted into the more stable amino alcohol, 5-aminopentan-1-ol. This compound was further deaminated by diamine oxidase and reduced leading to the formation of pentane-1,5-diol. Traces of the remaining aminoalcohol were removed by preparative TLC. Since stereospecific loss through two deaminations should lead to either complete loss or complete retention of tritium (depending on the nature of the label), the results were easy to interpret. Using the *(S)*-isomer as substrate, Battersby noted only trace amounts of tritium in the final product, whereas the product formed from the *(R)*-isomer showed complete retention of the label. Again, on the basis of these observations, Battersby concluded that the *pro-S* hydrogen was abstracted during the oxidation process.

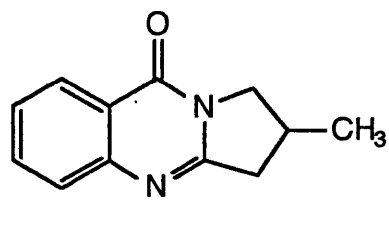
Since these findings were reported it has been shown that loss of the *pro-S* hydrogen is associated with pea DAO catalysed oxidations of a wide range of substrates.[12,97,98] This observed stereochemical consistency has led to the assumption that all oxidations catalysed by diamine oxidase result in the loss of the *pro-S* hydrogen. Indeed this assumption has formed the basis for configurational assignments of absolute stereochemistry.

2.17 Regioselectivity and Stereoselectivity in Reactions Catalysed by DAO

Santaniello *et al.* have examined the regio- and stereo-selectivity of the reaction catalysed by both pig kidney and pea DAO.[13] Initial studies, investigating the regioselectivity of the reaction, involved incubation of racemic 2-methylputrescine with the appropriate form of diamine oxidase. Trapping of the oxidation product thus formed with *o*-aminobenzaldehyde and subsequent oxidation led to the formation of 1'-or 2'-methyl-2,3-trimethylene-4(3*H*)-quinazolone (53) or (54).



(53)



(54)

The products obtained were analysed using ^{1}H NMR spectroscopy. The spectum of the product from the pig kidney DAO reaction showed two doublets (δ 1.24 and 1.46) of equal intensity whereas that obtained from the pea DAO reaction mix showed only one doublet (δ 1.24). On the basis of these results it was concluded that pea seedling DAO catalyses the oxidation of 2-methylputrescine in a regiospecific manner. Similar regioselective control was not observed for the mammalian form of the enzyme.

Santaniello *et al.* then studied the stereopecific nature of the reaction using *(R)*- and *(S)*-2-methylputrescines as substrates for both pea and pig kidney DAO. The products of the reactions were

trapped as before and a quantitative analysis was performed using HPLC. It was found that pea seedling diamine oxidase catalyses deamination at the less hindered group regardless of the configuration of substrate, since for both isomers compound (54) was essentially the only detectable product. With pig kidney DAO, the oxidation is dependent upon the chirality of the substrate. In the case of *(R)*-2-methylputrescine the aminoaldehyde formed from oxidation at C-1 is favoured (95%) whereas the (S)-isomer undergoes oxidation at the less hindered amine group. The above results indicate that pea DAO regioselectively oxidises 2-methylputrescine at C-4 and shows no stereochemical distinction between *(R)*- and *(S)*-forms. Pig kidney DAO catalyses the oxidation of *(R)*- and *(S)*-2-methylputrescines with opposite regioselectivity.

2.18 Inhibitors of Diamine Oxidase

Diamine oxidase is involved in the regulation of cellular levels of polyamines. Since polyamines are essential for cell growth and replication, inhibition of diamine oxidase may lead to some interesting activity.

Inhibitors of diamine oxidase can be divided into three main classes; (1) substances which are capable of disabling the cofactor; (2) substances which interfere with the catalytic role of Cu(II); and (3) substances which prevent the substrate gaining access to the active site.

(1) Enzyme inactivators- This mode of deactivation of the enzyme depends on the ability of the inhibitor to react with the carbonyl functionality of the cofactor. Hydrazine, semicarbazide and

hydroxylamine have all been shown to inhibit diamine oxidase by this mechanism.[22]

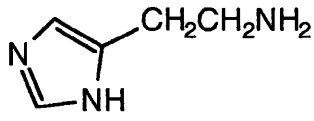
(2) Copper chelating agents- It has been shown that by the addition of substrates capable of disrupting the catalytic function of Cu(II) that partially and highly purified samples of both plant and animal DAO have been inhibited. These chelating agents bind to the copper present at the active site of the enzyme. 8-Hydroxyquinoline, sodium diethyldithiocarbamate, 2,2-bipyridyl, 1:10-phenanthroline, sodium azide and sodium cyanide act in this manner.

(3) Substrate analogues

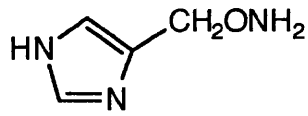
Very often, good inhibitors of an enzyme resemble the natural substrate in structure. A subtle change in the electronic or steric nature of a compound can bring about a dramatic change in the reactivity of this compound towards the enzyme.

Bieganski,[99] having noted that histamine (55) was a substrate for both plant and animal diamine oxidase, examined the effect of histamine analogues on the oxidation process. The studies undertaken concentrated on two structure types: (1) compounds possessing the imidazole ring and a further amine group such as (56) and (57); and (2) compounds possessing a histamine system combined with an aliphatic amine as in (58). Compounds (56) and (57) were found to be extremely good inhibitors of both pea seedling and pig kidney DAO. This result contradicted earlier work which suggested that imidazole and its derivatives inhibited only mammalian DAO. Compound (58) inhibited pea DAO selectively. This

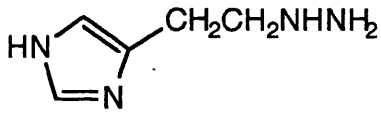
result again highlights the differences between the active sites of plant and mammalian DAO.



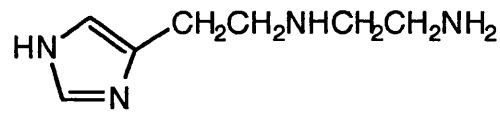
(55)



(56)

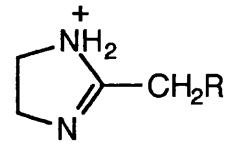


(57)

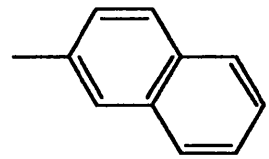


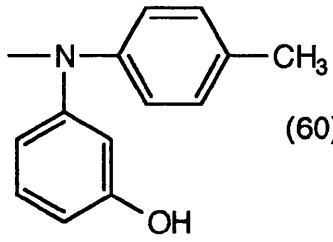
(58)

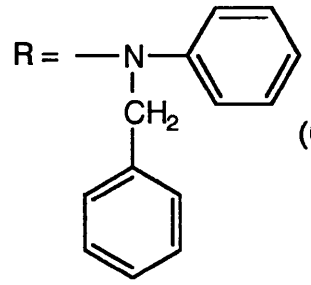
Pec and Hlidkova[100] examined the inhibitory effect of a range of 4,5-dihydroimidazole systems on the catalytic deamination of 1,4-diamino-2-butene by pig kidney DAO. It was shown that natazolin (59), fentolamin (60) and anatazolin (61) all function as noncompetitive inhibitors in the concentration range 0.5-5.0 mM. 2-Methyl-4,5-dihydroimidazole (62) showed no inhibitory effect, thus ruling out the possibility that the inhibition noted for the other analogues was due to Cu(II) chelation with the 4,5 dihydroimidazole ring.



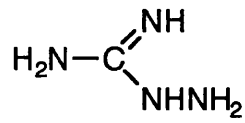
R = H (62)

R =  (59)

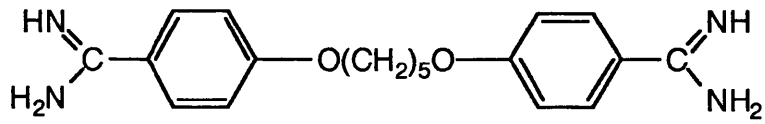
R =  (60)

R =  (61)

It has been shown that aminoguanidine (63) is a potent inhibitor of both plant and animal diamine oxidase.[101] The incorporation of an amidine grouping has led to the development of a range of DAO inhibitors including MGBG (64).



(63)



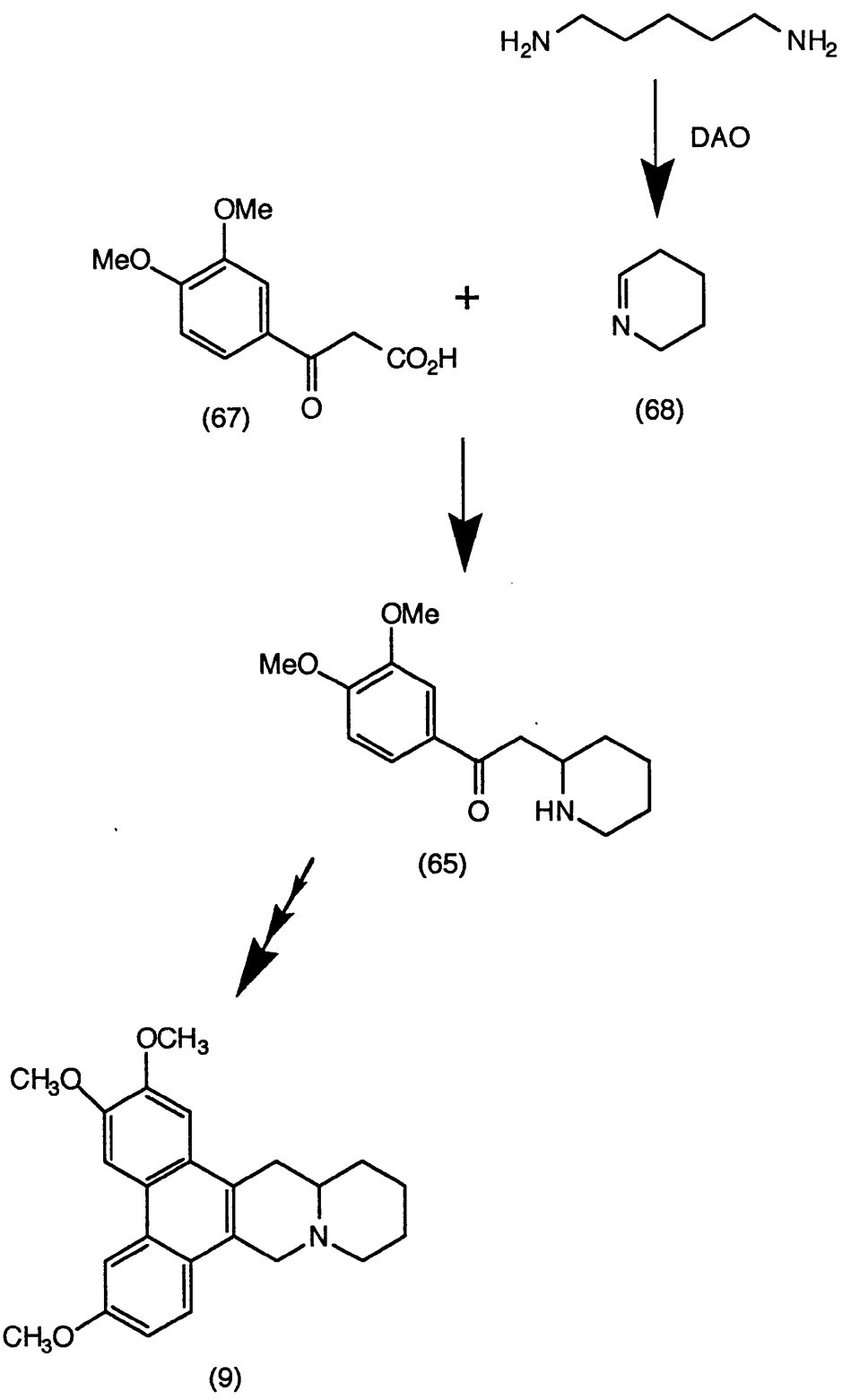
(64)

2.19 Synthetic Applications of Diamine Oxidase

Diamine oxidase catalyses a functional group transformation which is difficult to carry out by chemical means. Use of this enzyme, in a purified or partially purified form, is of increasing importance.

Cragg and Herbert[14] used partially purified pea seedling DAO as a convenient catalyst in the synthesis of a range of phenacyl derivatives. Enzymatic oxidation of a suitable diamine, followed by *in situ* condensation of the resultant cyclic imine with a benzoylacetic acid derivative, has led to the formation of a range of synthetically useful phenacyl intermediates.

Cragg and Herbert[15] used this approach in the synthesis of a range of biologically active alkaloids. 3',4'-Dimethoxy-2-(2-piperidyl)acetophenone (65) found in *Boehmeria platyphylla* is an important intermediate in the synthesis of the alkaloid cryptopleurine (9). Direct synthesis of this intermediate was achieved by the condensation of (3,4-dimethoxybenzoyl)acetic acid (67) with 3,4,5,6-tetrahydropyridine (68), formed *in situ* by the DAO catalysed oxidation of cadaverine (Scheme 2.19). Subsequent chemical manipulation led to the formation of cryptopleurine (9). By selecting substituted diamines with a range of carbon chain lengths, this route may enable the synthesis of a variety of alkaloid analogues.


H2N
NH2
DAO
OMe
MeO
CO2H
O
(67)
+
N
(68)
OMe
MeO
O
HN
(65)
OCH3
CH3O
N
CH3O
(9)

Scheme 2.19

The use of pea seedling DAO by Robins[16] to produce the pyrrolizidine alkaloid trachelanthamidine (12) has already been discussed (Chapter 1.7).

Further use of enzymatically generated 1-pyrroline or 1-piperideine may provide routes to other alkaloids and heterocyclic compounds containing pyrrolidine or piperideine rings.

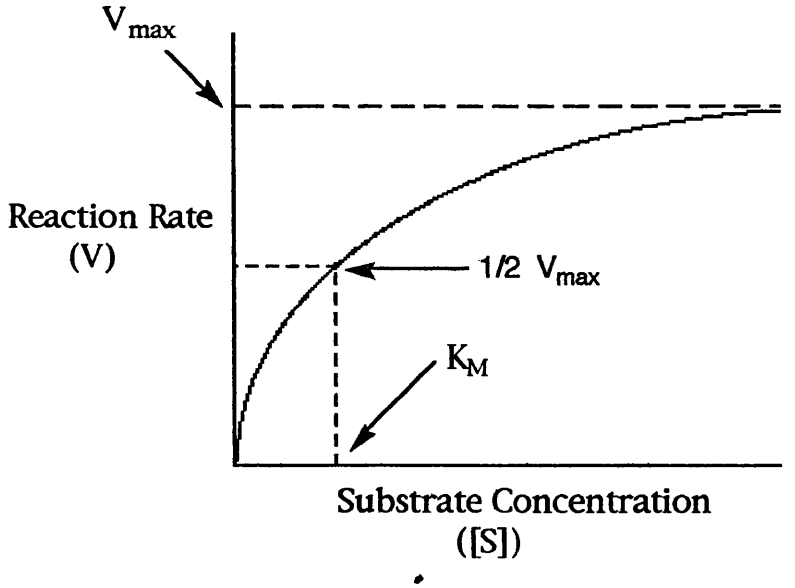
The enzyme kinetics necessary for an understanding of the work carried out in this project is summarised in the following Chapter.

CHAPTER 3

ENZYME KINETICS

3.1 Substrate-Enzyme Interactions

The kinetic feature that most distinguishes enzyme-catalysed reactions from most un-catalysed chemical reactions is that they show saturation. Almost all enzyme-catalysed reactions show a first order rate dependence at low substrate concentrations, but instead of increasing indefinitely as concentration increases, the rate approaches a limit, (V_{max}), which cannot be exceeded regardless of the concentration of substrate (Graph 3.1).



Graph 3.1- Reaction rate (V) *vs.* Substrate concentration ([S])

3.2 Michaelis-Menten Kinetics

Leonor Michaelis and Maud Menten are credited with providing the first major insight into enzyme kinetics. After a series of detailed experiments a mechanism was proposed to explain the observed kinetic characteristics. The mechanism (Scheme 3.1) supposes that the first step in the reaction is the binding of substrate (S) to the enzyme (E) to form an enzyme-substrate complex (ES). This intermediate can either go on to form the product or dissociate to regenerate the enzyme and substrate.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_{cat}}{\dashrightarrow} E + P$$

Scheme 3.1- The dotted arrow indicates the possible complexity of subsequent steps, including activated species (ES*) and enzyme -product complexes (EP).

Using this simple model it is possible to obtain an expression which relates the rate of catalysis to the concentration of enzyme and substrate and the rate of the individual steps.

Since product is formed only in the second step of the reaction, the rate of formation of product and hence the rate of the overall reaction is given by:

$$V = k_{cat}[ES] \qquad (3.1)$$

Using the steady-state assumption it is possible to derive an expression for [ES] in terms of known quantities. Briggs and Haldane[102] postulated that although at the instant of mixing the rate of formation of [ES] would be greater than the rate of breakdown,

since no [ES] is present initially, the rate of breakdown increases rapidly until it balances the rate of formation. A steady state will then exist in which the concentration of the intermediate remains constant while the concentrations of the starting material and products change.

Under steady state conditions:

Rate of formation of [ES] = Rate of breakdown of [ES]

$$k_1[E][S]=(k_2+k_{cat})[ES] \qquad (3.2)$$

Rearranging this equation gives an expression for [ES]:

$$[ES]=[E][S]/\{(k_2+k_{cat})/k_1\} \qquad (3.3)$$

This expression can be simplified by introducing a new constant K_M and defining it:

$$K_M= (k_2 + k_{cat})/k_1 \qquad (3.4)$$

Substituting this into (3.3) gives:

$$[ES]=[E][S]/K_M \qquad (3.5)$$

If the total concentration of enzyme is $[E_T]$ and the concentration of the enzyme-substrate complex is [ES] then the concentration of free enzyme, [E], is given by:

$$[E]=[E_T] - [ES] \qquad (3.6)$$

Similarly this argument can be applied to the substrate, leading to an expression for uncombined substrate, [S]:

$$[S]=[S_T]- [ES] \quad (3.7)$$

However, since under experimental conditions substrate concentrations are generally very large compared to enzyme concentrations, the concentration of free substrate is far greater than the concentration of bound substrate. Therefore, the concentration of free substrate is approximately equal to the total substrate concentration:

$$[S]=[S_T] \quad (3.8)$$

Substituting equation (3.6) into (3.5) gives:

$$[ES]=([E_T]-[ES])[S]/K_M \quad (3.9)$$

Solving this equation for [ES] gives:

$$[ES]=[E_T][S]/([S]+K_M) \quad (3.10)$$

Substituting this expression for [ES] into equation (3.1) gives the rate equation:

$$V=k_{cat}[E_T][S]/([S]+K_M) \quad (3.11)$$

At maximal rate, V_{max}, that is the rate when the enzyme sites are saturated, the concentration of substrate is much greater than K_M so,

$$[S]/([S]+K_M)= 1 \quad (3.12)$$

This gives equation (3.13):

$$V_{max}=k_{cat}[E_T] \quad (3.13)$$

Which on substitution into equation (3.11) gives the Michaelis-Menten equation (3.14),

$$V= V_{max}[S]/([S] + K_M) \quad (3.14)$$

This equation provides a clear explanation for the shape of Graph 3.1. At low concentrations $[S] << K_M$ and equation (3.14) becomes:

$$V = V_{max}[S]/K_M \quad (3.15)$$

It is clear to see that at low [S], the rate is directly proportional to the substrate concentration. At high substrate concentrations $[S] >> K_M$ and equation (3.14) becomes:

$$V = V_{max} \quad (3.16)$$

In this case the rate reaches a maximal rate which is independent of the concentration of substrate.

3.3 Significance of V_{max} and K_M Values

The V_{max} is the limiting rate for a particular reaction. It is, by definition, the rate of an enzyme catalysed reaction when saturation has occurred. It is not reached at any finite value of substrate concentration and indeed is only approached very slowly. Consider the Michaelis-Menten equation (3.14):

$$V = V_{max}[S]/([S] + K_M) \qquad (3.14)$$

When the concentration of substrate is 10 times the K_M then the observed rate is 0.91 times the V_{max}. At a substrate concentration equal to 20 times the K_M the observed rate is still only 0.95 times the V_{max}. Comparisons of V_{max} values for different substrates in relation to an enzymatic process may provide useful information on the structural requirements of the substrate for efficient turnover. This in turn can provide an insight into the nature of the active site of the enzyme.

The K_M is the Michaelis constant for a particular reaction. It is, by definition, the concentration of substrate at which the reaction rate is half the limiting rate. The definition is arrived at by setting $[S]=K_M$ in equation (3.14). This gives:

$$V=V_{max}/2 \qquad (3.17)$$

An expression relating the K_M for a reaction to the rate constants of the individual steps has already been considered (3.4). In general, it is found that during catalysis k_2 is much greater than k_{cat}. Under this condition (3.14) becomes:

$$K_M = k_2/k_1 \qquad (3.18)$$

This expression for K_M is identical to that for the dissociation constant of the ES complex:

$$K_{ES}=[E][S]/[ES]=k_2/k_1 = K_M \qquad (3.19)$$

It is clear that the K_M is a measure of the strength of the ES complex. A high K_M indicates weak binding whereas a low K_M indicates strong binding. By comparing the K_M values for different substrates in relation to an enzymatic process it is possible to examine the structural requirements of the substrate for efficient binding to the enzyme. This can give an insight into the nature of the active site of the enzyme and provide information on the mechanism of the transformation.

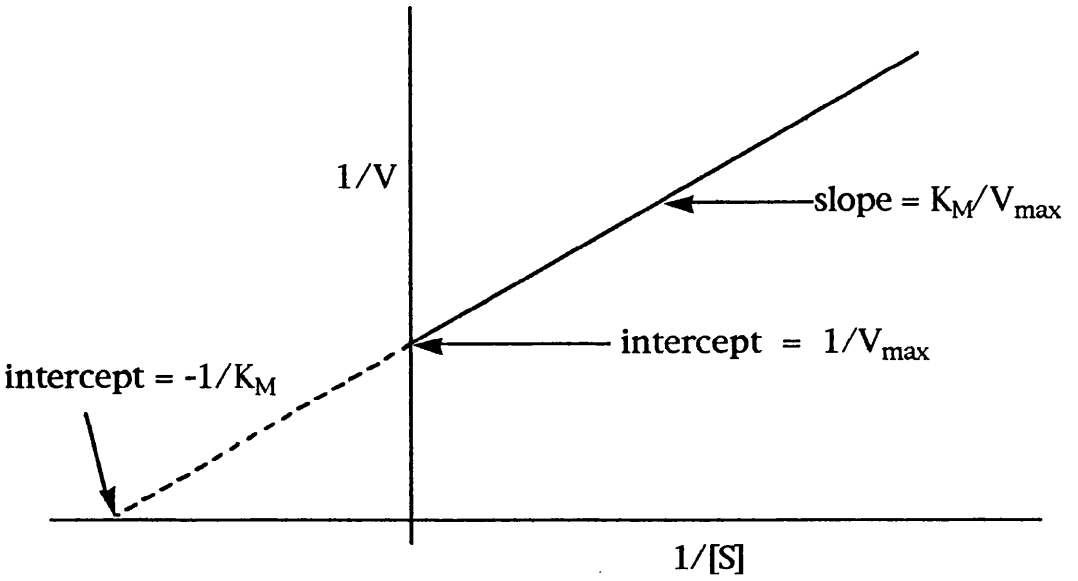
3.4 Experimental Determination of V_{max} and K_M

For the majority of enzymes the Michaelis-Menten model (Scheme 3.1) is an accurate representation of how they operate. When this is the case the Michaelis constant K_M and the limiting rate, V_{max}, can be derived from a measurement of the rates of catalysis at different substrate concentrations. The clearest way to display the data is in a plot of the observed rate (V) against substrate concentration [S] (Graph 3.2). This however is not a satisfactory way to determine the kinetic parameters. Since the line is a curve and only approaches the V_{max} slowly, it is difficult to judge exactly where the limit is. This is particularly relevant in the case where insufficient points, corresponding to saturation, have been plotted. An inaccurate determination of V_{max} will lead to an inaccurate determination of K_M. It was felt that more accuracy could be achieved by transforming

the Michaelis-Menten equation into the equation of a straight line. There are three popular ways of doing this, the first of which, devised by Lineweaver and Burk,[103] involves the inversion of the original Michaelis-Menten equation (3.14) giving:

$$(1/V)=K_M/V_{max}(1/[S]) + 1/V_{max} \qquad (3.20)$$

A plot of 1/V versus 1/[S] (Graph 3.2) leads to a straight line with gradient K_M/V_{max} and intercept on the y-axis $1/V_{max}$. Kinetic parameters can be obtained directly from the graph. Although the Lineweaver-Burk plot is widespread in the literature, its accuracy has been questioned. The main objection to this plot is that it gives undue weight to the least accurate values i.e. those corresponding to low substrate concentrations. Also, the double-reciprocal plot distorts the appearance of any experimental error in the primary observations of V, making it impossible to judge which points are most accurate.



Graph 3.2 Lineweaver-Burk Plot

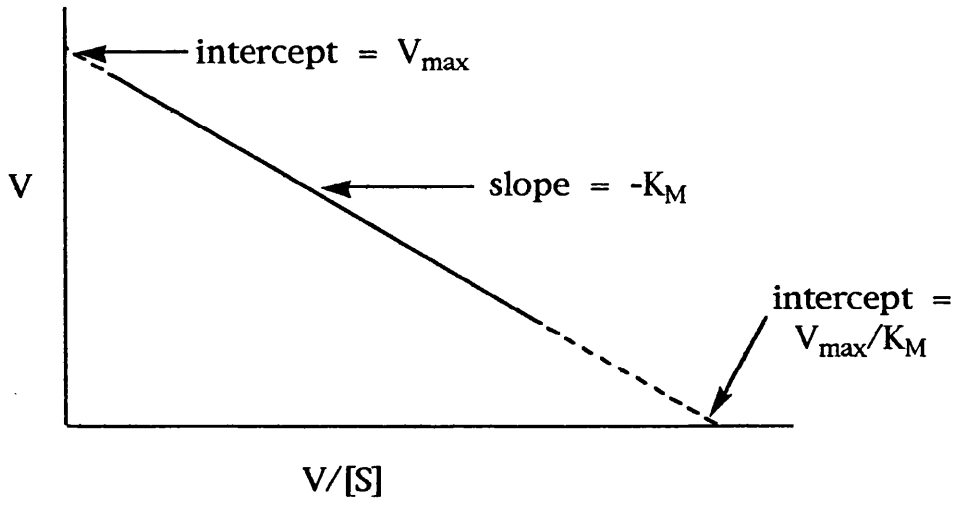
A second straight line plot commonly used is the Eadie-Hoftsee plot.[104,105] Although not entirely free from distortion it is less severely affected than the double-reciprocal plot. This plot (Graph 3.3) was arrived at by multiplying both sides of (3.20) by $V.V_{max}$ giving,

$$V.V_{max}(1/V)= \{(K_M/V_{max})(1/[S])\}V.V_{max} + (1/V_{max})V.V_{max}$$

which simplifies to,

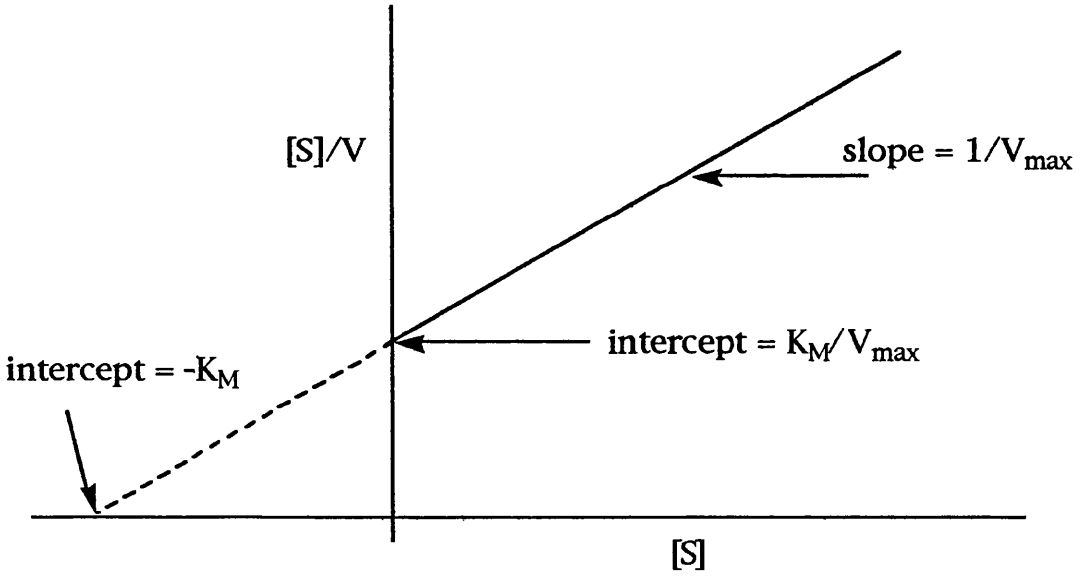
$$V= -K_M(V/[S]) + V_{max} \qquad (3.21)$$

Here a plot of V versus V/[S] will provide a straight line with gradient $-K_M$ and intercept on the y-axis, V_{max}.



Graph 3.3 Eadie-Hoftsee Plot

A third method which allows kinetic data to be presented in a linear fashion was devised by Hanes. The Hanes plot (Graph 3.4) is obtained by plotting [S]/V versus [S]. The straight line obtained has a gradient $1/V_{max}$ and an intercept at the y-axis of K_M/V_{max}. The desired kinetic parameters can be obtained directly.



Graph 3.4 Hanes Plot

In practise, the best estimate for the V_{max} and K_M values are found by considering all three linear plots. In order to minimise any error associated with the plots, it is important to obtain data covering a wide range of substrate concentrations which spread evenly over the plot used. It has been found that this is best achieved by selecting substrate concentrations ranging from 3 times K_M to 1/8 of K_M.

3.5 Inhibition of Enzyme Kinetics

Two major classes of inhibition may result from the binding of an inhibitor to an enzyme. Irreversible inhibition, often called modification, is usually the result of a covalent interaction between the enzyme and the inhibitor. This type of inhibition is characterised by an extremely slow dissociation of the enzyme-inhibitor complex. There are several types of reversible inhibition, including competitive, non-competitive and mixed inhibition processes, all of which show a fast dissociation of the enzyme-inhibitor complex. Competitive inhibition arises most simply when the inhibitor and the substrate compete for the same site on the enzyme, so that when one binds the other cannot. A competitive inhibitor reduces the rate of catalysis by reducing the proportion of enzyme molecules bound to a substrate at any given time. Competitive inhibition may be overcome at high concentrations of substrate.

In non-competitive inhibition the binding sites of the substrate and inhibitor do not overlap. This allows the substrate and inhibitor to bind simultaneously to the enzyme. A non-competitive inhibitor has no effect on the number of enzyme molecules bound to substrate, but instead acts by reducing the rate of turnover of the enzyme. Increasing the substrate concentration cannot overcome non-

competitive inhibition. In the more general case of mixed inhibition, both sets of characteristics may be observed.

It is possible to distinguish between competitive and non-competitive inhibition by measuring the rate of enzymatic catalysis at different concentrations of substrate and inhibitor. Consider the Lineweaver-Burk equation,

$$(1/V)= K_M/V_{max}(1/[S]) + 1/V_{max} \qquad (3.20)$$

In the presence of a competitive inhibitor this becomes;

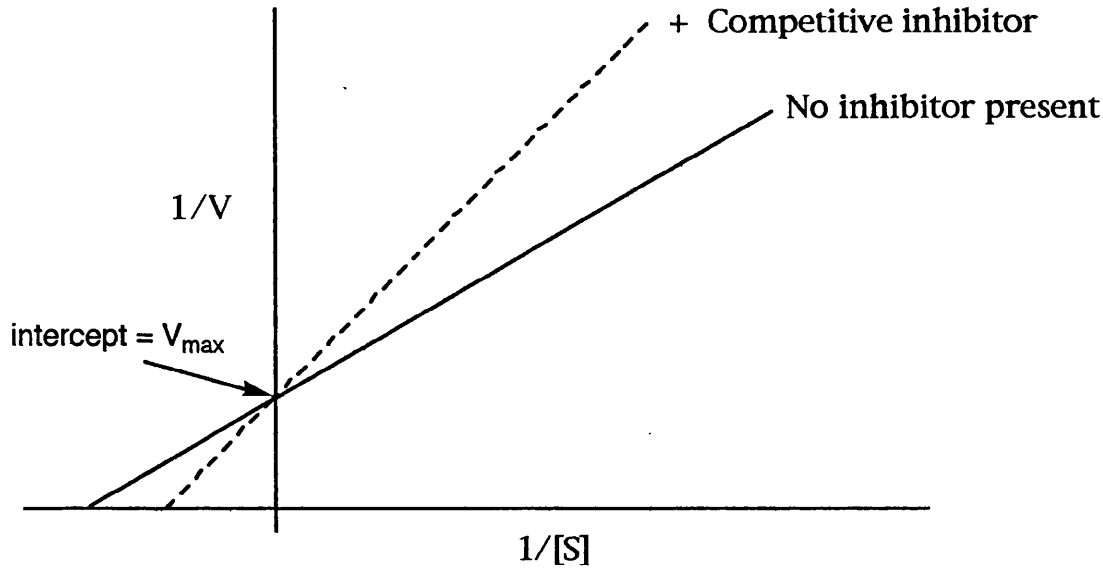
$$(1/V)=K_M/V_{max}(1+ [I]/K_i)1/[S] + 1/V_{max} \qquad (3.23)$$

where [I] is the concentration of inhibitor, and
K_i is the dissociation constant for the enzyme-inhibitor complex.

It is clear that a plot of 1/V versus 1/[S] for (1) substrate with no inhibitor and (2) substrate with competitive inhibitor present will have the same intercept at the y-axis, $1/V_{max}$, but a different gradient (Graph 3.5). This difference in gradient is represented by,

$$(slope^i)/(slope)=1 + [I]/K_i \qquad (3.24)$$

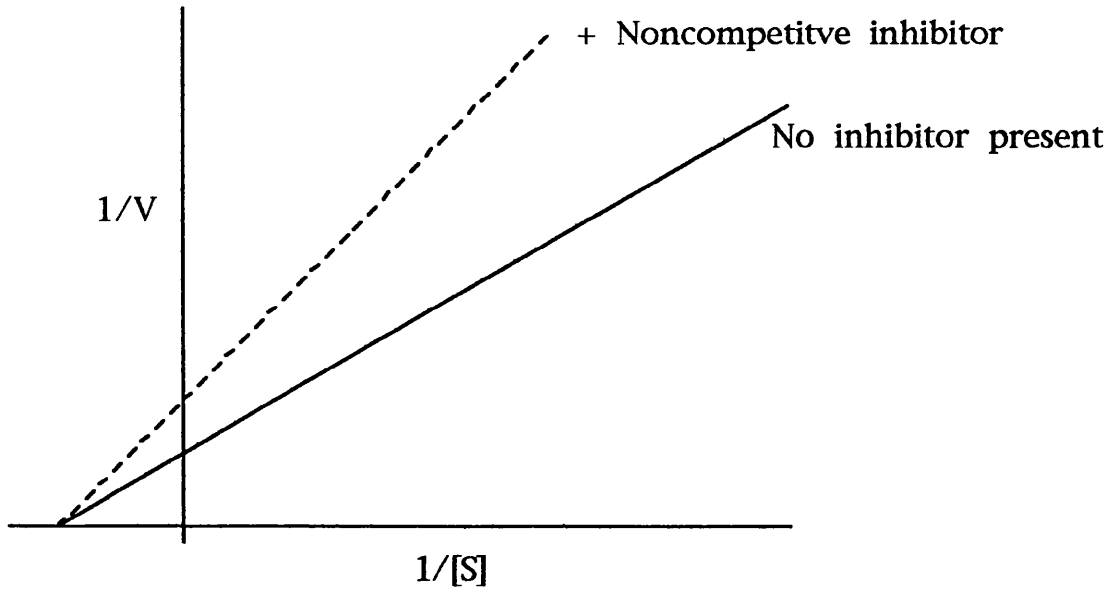
$(slope)^i$ is the gradient of the line with competitive inhibitor present.
(slope) is the gradient with no inhibitor present.



Graph 3.5 Competitive Inhibition: Double reciprocal plot of enzyme kinetics.

The difference in the gradient indicates the difference in the binding with inhibitor present. The V_{max} for an enzymatic reaction is unaltered by the addition of a competitive inhibitor, however the binding affinity of the enzyme for the substrate is reduced by a factor of $1 + [I]/K_i$.

Graph (3.6) shows a plot of 1/V versus 1/[S] in the presence of a non-competitive inhibitor. Here the V_{max} is decreased to V^i_{max} giving rise to an increase in the y-axis intercept. The gradient in the presence of inhibitor, K_M/V^i_{max}, differs from the gradient with no inhibitor present by a factor of V^i_{max}. In the simplest cases the Michaelis constant, K_M, is unaltered in the presence of a non-competitive inhibitor.



Graph 3.6 Simple non-competitive Inhibition: Double reciprocal plot of enzyme kinetics. In cases of mixed inhibition, the double-reciprocal lines do not necessarily intersect on either axis.

CHAPTER 4

OXIDATION OF CYCLIC DIAMINES BY PEA SEEDLING DIAMINE OXIDASE

4.1 Isolation and Partial Purification of the Enzyme

As mentioned previously, two sources of diamine oxidase are particularly convenient. However, due to the poor specific activity associated with the commercial pig product, accurate kinetic data have been difficult to obtain using this enzyme. For this reason our experiments have been restricted to the pea seedling form of diamine oxidase.

The extraction and purification of the enzyme was achieved using the method of Hill.[5] Pea seedlings from the "Fillbasket" variety were grown for *ca.* ten days. The initial stage of the treatment involved the precipitation of unwanted material by a 2:1 mixture of chloroform and ethanol. Subsequent saturation with ammonium sulphate led to the precipitation of the enzyme. Contrary to the observations of Hill, complete separation was not achieved by us after one hour therefore in an attempt to improve separation the mixture was left overnight. This modification was found to give rise to an improved yield of the isolated enzyme. Further ammonium sulphate precipitations followed by dialysis led to the isolation of the purified enzyme. As the purity of this enzyme was sufficient for our purposes, the chromatographic purification steps used by Hill were not undertaken. The enzyme was dissolved in phosphate buffer (pH 7) and stored in 0.5 ml aliquots in the freezer at -20 ^{o}C. Under these conditions the enzyme was found to be stable for several months.

It has been shown[20] by gel electrophoresis that pea seedling diamine oxidase purified in this manner consists of two main bands (molecular weights 70,000 and 20,000). It was not clear whether both bands were associated with diamine oxidase or whether an impurity was present.

Further purification of the enzyme is discussed in Chapter 7.

4.2 Determination of Protein Concentration

The protein concentrations of the isolated enzymes were measured using the procedure of Sedmark and Grossberg.[106] This method relies on the fact that in the presence of protein, Coomassie brilliant blue G in dilute acid is converted from a brownish-orange colour into an intense blue colour. The procedure was carried out using bovine serum albumin (BSA) as standard (1 mg of standard is equal to 1 mg of protein). Absorbances of the mixture were measured at A_{620} (blue) and A_{465} (brown-orange) for various concentrations of BSA and a standard graph was obtained by plotting A_{620}/A_{465} against protein concentration. [Note: The absorbance was measured approximately twenty minutes after the Coomassie reagent was added to the protein sample. The A_{620}/A_{465} was then measured for a range of enzyme samples of varying dilutions. Using the standard graph, these absorbance ratios enabled the protein concentrations in the enzyme samples to be determined (Appendix 1)].

This assay for the determination of protein concentrations was found to be highly reproducible.

4.3 The Assay System

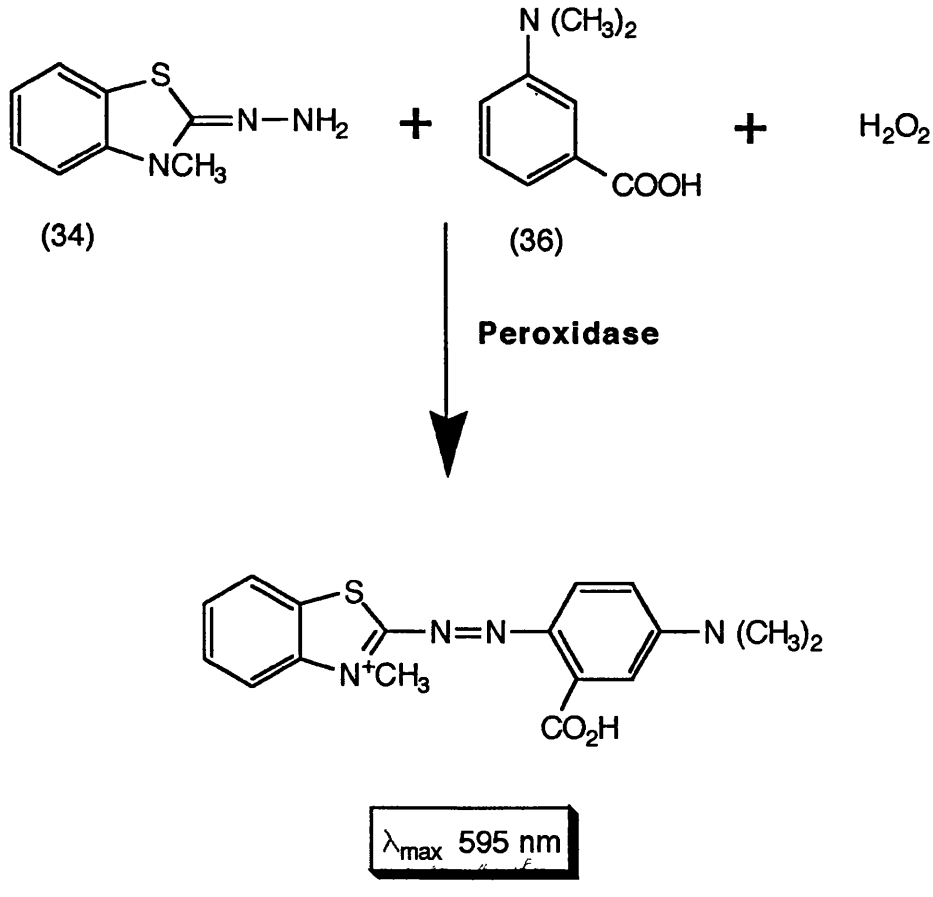
The many assay systems developed for the determination of diamine oxidase activity have been discussed (Chapter 2.14). The primary aim of our work relies on the accurate determination of the rate of enzymatic oxidation of a variety of substrates by pea seedling diamine oxidase. For this purpose we required an assay system which was not restricted by the requirement of 1-pyrroline (or 1-piperideine) as the oxidation product. It was found that the spectrophotometric method of Stoner[90] was a reliable and convenient method for our studies.

This assay depends on the fact that hydrogen peroxide is released during the catalytic deamination process. The hydrogen peroxide released, in the presence of peroxidase, oxidatively couples 3-methyl-2-benzothiazolinone hydrazone (MBTH) (34) to (dimethylamino)benzoic acid (DMAB) (36), generating a stoichiometric amount of an indamine dye with a characteristic absorbance maximum at 595 nm (Scheme 4.1). The rates of reaction can be obtained directly from the spectrophotometer. The validity of the assay system has been checked.[20] The original experiment carried out by Stoner[90] using histamine and pig kidney diamine oxidase was repeated. A K_M value was obtained which was in close agreement with the findings of Stoner. Formation of the indamine dye was also calibrated using standard solutions of hydrogen peroxide.

Stoner showed that MBTH (34) was an inhibitor of diamine oxidase. This inhibition was found to be both time and concentration dependant. The inhibitory effect of MBTH was kept to a minimum by selecting the lowest concentration of chromagen required for

coupling. This inhibitory effect was further reduced by adding the substrate immediately after the addition of enzyme, thus reducing the incubation time of the enzyme with MBTH. Using this spectrophotometric assay system, almost all of the compounds tested were shown to exhibit Michaelis-Menten behaviour. Rate data were analysed for V_{max} and K_M by least squares fitting of Lineweaver-Burk[103] (1/V against 1/[S]), Eadie-Hofstee[104,105] (V against V/[S]) and Hanes ([S]/V against [S]) plots. The experiments were carried out three times with each substrate and the data are quoted as an average of nine determinations.

2.



Scheme 4.1

4.4 Oxidation of Cyclic Diamines by Pea Seedling Diamine Oxidase

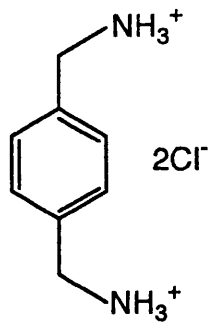
Investigations into the substrate specificity of pea seedling diamine oxidase have been considered (Chapter 2.15). The majority of published work has concerned systems which are analogues of the natural substrates.[19,21,91,94] It was our intention to examine further the catalytic deamination potential of the enzyme by studying the oxidation of a range of simple cyclic diamines. Using the sensitive peroxidase-coupled assay system of Stoner[91] data were collected by us for a variety of aromatic and six-membered alicyclic diamines. The kinetic parameters obtained from the enzyme catalysed reactions should provide an insight into the effectiveness of cyclic systems as substrates for pea seedling diamine oxidase as well as giving more information on the steric constraints at the active site.

4.5 Enzymatic Oxidation of bis(Aminomethyl)benzenes (Xylylenediamines) by Pea Seedling Diamine Oxidase

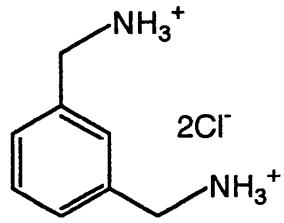
4.5(a) Introduction

Although benzylamine[93] has been examined as a substrate for pea seedling diamine oxidase, no kinetic data concerning the catalytic oxidation of aromatic systems containing two primary amine groups have been reported. In an attempt to gain an insight into the effectiveness of this type of compound as a substrate for pea seedling DAO, it was decided to synthesise 1,4-xylylenediamine dihydrochloride (69), 1,3-xylylenediamine dihydrochloride (70) and

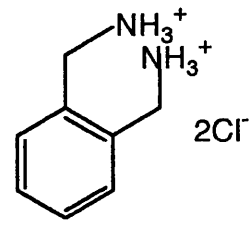
1,2-xylylenediamine dihydrochloride (71). Comparison of the kinetic parameters within the series should provide information on the optimum distance between the amine groups for (1) efficient oxidation and (2) strongest binding.



(69)



(70)

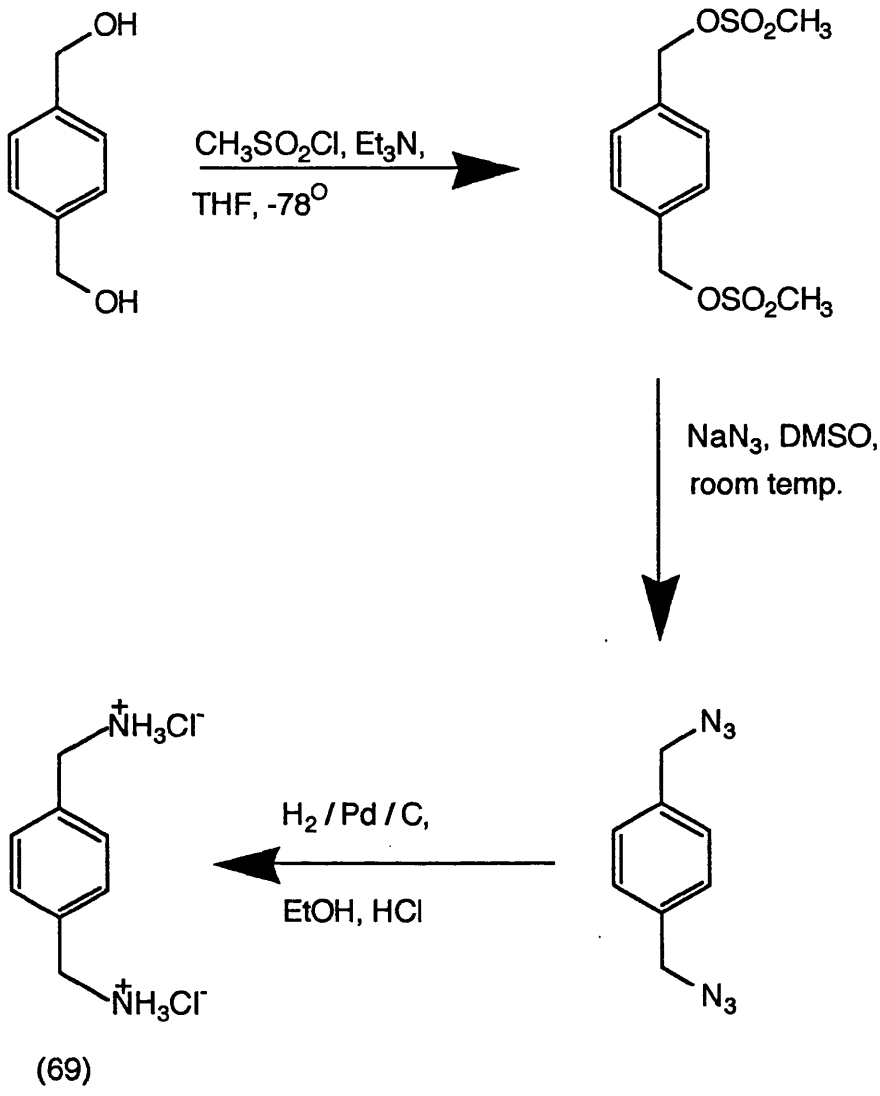


(71)

4.5 (b) Synthesis of Xylylenediamines

1,4-Xylylenediamine dihydrochloride (69) and 1,3-xylylenediamine dihydrochloride (70) were synthesised from the corresponding bis(hydroxymethyl)benzenes. The first step involved the conversion of the diol into the dimethanesulphonate (dimesylate) using methanesulphonyl chloride and triethylamine. Purification of the dimethanesulphonate was effected by crystallising from diethyl ether. Treating this intermediate with sodium azide introduced the nitrogen functionality *via* nucleophilic substitution. The resultant diazide was purified on a silica gel column. Catalytic hydrogenation of the diazide in the presence of palladium on carbon and concentrated hydrochloric acid produced the desired diamines, in the form of the

more stable dihydrochloride salts. The diamine dihydrochlorides were recrystallised from aqueous ethanol and acetone (Scheme 4.2).



(69)

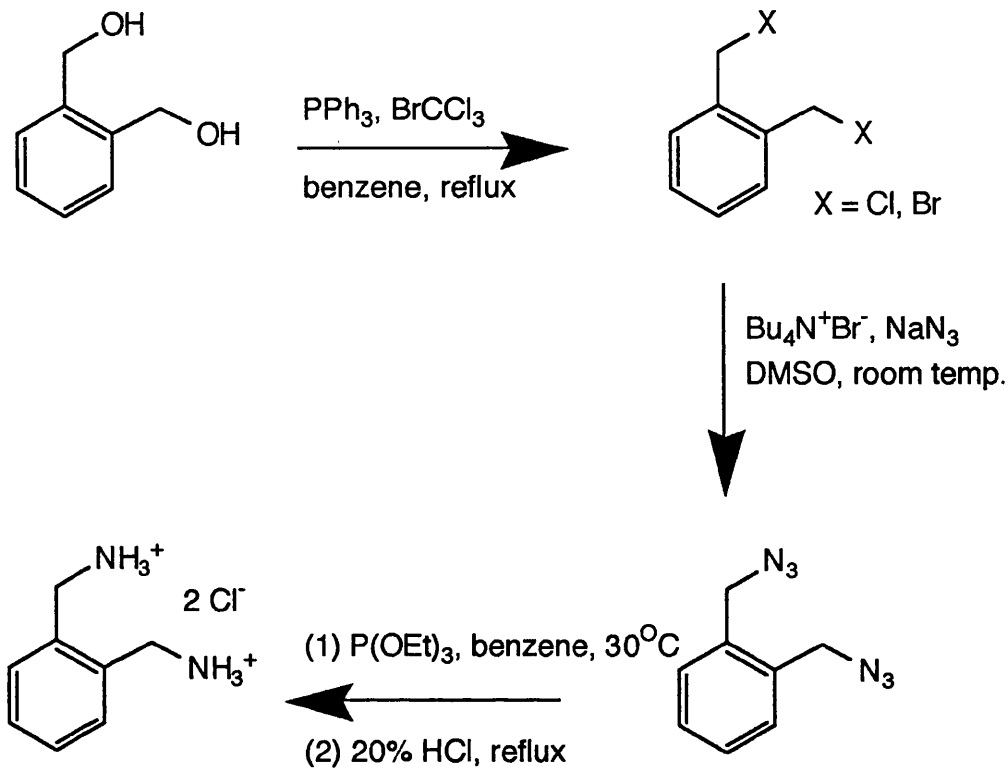
Scheme 4.2

Treatment of 1,2-bis(hydroxymethyl)benzene with methanesulphonyl chloride and triethylamine failed to produce the desired dimethanesulphonate and instead resulted in the formation of a brown tar which proved to be insoluble in all common organic solvents. Although the identity of this substance could not be

established, it is likely that it arises after initial intramolecular cyclisation of the monomesylate (Scheme 4.3).

Scheme 4.3

An alternative method was therefore used for the synthesis of 1,2-xylylenediamine dihydrochloride (71). The method of Koziara[107] for the conversion of primary alcohols into amines was adapted for use with diols (Scheme 4.4). Heating a mixture of the diol, triphenylphosphine and bromotrichloromethane at reflux produced the corresponding dihalide which was converted *in situ* into the diazide by treatment with sodium azide. Stirring the diazide in a solution of triethylphosphite in benzene produced the di-iminophosphorane which on hydrolysis with hydrochloric acid gave the dihydrochloride salt of the diamine. Although this method produced an excellent yield of 1,4-xylylenediamine dihydrochloride (69) from the corresponding diol, 1,2-xylylenediamine dihydrochloride (71) was only formed in approximately 10% yield. Despite this low yield, the method allowed the synthesis of enough material to carry out kinetic studies.



(71)

Scheme 4.4

4.5(c) Results and Discussion

Using the peroxidase-coupled spectrophotometric assay system, kinetic parameters for 1,4- and 1,3-xylylenediamine dihydrochlorides (69) and (70) with pea seedling diamine oxidase were obtained. 1,2-Xylylenediamine dihydrochloride (71) was found to be an extremely poor substrate for this enzyme. As such it did not display classical Michaelis-Menten behaviour, therefore accurate determinations of V_{max} and K_M were not possible. An estimation of the V_{max} for this substrate was found. The data are presented in

Table 4.1. Error values are reported as standard deviations over nine determinations.

Substrate	K_M	V_{max}
Putrescine	1.18 (±0.37)	1170 (±220)
Cadaverine	0.24 (±0.07)	2680 (±410)
1,4-Xylylenediamine (69)	0.06 (±0.02)	199 (±24)
1,3-Xylylenediamine (70)	0.03 (±0.01)	35 (±4)
1,2-Xylylenediamine (71)	———	1.2

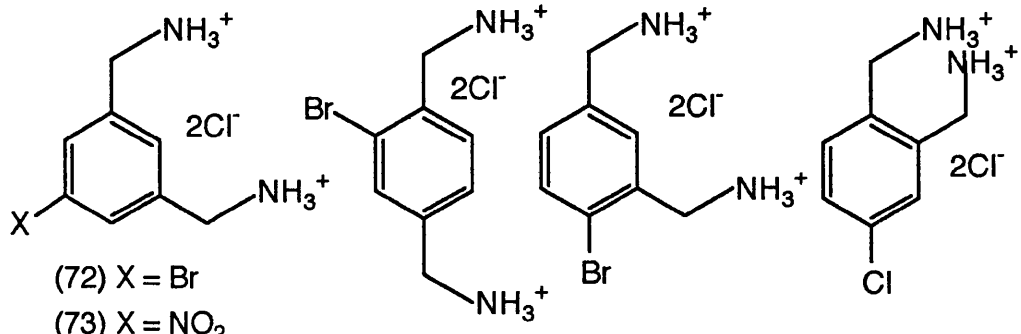
Table 4.1- All diamines were tested in the form of their more stable dihydrochloride salts to which the formulae numbers refer. K_M values are in units of mM and V_{max} values are in units of μmolmg^{-1}h^{-1}.

It is clear that all three compounds tested are oxidised significantly more slowly than the natural substrates, putrescine and cadaverine. However on consideration of the K_M values, it would appear that in the case of 1,4- and 1,3-xylylenediamine dihydrochlorides (69) and (70) at least, the aromatic substrates have a greater binding affinity for pea seedling DAO than either of the natural substrates. Within the series it was found that 1,4-xylylenediamine dihydrochloride (69) was oxidised approximately five times faster than the 1,3-isomer (70) which in turn was oxidised thirty times faster than 1,2-xylylenediamine dihydrochloride (71). As the distance between the amine groups decreases the rate of enzyme catalysed oxidation decreases. Both 1,4- and 1,3-xylylenediamine bind very strongly to the enzyme with the 1,3-isomer showing the greater affinity.

4.6 Catalytic Oxidation of Substituted Xylylenediamines by Pea Seedling DAO

4.6(a) Introduction

It has been reported that the incorporation of a substituent into the backbone of the natural substrates often leads to a dramatic effect on both the binding affinity and the rate of oxidation of these substrates with pea seedling diamine oxidase.[19,21,93] Having shown that the xylylenediamines were substrates for the enzyme we decided to examine the effects of placing a substituent onto the aromatic ring of these compounds. This was achieved by synthesising a range of compounds possessing substituents which would exert different steric and electronic effects on the system. Synthesis of 5-bromo-1,3-xylylenediamine (72), 5-nitro-1,3-xylylenediamine (73) and 5-hydroxy-1,3-xylylenediamine (74) (in the form of the dihydrochloride salts) would allow a direct comparison of substituent effects to be made. Consideration of 2-bromo-1,4-xylylenediamine (75), 4-bromo-1,3-xylylenediamine (76) and 4-chloro-1,2-xylylenediamine (77) would allow substituent effects to be examined within each form of the isomeric xylylenediamines.

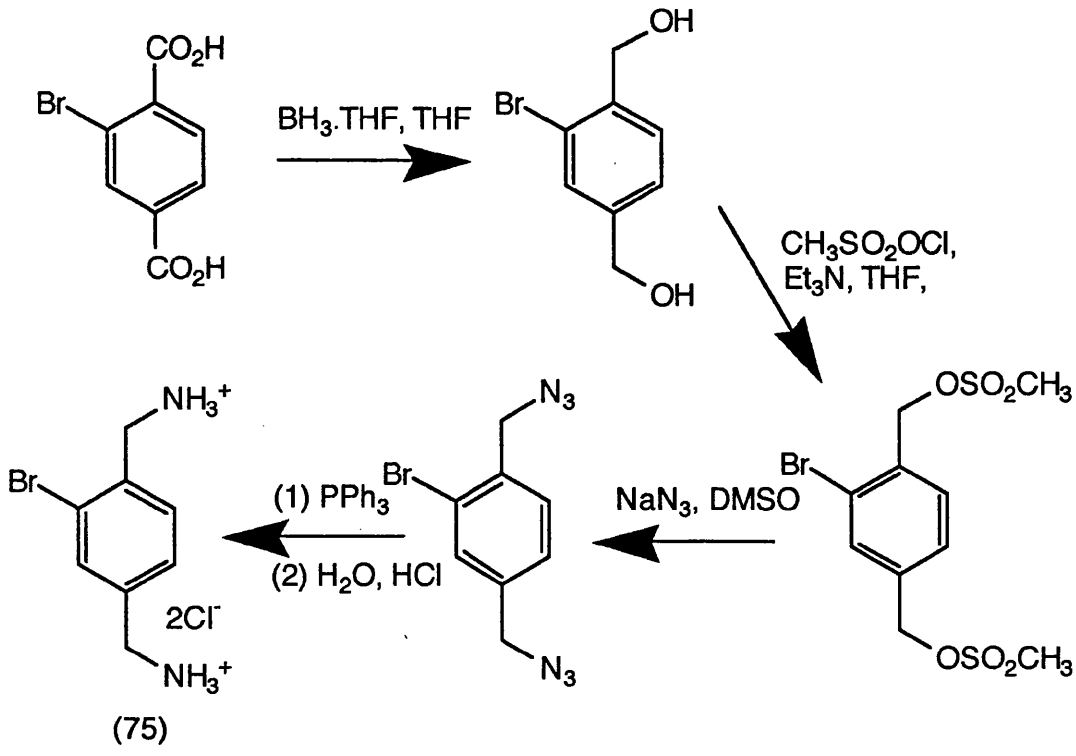


(72) X = Br
(73) X = NO_2
(74) X = OH

(75) (76) (77)

4.6(b) Synthesis of Substituted Xylylenediamines

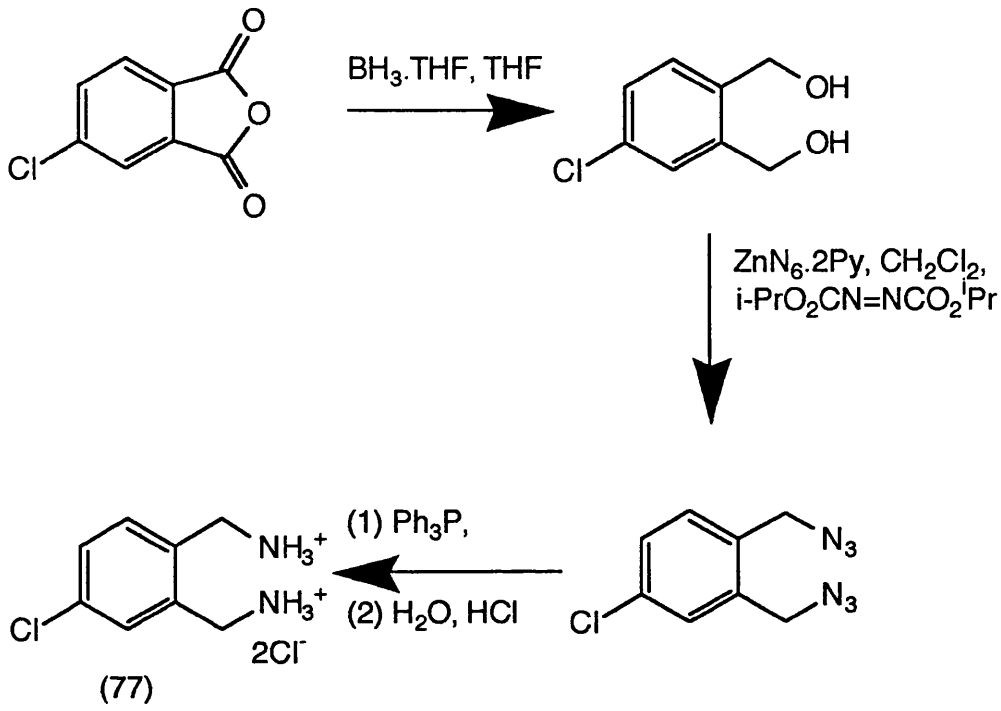
The substituted xylylenediamines were synthesised from the corresponding diacids. 2-Bromo-1,4-xylylenediamine dihydrochloride (75) was obtained from 2-bromoterephthalic acid. Reduction of this compound by borane-tetrahydrofuran (THF) complex in THF gave a diol which was converted into the dimethanesulphonate using methanesulphonyl chloride and triethylamine. Azide formation, followed by reaction with triphenylphosphine[108] produced the di-iminophosphorane which was hydrolysed using aqueous acid (Scheme 4.5). The diamine dihydrochloride (75) obtained *via* hydrolysis of the di-iminophosphorane was found to be more pure and of higher yield than that obtained from catalytic hydrogenation of the corresponding diazide.



(75)

Scheme 4.5

4-Bromoisophthalic acid, 5-bromoisophthalic acid and 4-chloroisophthalic acid were all converted directly into the corresponding diols by treatment with borane-THF. Under identical conditions 5-bromo- and 5-nitroisophthalic acid failed to give the desired diols. Synthesis of these diols was achieved by a two step process. The diacids were first converted into the diesters by heating at reflux in an acidic solution of methanol. Reduction of these dimethyl esters using di-isobutylaluminium hydride produced the diols in good overall yield. According to the previously described method of Koziara,[107] 4-bromo-1,3-xylylenediamine (76) , 5-bromo-1,3-xylylenediamine (72), 5-nitro-1,3-xylylenediamine (73) and 5-hydroxy-1,3-xylylenediamine (74) were then synthesised, as the hydrochloride salts, from the corresponding diols. Treatment of 4-chloro-1,2-bis(hydroxymethyl)benzene using the method of Koziara,[107] failed to produce the substituted xylylenediamine. An alternative route, avoiding a dimethanesulphonate intermediate (Scheme 4.3), was found. This route allowed direct coversion of the diol into the diazide by treatment with diisopropylazodicarboxylate and a zinc azide/bis pyridine complex.[109] The resultant diazide was purified by flash column chromatography on silica gel. Treatment of the diazide with triphenylphosphine followed by aqueous hydrolysis afforded the desired compound, 4-chloro-1,2-xylylenediamine dihydrochloride (77) (Scheme 4.6).



Scheme 4.6

4.6(c) Results and Discussion

Using the spectrophotometric assay system, kinetic parameters were obtained for all substrates except 4-chloro-1,2-xylylenediamine dihydrochloride (77) which was found to be a poor substrate for the enzyme. The data are reported in Table 4.2.

The remarkable similarities between the V_{max} and K_M values obtained for the 5-substituted-1,3-xylylenediamines and those of the unsubstituted compound (Table 4.1), suggest that the substrate is not sensitive to substitution at C-5. Neither activation of the aromatic ring by the incorporation of a hydroxy substituent nor deactivation of the ring by the introduction of a nitro substituent appear to have a significant effect on the rate of enzyme catalysed oxidation.

Substrate	K_M	V_{max}
Putrescine	1.18 (±0.37)	1170 (±220)
Cadaverine	0.24 (±0.07)	2680 (+410)
5-Bromo-1,3-xylylenediamine (72)	0.03 (+0.01)	39 (+6)
5-Nitro-1,3-xylylenediamine (73)	0.05 (+0.02)	29 (+6)
5-Hydroxy-1,3-xylylenediamine (74)	0.02 (+0.01)	31 (+4)
2-Bromo-1,4-xylylenediamine (75)	0.04 (+0.01)	473 (+50)
4-Bromo-1,3-xylylenediamine (76)	0.40 (+0.05)	11 (+1)
4-Chloro-1,2-xylylenediamine (77)	———	2.4

Table 4.2- All diamines were tested in the form of their more stable dihydrochloride salts to which the formulae numbers refer. K_M values are in units of mM and V_{max} values are in units of $\mu molmg^{-1}h^{-1}$.

In contrast to the kinetic parameters obtained for 5-bromo-1,3-xylylenediamine dihydrochloride (72), 4-bromo-1,3-xylylenediamine dihydrochloride (76) is a poorer substrate for the enzyme than 1,3-xylylenediamine dihydrochloride (70). The binding affinity also seems to be reduced by the incorporation of a bromo substituent onto C-4 of the system.

An interesting feature of the results is the fact that 2-bromo-1,4-xylylenediamine dihydrochloride (75) is both a better substrate and binder for pea seedling DAO than 1,4-xylylenediamine dihydrochloride (69). A rational explanation for this observation is difficult to obtain, since the steric effect of this bulky substituent might have been expected, if anything, to make binding more difficult. Also, if the electronic nature of the substituent was responsible for the increased rate of catalytic oxidation, then similar

trends in the data associated with the other bromo-substituted compounds might have been expected.

Like 1,2-xylylenediamine dihydrochloride (71), 4-chloro-1,2-xylylenediamine dihydrochloride (77) was found to be a poor substrate for pea seedling DAO. It would appear that for these aromatic systems, a distance of four carbon atoms between the amine groups does not favour efficient oxidation. This is in complete contrast to linear diamines since putrescine is one of the best substrates. The large discrepency between the V_{max} values found for these compounds and that of putrescine may be due to the inhibition of conformational changes in the catalytic step of the enzyme by the aromatic ring.

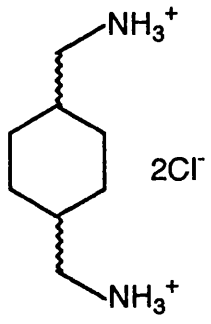
The presence of bulky substituents in compounds shown to be good binders to the enzyme suggests that the active site of pea seedling DAO is relatively uncrowded. Also, since the compounds examined are generally poorer substrates but better binders than the natural substrates, it would appear that substrate selectivity in the enzyme exists in the catalytic mechanism rather than the binding affinity.

4.7 Catalytic Oxidation of bis(Aminomethyl)cyclohexanes by pea seedling DAO

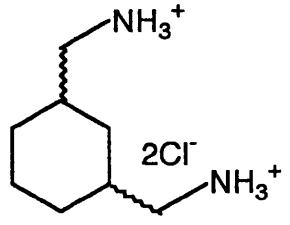
4.7(a) Introduction

Having shown that some simple aromatic diamines were substrates for pea seedling DAO we were interested in what effect replacement

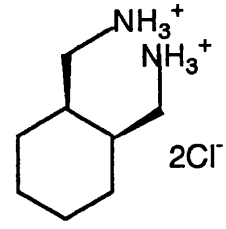
of the aromatic ring by a saturated six-membered ring might have on the rate of oxidation and the binding affinity of the substrate. This effect could be examined by the synthesis of 1,4-bis(aminomethyl)cyclohexane (78), 1,3-bis(aminomethyl)cyclohexane (79) and 1,2-bis(aminomethyl)cyclohexane (80) (all isolated as the dihydrochloride salts). Comparison of the kinetic parameters obtained for these compounds with those found for the xylylenediamines (Table 4.1) may provide an insight into whether the aromatic ring promotes or hinders efficient oxidation.



(78)



(79)



(80)

4.7(b) Synthesis of bis(Aminomethyl)cyclohexanes

The bis(aminomethyl)cyclohexanes were synthesised from the corresponding diols. Both 1,4-bis(hydroxymethyl)cyclohexane and 1,3-bis(hydroxymethyl)cyclohexane were commercially available as a mixture of diastereoisomers. 1,2-bis(Hydroxymethyl)cyclohexane was obtained exclusively in the *cis* form *via* reduction of the corresponding *cis*-anhydride with borane-THF. Conversion of the diols into the corresponding dimethanesulphonates followed by azide

displacement gave the diazides which were reduced using triphenylphosphine and hydrolysed with aqueous acid. The dihydrochloride salts of the *cis/trans* mixture of 1,4-bis(aminomethyl)cyclohexane (78), the *cis/trans* mixture of 1,3-bis(aminomethyl)cyclohexane (79) and *cis*- 1,2-bis(aminomethyl)cyclohexane (80) were all obtained in high yield.

4.7(c) Results and Discussion

The kinetic parameters, obtained using the spectrophotometric assay system, are reported in Table 4.3. Again, due to the low activity associated with the 1,2-isomer (80), full kinetic data could not be obtained for this compound.

Substrate	K_M	V_{max}
Putrescine	1.18 (±0.37)	1170 (±220)
Cadaverine	0.24 (±0.07)	2680 (±410)
1,4-bis(Aminomethyl)cyclohexane (78)	0.05 (±0.01)	7.7 (±2.4)
1,3-bis(Aminomethyl)cyclohexane (79)	0.22 (±0.07)	68 (±12)
cis-1,2-bis(Aminomethyl)cyclohexane (80)	———	0.2

Table 4.3- All diamines were tested in the form of their more stable dihydrochloride salts to which the formulae numbers refer. K_M values are in units of mM and V_{max} values are in units of $\mu molmg^{-1}h^{-1}$. 1,4-bis(Aminomethyl)cyclohexane dihydrochloride (78) and 1,3-bis(aminomethyl)cyclohexane dihydrochloride (79) were tested as a mixture of diastereoisomers.

Although the binding affinity of the substrates was the same, the *cis/trans* mixture of 1,4-bis(aminomethyl)cyclohexane dihydrochloride (78) was found to be a poorer substrate for pea seedling DAO than 1,4-xylylenediamine dihydrochloride (69) (Table 4.1). In this case it would appear that the presence of the aromatic ring promotes the rate of oxidation. In contrast to this, the *cis/trans* mixture of 1,3-bis(aminomethyl)cyclohexane dihydrochloride (79) was oxidised at twice the rate of 1,3-xylylenediamine dihydrochloride (70). The K_M value for this substrate mixture was found to be almost identical to that for the natural substrate cadaverine. This may not be all that surprising, since 1,3-bis(aminomethyl)cyclohexane dihydrochloride (79) may be thought of as a cadaverine analogue (i.e. a substituted C-5 diamine). The similar binding affinity but significantly different rate of oxidation found for 1,3-bis(aminomethyl)cyclohexane dihydrochloride (79) compared to cadaverine would again suggest that substrate selectivity in the enzyme is not determined by binding affinity. Like 1,2-xylylenediamine dihydrochloride (71), *cis*-1,2-bis(aminomethyl)cyclohexane dihydrochloride (80) was shown to be a poor substrate for the enzyme.

4.8 Regioselectivity in the Catalytic Deamination of 2-bromo-1,4-xylylenediamine by Pea Seedling DAO

With a view to examining possible regioselectivity in the pea seedling DAO catalysed oxidation of diamines, the enzymatic deamination of 2-bromo-1,4-xylylenediamine dihydrochloride (75) was considered in more detail. It was felt that this compound would

be a suitable model for our studies since: (1) the presence of the bromo substituent serves to distinguish between the two possible sites for deamination; (2) the relatively high rate of oxidation associated with this substrate should favour efficient conversion into an initial aminoaldehyde and (3) the steric constraints imposed on the aminoaldehyde product(s) should prevent formation of a cyclic imine, thus avoiding the problems associated with isolation of such compounds.

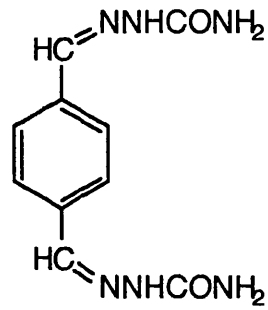
The dihydrochloride salt (75) of 2-bromo-1,4-xylylenediamine was dissolved in phosphate buffer (pH 6.3) and shaken with pea seedling DAO (the theoretical amount required to oxidise the substrate in one hour). A small amount of catalase was added to prevent the build up of hydrogen peroxide, which is thought to have an inhibitory effect on the enzyme. The reaction was allowed to proceed at room temperature and was monitored by TLC. After a relatively short period of time (twenty minutes), TLC evidence indicated the presence of the starting diamine dihydrochloride and two further compounds. After longer periods of time (two hours) it was clear that a third product was forming which was less polar in nature than the other products. In an attempt to react all of the starting material, a further batch of enzyme was added and the reaction vessel was shaken overnight. After this period TLC showed a faint spot corresponding to starting material and the same products but as three more intense spots. The solution was made basic and extracted with a range of organic solvents. No product could be isolated in this manner. The reaction was repeated on a larger scale, giving rise to the same products t.l.c. data. Again these compounds showed a high affinity for the aqueous phase and could not be extracted into organic solvents.

Having consumed our initial supplies of 2-bromo-1,4-xylylenediamine dihydrochloride (75), our attention turned to the oxidation of 1,4-xylylenediamine dihydrochloride (69). Although this is not a suitable substrate for the examination of regioselectivity in the process, it was hoped that, by optimising the conditions for the enzymatic oxidation and subsequent product isolation regarding this species, a method for the isolation of products from the oxidation of 2-bromo-1,4-xylylenediamine could be found.

The dihydrochloride salt (69) of 1,4-xylylenediamine was enzymatically oxidised as before. By monitoring the reaction by TLC it was clear to see that initially the oxidation of the substrate resulted in the formation of a single product, presumably the aminoaldehyde (82). At longer reaction times, however, it was found that a second product was formed. The addition of more enzyme failed to bring about complete consumption of starting material. Extraction of the aqueous phase using a variety of organic solvents failed to produce significant quantities of the oxidation products. In an attempt to ease isolation it was therefore decided to derivatise the aldehyde functionality expected in the products. Semicarbazone derivatives generally exhibit low solubilities in water and it was hoped that derivatisation in this manner would produce a precipitate which could be collected by filtration.

A solution of semicarbazide hydrochloride and sodium acetate in water was added to the buffered solution of enzyme and substrate (69). The mixture was shaken and allowed to stand at room temperature. After a short period of time a white solid began to crystallise from the solution. The solid was collected and dried. Although the compound was insoluble in most organic solvents, it was found to be partially soluble in DMSO. This allowed a structure

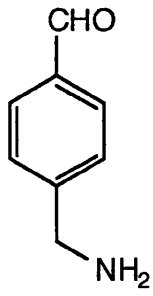
elucidation by NMR spectroscopy to be undertaken. The ^{13}C NMR spectrum of the compound showed only four carbon signals, two of which were due to quaternary carbons. The ^{1}H NMR spectrum indicated three environments of hydrogen at δ 6.54, 7.78-7.92 and 10.31 with relative intensities 1:3:2. On shaking with D_2O the broad singlets at δ 6.54 and δ 10.31 disappeared. On the basis of the above spectral charateristics the solid was assigned the structure of the disemicarbazone (81). Mass spectral data were consistent with the proposed structure.



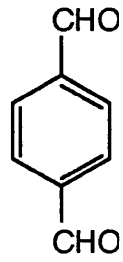
(81)

The ^{13}C NMR spectrum of the solid showed no sign of the mono-semicarbazone derivative. It is conceivable that the mono-semicarbazone derivative is water soluble and remains in the buffer after derivatisation. The catalytic deamination of 1,4-xylylenediamine by pea seedling DAO would appear to yield initially the aminoaldehyde (82). However this oxidation product itself appears to be a substrate for the enzyme and is oxidised to the corresponding dialdehyde (83). The appearance of a second compound, only after a relatively long period of time (approximately one hour), would suggest that the oxidation of the aminoaldehyde proceeds at a slower rate than the oxidation of the diamine. In this situation the aminoaldehyde (82) could competitively inhibit the

enzymatic deamination of 1,4-xylylenediamine dihydrochloride (69). This would explain the surprisingly long period of time observed for the complete consumption of starting material.

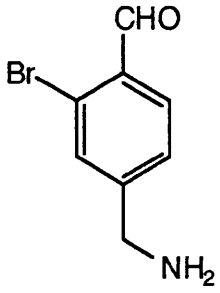


(82)

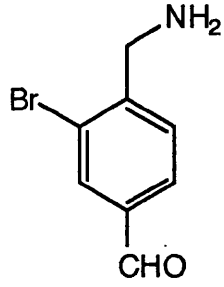


(83)

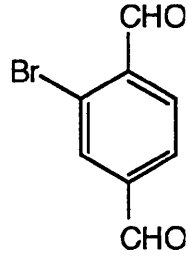
Time constraints prevented reinvestigation of the pea seedling DAO catalysed oxidation of 2-bromo-1,4-xylylenediamine dihydrochloride (75). The TLC evidence is, however, consitent with the above findings. The presence of two spots at short reaction time suggests that the oxidation of the diamine substrate takes place in a non-regiospecific manner, giving rise to two aldehyde products (84) and (85). On long standing a third product was produced. This may be the dialdehyde (86) which would form as a result of the catalytic deamination of the aminoaldehyde intermediates.



(84)



(85)



(86)

Further work is required in order to gain a more complete understanding into the nature of the DAO catalysed oxidation of 2-bromo-1,4-xylylenediamine dihydrochloride (75).

CHAPTER 5

PUTRESCINE DERIVATIVES AS SUBSTRATES FOR PEA SEEDLING DIAMINE OXIDASE

5.1 Introduction

A considerable amount of work has been undertaken with a view to investigating the effectiveness of putrescine analogues as substrates for pea seedling DAO. In particular the effect of placing a single substituent on the backbone of one of the natural substrates has been given much attention.[19,21,24] Much information has been gained on the active site of the enzyme by examining how structural deviations from that of the natural substrates effect the binding and rate of oxidation. However, to date, no work has been reported which examines haloputrescines as potential substrates for the enzyme.

Synthesis of a range of 2-haloputrescines would enable such a study to be undertaken as well as providing compounds which may be suitable for further examination of regioselectivity in the catalytic deamination. By synthesising homochiral material (Scheme 5.1) it may also be possible to investigate stereoselectivity in the process.

X = Cl
X = Br
X = F

Scheme 5.1

5.2 Attempted Synthesis of Homochiral 2-Haloputrescines

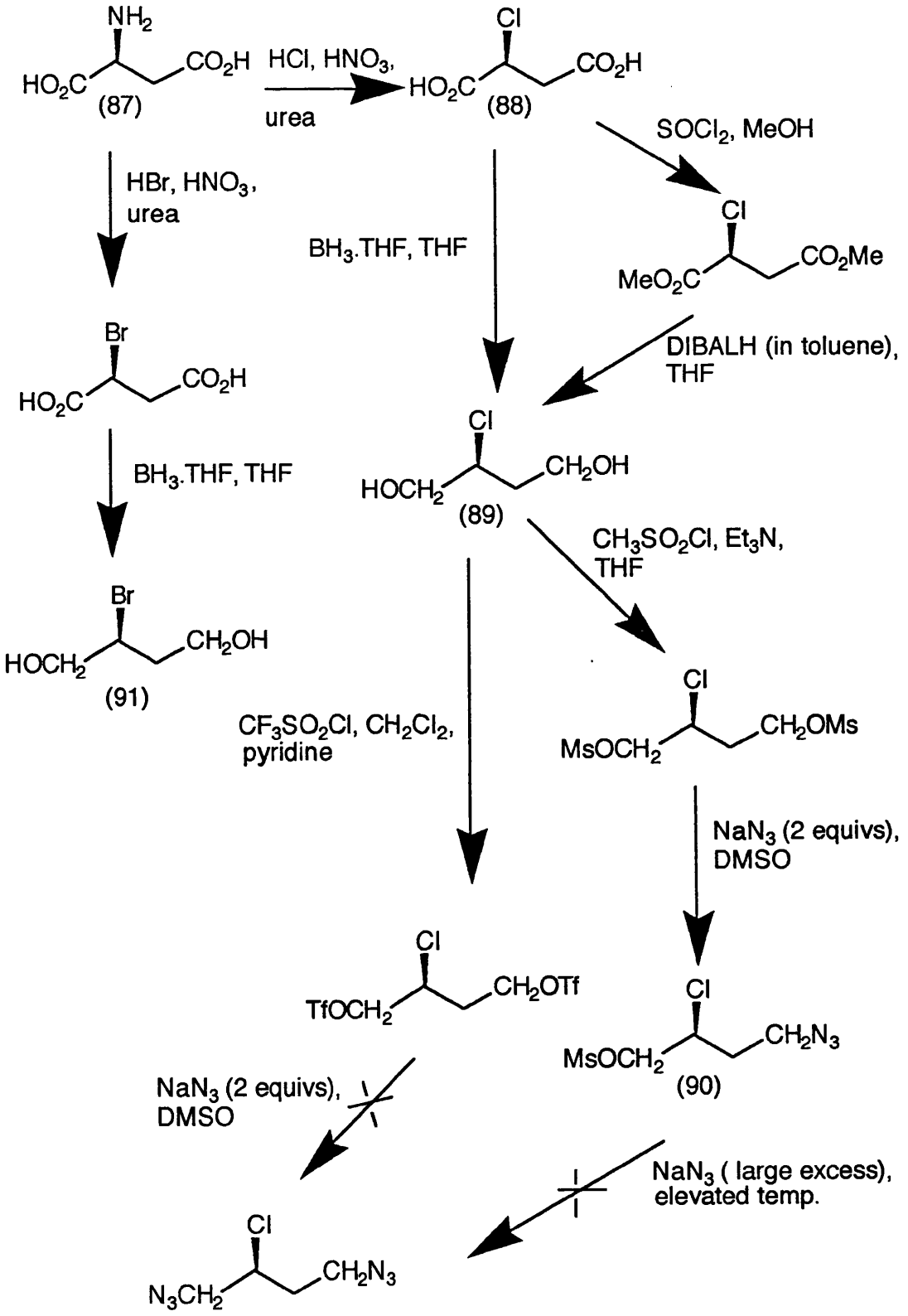
The starting materials for the proposed synthetic route (Scheme 5.2) to the enantiomeric 2-chlorobutane-1,4-diamines were the readilly available (2*R*)- and (2*S*)-aspartic acids. Treatment of (2*S*)-aspartic acid (87) with a mixture of 10M hydrochloric acid and concentrated nitric acid in the presence of urea led to replacement of the amino group by chlorine with retention of configuration.[110] Treatment of the resultant (2*S*)-chlorosuccinic acid (88) with borane-tetrahydrofuran complex gave (2*S*)-chlorobutane-1,4-diol (89) directly. The yield for the reduction after chromatographic purification, however, was only 55% and it was discovered that a higher overall yield of diol could be obtained by treating the diacid (88) with methanol and thionyl chloride and reducing the resultant diester with di-isobutylaluminium hydride. Having obtained the pure diol, several pathways seemed possible for further conversion into the desired diamine. The previously described methods involving the use of triphenylphosphine[107,108] could not be employed since it was known that under typical reaction conditions triphenylphosphine was capable of displacing chlorine from such molecules.[111] Instead it was decided to attempt the desired conversion *via* the dimesylate and diazide intermediates.

Although azide ions are generally capable of displacing primary halides, conditions for the displacement of secondary halides are required to be significantly more harsh.

Treatment of the diol (89) with methanesulphonyl chloride and triethylamine gave a clear oil which was characterised as the desired dimesylate. Despite the apparent purity of this intermediate (one spot TLC) all attempts to crystallise the oil met with failure. The

crude dimesylate was treated with two equivalents of sodium azide and stirred overnight at room temperature. On isolation, examination of the ^1H NMR spectrum of the product revealed that only one singlet (δ 3.1) corresponding to the methyl of a mesylate group remained and that a broad triplet (δ 3.6) indicative of a methylene adjacent to an azide group was now evident. The mono-azide (90) had been formed by attack at the less hindered mesylate. In an attempt to effect displacement of the second mesylate group the reaction was attempted again using a large excess of sodium azide and longer reaction times. No diazide was observed under these conditions. Azide displacement was attempted at elevated temperatures (50 ^oC). After a relatively short period of time it was clear by the absence of any proton resonance at δ 3.1 that the hindered mesylate had been displaced. However the characteristic multiplet associated with the CHCl proton was no longer in evidence indicating that under these harsher conditions azide displacement of the secondary chloride had taken place. No intermediate temperature could be found which allowed selective displacement of the mesylate.

A second approach to the target compound was attempted which involved treatment of (2*S*)-chlorobutane-1,4-diol (89) with trifluoromethanesulphonic (triflic) anhydride and pyridine. It was hoped that the increased reactivity associated with the triflate leaving group would promote formation of the desired diazide. A thick black oil was obtained which contained the ditriflate as the major product. Attempts to purify the oil by distillation led to decomposition of the material. Treatment of the crude ditriflate with sodium azide failed to produce significant quantities of the diazide.

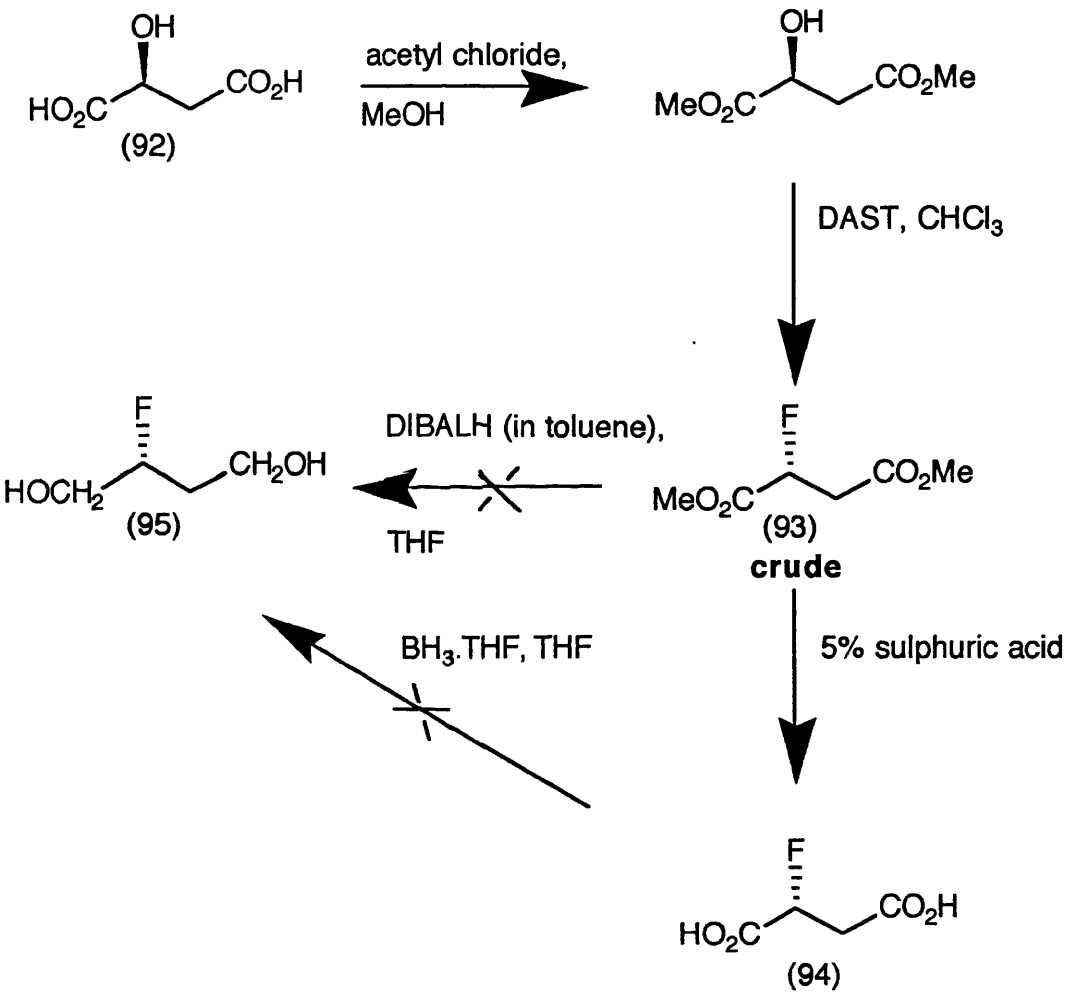

NH2
HO2C
CO2H
(87)
HCl, HNO3,
urea
Cl
HO2C
CO2H
(88)
SOCl2, MeOH
HBr, HNO3,
urea
BH3.THF, THF
Cl
MeO2C
CO2Me
DIBALH (in toluene),
THF
Br
HO2C
CO2H
BH3.THF, THF
Cl
HOCH2
CH2OH
(89)
CH3SO2Cl, Et3N,
THF
Br
HOCH2
CH2OH
(91)
CF3SO2Cl, CH2Cl2,
pyridine
Cl
MsOCH2
CH2OMs
NaN3 (2 equivs),
DMSO
Cl
TfOCH2
CH2OTf
Cl
MsOCH2
CH2N3
(90)
NaN3 (2 equivs),
DMSO
NaN3 (large excess),
elevated temp.
Cl
N3CH2
CH2N3

Scheme 5.2

Although (2*S*)-bromobutane-1,4-diol (91) was produced in an analogous fashion to (2*S*)-chlorobutane-1,4-diol (89) it was felt that the problems experienced with the chloro substituent would only be exaggerated with the increased steric bulk of the bromo substituent and the fact that bromide is a better leaving group. The attempted synthesis of these compounds was therefore discontinued.

A more direct route was sought to the enantiomeric 2-fluorobutane-1,4-diamines. It was shown by Brown[112] that 3-fluoropentane-1,5-diol could be converted through treatment with hydrazoic acid, di-isopropylazodicarboxylate and triphenylphosphine into the corresponding diamine. It was thought that an optically active 2-fluorobutane-1,4-diol could be converted into 2-fluorobutan-1,4-diamine by a similar procedure. The starting points for the attempted synthesis of the optically active 2-fluorobutane-1,4-diols were the readily available 2-hydroxysuccinic (malic) acids (Scheme 5.3). Protection of (2*S*)-hydroxysuccinic acid (92) as the corresponding diester was achieved using acetyl chloride and methanol. Treatment of this intermediate with diethylaminosulphur trifluoride (DAST) in ethanol-free chloroform gave dimethyl (2*R*)-fluorobutane-1,4-dioate (93) with inversion of configuration.[113] Attempted purification of this compound by column chromatography on silica gel resulted in racemisation. The crude fluorodiester was treated with di-isobutylaluminium hydride (DIBALH) in dichloromethane giving rise to a darkish oil which by TLC was shown to contain multiple products. No separation was attempted. Instead it was decided to hydrolyse the diester to the corresponding diacid, known to be a solid,[113] which could then be purified by recrystallisation. This was achieved by heating a solution of the diester in 5% sulphuric acid at reflux for 25h. Treatment of (2*R*)-

fluorosuccinic acid (94) with borane-THF complex failed to produce the diol as a major product. Other reducing agents were considered to bring about the conversion of dimethyl (2*R*)-fluorobutan-1,4-dioate (93) into (2*R*)-fluorobutane-1,4-diol (95) but with no success. Identical problems were encountered during the attempted synthesis of (2*S*)-fluorobutane-1,4-diol. Work on the synthesis of chiral 2-haloputrescines was terminated at this point.



Scheme 5.3

5.3 2,3-Dihaloputrescines as Substrates for Pea Seedling DAO

5.3(a) Introduction

Having failed in our attempt to produce optically active 2-haloputrescines our attention turned to the synthetically more viable 2,3-dihaloputrescines. Although these compounds are not suitable models for the examination of enzymatic selectivity, they may still provide useful kinetic data.

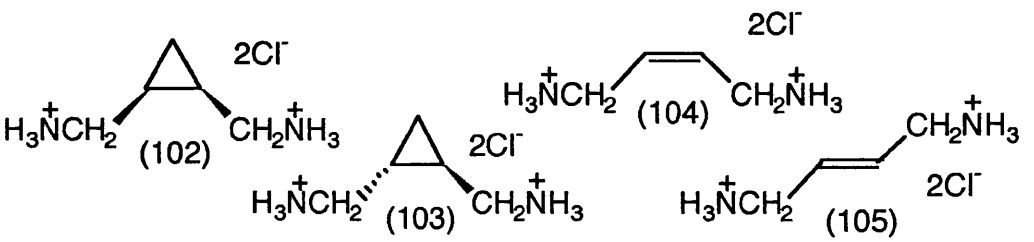
5.3(b) Synthesis of 2,3-Dihaloputrescines

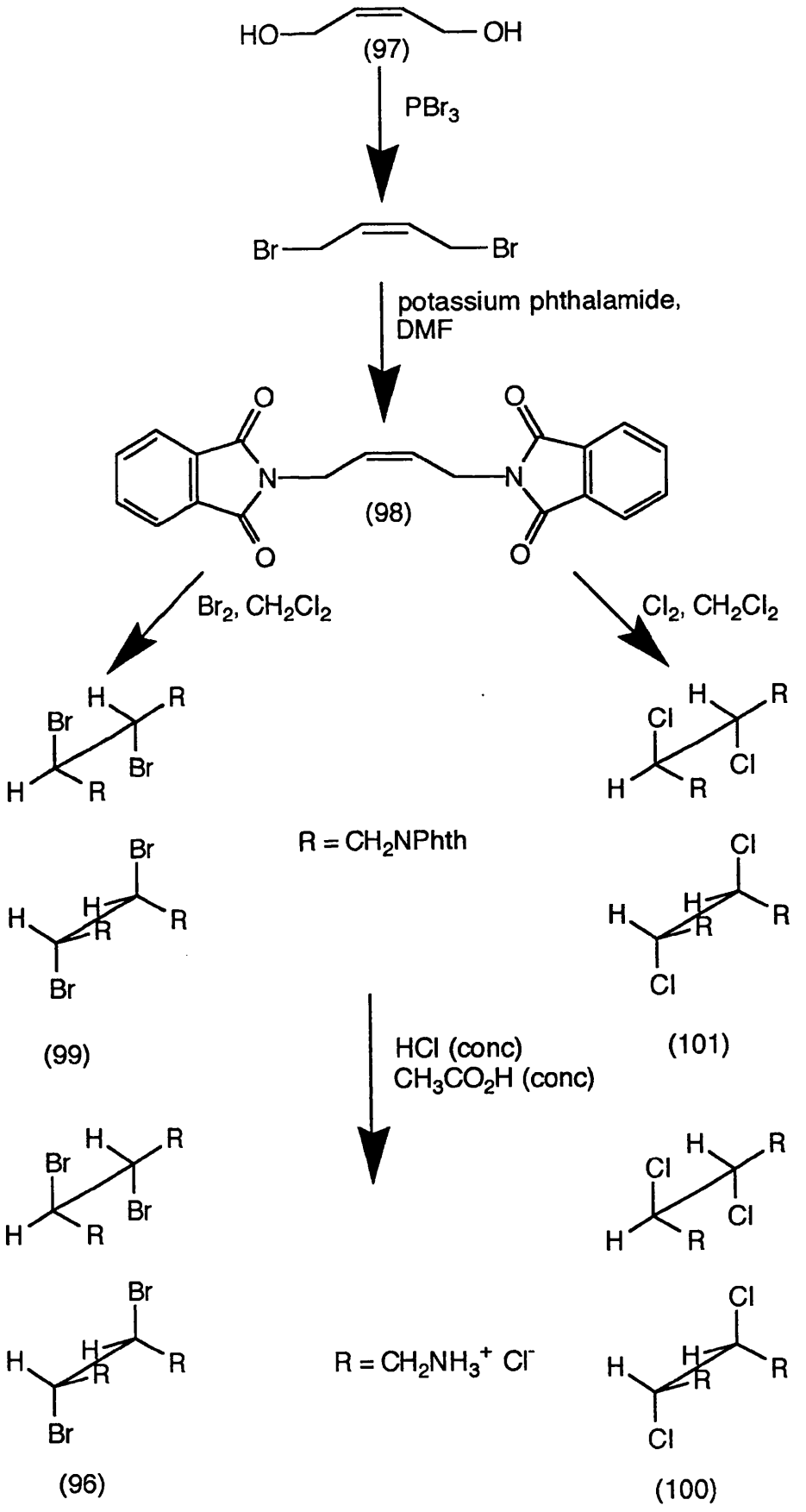
(±)-2,3-Dibromoputrescine dihydrochloride (96) was synthesised from *cis*-1,4-but-2-enediol (97). Treatment of the diol with phosphorus tribromide gave the corresponding dibromide which was converted into *cis*-1,4-diphthalimidobut-2-ene (98) using an excess of potassium phthalimide. Refluxing a solution of this diphthalimide with bromine gave (±)-2,3-dibromo-1,4-diphthalimidobutane (99), *via trans* addition of bromine, as a white crystalline solid. Cleavage of the phthalimide groups was best achieved by heating the intermediate (99) at reflux in a mixture of concentrated acetic and hydrochloric acids (Scheme 5.4). (±)-2,3-Dibromoputrescine was obtained in the form of the more stable dihydrochloride salt (96) with no appreciable amount of decomposition products present. *meso*-2,3-Dibromoputrescine dihydrochloride was synthesised in an analogous fashion. Treatment of *trans*-1,4-diphthalimidobut-2-ene, obtained from *trans*-1,4-dibromobut-2-ene, with bromine under reflux conditions yielded *meso*-2,3-dibromo-1,4-

diphthalimidobutane which was cleaved as before to yield the desired diamine dihydrochloride.

(±)-2,3-Dichloroputrescine dihydrochloride (100) could be formed in two steps from *cis*-1,4-diphthalimidobut-2-ene (98). Bubbling chlorine gas through a solution of the unsaturated diphthalimide in dichloromethane led to the formation of (±)-2,3-dichloro-1,4-diphthalimidobutane (101) which when hydrolysed in a mixture of conentrated acetic and hydrochloric acids gave the dihydrochloride (100) as a brown solid. Similarly *meso*-2,3-dichloroputrescine dihydrochloride was obtained from *trans*-1,4-diphthalimidobut-2-ene. The dichloroputrescine salts were less pure than the corresponding dibromoputrescine salts. This was believed to be a consequence of the harsher reaction conditions required to bring about successful cleavage of these diphthalimides encouraging some product decomposition. Repeated recrystallisations from ethanol/acetone, although improving the quality, failed to remove all of the impurities.

Despite the lack of purity associated with the 2,3-dichloroputrescine salts, kinetic studies were carried out on all of the 2,3-dihaloputrescine salts. Also tested were both *cis*-(102) and *trans*-bis(aminomethyl)cyclopropane (103), prepared as dihydrochlorides for our use by Dr M. Rodgers,[114] and *cis*-(104) and *trans*-but-2-ene-1,4-diamine dihydrochlorides (105) synthesised by W. Martin.




HO
OH
(97)
PBr_3
Br
Br
potassium phthalamide,
DMF
O
O
N
N
O
O
(98)
Br_2, CH_2Cl_2
Cl_2, CH_2Cl_2
R = CH_2NPhth
(99)
(101)
HCl (conc)
CH_3CO_2H (conc)
R = $CH_2NH_3^+$ Cl^-
(96)
(100)

Scheme 5.4

5.3(c) Results and Discussion

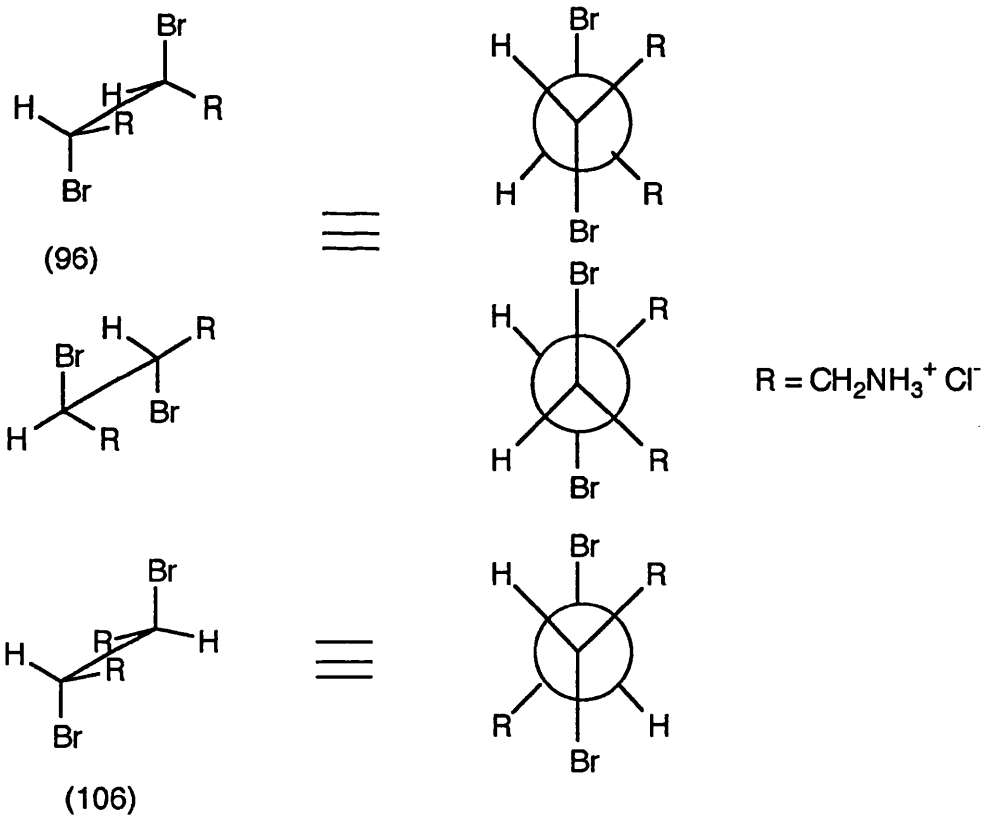
Using the spectrophotometric assay system, kinetic parameters were obtained for all compounds with the exception of the 2,3-dichloroputrescines. These compounds were found to be extremely poor substrates for pea seedling DAO and this, coupled with the fact that impure samples were tested, prevented reproducible kinetic data being obtained. The data are presented in Table 5.1.

Substrate	K_M	V_{max}
Putrescine	1.18 (±0.37)	1170 (±220)
Cadaverine	0.24 (±0.07)	2680 (±410)
meso-2,3-dibromoputrescine (106)	0.02 (±0.01)	1.1 (±0.1)
(+)-2,3-dibromoputrescine (96)	0.16 (±0.06)	10 (±1)
meso-2,3-dichloroputrescine (107)	———	———
(±)-2,3-dichloroputrescine (100)	———	———
cis-bis(aminomethyl)cyclopropane (102)	0.16 (±0.04)	146(±30)
trans-bis(aminomethyl)cyclopropane (103)	0.47 (±0.12)	202 (±17)
cis-but-2-ene-1,4-diamine (104)	0.18 (±0.08)	482 (±83)
trans-but-2-ene-1,4-diamine (105)	0.05 (±0.02)	646 (±77)

Table 5.1- All diamines were tested in the form of their more stable dihydrochloride salts to which the formulae numbers refer. K_M values are in units of mM and V_{max} values are in units of $\mu molmg^{-1}h^{-1}$.

meso-2,3-Dibromoputrescine dihydrochloride (106) was shown to be a poor substrate for the enzyme, suggesting that the bulky bromo-substituents hinder the molecule from obtaining the required

conformation for efficient oxidation. Although (±)-2,3-dibromoputrescine dihydrochloride (96) was enzymatically oxidised at a relatively slow rate, the V_{max} for this compound was ten times greater than that of the *meso*- form. A possible explanation for this observed difference in rate of oxidation may lie in the conformation that the molecules adopt at the active site of the enzyme. It is likely that the favoured (lowest energy) conformation that the 2,3-dibromoputrescines will adopt will be when the bromo-substituents are positioned *anti* to each other. When this is the case *meso*-2,3-dibromoputrescine dihydrochloride (106) has the amine groups positioned on opposite sides of the molecule whereas with (±)-2,3-dibromoputrescine dihydrochloride (96), the amine groups lie on the same side (Scheme 5.5).



Scheme 5.5

If for efficient oxidation the molecule is required to orientate itself such that the amine groups approach the active site from the same side of the molecule, then oxidation of (±)-2,3-dibromoputrescine dihydrochloride (96) would be more easily achieved than oxidation of *meso*-2,3-dibromoputrescine dihydrochloride (106). This theory, however, is not consistent with the V_{max} values obtained for the but-2-ene-1,4-diamines. The *trans*-isomer (amine groups on opposite sides) was oxidised at a greater rate than the *cis*-isomer. An explanation for these observed differences was not found. The incorporation of a double bond into the backbone of one of the natural substrates (putrescine) appears to half the rate of enzymatic oxidation, whilst significantly improving the binding affinity of the substrates for the enzyme. *cis*-(104) and *trans*-bis(aminomethyl)cyclopropane dihydrochlorides (105) were enzymatically oxidised at similar rates although the *cis*-isomer appears to be a better binder.

CHAPTER 6

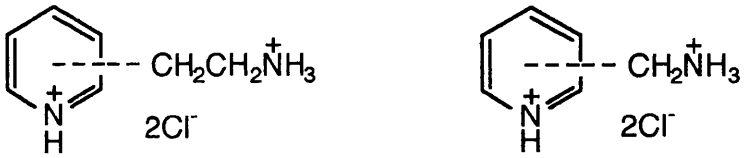
THE ENZYME CATALYSED OXIDATION OF PYRIDINE DERIVATIVES BY PEA SEEDLING DAO

6.1 Pyridine Derivatives as Substrates for Pea Seedling DAO

6.1(a) Introduction

Our work, to date, has concentrated solely on primary diamine molecules as substrates for pea seedling DAO. Having considered how various structural types and substituents effect both the rate of oxidation and the binding affinity of the diamine substrate, our attention turned to compounds which contain only one primary diamine. It has been shown,[93] that selected primary mono-amines can be enzymatically oxidised (albeit slowly) to the corresponding aldehyde. Our interest lay in diamine molecules which contained only one amine group capable of undergoing enzymatic oxidation. By undertaking kinetic studies on a range of such compounds it may be possible to gain an insight into the role of the second amine group during deamination; i.e. does the nature of the second amine group have a marked effect on the binding affinity of the substrate or has it a more relevant role to play in "triggering" the deamination process?

It was decided to attempt the synthesis of a range of (aminoalkyl)pyridine dihydrochlorides (Scheme 6.1).



Scheme 6.1

6.1 (b) Synthesis of Pyridine Derivatives

The starting points in the proposed synthetic pathway to the 2-pyridylethylamines were the corresponding pyridinecarboxaldehydes.

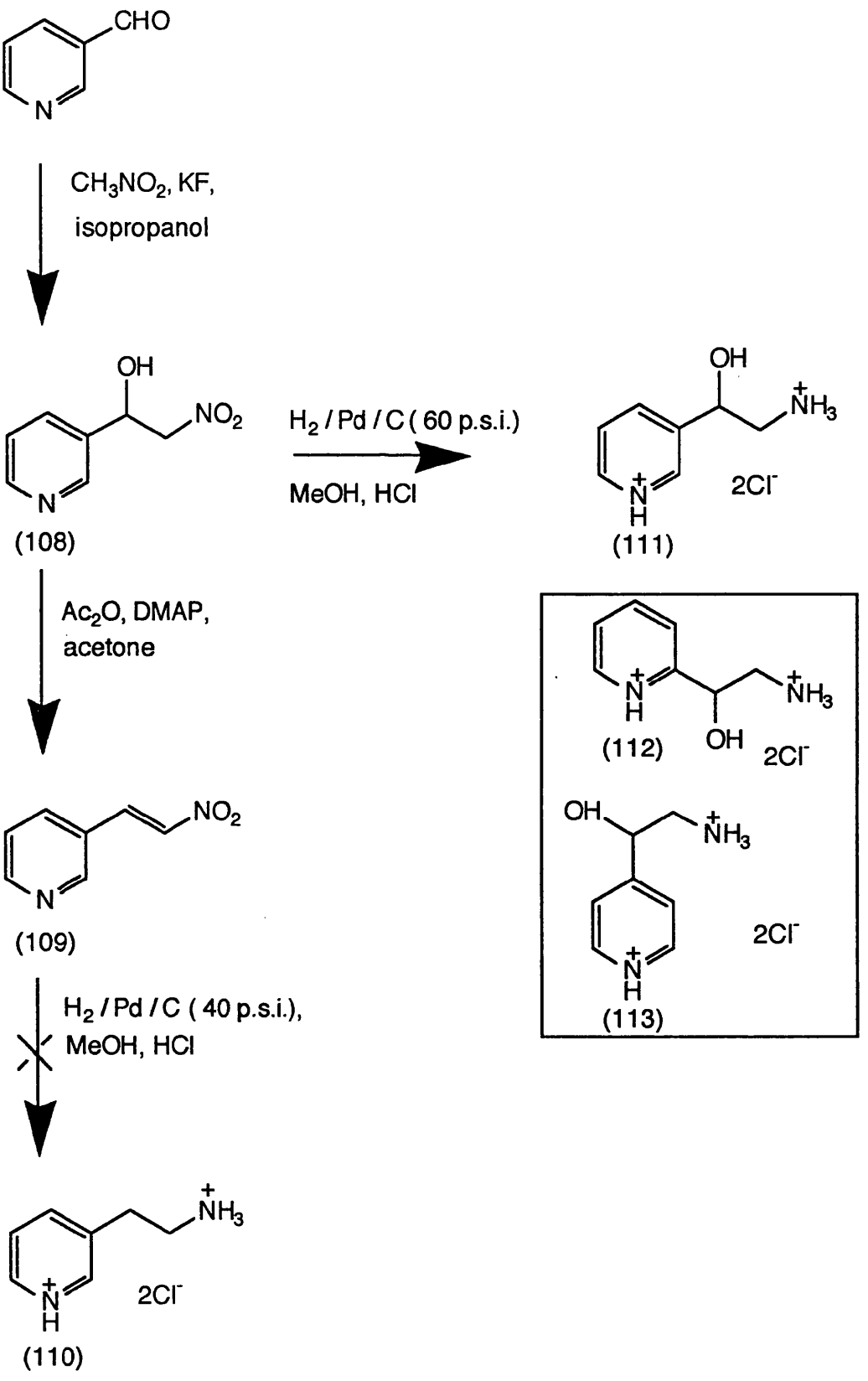
Treatment of 3-pyridinecarboxaldehyde with nitroethane in the presence of potassium fluoride gave racemic 2-hydroxy-2-(3'-pyridyl)nitroethane (108) as a yellow solid. It was thought that this compound, on elimination, would yield the corresponding nitro-olefin (109) which could be reduced in an acidic medium to give 2-(3'-pyridyl)ethylamine dihydrochloride (110) (Scheme 6.2).

Unfortunately all attempts to induce elimination in the nitro-alcohol (108) met with problems. Heating an acidic solution of the nitro-alcohol (108) at reflux in the presence of activated molecular sieves gave only the corresponding hydrochloride salt. Attempted base-catalysed elimination using sodium hydroxide also failed to bring about the desired conversion. Treatment of the nitro-alcohol (108) with $POCl_3$ yielded a dark solid which was shown to contain multiple products. A final attempt to produce the nitro-olefin involved treating a solution of the nitro-alcohol (108) with acetic acid and dimethylaminopyridine (DMAP). The reaction was monitored by ^1H NMR spectroscopy. At early stages of the reaction it was clear to see that the multiplet (δ 5.5 in d_6-acetone) associated with the C<u>H</u>OH proton was collapsing and was being replaced by a multiplet at δ 6.5.

Over longer reaction times the multiplet at δ 6.5 was seen to disappear, giving rise to extra resonances in the aromatic region. The characteristic resonance associated with the $C\underline{H}_2NO_2$ protons was no longer in evidence. The above data are consistent with the nitro-alcohol (108) undergoing initial acetylation at the alcohol functionality followed by elimination of acetic acid to give the desired nitro-olefin. A greenish solid, containing the nitro-olefin as the major product, was isolated from the reaction mixture. This compound, however, was found to be highly unstable, decomposing in a matter of minutes leaving a black tar. Storage of the compound at 0 °C in an atmosphere of nitrogen failed to prevent decomposition. A brief attempt was made to prepare 2-(3'-pyridyl)ethylamine by catalytically reducing the nitro-olefin immediately after isolation. No significant amount of the diamine was obtained by this procedure.

In view of the problems encountered with the proposed elimination, it was decided to reduce (±)-2-hydroxy-2-(3'-pyridyl)nitroethane (108) directly to the corresponding diamine. Catalytic hydrogenation (60 p.s.i.) of this intermediate in an acidic medium generated (±)-2-hydroxy-2-(3'-pyridyl)ethylamine in the form of the dihydrochloride salt (111). In an analogous fashion 2-pyridinecarboxaldehyde and 4-pyridinecarboxaldehyde were converted into the dihydrochlorides of (±)-2-hydroxy-2-(2'-pyridyl)ethylamine and (+)-2-hydroxy-2-(4'-pyridyl)ethylamine (112) and (113) respectively.

It was believed that these diamines would be suitable alternatives for studies into the precise role of the second amine group in the deamination process.

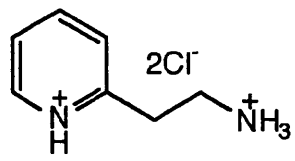

CHO
N
CH_3NO_2, KF,
isopropanol
OH
NO_2
N
(108)
H_2 / Pd / C (60 p.s.i.)
MeOH, HCl
OH
NH_3^+
N^+H
2Cl⁻
(111)
Ac_2O, DMAP,
acetone
NO_2
N
(109)
H_2 / Pd / C (40 p.s.i.),
MeOH, HCl
NH_3^+
N^+H
2Cl⁻
(110)
N^+H
NH_3^+
OH
2Cl⁻
(112)
OH
NH_3^+
N^+H
2Cl⁻
(113)

Scheme 6.2

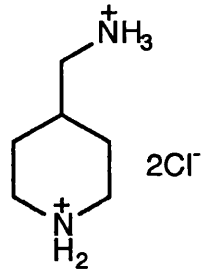
The pyridylmethylamines were synthesised in a straightforward manner from the corresponding pyridinecarboxaldehydes.

Treatment of 2-pyridinecarboxaldehyde with a mixture of hydroxylamine hydrochloride and formic acid (98%)[115] gave 2-cyanopyridine (114) as a sticky white solid. Catalytic hydrogenation (60 p.s.i) of this intermediate in the presence of Raney nickel[116] gave, on acidification, (2'-pyridyl)methylamine dihydrochloride (115) (Scheme 6.3). In analogous fashion (3'-pyridyl)methylamine dihydrochloride (116) and (4'-pyridyl)methylamine dihydrochloride (117) were prepared from 3-pyridinecarboxaldehyde and 4-pyridinecarboxaldehyde respectively.

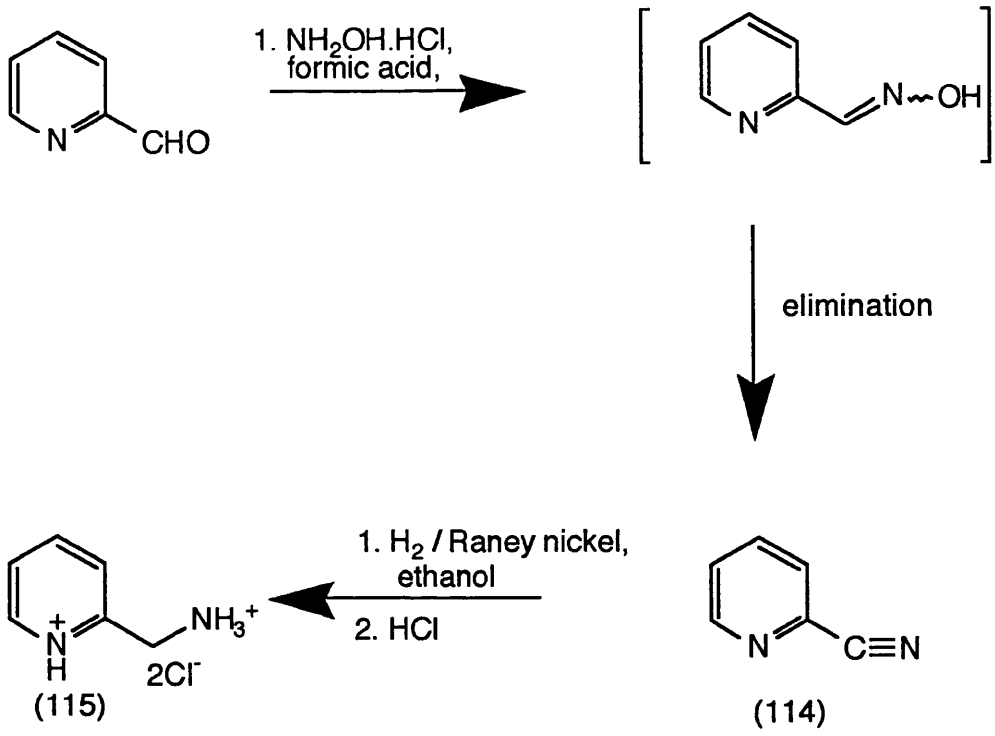
2-(2'-Pyridyl)ethylamine and (4'-piperidyl)methylamine were available from Aldrich and were converted into the corresponding dihydrochlorides (118) and (119) and purified by recrystallisation.

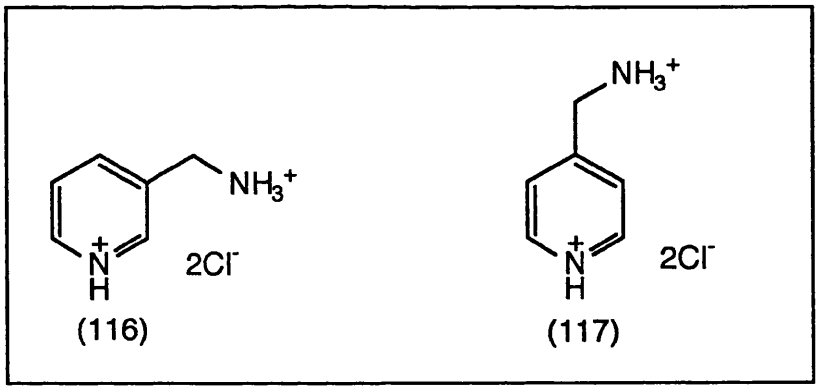


(118)



(119)





Scheme 6.3

6.1 (c) Results and Discussion

Using the spectrophotometric assay system, Michaelis-Menten kinetics were observed for all pyridine derivatives. Kinetic parameters for the enzymatic deamination of benzylamine are also reported. The data are summarised in Table 6.1.

Substrate	K_M	V_{max}
Putrescine	1.18 (±0.37)	1170 (±220)
Cadaverine	0.24 (±0.07)	2680 (±410)
Benzylamine	0.35 (±0.09)	4.8 (±0.3)
(+)-2-hydroxy-2-(2'-pyridyl)ethylamine (112)	0.22 (±0.04)	3.9 (±0.3)
(+)-2-hydroxy-2-(3'-pyridyl)ethylamine (111)	0.26 (±0.04)	0.7 (±0.1)
(+)-2-hydroxy-2-(4'-pyridyl)ethylamine (113)	0.95(±0.11)	3.8 (±0.2)
2-(2'-pyridyl)ethylamine (118)	0.22 (±0.05)	3.0 (±0.2)
(2'-pyridyl)methylamine (115)	0.04 (±0.01)	8.6 (±0.2)
(3'-pyridyl)methylamine (116)	0.40 (±0.06)	19 (±2)
(4'-pyridyl)methylamine (117)	0.06 (±0.03)	10 (±2)
(4'-piperidyl)methylamine (119)	0.59 (±0.13)	98 (±17)

Table 6.1- All diamines were tested in the form of their more stable dihydrochloride salts to which the formulae numbers refer. K_M values are in units of mM and V_{max} values are in units of $\mu molmg^{-1}h^{-1}$.

The most striking feature of the above results is the fact that all of the compounds tested appear to bind strongly to pea seedling DAO. This result suggests that the presence of a second primary amine group is not required for substrate binding. Indeed the fact that benzylamine appeared to bind to DAO as efficiently as most of the diamines examined suggests that the K_M value for such a substrate is dependent only on the initial interaction of one amine group with the enzyme and is independent of the nature of the second amine.

Greater variation existed in the rates at which the pyridine derivatives were oxidised. Comparison of the kinetic parameters for the substrates benzylamine and (3'-pyridyl)methylamine dihydrochloride (116) suggest that whilst the presence of a ring nitrogen may have no influence on the binding affinity it does appear to have a significant role to play in promoting the deamination. This result is further illustrated by considering the V_{max} values obtained for (4'-pyridyl)methylamine dihydrochloride (117) and (4'-piperidyl)methylamine dihydrochloride (119). The aliphatic diamine was oxidised at ten times the rate of the corresponding aromatic compound. Although the reason for this large difference may be due to the different spatial and electronic natures of the ring systems involved, it would seem likely that the differing electronic environments of the ring nitrogens is a contributing factor.

The pyridylmethylamines as a group were better substrates for pea seedling DAO than were the racemic 2-hydroxy-2-pyridylethylamines.

The remarkable similarities in the kinetic parameters obtained for (±)-2-hydroxy-2-(2'-pyridyl)ethylamine dihydrochloride (112) and 2-(2'-pyridyl)ethylamine dihydrochloride (118) suggest that the hydroxy substituent (capable of hydrogen bonding) on C-2 of (112) has no influence on the catalytic deamination of this substrate.

6.2 Pyridine Derivatives as Inhibitors of Pea Seedling DAO

6.2 (a) Introduction

Enzyme inhibitors may be defined as substances which when present in the reaction mixture cause enzyme-catalysed reactions to

proceed more slowly. Many types of inhibition are possible and each is characterised by a particular interaction between the inhibitor, the substrate and the active site of the enzyme. An increased understanding of enzyme inhibition has led to major advances in the area of drug design. The action of many pharmaceutical drugs depend on the inhibition of biologically important enzymes and much emphasis is currently being placed on the development of compounds which show a high degree of specificity towards the target enzyme. Enzyme inhibitors have also been employed in the mechanistic study of biotransformations. By using specific enzymes it has been possible to obtain much information on the critical residues required for catalysis in many enzymes.

The importance of polyamines and the role of pea seedling DAO in regulating their cellular levels has been discussed previously (Chapter 1.5). It was thought that good inhibitors of pea seedling DAO may possess useful antifungal activity. In the course of our studies a number of compounds have been shown to be efficient binders (low K_M) for the enzyme but poor substrates (low V_{max}). These compounds therefore possess the requirements for a competitive inhibitor. Several compounds were selected for testing as inhibitors of the DAO-catalysed deamination of putrescine.

6.2 (b) Assay System for Inhibition Studies

Inhibition studies were carried out using the spectrophotometric method of Stoner[90] (See Chapter 4.3). The only alteration made to the method used in the determination of the kinetic paramaters for the oxidation of putative substrates was the addition of various concentrations of inhibitor to the initial reaction

mixture. A separate experiment was carried out for each concentration of inhibitor. The potential inhibitor was added to the reaction mixture after the enzyme but before the addition of substrate.

6.2 (c) Kinetics of Inhibition

The kinetics of inhibition have been discussed in Chapter 3 (3.5).

It has been shown that for competitive inhibition plots of 1/V versus 1/[S] for (1) substrate with no inhibitor, and (2) substrate with inhibitor present differ in gradient. This difference in gradient is represented by,

$$(\text{slope}^{i})/(\text{slope}) = 1 + [I]K_i$$

Since in the Lineweaver-Burk plot the gradient gives an indication of the K_M, this equation can be represented,

$$K_M^*/K_M = 1 + [I]K_i \qquad (6.1)$$

where K_M^* is the apparent binding constant i.e the binding constant in the presence of a competitive inhibitor. K_M is the binding constant with no inhibitor present. [I] is the fixed concentration of inhibitor. K_i is the inhibition constant.

A value for the apparent binding constant can be obtained by measuring the rate for substrate oxidation at various concentrations of substrate in the presence of a fixed concentration of inhibitor. This

value for K_M^* can be substituted into equation 6.1 along with the relevant values for [I] and K_M to obtain a value for the inhibition constant.

The value of the inhibition constant is a measure of the strength of the enzyme-inhibitor complex and hence provides an indication of the effectiveness of the inhibitor. In practice K_M^* values are calculated at three different inhibitor concentrations and the K_i value is quoted as an average of nine determinations.

6.2 (d) Compounds Selected for Inhibitor Studies

When searching for potential inhibitors we were interested in compounds which bind well to the enzyme but are not oxidatively deaminated at a high rate. It is clear, by considering the data reported in Table 6.1, that the majority of pyridine amines examined satisfy these conditions. Of these compounds only (4'piperidyl)methylamine (119) was not tested as an inhibitor of the DAO catalysed deamination of putrescine. It was felt that the relatively high rate of oxidation of this compound would effect the kinetic behaviour of the enzymic reaction to such an extent that an accurate K_i value could not be obtained.

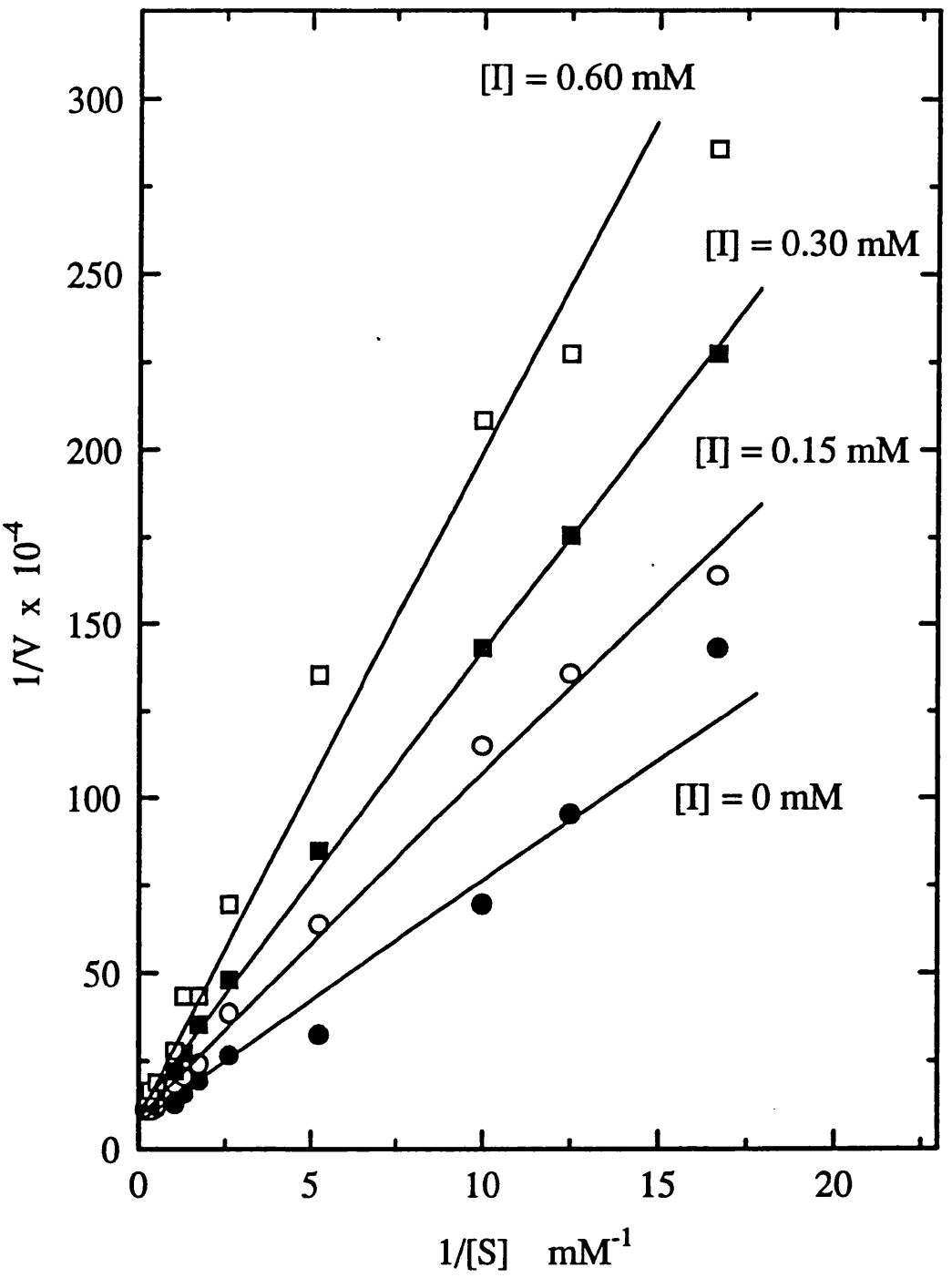
Other compounds considered in the course of our studies satisfy the criteria for competitive inhibition. However, of these, only (±)-2,3-dibromoputrescine dihydrochloride (96) and *meso*-2,3-dibromoputrescine dihydrochloride (106) were available in large enough quantities to enable full inhibitor studies to be carried out.

6.2 (e) Results and Discussion

Using the peroxidase-coupled assay with putrescine as substrate, in the presence of a fixed concentration of inhibitor, all systems were found to exhibit Michaelis-Menten kinetics. All inhibitor studies involved an initial experiment in which putrescine was enzymatically deaminated with no inhibitor present. The deamination was repeated a further three times but in the presence of a fixed concentration of inhibitor which was added prior to the addition of putrescine. The concentrations chosen were determined by the binding affinity of the inhibitor and were generally equal to the K_M, 2 x K_M and 4 x K_M of the inhibitor. K_i values were evaluated as discussed in Chapter 6.3 (c) and are reported as an average of nine determinations. The data are summarised in Table 6.2.

Under the experimental conditions all compounds were found to be competitive inhibitors of DAO with putrescine as substrate. This is illustrated in Graph 6.1 with reference to (2'-pyridyl)methylamine dihydrochloride (115). It is clear from the plot that the V_{max} (intercept on the Y-axis) is relatively unaffected by the presence of inhibitor whereas the K_M (slope of line) consistently rises with increasing concentrations of (2'-pyridyl)methylamine dihydrochloride.

A wide range of K_i values were obtained for the compounds tested. The most significant inhibitors of DAO were found to be (2'-pyridyl)methylamine dihydrochloride (115), (4'-pyridyl)methylamine dihydrochloride (117) and *meso*-2,3-dibromoputrescine dihydrochloride (106). These compounds had a marked effect in decreasing the rate of enzymatic deamination of putrescine at low substrate concentrations. The K_i values for these



Graph 6.1: Double reciprocal plot of enzyme kinetics. Inhibition of oxidative deamination of putrescine by pea seedling diamine oxidase with (2'-pyridyl)methylamine as inhibitor. [I] = inhibitor concentration.

compounds were lower than the K_M value (> 1mM) measured for putrescine indicating, that when present in comparable concentrations, the inhibitor has a greater affinity than putrescine for the active site of the enzyme. This effect, however, was overcome at high substrate concentrations.

Inhibitor	K_i
(±)-2-hydroxy-2- (2'-pyridyl)ethylamine (112)	1.58
(±)-2-hydroxy-2- (3'-pyridyl)ethylamine (111)	1.86
(±)-2-hydroxy-2-(4'-pyridyl)ethylamine (113)	2.56
2-(2'-pyridyl)ethylamine (118)	1.40
(2'-pyridyl)methylamine (115)	0.32
(3'-pyridyl)methylamine (116)	1.55
(4'-pyridyl)methylamine (117)	0.54
meso-2,3-dibromoputrescine (106)	0.36
(+)-2,3-dibromoputrescine (96)	2.42

Table 6.2- The diamines were in the form of their more stable dihydrochloride salts to which the formulae numbers refer. All compounds were tested as inhibitors of the DAO catalysed deamination of putrescine. K_i values are reported in units of mM.

In contrast to the low inhibition constants observed for (2'-pyridyl)methylamine dihydrochloride (115) and (4'-pyridyl)methylamine dihydrochloride (117), the inhibitory effect shown by (3'-pyridyl)methylamine dihydrochloride (116) was less dramatic. An explanation for these observed differences can be given with reference to the K_M values otained when the compounds were assayed as substrates for DAO (Table 6.1). Although all three bound well to the enzyme, the K_M value for (3'-pyridyl)methylamine

dihydrochloride (116) was approximately ten times greater than the binding constants obtained for the other compounds. It is therefore not surprising that this compound would show a lesser affinity for the enzyme when competing with one of the natural substrates for the active site.

An added complication that arises when determining an inhibition constant for (3'-pyridyl)methylamine dihydrochloride is that the compound is a substrate for the enzyme in its own right, being oxidised at twice the rate of the other pyridinemethylamines. At extremely low substrate concentrations, (3'-pyridyl)methylamine dihydrochloride will behave as a competing substrate for the enzyme. The observed rate of oxidation under these conditions will be a consequence, not only of the rate of deamination of putrescine, but also of the rate of deamination of (3'-pyridyl)methylamine dihydrochloride. This will lead to an inaccurate K_i value being obtained.

Although not as effective as the pyridylmethylamines (probably as a consequence of their lower binding affinities for the enzyme), the 2-hydroxy-2-pyridylethylamine dihydrochlorides had a significant effect in reducing the rate of DAO-catalysed deamination of putrescine at low substrate concentrations. Again it was clear that the variations in K_i values within the series could be explained in terms of the binding affinities of the compounds involved (Table 6.1). (±)-2-Hydroxy-2-(4'-pyridyl)ethylamine dihydrochloride (113), shown to have the lowest binding affinity for the enzyme, was found to be the poorest inhibitor of the DAO-catalysed deamination of putrescine.

meso-2,3-Dibromoputrescine dihydrochloride (106), shown to be a good binder but poor substrate for DAO, was confirmed as an

excellent ihibitor of the enzyme-catalysed deamination of putrescine. In contrast, (±)-2,3-dibromoputrescine dihydrochloride (96), rather surprisingly, showed only a moderately low inhibitory effect on the deamination process. The lower binding affinity of the (±)-form would not seem to explain fully the large discrepancies in inhibition constants found for these two compounds. No other explanation could be found.

Time constraints prevented us testing these compounds (Table 6.2) as inhibitors of pea seedling DAO with cadaverine as substrate.

6.3 Inhibitors of Putrescine Methyl Transferase

A range of diamine dihydrochlorides were supplied to Dr. N. Walton (Institute of Food Research, Norwich) to aid him in his studies concerning inhibition of the enzyme putrescine methyl transferase. Although full kinetic data have not been determined results suggest that when present in equal concentration to the natural substrate putrescine (2 mM), (2'-pyridyl)methylamine dihydrochloride (115) and (4'-pyridyl)methylamine dihydrochloride (117) significantly inhibit the reaction. In contrast (3'-pyridyl)methylamine dihydrochloride (116) was found to be a poor inhibitor of the biotransformation. These results clearly parallel our own observations.

CHAPTER 7

PURIFICATION OF PEA SEEDLING DAO

The following work was carried out during a CASE placement at Shell Research, Sittingbourne.

7.1 Introduction

Throughout our work, we have used enzyme which was partially purified by a series of precipitations.[5]

Several investigators have undertaken the purification of pea seedling DAO.[22,117] Although, in many cases, highly purified preparations were obtained, the methods used were generally long, multi-step processes. A more direct method for the production of homogeneous pea seedling DAO was reported.[25] However this method resulted in only a 32-fold increase in specific activity. It was our aim to obtain a simple, direct route to homogeneous pea seedling DAO which utilised the comparatively new technique of Fast Protein Liquid Chromatography (FPLC).

Our interest in the preparation of homogeneous enzyme was two-fold. Firstly, it would be interesting to compare the kinetic parameters obtained for substrates using this enzyme with those obtained using the partially purified form. Secondly, if sufficient material could be isolated it might be possible to crystallise the enzyme to enable an X-ray crystallographic structure elucidation to be undertaken. This could provide much information on the nature of the active site and cofactor of pea seedling DAO.

7.2 (a) Methods Used to Determine the Activity/Protein Concentration of Enzyme Preparations

In order to assess the effectiveness of the purification process we required accurate methods for determining the activity and the protein concentrations at the various stages of purification.

7.2 (b) Determination of Enzyme Activity

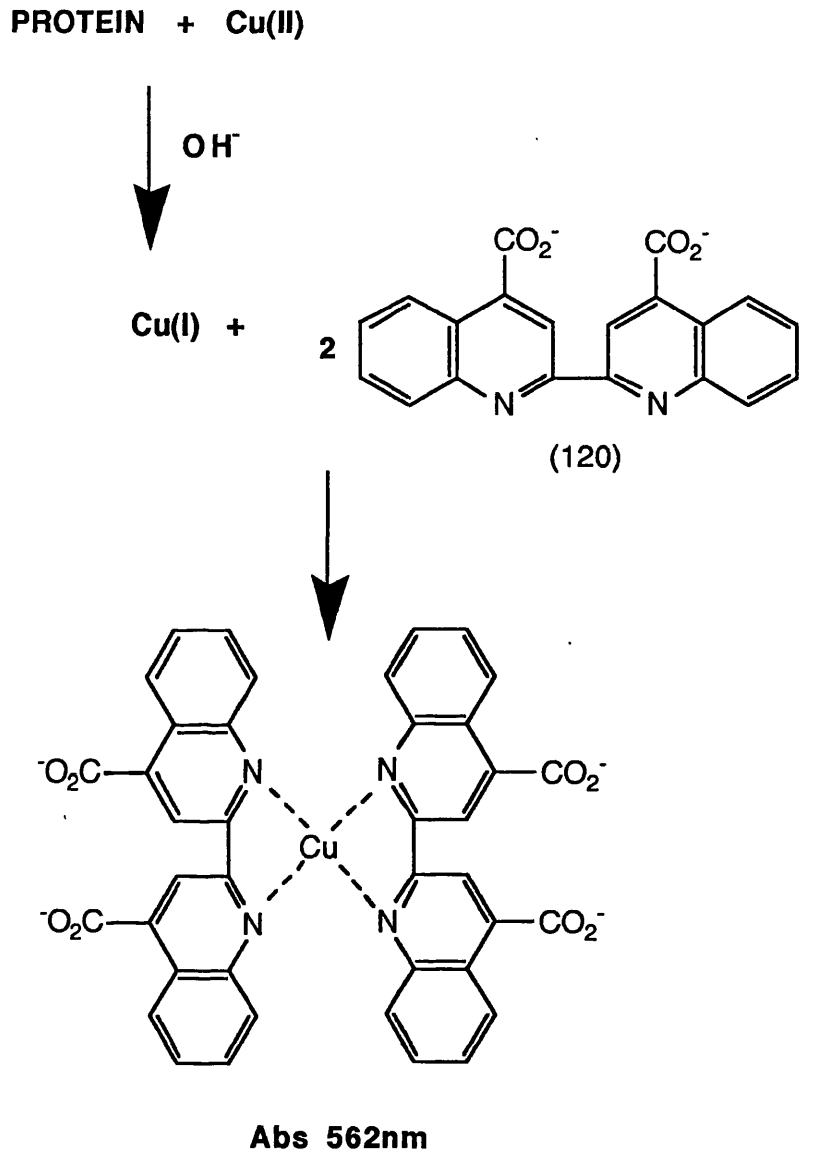
Here, activity is defined as the rate at which the enzyme was found to oxidise the natural substrate putrescine.

The rate of oxidation was determined directly using the Oxygen Electrode.[118] The Oxygen Electrode is sensitive to the amount of oxygen present in the surrounding cell. Since the enzymatic reaction requires the uptake of oxygen, the rate at which the oxygen level is depleted gives an indication of the activity of the enzyme.

7.2 (c) Determination of Protein Concentration

The protein concentrations were determined according to the method of Pierce.[119] The method (Scheme 7.1) involves treatment of the enzyme solution with a mixture of bicinchoninic acid (BCA) (120), copper(II) sulphate and sodium hydroxide. The first stage involves the reduction of the Cu(II) ions by the action of protein/base. The Cu(I) ions thus formed react with BCA, forming a water soluble complex with an absorbance maximum at 562 nm. By measuring the absorbance at 562 nm and comparing it to the absorbances for a range of standards of known protein concentration it is possible to

obtain an estimate of the protein concentration in the enzyme preparation.



Scheme 7.1

7.3 (a) Purification of Diamine Oxidase from Pea Epicotyls

The attempted purification of DAO from pea epicotyls involved a five step process. The methods described for the preparation of the crude extract, subsequent treatment with protamine sulphate and ammonium sulphate precipitation are similar to those reported by Yanagisawa *et al.*[25] The remaining steps involve chromatography on a cellulose phosphate column followed by final purification using the afore-mentioned technique of FPLC. Several attempts were made before a suitable FPLC method could be obtained. The following discussion gives details of the most successful preparation only. Protein concentrations and activities were measured at all stages of the purification.

Step 1- Preparation of the Crude Extract

The peas were washed with water, sown in vermiculite and allowed to germinate and grow for a period of 9-12 days. The stems (50 g) were then collected and homogenised in 0.1 M phosphate buffer (pH 6.4). The crude mix was then squeezed through muslin and centrifuged to produce the crude buffered enzyme solution.

Step 2- Protamine Sulphate Treatment

This technique is commonly used during enzyme purifications. Protamine sulphate forms an insoluble precipitate with nucleic acids which can be removed from the buffered enzyme solution by centrifugation.

Treatment of the enzyme solution with a 5% protamine sulphate solution followed by centrifugation led to the selective removal of nucleic acids from the protein mixture.

Step 3- Ammonium Sulphate Precipitation/Gel Filtration

This technique utilises the different solubilities of proteins to purify further the enzyme.

For DAO the active protein precipitated out of the buffered solution at between 35% and 65% saturation with ammonium sulphate.

Solid ammonium sulphate was added until the buffered solution was 35% saturated. The cloudy solution was then centrifuged and the solid collected, containing the least soluble proteins, was discarded. The remaining aqueous solution was then taken to 65% saturation by the addition of further ammonium sulphate. This time the solid collected on centrifugation was found to contain the active protein. The remaining aqueous solution, containing the most soluble proteins, was discarded.

Although selective precipitation in this manner usually gives rise to a significant increase in the specific activity of the enzyme preparation, contamination with ammonium sulphate is an unavoidable drawback.

Gel filtration offers a method for the separation of ammonium and sulphate ions (and other such species) from proteins based on size. The method involves passing the buffered mix through a gel, consisting of an open, cross-linked three dimensional network in bead form. These gels are designed in such a way that not all the pores within the beads are accessible by large molecules whereas all

are accessible by the smaller molecules. The result is that when the buffered enzyme solution is washed through the gel, the large molecules pass through faster and can be collected in the early fractions. By choosing an appropriate gel it is possible to separate proteins of different size.

The precipitate containing the active protein was dissolved in a minimum volume of phosphate buffer (20 mM), applied to a Sephadex G25 column and eluted with 20 mM phosphate buffer (pH 6.4). The material washed from the column was monitored by UV (254 nm). The active protein was collected in the first fraction.

Step 4- Cellulose Phosphate Column

This technique enables proteins to be separated on the basis of their charge characteristics.

The buffered enzyme solution, having been freed from ammonium sulphate, was placed on a Celex P column (cellulose phosphate; cationic exchanger) and eluted with 20 mM phosphate buffer (pH 6.4). The fractions collected were monitored by UV. It was clear that a large amount of material was washed straight through the column. This material was collected as a single fraction and checked for activity using the Oxygen Electrode. No substantial activity was observed for this fraction. Continuous elution with 20 mM phosphate buffer failed to remove any further material from the column. The strength of the buffer was increased to 0.1 M (pH 7.0). Elution of the column using this buffer produced material with a UV absorbance at the selected wavelength. Three fractions were collected, of which only the first two showed any appreciable activity. The active fractions were combined.

Step 5- Fast Protein Liquid Chromatography (FPLC)

An extension of liquid chromatography, FPLC allows the separation of proteins based on the charge characteristics of the biomolecules involved. Since nearly all biomolecules can exist in a charged state, this ion exchange method is applicable to many different types of macromolecule.

The method involves the interaction of the biomolecule with an ion exchange column (through electrostatic forces). The charged groups on the surface of the biomolecule interact with groups of opposite charge on the ion-exchanger. Elution of the ion-exchange column with an ionic salt (of varying concentrations) diminishes the interaction between the protein and the exchanger with the result that the proteins are washed from the column in order of increasing overall charge. The presence of an auto-sampler allows the collection of a set number of fractions of fixed volume over a specified period of time.

A cationic exchange column (mono-S) was equilibrated in 20 mM phosphate buffer (pH 6.4). The buffered enzyme solution was then injected onto the column and continuously washed with 20 mM buffer. The eluant passing from the column was monitored by UV spectroscopy (254 nm). A large amount of protein was seen to pass through the column on elution with the weak buffer. No substantial activity was associated with this fraction. [Note : when an anionic exchange column was used the protein which passed straight through was found to contain >95% of the total activity]. An automated programme was then selected which gradually increased the percentage of 1M KCl in the eluant. Three different programmes were considered in an attempt to isolate the homogeneous enzyme.

Programme 1

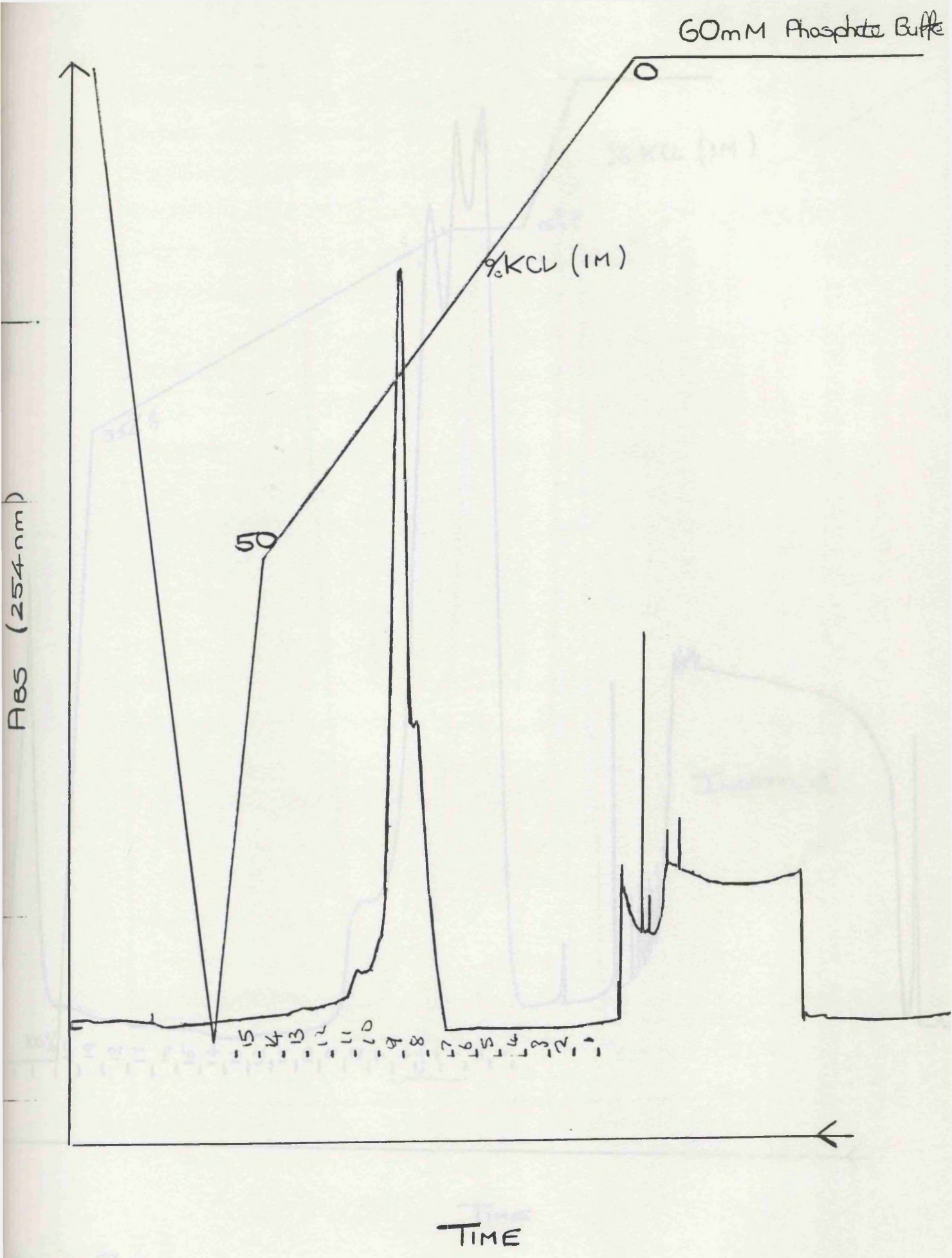
Gradient	Time
0 - 50% 1 M KCl (in 20 mM buffer)	15 min (15 fractions)
50 - 100%	1 min

The initial programme was intended to provide an estimate of the proportion of KCl (1 M) required to remove the active protein from the column. The proportion of KCl in the eluant (consisting initially of 100% 20 mM phosphate buffer) was gradually increased to 15% over a period of 15 minutes with a fraction (1 ml) being collected every minute. The proportion of KCl (1M) was then increased sharply until the column was eluted with 100% KCl. The eluant passing through the column was monitored by UV spectroscopy (Scan 7.1). It was clear from the scan that the remaining protein could be eluted from the column by washing with a mixture which was 20-30% in KCl (1M).

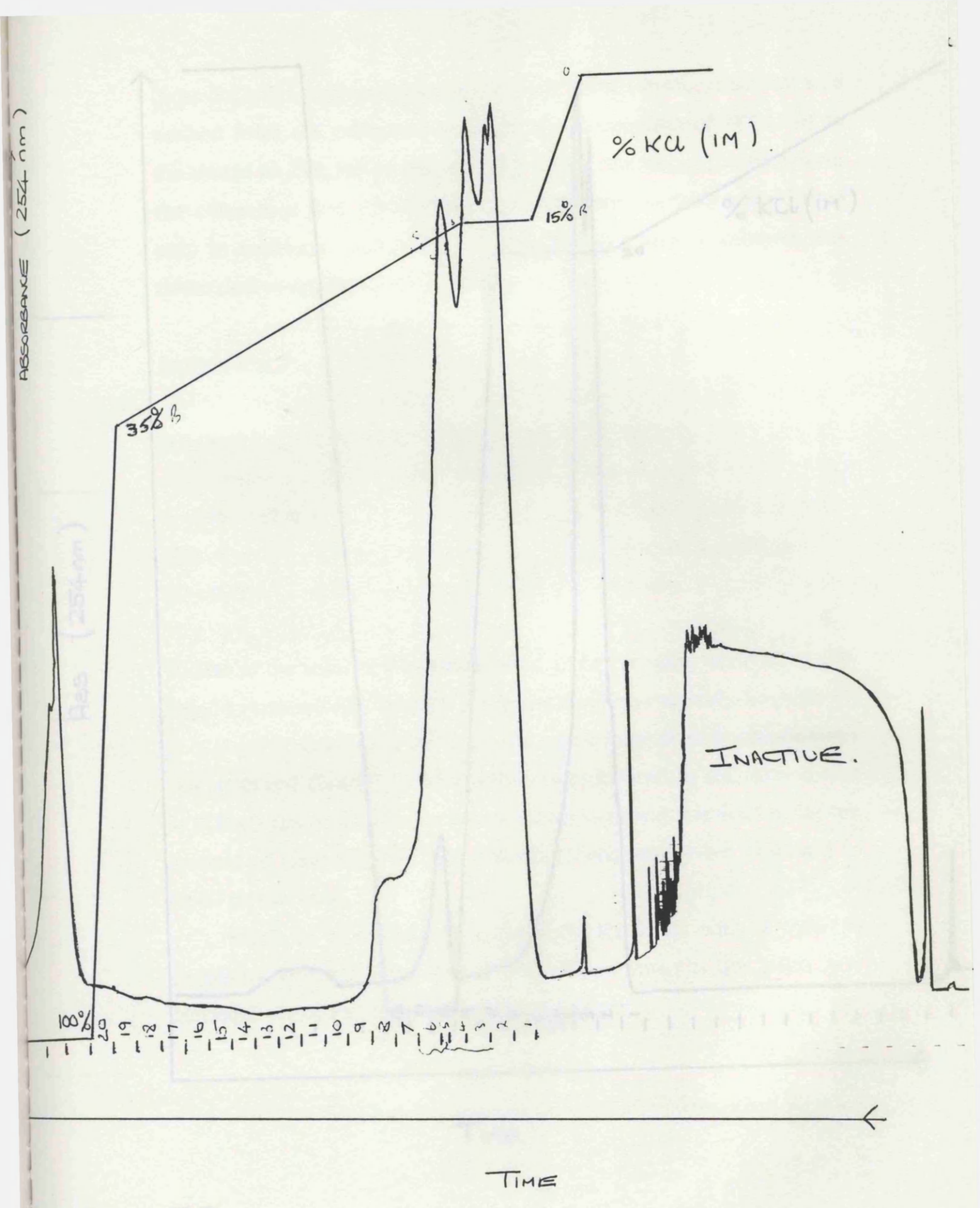
Programme 2

Gradient	Time
0 - 15% 1 M KCl (in 20mM buffer)	3 min
15%	3 min (3 fractions)
15 - 35%	15 min (15 fractions)
35 - 100%	1 min

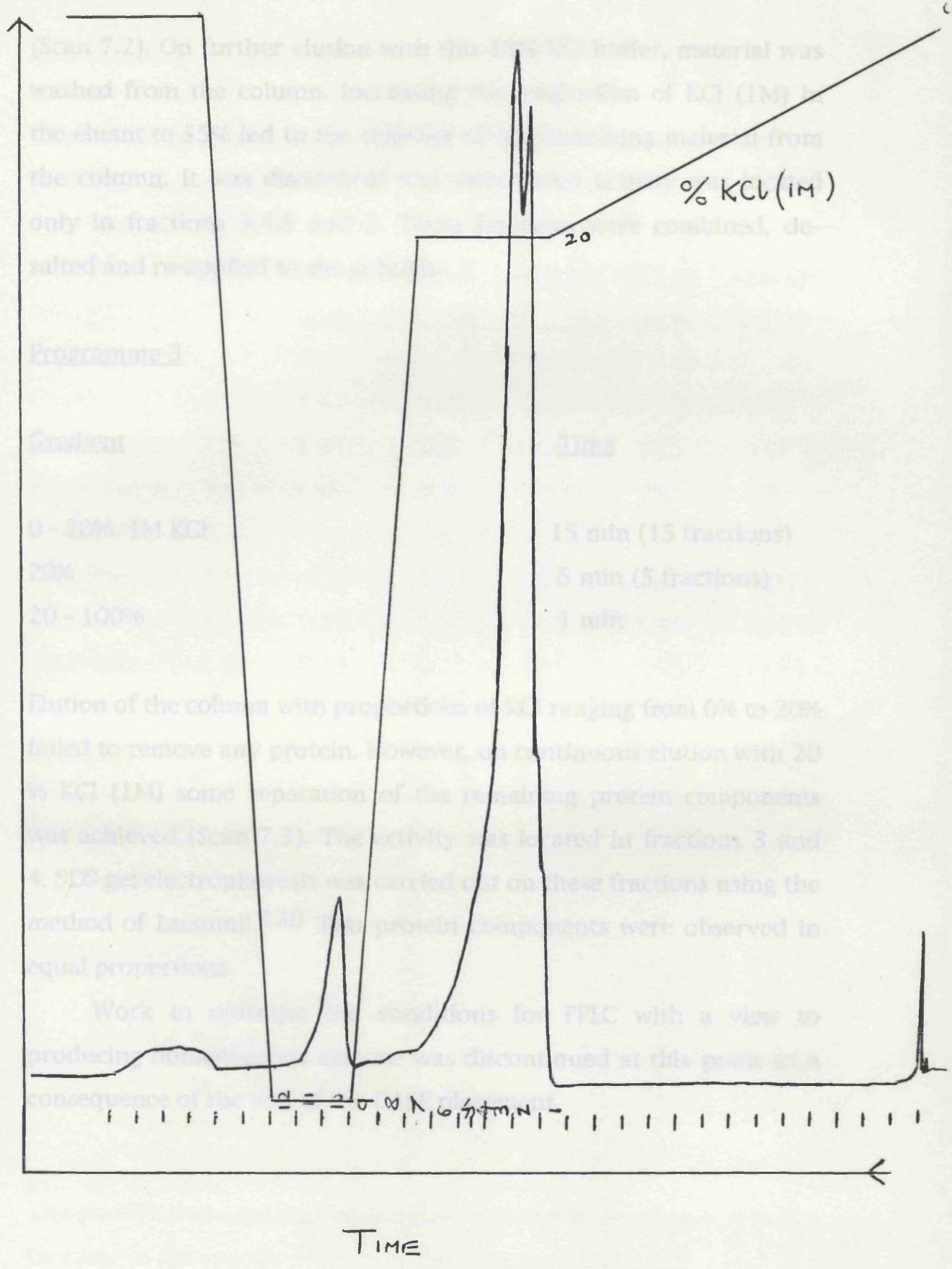
Allowing the proportion of KCl in the eluant to rise from 0% to 15% over a 3 minute period failed to remove any protein from the column



SCAN 7.1



SCAN 7.2.



SCAN 7.3

(Scan 7.2). On further elution with this 15% KCl buffer, material was washed from the column. Increasing the proportion of KCl (1M) in the eluant to 35% led to the removal of the remaining material from the column. It was discovered that substantial activity was located only in fractions 3,4,5 and 6. These fractions were combined, de-salted and re-applied to the column.

Programme 3

Gradient	Time
0 - 20% 1M KCl	15 min (15 fractions)
20%	5 min (5 fractions)
20 - 100%	1 min

Elution of the column with proportions of KCl ranging from 0% to 20% failed to remove any protein. However, on continuous elution with 20 % KCl (1M) some separation of the remaining protein components was achieved (Scan 7.3). The activity was located in fractions 3 and 4. SDS gel electrophoresis was carried out on these fractions using the method of Laemmli.[120] Two protein components were observed in equal proportions.

Work to optimise the conditions for FPLC with a view to producing homogeneous enzyme was discontinued at this point as a consequence of the end of the CASE placement.

7.3 (b) Results and Discussion

The following results (Table 7.1) were obtained from an input of 50 g of frozen pea epicotyls.

	Vol	Protein Conc.	Total prot.	Activity	Total Act.	Specific Act.
Crude	112	4.87	545	0.35	39.2	0.07
Prot. Sulph.	112	3.24	363	0.29	32.5	0.09
Amm. Sulph. /Gel Filt.	3.6	8.56	30.8	7.48	26.9	0.87
Cell. Phos.	24.1	0.75	18.1	1.10	26.5	1.47
FPLC (f3)	1.0	0.18	0.18	3.45	3.45	19.17
FPLC (f4)	1.0	0.11	0.11	2.15	2.15	19.55

Table 7.1

$$\text{Specific Activity} = \frac{\text{Activity}}{\text{Protein Conc.}}$$

Units : Volume (ml)
Protein Conc. (mg/ml)
Total Protein (mg)
Activity (μmol/min/ml)
Specific Activity (μmol/min/mg)

The purification sequence undertaken resulted in an overall 280-fold increase in the specific activity of the enzyme preparation.

Treatment with protamine sulphate whilst reducing the amount of inactive protein in the sample, led to only a relatively small increase in specific activity. Selective precipitation of the active protein with ammonium sulphate followed by gel filtration led to a more significant improvement in the purity of the enzyme. A major reduction in the total protein present was achieved with only a relatively small loss in the total activity. A 10-fold increase in specific activity was achieved during this stage. Chromatography on a Cellulose Phosphate column led to an improvement in the purity of the enzyme with no significant loss in activity.

The FPLC method was only a partial success. Although a definite advantage could be gained in terms of the specific activity of the final product, the yield obtained for the final preparation (fractions 3 and 4) was low, with most of the activity being lost during this final stage. Although it is likely that the method could be modified to allow the isolation of homogeneous enzyme, it is unlikely even with increased inputs that the quantity of enzyme required for comparative kinetic studies and enzyme crystallisation could be obtained without multiple runs. Further work is required to improve the purification technique.

CHAPTER 8

EXPERIMENTAL

8.1 GENERAL

Melting points were measured on a Kofler hot-stage apparatus. Boiling points refer to the oven temperature using a Kugelrohr apparatus. Optical rotations were measured with an Optical Activity Ltd. AA 10 Polarimeter. Infra red spectra were obtained on a Perkin Elmer 580 spectrometer. Nuclear magnetic resonance spectra were recorded on a Perkin Elmer R32 spectrometer operating at 90 MHz, or a Bruker WP200-SY spectrometer operating at 200 MHz (δ_H) or 50.3 MHz (δ_C). The multiplicities of the ^{13}C NMR spectrum were determined using DEPT spectra with pulse angles of $\theta = 90^o$ and $\theta = 135^o$. Spectra were recorded with either tetramethylsilane at 0 p.p.m or the NMR solvent as the internal standard. Mass spectra were obtained using A.E.I MS 12 or 902 spectrometers. Elemental analyses were performed with a Carlo-Erba 1106 elemental analyser.

TLC was carried out on Merck Kieselgel G plates of 0.25 mm thickness in the solvent stated. Diamine dihydrochlorides were detected using ninhydrin and all other compounds by iodine. Chromatographic purification was carried out by dry-column flash chromatography using Kieselgel 60 (Merck, 70-230 mesh).

Tetrahydrofuran (THF) and diethyl ether were dried by distillation from sodium-benzophenone under argon immediatley before use.

Organic solvents were dried using either anhydrous sodium sulphate or anhydrous magnesium sulphate.

8.2 EXPERIMENTAL TO CHAPTER 4

Extraction of Diamine Oxidase (EC 1.4.3.6) from Pea Seedlings[5]

Step 1

Pea seeds (500 g), variety 'Fillbasket', were soaked in tap water for 24h. The water was changed *ca.* 4 times. The pea seeds were then sown thickly in Perlite (4-6 cm deep) and covered in Perlite (1-2 cm). They were allowed to germinate and grow in the dark for 10-14 days until the shoots were 5-10 cm tall. Note: the Perlite was kept moist throughout but not too wet since this was found to reduce germination. The shoots were stripped of their roots, washed free of growing medium, drained and weighed (1-1.5 kg). The harvested shoots were kept cool throughout the following operation. The peas were minced using a pre-cooled Waring blender. They were then strained through cotton mesh and the juice was squeezed out. The solid residue was mixed with 0.1 M potassium phosphate buffer (pH 7, 1 ml/g of material) and the juice was squeezed out as before. A second extraction using the same potassium phosphate buffer (0.5 ml/g of material) was performed. The total extract (2-3 l) was cooled to $< 5\ ^{o}C$

Step 2

Ethanol/chloroform (2:1 v/v, 30 ml per 100 ml of extract) was cooled to -10 ^{o}C and added to the extract over 30 min. Care was taken to ensure that the temperature of the extract did not rise above 5 ^{o}C

during this addition. The mixture was allowed to stand for *ca.* 1h at 0 to +5 °C after which the inactive precipitate was removed by centrifugation at 3000-4000 g for 20 min. The supernatant liquid was collected and saturated with ammonium sulphate (45 g/100 ml) and the temperature was allowed to rise to 10 °C. A solid separated and floated . The lower liquid was siphoned off and discarded. The slurry was centrifuged at 3000 g for 10-15 min. The curd collected was mixed with 0.02 M phosphate buffer (pH 7, 400-500 ml) and allowed to stand overnight.

Step 3

The dialysis tubing was pre-soaked in distilled water for *ca.* 2h. The solution was stirred for 1.5h at 15-18 °C and the precipitate was removed by centrifugation at 3000-4000 g for 20 min. The supernatant was again saturated with ammonium sulphate (200-300 g) and left for 1.5h at 8-10 °C. It was then centrifuged at 3000-4000 g for 20 min. The curd was mixed with 0.2 M phosphate buffer (pH 7, 20ml). The solution was dialysed in a 30 cm tube (diameter 15 mm) for 2-3h with cold running water. Dialysis was then carried out with 0.005 M phosphate buffer (pH 7, 1 l) over 36 h at 0-4 °C. The buffer was changed twice during this period.

Step 4

The dialysed material was centrifuged at 3000 g for 10-20 min to remove inactive precipitate. The supernatant liquid was adjusted to pH 5 by slow addition of 0.05 M acetic acid at *ca.* 5 °C then allowed

to stand for 1h at 0-4 °C. The precipitate was collected by centrifugation and triturated with water (20 ml). The pH was adjusted to pH 7 using 0.05 M potassium hydroxide to dissolve the precipitate and then to pH 5 with 0.05 M acetic acid. The solution was left for 1h and centrifuged to collect the precipitate. The precipitate obtained was taken up in 0.01 M phosphate buffer (pH 7, 1 ml/100 g of seedlings harvested). It was stored in the freezer (in 0.5 ml batches) at *ca.* -20 °C and was stable for many months.

The yield was *ca.* 30 mg per kg of peas. Protein concentration was *ca.* 8 mg per ml of enzyme solution.[See Appendix 1 for calculation.]

Determination of Protein Concentration[106]

Coomassie brilliant blue G was prepared as a 0.06% (w/v) solution in 3% perchloric acid. The solution was stirred overnight and filtered to remove any undissolved material.

A standard graph was determined using Bovine Serum Albumin (BSA, 1 mg/ml phosphate buffer pH 6.3).

A typical cuvette contained;

1 ml	Dye
1000 µl - x µl	Distilled water
x µl	BSA

where x = 5 to 50 µl

The experiment was carried out twice and the average plot was used to determine the protein concentration of the unknown DAO samples (replacing BSA with DAO in the cuvette). [See Appendix 1.]

Spectrophotometric Assay[90]

The kinetics of the DAO-catalysed oxidation of putative substrates were determined according to the method of Stoner.[90] This involved a peroxidase-coupled assay (horseradish peroxidase, EC 1.11.1.7, from Sigma) to monitor continuously the hydrogen peroxide released during diamine oxidation at 25 °C, 70 mM phosphate buffer (pH 6.3), in the presence of 3-methyl-2-benzothiazolinone hydrazone (MBTH) and 3-(dimethylamino)benzoic acid (DMAB). Oxidative coupling generated stoichiometric quantities of an indamine dye with a characteristic absorbance maximum at 595 nm. Rates were determined directly in the spectrophotometer.

Stock solutions were prepared as follows;

DMAB	18 mM (29.7 mg/10 ml phosphate buffer pH 6.3)
MBTH	0.6 mM (12.9 mg/100 ml distilled water)
Peroxidase	0.68 mg/2 ml phosphate buffer pH 6.3
Pea Seedling DAO	0.03-0.06 mg/ml phosphate buffer pH 6.3

A typical 1 cm pathlength cuvette contained;

2.5 ml	Phosphate buffer pH 6.3
100 µl	MBTH
170 µl	DMAB
50 µl	peroxidase
25 µl	pea seedling DAO
300 µl	substrate of varying concentrations

The reaction was initiated by the addition of standard enzyme to the thermally equilibrated reaction mixture, followed immediately by substrate addition, therefore minimising the possible inhibitory effects of extensive preincubation of DAO with the chromogenic agents.[90] Initial rates were determined over a range of substrate concentrations from the linear absorbance changes observed during the first minute of the reaction. Rate data were analysed for K_M and V_{max} by least squares fitting of Eadie-Hofstee[104,105](V against V/[S]), Lineweaver-Burk[103](1/V against 1/[S]) and Hanes([S]/V against [S]) plots. [See appendix 2.]

All experiments were carried out three times with all data quoted being the mean of nine determinations.

The validity of the assay system had previously been checked,[20] with the formation of the indamine dye being calibrated using standard solutions of hydrogen peroxide.

Inhibition Studies

The assay system used for inhibition studies was as above but with the addition of the various concentrations of inhibitor being made after the addition of DAO but before the addition of substrate.

The typical reaction mixture in the cuvette was ;

2.5 ml	Phosphate buffer pH 6.3
100 µl	MBTH
170 µl	DMAB
50 µl	peroxidase
25 µl	pea seedling DAO
100 µl	inhibitor
300 µl	substrate of varying concentrations

[See Appendix 3]

8.2 (a) GENERAL PROCEDURES

General Procedure (A) for Esterification of a Diacid to a Diester

A solution of the diacid (22 mmol) in methanol (100 ml) and concentrated H_2SO_4 (5 ml) was heated at reflux for *ca.* 40h. At the end of this period the solution was filtered, stripped of solvent and the resultant residue was taken up in ethyl acetate and washed free of acid with an aqueuos solution of sodium bicarbonate. The solution was dried ($MgSO_4$) and evaporated to give the desired product.

Dimethyl 5-nitroisophthalate

5-Nitroisophthalic acid was esterified to the corresponding diester using general procedure (A). A white crystalline solid was obtained (87%); m.p. 120-122 °C; ν_{max} (KBr disc) 3100, 2900, 1735, 1540 and 730 cm^{-1}; δ_H ($CDCl_3$) 4.00 (6H, s) and 8.87 (3H, s); *m/z* 239 (M^+) and 180 (100 %).

Dimethyl-5-hydroxyisophthalate

5-Hydroxyisophthalic acid was esterified to the corresponding diester using general procedure (A). A white crystalline solid was obtained (80%); m.p. 160-162 °C; ν_{max} (KBr disc) 3360, 2900, 1720, 1600 and 1470 cm^{-1}; δ_H (CD_3OD) 3.85 (6H, s), 7.60 (2H, m) and 8.05 (1H, m); *m/z* 211 ($M^+ + 1$), 210 (M^+) and 179 (100 %).

General Procedure (B) for the Reduction of a Diester to a Diol

A solution of the diester (50 mmol) in toluene (100 ml) was cooled to 0 °C and di-isobutylaluminium hydride (1.5M in toluene; 150 ml) was added over 30 min with cooling. After a further 30 min ethyl acetate (10 ml) was added and the mixture was added to a suspension of Celite (70 g) in acetone (150 ml). Methanol was added in portions over 15 min and the mixture was stirred vigorously. Stirring was continued until gelling had occured at which point the mixture was left for 30 min. Water (150 ml) was added and the mixture was filtered. The filtrate was concentrated under reduced pressure and the final traces of water were removed by azeotropic distillation with benzene. A subsequent chromatographic purification step produced the purified product.

5-Nitro-1,3-bis(hydroxymethyl)benzene

Dimethyl-5-nitroisophthalate was reduced to the corresponding diol using general procedure (B). Chromatographic purification on a silica gel column (hexane/ethyl acetate 1:3) afforded the desired compound (62%); m.p. 95-97 °C; ν_{max} (KBr disc) 3410-3170, 2900, 1430, 1330, 775 and 745 cm^{-1}; δ_H $(CD_3)_2SO$ 4.63 (4H, s), 5.36 (2H, bs, OH), 7.58 (1H, s) and 7.98 (2H, s); *m/z* 183 (M^+) and 66 (100 %).

5-Hydroxy-1,3-bis(hydroxymethyl)benzene

Dimethyl-5-hydroxyisophthalate was reduced to the corresponding triol using general procedure (B). Chromatographic purification on a silica gel column (hexane/ethyl acetate 1:3) afforded the desired compound (56%); Rf = 0.31 (EtOAc); ν_{max} (KBr disc) 3600-3100, 2920 and 1600 cm^{-1}; δ_H ($CDCl_3$) 4.50 (4H, s), 5.38 (3H, bs, OH) and 6.73-7.10 (3H, complex); *m/z* 155 ($M^+ + 1$), 154 (M^+) and 66 (100%).

General Procedure (C) for the Reduction of a Diacid or Anhydride to a Diol

Diacid or anhydride (7.2 mmol) was dissolved in anhydrous THF (20 ml) under a dry nitrogen atmosphere and cooled in an ice bath to 0 °C. 1 M Borane.THF (26.5 ml, 26.5 mmol) was then added dropwise over a period of 10-15 min. The solution was stirred at room temperature for a further 1h. Excess borane was destroyed by the slow addition of water. The mixture was separated by salting out the organic layer using K_2CO_3. The aqeous layer was extracted with ether (3 x 20 ml). The combined organic extracts were dried (Na_2SO_4) and evaporated to dryness under reduced pressure to yield the product.

2-Bromo-1,4-bis(hydroxymethyl)benzene

2-Bromoterephthalic acid was reduced to the corresponding diol using general procedure (C). Chromatographic purification on a silica gel column (hexane/ethyl acetate 1:3) afforded the desired compound (79%); Rf = 0.46 (EtOAc); ν_{max} (KBr disc) 3390-3240, 2940, 1600 and 460 cm^{-1}; δ_H (D_2O) 4.45 (2H, s), 4.55 (2H, s) and 7.15-7.45 (3H, complex); *m/z* 218 (^{81}Br M^+), 216 (^{79}Br M^+) and 91 (100%).

4-Bromo-1,3-bis(hydroxymethyl)benzene

4-Bromoisophthalic acid was reduced to the corresponding diol using general procedure (C). Chromatographic purification on a silica gel column (hexane/ethyl acetate 1:3) afforded the desired compound (70%); Rf = 0.41 (EtOAc); ν_{max} (KBr disc) 3450-3200, 2940, 1600, 1570 and 440 cm^{-1}; δ_H (CD_3OD) 4.52 (2H, s), 4.61 (2H, s), 7.05-7.15 (1H, complex) and 7.40-7.55 (2H, complex); *m/z* 218 (^{81}Br M^+), 216 (^{79}Br M^+) and 91 (100%).

5-Bromo-1,3-bis(hydroxymethyl)benzene

5-Bromoisophthalic acid was reduced to the corresponding diol using general procedure (C). Chromatographic purification on a silica gel column (hexane/ethyl acetate 1:3) afforded the desired compound (76%); Rf = 0.42 (EtOAc); ν_{max} (KBr disc) 3400-3200, 2960, 1600 and

440 cm^{-1}; δ_H (CD_3OD) 4.55 (4H, s) and 7.15-7.35 (3H, complex); *m/z* 218 (^{81}Br M^+), 216 (^{79}Br M^+) and 91 (100%).

cis-*1,2-bis(Hydroxymethyl)cyclohexane*

cis-1,2-Cyclohexanedicarboxylic anhydride was reduced to the corresponding diol using general procedure (C). Chromatographic purification on a silica gel column (hexane/ethyl acetate 1:3) afforded the desired compound (69%); Rf = 0.27 (EtOAc); ν_{max} (KBr disc) 3600 (sharp), 3450-3150, 2960, 1450 and 1035 cm^{-1}; δ_H (CD_3OD) 1.0-2.0 (10H, complex) and 3.65 (4H, d); *m/z* 145 ($M^+ + 1$), 144 (M^+) and 68 (100%).

4-Chloro-1,2-bis(hydroxymethyl)benzene

4-Chlorophthalic anhydride was reduced to the corresponding diol using general procedure (C). Chromatographic purification on a silica gel column (ethyl acetate 100%) afforded the desired compound (52%); Rf = 0.46 (EtOAc); ν_{max} (KBr disc) 3540 (sharp), 3460-3200, 2960, 1600 and 650 cm^{-1}; δ_H ($CDCl_3$) 4.05 (2H, bs, OH), 4.48 (2H, s), 4.57 (2H, s) and 7.18-7.27 (3H, complex); *m/z* 172 (M^+), 155, 120 and 91 (100%).

General Procedure (D) for the Mesylation of a Diol

The diol (26 mmol) was dissolved in anhydrous THF (50 ml) under a dry nitrogen atmosphere and the solution was cooled to -78 °C

Methanesulphonyl chloride (52 mmol) was added with stirring and triethylamine (52 mmol) was then added slowly over a 5 min period. The mixture was allowed to reach room temperature overnight. The mixture was then poured into iced water (100 ml) and extracted with dichloromethane (3 x 75 ml). The combined extracts were dried (Na_2SO_4), filtered and evaporated to dryness under reduced pressure to leave an oil which could be crystallised from diethyl ether.

1,4-bis(Hydroxymethyl)benzene dimethanesulphonate

Using general procedure (D), 1,4-bis(hydroxymethyl)benzene gave the corresponding dimethanesulphonate as a white crystalline solid (72%); m.p. 120-121 °C; ν_{max} (KBr disc) 2950, 1350 and 1175 cm^{-1}; δ_H ($CDCl_3$) 3.05 (6H, s), 5.25 (4H, s) and 7.43 (4H, s); *m/z* 294 (M^+), 199, 119, 104 and 79 (100%).

1,3-bis(Hydroxymethyl)benzene dimethanesulphonate

Using general procedure (D), 1,3-bis(hydroxymethyl)benzene gave the corresponding dimethanesulphonate as a white crystalline solid (68%); m.p. 116-117 °C; ν_{max} (KBr disc) 2970, 1340 and 1160 cm^{-1}; δ_H ($CDCl_3$) 3.00 (6H, s), 5.20 (4H, s) and 7.43 (4H, s, with fine splitting); *m/z* 294 (M^+), 199, 119, 104 and 79 (100%).

2-Bromo-1,4-bis(hydroxymethyl)benzene dimethanesulphonate

Using general procedure (D), 2-bromo-1,4-bis(hydroxymethyl)benzene gave the corresponding dimethanesulphonate as a white crystalline solid (65%); ν_{max} (KBr disc) 2950, 1350, 1175 and 480 cm^{-1}; δ_H ($CDCl_3$) 2.95 (3H, s), 3.05 (3H, s), 5.18 (2H, s), 5.30 (2H, s) and 7.25-7.70 (3H, complex); *m/z* 374 (^{81}Br M^+), 372 (^{79}Br M^+) and 79 (100%).

cis/trans-*1,4-bis(Hydroxymethyl)cyclohexane dimethanesulphonate*

Using general procedure (D), *cis/trans*-1,4-bis(hydroxymethyl)cyclohexane gave the corresponding dimethanesulphonates as white crystalline solids (70%); Rf = 0.17 ($CHCl_3$); ν_{max} (KBr disc) 2940, 1325 and 1180 cm^{-1}; δ_H ($CDCl_3$) 1.00-2.05 (10H, complex), 3.00 (6H, s) and 4.05 (4H, d, with fine splitting); *m/z* 300 (M^+), 205 and 93 (100%).

cis/trans-*1,3-bis(Hydroxymethyl)cyclohexane dimethanesulphonate*

Using general procedure (D), *cis/trans*-1,3-bis(hydroxymethyl)benzene gave the corresponding dimethanesulphonates as white crystalline solids (72%); Rf = 0.15 ($CHCl_3$); ν_{max} (KBr disc) 2950, 1345 and 1155 cm^{-1}; δ_H ($CDCl_3$) 1.00-2.05 (10H, complex), 3.00 (6H, s) and 4.05 (4H, d, with fine splitting); *m/z* 300 (M^+), 205 and 93 (100%).

cis-1,2-bis(Hydroxymethyl)cyclohexane dimethanesulphonate

Using general procedure (D), *cis*-1,2-bis(hydroxymethyl)benzene gave the corresponding dimethanesulphonate as a white crystalline solid (61%); ν_{max} (KBr disc) 2960, 1340 and 1180 cm^{-1}; δ_H ($CDCl_3$) 1.58-2.44 (10H, complex), 3.05 (6H, s) and 4.25 (4H, d); *m/z* 126, 79 (100%).

General Procedure (E) for Converting Dimethanesulphonates into Diazides

The dimethanesulphonate (1.8 mmol) in dry DMSO (2 ml) was added to a solution of NaN_3 (4.5 mmol) in dry DMSO (8 ml) under a dry nitrogen atmosphere. The mixture was then stirred at room temperature and monitored by TLC. When the reaction was complete the solution was poured into water (80 ml) and extracted with diethyl ether (2 x 30 ml). The organic layers were combined and washed with water (2 x 50 ml). The organic extracts were dried ($MgSO_4$), filtered and evaporated to dryness under reduced pressure to yield the product. Extraction of the aqueous layer was repeated to increase yield.

Note : Due to the potentially explosive nature of the diazide product, no mass spectrum was collected.

1,4-bis(Azidomethyl)benzene

Using general procedure (E) and stirring for 16h, 1,4-bis(hydroxymethyl)benzene dimethanesulphonate gave the corresponding diazide as a yellow oil which was purified on a silica gel column (hexane/ chloroform 1:1) (61%); Rf = 0.75 ($CHCl_3$); ν_{max} (thin film) 2960, 2100 (strong), 1600, 1475 and 1250 cm^{-1}; δ_H ($CDCl_3$) 4.33 (4H, s) and 7.33 (4H, s).

1,3-bis(Azidomethyl)benzene

Using general procedure (E) and stirring for 48h, 1,3-bis(hydroxymethyl)benzene dimethanesulphonate gave the corresponding diazide as a yellow oil which was purified on a silica gel column (hexane/ chloroform 1:1) (64%); Rf = 0.74 ($CHCl_3$); ν_{max} (thin film) 2940, 2100 (strong), 1600, 1470 and 1230 cm^{-1}; δ_H ($CDCl_3$) 4.35 (4H, s) and 7.43 (4H, s).

2-Bromo-1,4-bis(azidomethyl)benzene

Using general procedure (E) and stirring for 7 days, 2-bromo-1,4-bis(hydroxymethyl)benzene dimethanesulphonate gave the corresponding diazide as a yellow oil which was purified on a silica gel column (hexane /ethyl acetate 4:1) (49%); Rf = 0.79 ($CHCl_3$); ν_{max} (thin film) 2990, 2110 (strong), 1610, 1410, 1050 and 670 cm^{-1}; δ_H ($CDCl_3$) 4.33 (2H, s), 4.46 (2H, s) and 7.22-7.57 (3H, complex).

cis/trans-*1,4-bis(Azidomethyl)cyclohexane*

Using general procedure (E) and stirring for 6 days, *cis/trans*-1,4-bis(hydroxymethyl)cyclohexane dimethanesulphonate gave the corresponding diazide as a yellow oil which was purified on a silica gel column (hexane/chloroform 1:1) (55%); Rf = 0.61 ($CHCl_3$); ν_{max} (thin film) 2920, 2100 (strong), 1450 and 1270 cm^{-1}; δ_H ($CDCl_3$) 0.87-1.98 (10H, complex) and 3.14 (4H, d, with fine splitting).

cis/trans-*1,3-bis(Azidomethyl)cyclohexane*

Using general procedure (E) and stirring for 7 days, *cis/trans*-1,3-bis(hydroxymethyl)cyclohexane dimethanesulphonate gave the corresponding diazide as a yellow oil which was purified on a silica gel column (hexane/chloroform 1:1) (58%); Rf = 0.68 ($CHCl_3$); ν_{max} (thin film) 2950, 2100 (strong), 1450 and 1275 cm^{-1}; δ_H ($CDCl_3$) 1.17-2.21 (10H, complex) and 3.18 (4H, d, with fine splitting).

cis-*1,2-bis(Azidomethyl)cyclohexane*

Using general procedure (E) and stirring for 14 days, 1,2-bis(hydroxymethyl)cyclohexane dimethanesulphonate gave the corresponding diazide as a yellow oil which was purified on a silica gel column (hexane/chloroform 1:1) (64%); Rf = 0.70 ($CHCl_3$); ν_{max} (thin film) 2960, 2100 (strong), 1450 and 1280 cm^{-1}; δ_H ($CDCl_3$) 1.48-2.20 (10H, complex) and 3.29 (4H, d).

General Procedure (F) for Reduction of Diazides to Diamines by Catalytic Hydrogenation

The diazide (2 mmol) was added to a suspension of 10% palladium on charcoal (10% w/w) in absolute alcohol (15 ml). A few drops of conc. hydrochloric acid were added and the suspension was hydrogenated at 1 atmosphere for *ca.* 18h. The catalyst was filtered through Celite and the Celite was washed thoroughly with a few drops of conc. hydrochloric acid. The solution was evaporated to dryness under reduced pressure to yield the diamine dihydrochloride. The product was recrystallised from aqueous ethanol (95%) and acetone.

1,4-Xylylenediamine dihydrochloride (69)

1,4-bis(Azidomethyl)benzene was reduced to the corresponding diamine dihydrochloride (69) using general procedure (F). The product was obtained as a white crystalline solid (98%); m.p. > 250 ^{o}C; ν_{max} (KBr disc) 3600-3400, 3200-2800, 1600 and 1480 cm^{-1}; δ_H (200 MHz, D_2O) 4.04 (4H, s) and 7.35 (4H, s); *m/z* 137 ($M^+ + 1$), 136 (M^+) and 106 (100%); (Found: C, 45.86; H, 6.71; N, 13.38; Cl, 33.91. $C_8H_{14}N_2Cl_2$ requires C, 45.93; H, 6.70; N, 13.40; Cl, 33.97%).

1,3-Xylylenediamine dihydrochloride (70)

1,3-bis(Azidomethyl)benzene was reduced to the corresponding diamine dihydrochloride (70) using general procedure (F). The

product was obtained as a white crystalline solid (91%); m.p. > 250 ^{o}C; ν_{max} (KBr disc) 3570-3360, 3200-2800, 1600 and 1475 cm^{-1}; δ_H (200 MHz, D_2O) 4.07 (4H, s) and 7.38 (4H, s); *m/z* 137 ($M^+ + 1$), 136 (M^+) and 30 (100%); (Found: C, 45.84; H, 6.66; N, 13.40; Cl, 33.93. $C_8H_{14}N_2Cl_2$ requires C, 45.93; H, 6.70; N, 13.40; Cl, 33.97%).

General Procedure (G) for a Staudinger Reaction of a Diazide to a Diamine[108]

The diazide (2 mmol) was dissolved in dry THF (3 ml) and to this solution was added Ph_3P (4.4 mmol) in 5 ml of dry THF. The reagents were stirred at room temperature for 1h and then heated at 50 ^{o}C for a further 3h. Water (4 ml) was then added and heating was continued for 3h. At the end of this period the solvents were removed *in vacuo* and the residue was partitioned between 1 M hydrochloric acid (15 ml) and dichloromethane (15 ml). After separation the aqueous layer was further washed with dichloromethane (2 x 15 ml). The aqueous layer was then evaporated to leave the product which was recrystallised from aqueous ethanol (95%) and acetone.

cis/trans-*1,4-Cyclohexane bis(methylamine) dihydrochloride (78)*

Using general procedure (G), *cis/trans*-1,4-bis(azidomethyl)cyclohexane was reduced to the corresponding diamine and isolated as the dihydrochloride salt (78). The product

was obtained as a white crystalline solid (84%); m.p. > 250 °C; ν_{max} (KBr disc) 3600-3400, 3100-2900, 2050, 1600 and 1500 cm^{-1}; δ_H (D_2O) 0.95-2.10 (10 H, complex) and 2.97 (4H, d, with fine splitting); *m/z* 143 ($M^+ + 1$), 142 (M^+) and 30 (100%); (Found: C, 44.56; H, 9.18; N, 13.01; Cl, 32.89. $C_8H_{20}N_2Cl_2$ requires C, 44.65; H, 9.30; N, 13.02; Cl, 33.02%).

cis/trans-*1,3-Cyclohexane bis(methylamine) dihydrochloride (79)*

Using general procedure (G), 1,3-bis(azidomethyl)cyclohexane was reduced to the corresponding diamine and isolated as the dihydrochloride salt (79). The product was obtained as a white crystalline solid (76%); m.p. > 250 °C; ν_{max} (KBr disc) 3600-3400, 3100-2900, 1600 and 1500 cm^{-1}; δ_H (D_2O) 0.72-2.25 (10 H, complex) and 2.95 (4H, d, with fine splitting); *m/z* 126 and 30 (100%); (Found: C, 44.60; H, 9.20; N, 12.92; Cl, 32.95. $C_8H_{20}N_2Cl_2$ requires C, 44.65; H, 9.30; N, 13.02; Cl, 33.02%).

cis-*1,2-Cyclohexane bis(methylamine) dihydrochloride (80)*

Using general procedure (G), *cis*-1,2-bis(azidomethyl)cyclohexane was reduced to the corresponding diamine and isolated as the dihydrochloride salt (80). The product was obtained as a white crystalline substance (72%); m.p. > 250 °C; ν_{max} (KBr disc) 3600-3400, 3100-2900, 1590 and 1495 cm^{-1}; δ_H (D_2O) 1.52-2.25 (10 H, complex) and 2.89 (4H, d,); *m/z* 126 and 30 (100%); (Found: C, 44.52;

H, 9.21; N, 13.06; Cl, 32.91. $C_8H_{20}N_2Cl_2$ requires C, 44.65; H, 9.30; N, 13.02; Cl, 33.02%).

2-Bromo-1,4-xylylenediamine dihydrochloride (75)

Using general procedure (G), 2-bromo-1,4-bis(azidomethyl)benzene was reduced to the corresponding diamine dihydrochloride (75). The product was obtained as a white crystalline solid (84%); m.p. > 250 °C; ν_{max} (KBr disc) 3600-3400, 3100-2700, 1610, 1465 and 600 cm^{-1}; δ_H (D_2O) 4.23 (2H, s), 4.40 (2H, s) and 7.60-7.85 (3H, complex); *m/z* 217 (^{81}Br $M^+ + 1$), 216 (^{81}Br M^+), 215 (^{79}Br $M^+ + 1$), 214 (^{79}Br M^+) and 28 (100%); (Found: C, 33.22; H, 4.37; N, 9.60. $C_8H_{13}N_2BrCl_2$ requires C, 33.36; H, 4.52; N, 9.73 %).

General Procedure (H) for the One-Pot Conversion of Diols into Diamines[107]

A mixture of the diol (20 mmol), triphenylphosphine (40 mmol), bromotrichloromethane (40 mmol) and benzene (20 ml) was refluxed with stirring for 3h. It was then cooled to room temperature and after addition of sodium azide (80 mmol), tetrabutyl ammonium bromide (5 mol %) and dimethylformamide (DMF) (20 ml), refluxed again with stirring for 6h. The resultant mixture was then poured onto water (200 ml) and extracted with benzene (2 x 20 ml). The organic phase was then dried ($MgSO_4$) and treated with triethylphosphite (40 mmol) at 30 °C for 2h. After this solution had been stood overnight at room temperature, 20% hydrochloric acid

(25 ml) was added and the mixture was refluxed for 2h. At the end of this period the aqueous phase was separated, made strongly alkaline with solid sodium hydroxide and extracted with dichloromethane (3 x 25 ml). The combined extracts were shaken with 20% hydrochloric acid (40 ml) and evaporation of the aqueous layer left the desired diamine dihydrochloride as a solid which was recrystallised from ethanol and acetone to give the white crystalline salt.

1,2-Xylylenediamine dihydrochloride (71)

According to general procedure (H), 1,2-bis(hydroxymethyl)benzene gave the corresponding diamine dihydrochloride (71) as a brown crystalline solid (10%); m.p. > 250 °C; ν_{max} (KBr disc) 3600-3320, 3200-2870, 1640, 1580, 1490 and 1150 cm^{-1}; δ_H (D_2O) 4.37 (4H, s) and 7.60 (4H, s); *m/z* 137 ($M^+ + 1$) and 119 (100%); (Found: C, 45.64; H, 6.54; N, 13.47; Cl, 33.79. $C_8H_{14}N_2Cl_2$ requires C, 45.93; H, 6.70; N, 13.40; Cl, 33.97%).

5-Nitro-1,3-xylylenediamine dihydrochloride (73)

According to general procedure (H), 5-nitro-1,3-bis(hydroxymethyl)benzene gave the corresponding diamine dihydrochloride (73) as a white crystalline solid (36%); m.p. > 250 °C; ν_{max} (KBr disc) 3500-3370, 3200-2700, 1600, 1520 and 1350 cm^{-1}; δ_H (D_2O) 4.35 (4H, s), 7.62 (2H, complex) and 8.27 (1H, bs); *m/z* 182 ($M^+ + 1$) and 28 (100%); (Found: C, 37.77; H, 5.19; N, 16.53; Cl, 27.99.

$C_8H_{13}N_3O_2Cl_2$ requires C, 37.81; H, 5.12; N, 16.54; O, 12.60; Cl, 27.92%).

4-Bromo-1,3-xylylenediamine dihydrochloride (76)

According to general procedure (H), 4-bromo-1,3-bis(hydroxymethyl)benzene gave the corresponding diamine dihydrochloride (76) as a white crystalline solid (45%); m.p. > 250 °C; ν_{max} (KBr disc) 3620-3360, 3200-2800, 1590, 1550, 1500 and 830 cm^{-1}; δ_H (200 MHz, D_2O) 4.18 (2H, s), 4.33 (2H, s), 7.39 (1H, dd), 7.50 (1H, d) and 7.75 (1H, d); δ_C (D_2O) 43.06 (t, $\underline{C}H_2N$), 43.87 (t, $\underline{C}H_2N$), 125.72 (s, Ar-C), 132.43 (d, Ar-C), 132.50 (d, Ar-C), 133.66 (s, Ar-C), 133.81 (s, Ar-C) and 134.95 (d, Ar-C); *m/z* 217 (^{81}Br $M^+ + 1$), 216 (^{81}Br M^+), 215 (^{79}Br $M^+ + 1$), 214 (^{79}Br M^+) and 28 (100%); (Found: C, 33.41; H, 4.39; N, 9.71; Cl, 24.45. $C_8H_{13}N_2BrCl_2$ requires C, 33.36; H, 4.52; N, 9.73; Cl, 24.64%).

5-Bromo-1,3-xylylenediamine dihydrochloride (72)

According to general procedure (H), 5-bromo-1,3-bis(hydroxymethyl)benzene gave the corresponding diamine dihydrochloride (72) as a white crystalline solid (54%); m.p. > 250 °C; ν_{max} (KBr disc) 3600-3350, 3180-2700, 1600, 1520 and 620 cm^{-1}; δ_H (D_2O) 4.27 (4H, s), 7.47 (2H, complex) and 7.82 (1H, complex); *m/z* 217 (^{81}Br $M^+ + 1$), 216 (^{81}Br M^+), 215 (^{79}Br $M^+ + 1$), 214 (^{79}Br M^+) and 28 (100%); (Found: C, 33.19; H, 4.71; N, 9.65. $C_8H_{13}N_2BrCl_2$ requires C, 33.36; H, 4.52; N, 9.73%).

5-Hydroxy-1,3-xylylenediamine dihydrochloride (74)

According to general procedure (H), 5-hydroxy-1,3-bis(hydroxymethyl)benzene gave the corresponding diamine dihydrochloride (74) as a white crystalline solid (47%); m.p. > 250 °C; ν_{max} (KBr disc) 3600, 3290-2850, 1600, 1510, and 1420 cm^{-1}; δ_H (D_2O) 4.23 (4H, s), 7.31 (2H, complex) and 7.65 (1H, complex); *m/z* 153 (M^+ + 1) and 28 (100%); (Found: C, 42.51; H, 6.20; N, 12.43; $C_8H_{13}N_2BrCl_2$ requires C, 42.69; H, 6.22; N, 12.45%).

4-Chloro-1,2-xylylenediamine dihydrochloride (77)

4-Chloro-1,2-bis(azidomethyl)benzene

Step 1

Preparation of Zinc Azide/bis-Pyridine complex[109]

To a stirred 2 M aqueous solution of $Zn(NO_3)_2$ (20 ml, 40 mmol) was added dropwise a 2 M aqueous solution NaN_3 (40 ml, 80 mmol). The white suspension was brought to *ca.* 50 °C then a slight excess of pyridine (6.7 ml; 82.35 mmol) was added dropwise, forming a dense white precipitate. Stirring was continued while the mixture was slowly cooled to room temperature. The salt was filtered, washed with iced water and dried to give a white crystalline solid(85%).

Step 2

Reaction of the Zinc Azide Complex with the Diol[109]

The zinc azide/pyridine complex (0.72 g, 2.35 mmol) was suspended in a solution of 4-chloro-1,2-bis(hydroxymethyl)benzene (0.27g, 1.57 mmol) in dry dichloromethane (10 ml). This mixture was stirred at room temperature and to it was added dropwise di-isopropyl azodicarboxylate (1.25 ml, 6.25 mmol) . The reaction mixture was allowed to stir overnight and was then filtered over a Celite pad and concentrated *in vacuo* to yield the crude mix. Purification on a silica gel column (hexane/ethyl acetate 4:1) gave 4-chloro-1,2-bis(azidomethyl)benzene as a yellow oil (69%); Rf = 0.74 ($CHCl_3$); ν_{max} (thin film) 2920, 2100 (strong), 1600 and 1530 cm^{-1}; δ_H ($CHCl_3$) 4.40 (2H, s), 4.43 (2H, s) and 7.23-7.50 (3H, complex).

4-Chloro-1,2-xylylenediamine dihydrochloride (77)

4-Chloro-1,2-xylylenediamine dihydrochloride (77) was obtained by reduction of 4-chloro-1,2-bis(azidomethyl)benzene according to general procedure (G). The product was a white crystalline solid (87%); m.p. > 250 °C; ν_{max} (KBr disc) 3610-3350, 3190-2550, 1580, 1490, and 770 cm^{-1}; δ_H (D_2O) 4.36 (2H, s), 4.38 (2H, s) and 7.59 (3H, s, with fine splitting); *m/z* 156, 154 and 35 (100%); (Found: C, 39.52; H, 5.37; N, 11.50. $C_8H_{13}N_2Cl_3$ requires C, 39.45; H, 5.34; N, 11.51%).

Isolation of Products from the DAO-Catalysed Oxidation of 1,4-Xylylenediamine Dihydrochloride (69)

1,4-Xylylenediamine dihydrochloride (69) (50 mg; 0.24 mmol) was dissolved in 0.2 M potassium phosphate buffer, pH 7.0 (30 ml). To this stirred solution was added catalase (2 mg) and pea seedling DAO (400 μl; 1.52 mg of protein). The reaction was monitored by TLC [methanol/isopropanol/conc. ammonia] (80:19:1) . After stirring for two hours it was clear that the solution contained starting material (R_f = 0.12) and two products (R_f = 0.38 and 0.67). Further enzyme (400 μl; 1.52 mg of protein) was added at this point and the solution was allowed to stir for a further 24 h. A solution of semicarbazide hydrochloride (83 mg; 0.74 mmol) in water (5 ml) was added and the solution was shaken vigorously. On longstanding a white solid was seen to precipitate. The precipitate was filtered and dried giving 24 mg of the disemicarbazone (81); ν_{max} (KBr disc) 3500-3420, 3350, 2940, 1610 and 1380 cm^{-1}; δ_H [200 MHz, $(CD_3)_2SO$] 6.54 (4H, bs, NH_2), 7.78-7.92 (6H, complex) and 10.31 (2H, bs, NH); δ_C [$(CD_3)_2SO$] 126.76 (d, Ar-C), 135.31 (s, Ar-C), 138.60 (d, HCNN) and 156.75 (s, HNCONH$_2$); *m/z* 247 (M^+-1) and 42 (100%).

8.3 EXPERIMENTAL TO CHAPTER 5

Attempted Synthesis of 2-Halobutane-1,4-diamines

(2S)-Chlorosuccinic acid (88) [110]

(2S)-Aspartic acid (10.0 g; 75 mmol) and urea (1.0 g; 17.2 mmol) were dissolved in a mixture of 10 M hydrochloric acid (16 ml) and concentrated nitric acid (16 ml). The solution was heated at 70 °C for 5 h with vigorous evolution of gas being noted. After this period the solution was allowed to cool overnight. The crystals of (2S)-chlorosuccinic acid (88) were filtered, washed with water and dried in vacuo (56%); $[\alpha]_D^{19}$ -20.8° (c 1, H_2O) {lit.,[117] $[\alpha]_D^{19}$ -21.5° (H_2O)}; υ_{max} (KBr disc) 3150-2950, 1720 and 670 cm^{-1}; δ_H [200 MHz, $(CD_3)_2SO$] 2.88 (2H, ABX, J_{AB} 17 Hz J_{AX} 8 Hz) and 4.52 (1H, t, J 8 Hz); δ_C [$(CD_3)_2SO$] 39.95 (t, $\underline{C}H_2CO_2H$), 53.19 (d, $\underline{C}HCl$), 170.45 (s, $\underline{C}O_2H$) and 171.46 (s, $\underline{C}O_2H$); *m/z* 153 (^{35}Cl M^+ + 1) and 73 (100%).

Dimethyl (2S)-chlorobutane-1,4-dioate

(2S)-Chlorosuccinic acid (88) (7.0 g; 45.9 mmol) was dissolved in methanol (50 ml) and the solution was cooled to 0 °C. Thionyl chloride (11.0 g; 92.4 mmol) was then added and the solution was stirred for 90 min and allowed to reach room temp. The excess reagents were removed under reduced pressure to yield dimethyl-(2S)-chlorobutane-1,4-dioate (149) as a greenish oil (95 %); $[\alpha]_D^{19}$ -41.9° (c 1, $CHCl_3$) {lit.,[117] $[\alpha]_D^{19}$ -42.8° ($CHCl_3$)}; υ_{max} (thin film)

3100-2920, 1755 and 655 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 3.05 (2H, ABX, J_{AB} 17 Hz J_{AX} 8 Hz) and 4.61 (1H, t, J 8 Hz); δ_C ($CDCl_3$) 38.94 (t, $\underline{C}H_2CO_2Me$), 51.52 (q, $CO_2\underline{C}H_3$), 52.73 (q, $CO_2\underline{C}H_3$), 53.24 (d, $\underline{C}HCl$), 169.12 (s, $\underline{C}O_2Me$) and 169.82 (s, CO_2Me); *m/z* 181 (^{35}Cl $M^+ + 1$) and 149 (100%).

(2S)-Chlorobutane-1,4-diol (89)

To a solution of (2S)-chlorobutane-1,4-dioate (8.0 g; 44.5 mmol) in dry THF (100 ml), cooled to 0 °C, was added di-isobutylaluminium hydride (200 ml; 1.0 M in toluene). The solution was stirred for 30 min after which time ethyl acetate (15 ml) was added. The solution was then poured onto a suspension of Celite (50 g) in acetone (100 ml). Methanol (8 ml) was then added slowly until gelling had occured. After 30 min water (120 ml) was added and the mixture was filtered. The solution was evaporated to give (2S)-chlorobutane-1,4-diol (89) as a green oil (51%); $[\alpha]_D^{19}$ -43.2° (c 1.2, MeOH) {lit.,[117] $[\alpha]_D^{19}$ -45.0° (MeOH)}; υ_{max} (thin film) 3540-3030, 2970-2910, 1400, 1050 and 620 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 1.55-2.15 (2H, complex), 3.45-3.67 (4H, complex), 3.95 (1H, complex) and 4.48 (2H, bs, OH); δ_C ($CDCl_3$) 36.56 (t, $\underline{C}H_2CH_2OH$), 58.30 (t, $CH_2\underline{C}H_2OH$), 59.83 (d, $\underline{C}HCl$) and 66.13 (t, $CH(Cl)\underline{C}H_2OH$); *m/z* 125 (^{35}Cl $M^+ + 1$) and 31 (100%).

(2S)-Chlorobutane-1,4-diol dimethanesulphonate

(2S)-Chlorobutane-1,4-diol (89) (2.0 g; 16.1 mmol) was dissolved in dry THF (30 ml) and the solution was cooled to -78 ^{o}C. Methanesulphonyl chloride (2.67 ml; 32.1 mmol) was added with stirring followed by triethylamine (3.22 g; 32.1 mmol). The mixture was stirred overnight at room temperature after which time it was poured into iced water (60 ml) and extracted with dichloromethane (3 x 50 ml). The combined extracts were dried ($MgSO_4$) and evaporated to dryness to leave the crude product as a viscous clear oil which could not be crystallised (82%); Rf = 0.18 (EtOAc); $[\alpha]_D^{19}$ -19.3^{o} (c 1, $CHCl_3$); υ_{max} (thin film) 2950, 1380 and 1145 cm^{-1}; δ_H ($CDCl_3$) 1.95-2.52 (2H, complex), 3.03 (3H, s), 3.07 (3H, s) and 4.33 (5H, complex); *m/z* 252, 175 and 79 (100%).

(2S)-4-Azido-2-chlorobutan-1-ol methanesulphonate (90)

(2S)-Chlorobutane-1,4-diol dimethanesulphonate (2 g; 7.12 mmol) in dry dimethylsulphoxide (10 ml) was added to a solution of sodium azide (0.94 g; 14.5 mmol) in dimethylsulphoxide (20 ml) and the mixture was stirred overnight at room temperature under an atmosphere of nitrogen. The solution was then poured onto water (100 ml) and extracted with diethyl ether (2 x 50 ml). The organic layers were combined, washed with water (50 ml), dried ($MgSO_4$) and evaporated to dryness to yield crude *(2S)*-4-azido-2-chlorobutan-1-ol methanesulphonate (90) as an oil; Rf = 0.41 (EtOAc); υ_{max} (thin film) 3020-2970, 2100 (strong), 1460, 1375 and 1185

cm^{-1}; δ_H ($CDCl_3$) 1.85-2.30 (2H, complex), 3.08 (3H, s), 3.60 (2H, t) and 4.36 (3H, complex).

Note: Further attempts to produce the desired diazide using more equivalents of sodium azide and higher reaction temperatures failed.

(2S)-Chlorobutane-1,4-diol di-trifluoromethanesulphonate

To a stirred solution of trifluoromethanesulphonic anhydride (0.54 ml; 3.2 mmol) in dichloromethane (20 ml), cooled to 0 °C, was added a mixture of (2S)-chlorobutane-1,4-diol (89) (0.2 g; 1.6 mmol) and pyridine (0.25 ml; 3.2 mmol) in dichloromethane (10 ml). After stirring for 30 min, allowing the temperature to rise to room temp., the solution was filtered, washed with water (3 x 20 ml) and dried over Na_2SO_4. The solvents were removed under reduced pressure, leaving the crude product as a black oil (77%) which decomposed on distillation; Rf = 0.11 (EtOAc); υ_{max} (thin film) 3050, 1420, 1240, 1140 and 600 cm^{-1}; δ_H ($CDCl_3$) 2.05-2.70 (2H, complex), 4.05-4.51 (1H, complex) and 4.10-4.45 (4H, complex); *m/z* 239 and 149 (100%).

Note: All attempts to convert the ditriflate into the corresponding azide met with failure.

(2S)-Bromosuccinic acid

(2S)-Aspartic acid (2.0 g; 15 mmol) and urea (0.2 g; 3.4 mmol) were dissolved in a mixture of concentrated hydrobromic acid (5 ml) and concentrated nitric acid (5 ml). Diethyl ether (10 ml) was then added and the solution was heated gently (30 °C) for 20 min. The mixture was cooled and extracted with diethyl ether (3 x 25 ml), dried ($MgSO_4$) and evaporated to leave an oily residue. The residue was crystallised from a minimum of diethyl ether, giving (2S)-bromosuccinic acid as a crystalline solid (42%); υ_{max} (KBr disc) 3430-2910, 1715, 1420, 940 and 650 cm^{-1}; δ_H ($CDCl_3$) 3.15 (2H, ABX, J_{AB} 17 Hz J_{AX} 7 Hz) and 4.59 (1H, t, J 7 Hz); *m/z* 180 and 27 (100%).

(2S)-Bromobutane-1,4-diol (91)

(2S)-Bromosuccinic acid (5.0 g; 25.4 mmol) was dissolved in dry THF (20 ml) and the solution was cooled to 0 °C. 1 M Borane.THF (82 ml; 82 mmol) was added dropwise over a period of 30 mins after which time the solution was allowed to warm to room temperature (1 h). Water (10 ml) was cautiously added and the organic layer was "salted out" using K_2CO_3. After separation, the aqueous layer was extracted with ethyl acetate (2 x 10 ml). The combined organic extracts were dried ($MgSO_4$) and evaporated to dryness to leave (2S)-bromobutane-1,4-diol (91) as a pale green oil (66%); $[\alpha]_D^{19}$ -12.6° (c 1.2, MeOH); υ_{max} (thin film) 3650-3100, 2950, 1410 and 1050 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 1.52-2.19 (2H, complex), 3.45 (2H, bs, OH), 3.72-3.86 (2H, complex) and 4.29 (1H, complex); δ_C ($CDCl_3$)

37.11 (t, $\underline{C}H_2CH_2OH$), 58.85 (t, $CH_2\underline{C}H_2OH$), 60.42 (d, $\underline{C}HBr$) and 66.67 (t, $CH(Br)\underline{C}H_2OH$); *m/z* 170 (^{81}Br M^+), 168 (M^+) and 31 (100%).

Dimethyl (2S)-hydroxybutane-1,4-dioate

(2S)-Malic acid (92) (15.0 g; 112 mmol) was dissolved in cooled (0 °C) methanol (100 ml) containing acetyl chloride (5 ml). The solution was allowed to warm to room temperature overnight after which time the solvent was removed *in vacuo.* The pure diester was obtained on distillation under reduced pressure (86%), b.p. 121-124 °C at 15 mmHg; $[\alpha]_D^{19}$ -29.4° (c 5, pyridine) {lit.,[120] $[\alpha]_D^{19}$ -28.3° (pyridine)}; υ_{max} ($CHCl_3$) 3545-3440, 3020, 2980, 1745 and 1445 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 2.69-2.74 (2H, complex), 3.59 (3H, s), 3.67 (3H, s), 4.44 (1H, complex) and 5.26-5.52 (1H, bs, OH); δ_C ($CDCl_3$) 38.18 (t, $\underline{C}H_2CO_2Me$), 51.74 (q, $CO_2\underline{Me}$), 52.44 (q, $CO_2\underline{Me}$), 66.98 (d, $\underline{C}HOH$), 170.93 (s, $\underline{C}O_2Me$) and 171.09 (s, $\underline{C}O_2Me$); *m/z* 132, 103 and 43 (100%).

Dimethyl (2R)-fluorobutane-1,4-dioate (93)[113]

To a stirred solution of diethylaminosulphur trifluoride (DAST) (2.5 g; 15.5 mmol) in dry ethanol-free chloroform (20 ml), cooled to 0 °C, was added dropwise a solution of dimethyl (2S)-hydroxybutane-1,4-dioate (153) (2.5 g; 15.5 mmol) in ethanol-free chloroform (20 ml). The mixture was allowed to reach room temperature and water (20 ml) was then added to the vigorously stirred solution. The organic

layer was separated, washed with $NaHCO_3$ solution then brine and dried over $MgSO_4$. The solvent was evaporated, leaving the crude product as a yellow oil (67.2 %); $[\alpha]_D^{19}$ +9.4° (c 5, pyridine); υ_{max} ($CHCl_3$) 2950, 1755 and 1455 cm^{-1}; δ_H (250 MHz, $CDCl_3$) 2.91-2.99 (2H, dd $^3J_{HF}$ 24 Hz J_{HH} 6 Hz), 3.67 (3H, s), 3.77 (3H, s), 5.19 and 5.36 (1H, dt $^2J_{HF}$ 48 Hz J_{HH} 6 Hz); *m/z* 165 (M^+ + 1), 145 and 131 (100%).

(2R)-Fluorosuccinic acid (94)[113]

Dimethyl (2R)-fluorobutane-1,4-dioate (93) (1.64 g; 10 mmol) was heated at reflux in 5% sulphuric acid (10 ml) for 25 h. The solution was then made strongly acid by the addition of concentrated sulphuric acid (10 ml) and extracted with diethyl ether (2 x 20 ml). The solution was dried and the solvent removed to leave (2R)-fluorosuccinic acid (94) as a white solid that was recrystallised from ethyl acetate (65%); $[\alpha]_D^{19}$ -39.1 (c 1, H_2O); υ_{max} (KBr disc) 3160-2850, 1725 and 1420 cm^{-1}; δ_H ($CDCl_3$) 2.69-3.00 (2H, complex) and 5.21 and 5.35 (1H, dt); *m/z* 136 (M^+) and 91 (100%).

Synthesis of 2,3-Dihaloputrescines

cis-*1,4-Dibromobut-2-ene*

Phosphorus tribromide (5 ml; 53 mmol) was cooled to 0 °C and *cis*-1,4-but-2-enediol (97) (6 g; 68 mmol) was added slowly over a 2 h period. The dark mixture which formed was stirred overnight. The

mixture was cooled to 0 °C and water (50 ml) was added slowly to hydrolyse the excess phosphorus tribromide. The biphasic system was separated and diethyl ether (50 ml) was added to the organic layer. After washing with $NaHCO_3$ solution (3 x 20 ml) and drying over Na_2SO_4 the ether was evaporated to give *cis*-1,4-dibromobut-2-ene as a colourless oil (72 %); υ_{max} (thin film) 2950, 1630 and 690 cm^{-1}; δ_H ($CDCl_3$) 4.10 (4H, d) and 5.97 (2H, complex); *m/z* 133 and 104 (100%).

cis-*1,4-Diphthalimidobut-2-ene (98)*

cis-1,4-Dibromobut-2-ene (4.28 g; 20 mmol) was stirred in DMF (75 ml) and to this was added, in portions, potassium phthalimide (8.0 g; 43 mmol). The suspension was stirred at room temperature for 48 h then poured onto water (75 ml) and extracted with dichloromethane (7 x 100 ml). The dichloromethane was removed under reduced pressure, leaving behind residual DMF from which *cis*-1,4-diphthalimidobut-2-ene (98) precipitated. The solid was filtered, washed free of DMF with diethyl ether (20 ml) and dried (78 %); υ_{max} (KBr disc) 3200-2950, 1765, 1710 and 720 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 4.56 (4H, d), 5.70 (2H, complex) and 7.78 (8H, complex); δ_C ($CDCl_3$) 34.61 (t, $N\underline{C}H_2$), 123.30 (d, Ar-C), 123.61 (d, Ar-C), 127.64 (d, $CH_2\underline{C}H$), 132.43 (s, Ar-C), 133.95 (d, Ar-C), 134.33 (d, Ar-C) and 167.87 (s, $N\underline{C}O$); *m/z* 204 and 28 (100%).

trans-*1,4-Diphthalimidobut-2-ene*

trans-1,4-Dibromobut-2-ene was converted into *trans*-1,4-diphthalimidobut-2-ene (155) according to the above procedure (75%); υ_{max} (KBr disc) 3100-2950, 1765, 1710 and 730 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 4.23 (4H, d), 5.77 (2H, complex) and 7.76 (8H, complex); δ_C ($CDCl_3$) 38.68 (t, $N\underline{C}H_2$), 123.27 (d, Ar-C), 127.28 (d, $CH_2\underline{C}H$), 132.01 (s, Ar-C), 133.93 (d, Ar-C) and 167.72 (s, $N\underline{C}O$); *m/z* 201 and 199 (100%).

(±)-2,3-Dibromo-1,4-diphthalimidobutane (99)

cis-1,4-Diphthalimidobut-2-ene (98) (0.5 g; 1.5 mmol) and bromine (0.24 g; 1.5 mmol) were dissolved in dry dichloromethane (50 ml) and the solution was heated overnight at reflux. The solvent was removed under reduced pressure, leaving (±)-2,3-dibromo-1,4-diphthalimidobutane (99) as a white crystalline solid (80%); υ_{max} (KBr disc) 1770, 1710, 1400 and 710 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 4.05-4.36 (4H, complex), 4.69-4.82 (2H, complex) and 7.79 (8H, complex); δ_C ($CDCl_3$,) 43.62 (t, $N\underline{C}H_2$), 50.71 (d, $\underline{C}HBr$), 123.67 (d, Ar-C), 131.64 (s, Ar-C), 134.34 (d, Ar-C), 167.74 (s, $N\underline{C}O$) and 43.19, 51.17, 123.50, 131.64, 133.97 and 167.74; *m/z* 426, 424 and 76 (100%).

meso-*2,3-Dibromo-1,4-diphthalimidobutane*

According to the above procedure *trans*-1,4-diphthalimidobut-2-ene was converted into *meso*-2,3-dibromo-1,4-diphthalimidobutane (83%); υ_{max} (KBr disc) 1770, 1710, 1400 and 710 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 4.02-4.39 (4H, complex), 4.73 (2H, complex) and 7.79 (8H, complex); δ_C ($CDCl_3$) 43.61 (t, N<u>C</u>H_2), 50.72 (d, <u>C</u>HBr), 123.67 (d, Ar-C), 131.63 (s, Ar-C), 134.34 (d, Ar-C) and 167.73 (s, N<u>C</u>O); *m/z* 426, 424 and 76 (100%).

(±)-2,3-Dichloro-1,4-diphthalimidobutane (101)

cis-1,4-Diphthalimidobut-2-ene (98) (0.5 g; 1.45 mmol) was dissolved in dry dichloromethane (20 ml). Chlorine gas was then bubbled through until the solution became green solution, indicating that saturation had been achieved. The solution was then heated to reflux for 16 h after which time the solvent was removed leaving crude (±)-2,3-dichloro-1,4-diphthalimidobutane (101) as a white solid (94%); υ_{max} (KBr disc) 3570-3340, 1775, 1720, 1400 and 720 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 3.76-4.41 (4H, complex), 4.69 (2H, complex) and 7.79 (8H, complex); δ_C ($CDCl_3$) 40.34 (t, N<u>C</u>H_2), 59.92 (d, <u>C</u>HBr), 123.52 (d, Ar-C), 131.69 (s, Ar-C), 134.15 (d, Ar-C), 168.85 (s, N<u>C</u>O) and 41.20, 71.19, 123.71, 131.96, 134.39 and 168.85; *m/z* 382, 380 and 160 (100%).

meso-*2,3-Dichloro-1,4-diphthalimidobutane*

According to the above procedure *trans*-1,4-diphthalimidobut-2-ene was converted into *meso*-2,3-dichloro-1,4-diphthalimidobutane (96%); υ_{max} (KBr disc) 3570-3340, 1775, 1720, 1400 and 720 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 3.92-4.40 (6H, complex) and 7.80 (8H, complex); δ_C ($CDCl_3$) 40.52 (t, N$\underline{C}H_2$), 59.90 (d, $\underline{C}$HBr), 123.50 (d, Ar-C), 131.65 (s, Ar-C), 134.15 (d, Ar-C), 168.84 (s, N$\underline{C}$O); *m/z* 382, 380 and 160 (100%).

General Procedure (I) for Cleavage of a Diphthalimide to Yield a Diamine Dihydrochloride

A suspension of the diphthalimide (2.3 mmol) in concentrated hydrochloric acid (15 ml) and glacial acetic acid (15 ml) was heated at 130 °C for 30 h. The reaction mixture was allowed to cool and the precipitated phthalic acid was removed by filtration. The filtrate was concentrated to a volume of 5 ml and further phthalic acid was filtered off. Glacial acetic acid (25 ml) was then added and the dihydrochloride salt was seen to precipitate on addition of diethyl ether (25 ml).

(±)-2,3-Dibromoputrescine dihydrochloride (96)

According to general procedure (I) (±)-2,3-dibromo-1,4-diphthalimidobutane (99) gave (±)-2,3-dibromoputrescine in the form of the dihydrochloride salt (96) (65%); m.p. > 250 °C; R_f = 0.61

[methanol, isopropylalcohol, ammonia (9:7:4)]; υ_{max} (KBr disc) 3540-3350, 3150-2950, 1605, 1510 and 875 cm^{-1}; δ_H (200 MHz, D_2O) 3.21-3.80 (4H, complex) and 4.37-4.56 (2H, complex); δ_C (D_2O, with dioxan as reference at 67.4 ppm) 45.35 (t, N$\underline{C}H_2$), 50.68 (d, $\underline{C}$HBr) and 44.58, 59.12; *m/z* 167, 165 and 30 (100%).

meso-*2,3-Dibromoputrescine dihydrochloride (106)*

According to general procedure (I) *meso*-2,3-dibromo-1,4-diphthalimidobutane gave *meso*-2,3-dibromoputrescine in the form of the dihydrochloride salt (106) (61%); m.p. > 250 °C; Rf = 0.65 [methanol, isopropylalcohol, ammonia (9:7:4)]; υ_{max} (KBr disc) 3420-3300, 1970, 1600, 1520 and 880 cm^{-1}; δ_H (200 MHz, D_2O) 3.29-3.73 (4H, complex) and 4.41-4.49 (2H, complex); δ_C (D_2O, with dioxan as reference at 67.4 ppm) 45.36 (t, N$\underline{C}H_2$) and 50.67 (d, $\underline{C}$HBr); *m/z* 150, 148 and 30 (100%).

(±)-2,3-Dichloroputrescine dihydrochloride (100)

According to general procedure (I) (±)-2,3-dichloro-1,4-diphthalimidobutane (101) gave (±)-2,3-dichloroputrescine in the form of the dihydrochloride salt (100) (52%); m.p. > 250 °C; υ_{max} (KBr disc) 3400-3000, 1980, 1600, 1520 and 675 cm^{-1}; δ_H (D_2O) 3.11-3.72 (4H, complex) and 4.27-4.42 (2H, complex); *m/z* 120 and 30 (100%).

meso-*2,3-Dichloroputrescine dihydrochloride (107)*

According to general procedure (I) *meso*-2,3-dichloro-1,4-diphthalimidobutane gave *meso*-2,3-dichloroputrescine in the form of the dihydrochloride salt (107) (59%); m.p. > 250 ^{o}C; υ_{max} (KBr disc) 3400-3130, 1975, 1600, 1520 and 680 cm^{-1}; δ_H (D_2O, 200 MHz) 3.09-3.74 (4H, complex) and 4.38-4.44 (2H, complex); δ_C (D_2O, with dioxan as reference at 67.4 ppm) 44.06 (t, N$\underline{C}H_2$) and 58.84 (d, $\underline{C}$HBr); *m/z* 120 and 30 (100%).

8.4 EXPERIMENTAL TO CHAPTER 6

Synthesis of Pyridine Amines

(±)-2-Hydroxy-2-(2'-pyridyl)ethylamine dihydrochloride (112)

(±)-2-Hydroxy-2-(2'-pyridyl)nitroethane

2-Pyridinecarboxaldehyde (1.0 g, 9.35 mmol) was dissolved in isopropanol (10 ml) and to this solution was added KF (25 mg) and nitromethane (1.12 ml, 18.7 mmol). After stirring at room temperature for 3h the solution was heated to 40 °C for a further 1h. The solvent was removed *in vacuo* to yield (±)-2-hydroxy-2-(2'-pyridyl)nitroethane as a red oil. Purification on a silica gel column (hexane/ethyl acetate 2:3) produced the pure product as a orange solid (86%); υ_{max} (KBr disc) 3600-2950, 1600, 1550 (strong), 1435 and 1380 (strong) cm^{-1}; δ_H (200 MHz, $CDCl_3$) 4.67 (2H, ABX, J_{AB} 14 Hz J_{AX} 4 Hz J_{BX} 8 Hz), 5.43 (1H, dd, J_{AX} 4 Hz J_{BX} 8 Hz) and 7.22-8.72 (4H, complex); δ_C ($CDCl_3$) 70.35 (d, $\underline{C}HOH$), 80.71 (t, $\underline{C}H_2NO_2$), 120.86 (d, Ar-C), 123.23 (d, Ar-C), 137.27 (d, Ar-C), 148.85 (d, Ar-C) and 156.67 (s, Ar-C); *m/z* 168 (M^+), 121, 103 and 36 (100%).

(±)-2-Hydroxy-2-(2'-pyridyl)ethylamine dihydrochloride (112)

2-Hydroxy-2-(2'-pyridyl)nitroethane (0.5 g, 2.98 mmol) was dissolved in methanol (50 ml) . To this was added 10% Pd/C catalyst (0.2 g) and the mixture was hydrogenated at *ca.* 60 p.s.i. at 25 °C for

18h. The solution was then filtered through a Celite pad and 5 M hydrochloric acid (25 ml) was added. Evaporation of the solvent *in vacuo* gave the crude dihydrochloride salt (112) which was recrystallised from ethanol and acetone (90%); m.p. > 250 °C; υ_{max} (KBr disc) 3620, 3100-2650, 1640 and 1580 cm^{-1}; δ_H (200 MHz, D_2O) 3.59 (2H, ABX, J_{AB} 14 Hz J_{AX} 4 Hz J_{BX} 8 Hz), 5.54 (1H, dd, J_{AX} 4 Hz J_{BX} 8 Hz) and 7.82-8.61 (4H, complex); δ_C (D_2O) 55.02 (t, $\underline{C}H_2NH_2$), 64.33 (d, $\underline{C}HOH$), 125.64 (d, Ar-C), 127.60 (d, Ar-C), 142.10 (d, Ar-C), 148.02 (d, Ar-C) and 154.56 (s, Ar-C); *m/z* 139 (M^++1), 109 and 36 (100%); (Found: C, 39.80; H, 5.62; N, 13.28. $C_7H_{12}N_2OCl_2$ requires C, 39.81; H, 5.69; N, 13.27%).

(±)-2-Hydroxy-2-(3'-pyridyl)ethylamine dihydrochloride (111)

(±)-2-Hydroxy-2-(3'-pyridyl)nitroethane (108)

3-Pyridinecarboxaldehyde (1.0 g, 9.35 mmol) was dissolved in isopropanol (10 ml) and to this solution was added KF (25 mg) and nitromethane (1.12 ml, 18.7 mmol). The mixture was stirred overnight at room temperature after which time the solution had turned yellow. The solvent was removed *in vacuo* leaving (±)-2-hydroxy-2-(3'-pyridyl)nitroethane (108) as a yellow solid which was used without further purification(98%); υ_{max} (KBr disc) 3610-3150, 2980-2810, 1600, 1575 (strong), 1380 (strong) and 620cm^{-1}; δ_H (200 MHz, $CDCl_3$) 4.60 (2H, ABX, J_{AB} 13 Hz J_{AX} 4 Hz J_{BX} 9 Hz), 5.43 (1H, dd, J_{AX} 4 Hz J_{BX} 9 Hz) and 7.76-8.58 (4H, complex); *m/z* 168 (M^+), 121 and 36 (100%).

(±)-2-Hydroxy-2-(3'-pyridyl)ethylamine dihydrochloride (111)

(±)-2-Hydroxy-2-(3'-pyridyl)nitroethane (108) (1.2 g, 7.15 mmol) was dissolved in methanol (120 ml). To this was added 10% Pd/C catalyst (0.48 g) and the mixture was hydrogenated at *ca.* 60 p.s.i. at 25 °C for 14h. The solution was then filtered through a Celite pad and 5 M hydrochloric acid (50 ml) was then added. Evaporation of the solvent gave the crude dihydrochloride salt (111) which was recrystallised from ethanol and acetone (78%); m.p. > 250 °C; υ_{max} (KBr disc) 3490-3280, 3050-2740, 1630, 1600 and 640 cm^{-1}; δ_H (200 MHz, D_2O) 3.24 (2H, ABX, J_{AB} 13 Hz J_{AX} 4 Hz J_{BX} 9 Hz), 5.15 (1H, dd, J_{AX} 4 Hz J_{BX} 9 Hz) and 7.94 (1H, complex), 8.51 (1H, d, with fine splitting), 8.61 (1H, d, with fine splitting) and 8.72 (1H, s, with fine splitting); δ_C (D_2O) 45.40 (t, $\underline{C}H_2NH_2$), 67.17 (d, $\underline{C}HOH$), 128.35 (d, Ar-C), 140.00 (d, Ar-C), 141.55 (s, Ar-C), 141.88 (d, Ar-C) and 145.52 (d, Ar-C); *m/z* 139 (M^++1), 109 and 36 (100%); (Found: C, 39.69; H, 5.57; N, 13.34. $C_7H_{12}N_2OCl_2$ requires C, 39.81; H, 5.69; N, 13.27%).

(±)-2-Hydroxy-2-(4'-pyridyl)ethylamine dihydrochloride (113)

(±)-2-Hydroxy-2-(4'-pyridyl)nitroethane

4-Pyridinecarboxaldehyde (2.0 g, 18.7 mmol) was dissolved in isopropanol (20 ml) and to this solution was added KF (25 mg) and nitromethane (2.24 ml, 37.4 mmol). The mixture was stirred overnight at room temperature giving rise to a yellow solution. The solvent was removed *in vacuo* leaving (±)-2-hydroxy-2-(4'-

pyridyl)nitroethane as a yellow solid which was used without further purification (90%); υ_{max} (KBr disc) 3550-2720, 1605, 1550 (strong), 1380 (strong) and 610 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 4.56 (2H, ABX, J_{AB} 13 Hz J_{AX} 4 Hz J_{BX} 9 Hz), 5.47 (1H, dd, J_{AX} 4 Hz J_{BX} 9 Hz), 7.37 (2H, d, with fine splitting) and 8.52 (2H, d, with fine splitting); δ_C ($CDCl_3$) 69.47 (d, $\underline{C}HOH$), 81.04 (t, $\underline{C}H_2NO_2$), 121.12 (d, Ar-C) 148.63 (s, Ar-C) and 149.92; *m/z* 168 (M^+), 121 and 36 (100%).

(±)-2-Hydroxy-2-(4'-pyridyl)ethylamine dihydrochloride (113)

2-Hydroxy-2-(4'-pyridyl)nitroethane (1.0 g, 5.96 mmol) was dissolved in methanol (75 ml). To this was added 10% Pd/C catalyst (0.4 g) and the mixture was hydrogenated at *ca.* 40 p.s.i. at 25 °C for 25h. The solution was then filtered through a Celite pad and 5 M hydrochloric acid (40 ml) was added. Evaporation of the solvent *in vacuo* gave the crude dihydrochloride salt (113) which was recrystallised from ethanol and acetone (90%); m.p. > 250 °C; υ_{max} (KBr disc) 3480-3260, 3100-2850, 1630, 1610, 1460 and 680 cm^{-1}; δ_H (200 MHz, D_2O) 3.15 (2H, ABX, J_{AB} 13 Hz J_{AX} 4 Hz J_{BX} 9 Hz), 5.16 (1H, dd, J_{AX} 4 Hz J_{BX} 9 Hz), 7.98 (2H, d, with fine splitting) and 8.63 (2H, d, with fine splitting); δ_C (D_2O) 45.03 (t, $\underline{C}H_2NH_2$), 68.77 (d, $\underline{C}HOH$), 125.40 (d, Ar-C), 142.28 (d, Ar-C) and 161.84 (s, Ar-C); *m/z* 139 (M^++1) and 109 (100%); (Found: C, 39.87; H, 5.59; N, 13.21. $C_7H_{12}N_2OCl_2$ requires C, 39.81; H, 5.69; N, 13.27%).

Attempted Synthesis of 2-(3'-pyridyl)ethylamine dihydrochloride (110)

Stage 1 : Elimination Reactions on (±)-2-hydroxy-2-(3'-pyridyl)nitroethane (108)

2-(3'-Pyridyl)nitroethene (109)

1st Attempt

A solution of 2-hydroxy-2-(3'-pyridyl)nitroethane (108) (0.1 g, 0.6 mmol) in pyridine (6 ml) was cooled to 0 °C and treated dropwise with $POCl_3$ (1.0 ml, 10.7 mmol). The mixture was allowed to stir overnight at room temperature. At the end of this period the mixture was decomposed by the cautious addition of water (5 ml). The solution was extracted with diethyl ether (2 x 10 ml) and washed with water (2 x 10 ml) then brine (2 x 10 ml). After drying ($MgSO_4$) the solution was evaporated leaving a dark, sticky solid which was insoluble in all common solvents. TLC (ethyl acetate) showed the presence of at least 5 compounds. Further purification was not undertaken.

2nd Attempt

2-Hydroxy-2-(3'-pyridyl)nitroethane (108) (0.2 g, 1.2 mmol) was dissolved in dry THF (15 ml) containing activated molecular sieves and the solution was heated at reflux overnight. The solution was then cooled and 5 M hydrochloric acid was added. The stirred solution was again refluxed for a further 4h. After cooling the

solution was filtered and evaporated to dryness under reduced pressure leaving only the hydrochloride salt of the starting material (108). (Found: C, 41.02; H, 4.45; N, 13.60. $C_7H_9N_2O_3Cl$ requires C, 41.08; H, 4.40; N, 13.69%).

3rd Attempt

2-Hydroxy-2-(3'-pyridyl)nitroethane (108) (0.2 g, 1.2 mmol) was dissolved in THF (15 ml) and NaOH (10 ml, 5 M) was then added. This solution was then refluxed for 20h, cooled and evaporated leaving starting material (108) as the only detectable product.

4th Attempt

2-Hydroxy-2-(3'-pyridyl)nitroethane (108) (0.5 g, 2.98 mmol) was dissolved in acetone (15 ml) and to this was added acetic anhydride (0.31 g, 3.04 mmol) and DMAP (5 mg). After stirring at room temperature for 16h, the dark solution was filtered and evaporated under reduced pressure leaving a black solid. Chromatographic purification on a silica gel column (hexane/ethyl acetate 1:1) gave a dark green solid believed to be 2-(3'-pyridyl)nitroethene (109) (65%); R_f = 0.71 (EtOAc); δ_H ($CDCl_3$) 7.29-8.69 (complex).

[Note : On standing for *ca.* 10 minutes at room temperature the green solid decomposed leaving a black tar which was insoluble in all common organic solvents. This prevented any further analysis being carried out on the solid.]

Stage 2 : Catalytic Hydrogenation of 2-(3'-pyridyl)nitroethene (109)

2-(3'-Pyridyl)ethylamine dihydrochloride (110)

[Note : A dilute solution of 2-(3'-pyridyl)nitroethane (109) in absolute alcohol was used immediately after purification.]

A solution of 2-(3'-pyridyl)nitroethene (109) (0.2 g, 1.33 mmol) in absolute alcohol (100 ml) was hydrogenated at *ca.* 40 p.s.i. at 25 °C for 18h. At the end of this period the dark solution was filtered and 5 M hydrochloric (40 ml) was added. Evaporation of the solvent to dryness left a black tar which could not be purified.

[Note : Identical problems were encountered in an attempted synthesis of 2-(4'-pyridyl)ethylamine by the procedure outlined above].

General Procedure (I) for the Conversion of Aldehydes into Nitriles[115]

A solution of the aldehyde (4.67 mmol) and hydroxylamine hydrochloride (5 mmol) in 98% formic acid (10 ml) was heated at reflux for 2h. The mixture was then diluted with ice water (50 ml), neutralised with 5% NaOH solution and extracted with diethyl ether (2 x 30 ml). After drying ($MgSO_4$) the solution was evaporated to

dryness leaving the product as a white solid which was recrystallised from toluene.

2-Cyanopyridine (114)

Using general procedure (I), 2-pyridinecarboxaldehyde gave 2-cyanopyridine (114) as a white crystalline solid (77%); m.p. 25-26 °C; υ_{max} (KBr disc) 2950, 2235 (strong), 1590 and 1510 cm^{-1}; δ_H ($CDCl_3$) 7.52-8.16 (3H, complex) and 8.72-8.91 (1H, complex).

3-Cyanopyridine

Using general procedure (I), 3-pyridinecarboxaldehyde gave 3-cyanopyridine as a white crystalline solid (62%); m.p. 51-52 °C; υ_{max} (KBr disc) 2960, 2230 (strong), 1600 and 1520 cm^{-1}; δ_H ($CDCl_3$) 7.43-7.58 (1H, complex), 8.02-8.18 (1H, complex) and 8.78-8.94 (2H, complex).

4-Cyanopyridine

Using general procedure (I), 4-pyridinecarboxaldehyde gave 4-cyanopyridine as a white crystalline solid (71%); m.p. 77-79 °C; υ_{max} (KBr disc) 2960, 2230 (strong), 1600 and 1520 cm^{-1}; δ_H ($CDCl_3$) 7.60 (2H, d, with fine splitting), 8.96 (2H, d, with fine splitting).

General Procedure (J) for the Reduction of a Nitrile to a Diamine[116]

Raney nickel (0.5 g) was added to a solution of 2-cyanopyridine (1 g, 9.63 mmol) in a mixture of 95% ethanol (20 ml) and concentrated ammonium hydroxide (5 ml). The mixture was then hydrogenated for 24h at room temperature at *ca.* 60 p.s.i. after which time the catalyst was filtered and washed with ethanol. The solution was carefully acidified (5 M HCl, 0 °C) and the solvents were removed *in vacuo* to leave the diamine dihydrochloride salt as a white solid which was recrystallised from ethanol and acetone.

(2'-Pyridyl)methylamine dihydrochloride (115)

2-Cyanopyridine (114) was reduced to the corresponding diamine dihydrochloride (115) using general procedure (J). The product was isolated as a white crystalline solid (44%); m.p. > 250 °C; υ_{max} (KBr disc) 3340-3200, 2950, 1605, 1575 and 1490 cm^{-1}; δ_H (D_2O) 4.61 (2H, s), 8.19-8.65 (3H, complex) and 8.92 (1H, complex); *m/z* 109 (M^+ + 1) and 80 (100%); (Found: C, 39.79; H, 5.49; N, 15.40. $C_6H_4N_2Cl_2$ requires C, 39.78; H, 5.52; N, 15.47%).

(3'-Pyridyl)methylamine dihydrochloride (116)

3-Cyanopyridine was reduced to the corresponding diamine dihydrochloride (116) using general procedure (J). The product was isolated as a white crystalline solid (51%); m.p. > 250 °C; υ_{max} (KBr

disc) 3290-3170, 2950, 1610, 1575 and 1490 cm^{-1}; δ_H (200 MHz, D_2O) 4.49 (2H, s), 8.13 (1H, dd), 8.76 (1H, d), 8.86 (1H, d) and 9.00 (1H, s); δ_C (D_2O, with dioxan as reference at 67.4 ppm) 40.66 (t, $\underline{C}H_2N$), 128.81 (d, Ar-C), 133.77 (s, Ar-C), 142.71 (d, Ar-C) and 148.90 (d, Ar-C); *m/z* 109 ($M^+ + 1$) and 80 (100%); (Found: C, 39.84; H, 5.44; N, 15.36. $C_6H_4N_2Cl_2$ requires C, 39.78; H, 5.52; N, 15.47%).

(4'-Pyridyl)methylamine dihydrochloride (117)

4-Cyanopyridine was reduced to the corresponding diamine dihydrochloride (117) using general procedure (J). The product was isolated as a white crystalline solid (55%); m.p. > 250 °C; υ_{max} (KBr disc) 3390-2960, 1620, 1580 and 1485 cm^{-1}; δ_H (200 MHz, D_2O) 4.59 (2H, s), 8.15 (2H, d, with fine splitting) and 8.86 (2H, d, with fine splitting); δ_C (D_2O, with dioxan as reference at 67.4 ppm) 42.83 (t, $\underline{C}H_2N$), 127.54 (d, Ar-C), 142.55 (d, Ar-C), and 154.28 (s, Ar-C); *m/z* 109 ($M^+ + 1$) and 80 (100%); (Found: C, 39.72; H, 5.42; N, 15.58. $C_6H_4N_2Cl_2$ requires C, 39.78; H, 5.52; N, 15.47%).

General Procedure (K) for the Conversion of Diamines into Diamine Dihydrochlorides

The diamine (2 mmol) was partitioned between dichloromethane (80 ml) and 1M hydrochloric acid (80 ml). The aqueous layer was further washed with dichloromethane (2 x 80 ml). The aqueous layer was evaporated to dryness under reduced pressure leaving the diamine

dihydrochloride which was recrystallised from aqueous ethanol (95%) and acetone.

Putrescine (5) dihydrochloride

Using butane-1,4-diamine, the corresponding dihydrochloride was obtained as a white crystalline solid after recrystallisation (87%); m.p > 250 °C; υ_{max} (KBr disc) 3400-3300, 3100-2900, 2040, 1485 and 1450 cm^{-1}; δ_H (D_2O) 1.60-1.90 (4H, complex) and 2.95-3.10 (4H, complex); *m/z* 89 (M^++1), 88 (M^+) and 30 (100%); (Found: C, 29.82; H, 8.84; N, 17.49. $C_4H_{14}N_2Cl_2$ requires C, 29.81; H, 8.70; N, 17.39%).

2-(2'-Pyridyl)ethylamine dihydrochloride (118)

Using 2-(2'-pyridyl)ethylamine, the corresponding dihydrochloride (118) was obtained as a white crystalline solid after recrystallisation (77%); m.p. > 250 °C; υ_{max} (KBr disc) 3180-2950, 1605, 1575 and 1480 cm^{-1}; δ_H (200 MHz, D_2O) 3.39 (4H, s, with fine splitting), 7.90 (2H, complex), 8.45 (1H, complex) and 8.64 (1H, complex); δ_C (D_2O, with dioxan as reference at 67.4 ppm) 31.49 (t, $\underline{C}H_2CH_2N$), 38.67 (t, $CH_2\underline{C}H_2N$), 126.94 (d, Ar-C), 128.85 (d, Ar-C), 132.46 (d, Ar-C), 148.43 (d, Ar-C) and 151.91 (s, Ar-C); *m/z* 123 (M^++1), 122 (M^+) and 93 (100%); (Found: C, 43.07; H, 6.11; N, 14.39. $C_7H_{12}N_2Cl_2$ requires C, 43.08; H, 6.15; N, 14.36%).

(4'- Piperidyl)methylamine dihydrochloride (119)

Using 4-(aminomethyl)piperidine, the corresponding dihydrochloride (119) was obtained as a white crystalline solid after recrystallisation (77%); m.p. > 250 °C; υ_{max} (KBr disc) 3190-3090, 2960, 1590 and 1550cm^{-1}; δ_H (200 MHz, D_2O) 1.34 (2H, complex), 1.84 (3H, complex), 2.85 (4H, complex) and 3.27 (2H, complex); δ_C (D_2O, with dioxan as reference at 67.4 ppm) 26.47 (t, ring $\underline{C}H_2CH_2N$), 32.28 (d, ring $\underline{C}H$), 44.12 (t, $\underline{C}H_2N$) and 44.47 (t, $\underline{C}H_2N$); *m/z* 115 (M^++1), 114 (M^+) and 56 (100%); (Found: C, 38.60; H, 8.53; N, 14.91. $C_6H_{16}N_2Cl_2$ requires C, 38.50; H, 8.56; N, 14.97%).

8.5 EXPERIMENTAL TO CHAPTER 7

Purification of Pea Seedling Diamine Oxidase

Step 1

Pea seeds (500 g) were soaked in tap water for 18-24 h and then sown into vermiculite and allowed to germinate and grow for a period of 9-12 days. At the end of this period the stems were collected, weighed into bags (50 g) and frozen until required.

Step 2

Frozen pea epicotyls (50 g) were added to 0.1 M, pH 6.4 potassium phosphate buffer (100 ml) and homogenised in a pre-cooled Waring blender. The juice was then squeezed through muslin (2 layers) and centrifuged at 12000 g for 15 min. The inactive precipitate was discarded, leaving a total volume of 112 ml which was held at $< 5\ ^{o}C$.

Step 3

The crude buffered enzyme preparation (112 ml) was placed in a beaker and to this was added dropwise, with stirring, 5% protamine sulphate solution (1 ml; 50 mg). After the addition was complete stirring was continued for a further 15 min. The precipitate was removed by centrifugation (12000 g for 15 min) leaving 112 ml of the enzyme preparation which was kept between 0 ^{o}C and 5 ^{o}C.

Step 4

The buffered material (112 ml) was placed in a beaker and taken to 35% saturation by the addition of solid ammonium sulphate (23.4 g; 209 mg/ml). The inactive precipitate was then removed by centrifugation (12000 g for 15 min) leaving 120 ml of the enzyme preparation. This supernatant was placed in a beaker and taken to 65% saturation by the addition of further ammonium sulphate (24.0 g; 200 mg/ml). After centrifugation (12000 g for 15 min) the active precipitate was dissolved in a minimum amount of 20 mM, pH 6.4 potassium phosphate buffer (1.5 ml) and applied onto a G25 Sephadex column. The column was elueted with 20 mM, pH 6.4 potassium phosphate buffer. The material being washed from the column was monitored by UV (254 nm). The activity was collected in a single fraction (3.6 ml).

Step 5

The remaining volume (3.6 ml) was placed on a Celex P (cellulose phosphate; cationic exchange) column and eluted with 20 mM, pH 6.4 potassium phosphate buffer. Continuous elution with this buffer produced a protein-containing fraction which showed no appreciable activity. The column was then eluted with 0.1 M, pH 7.0 potassium phosphate buffer with a further three protein-containing fractions being collected in chilled measuring cylinders, the first two of which were found to be active. These fractions were combined giving a total volume of 24.1 ml. This was concentrated to a volume of 4.9 ml (Amicon cell) adjusted to pH 6.4 and held at $< 5\ ^{o}C$

Step 6 (FPLC)

A mono-S (cationic exchange) column was allowed to equilibrate in potassium phosphate buffer, 20 mM (pH 6.4) for a period of 30 min. The chilled enzyme preparation (4.9 ml) was then injected onto the column which was continuously eluted with 20 mM, pH 6.4 potassium phosphate buffer. The inactive protein which was washed from the column was collected as a single fraction. The percentage of 1 M potassium chloride (in phosphate buffer) in the eluant was then allowed to increase to 15% over a 3 min period. After eluting the column with this mixture for a further 3 min the percentage of potassium chloride solution in the eluant was increased to 35% over a 15 min period after which time the level of potassium chloride solution was rapidly increased to 100% (1 min). A fraction (1 ml) was collected every minute by means of an auto-sampler. Scan 7.2 indicates the separation achieved using the above method.

Re-application :-

The active fractions were combined and diluted using de-ionised water (20 ml). The solution was then concentrated (Amicon cell) to a volume of *ca.* 5 ml after which further de-ionised water (20 ml) was added. Concentration as before left 4.6 ml of the buffered enzyme solution which was ajusted to pH 6.4 and re-applied to the column. Continuous elution of the column with 20 mM phosphate buffer, pH 6.4 failed to remove any inactive protein. The level of 1 M potassium chloride solution in the eluant was then allowed to rise to 20% over a 15 min interval. The column was washed with this eluant for a further 5 min period after which the percentage of 1 M potassium

chloride solution was increased to 100%. The principal activity was located in two fractions which were combined. Scan 7.3 gives an indication of the separation achieved using the above method.

Appendix 1

See Experimental Section for method

Table 1 (a): Standard Protein Concentrations and A_{620}/A_{465} Readings (1)

Protein Content (mg)	A_{465}	A_{620}	A_{620}/A_{465} - A_{620}/A_{465} (Blank)
50	0.317	0.971	2.54
40	0.366	0.886	1.90
30	0.476	0.783	1.12
25	0.512	0.740	0.92
20	0.526	0.623	0.66
15	0.555	0.638	0.63
10	0.606	0.550	0.39
5	0.637	0.479	0.23
Blank	0.613	0.320	0.00

Appendix 1 (contd)

See Experimental Section for method

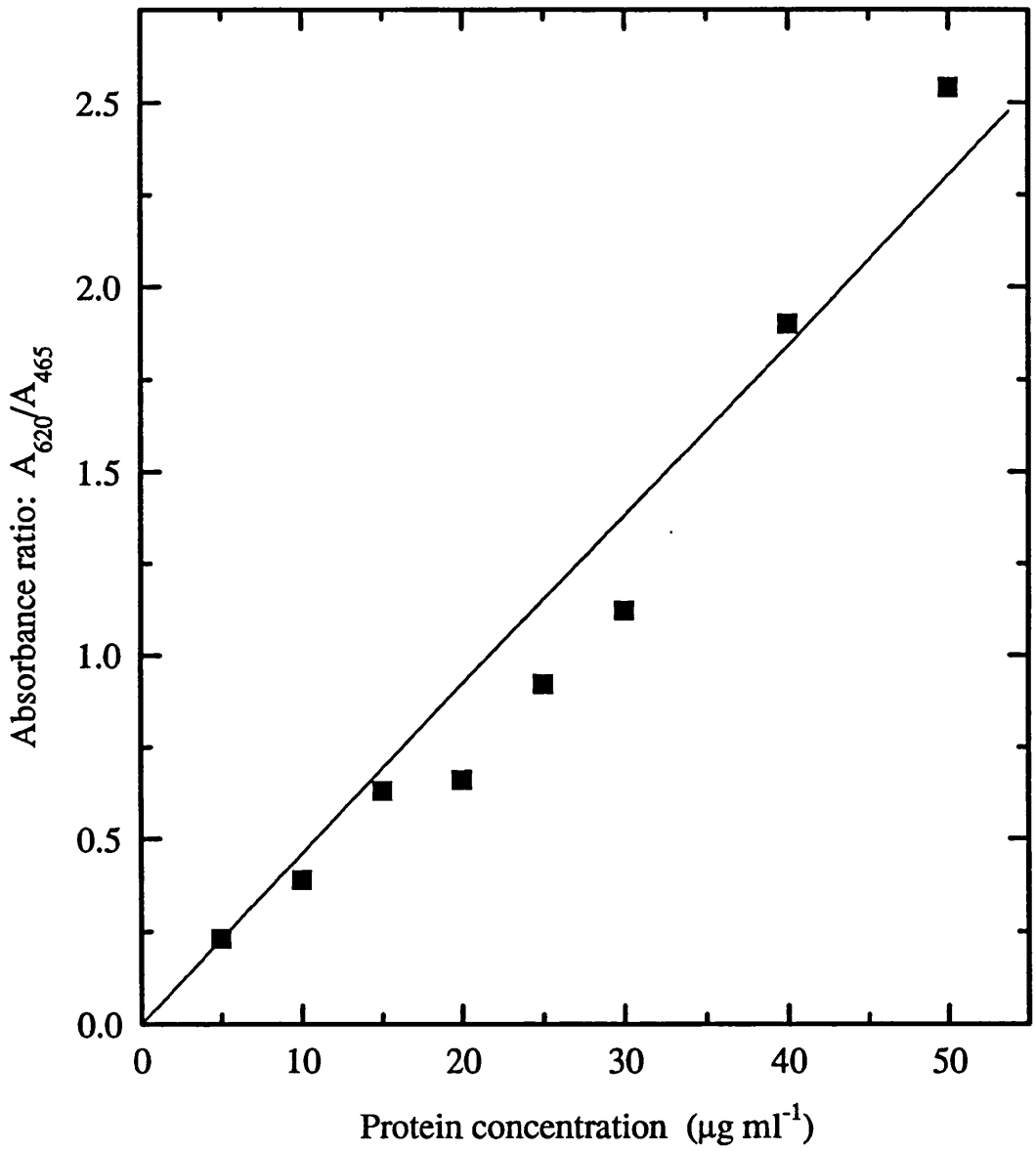
Table 1 (b): Standard Protein Concentrations and A_{620}/A_{465} Readings (2)

Protein Content (mg) (Blank)	A_{465}	A_{620}	A_{620}/A_{465} - A_{620}/A_{465}
50	0.424	1.003	1.80
40	0.445	0.920	1.50
30	0.463	0.868	1.31
25	0.490	0.782	1.03
20	0.504	0.737	0.90
15	0.589	0.662	0.56
10	0.598	0.578	0.40
5	0.682	0.486	0.23
Blank	0.681	0.384	0.00

See Experimental Section for method

Table 1 (c): Standard Protein Concentrations and Average A_{620}/A_{465} Readings from Table 1 (a) and 1 (b)

Protein Content (mg)	A_{620}/A_{465} - A_{620}/A_{465} (Blank)
50	2.17
40	1.70
30	1.22
25	0.98
20	0.78
15	0.60
10	0.40
5	0.23
Blank	0.00



Graph 1A: Typical standard calibration curve for the estimation of protein concentrations by the Coomassie Blue method.

Appendix 2

Spectrophotometric Assay

Calculation of Results

For Example : 2-Bromo-1,4-xylylenediamine dihydrochloride (75) as Substrate

Assay:	2500 ml	Phosphate Buffer pH 6.3
	170 ml	DMAB
	100 ml	MBTH
	50 ml	Peroxidase
	25 ml	Pea Seedling DAO (1/10 dilute)
	300 ml	Substrate (varying concentrations)

See Experimental Section for more detail

Appendix 2 (contd)

Table 2 (a) Rate of Enzymic Oxidation v's Substrate Concentration for 2-bromo-1,4-xylylenediamine dihydrochloride (75)

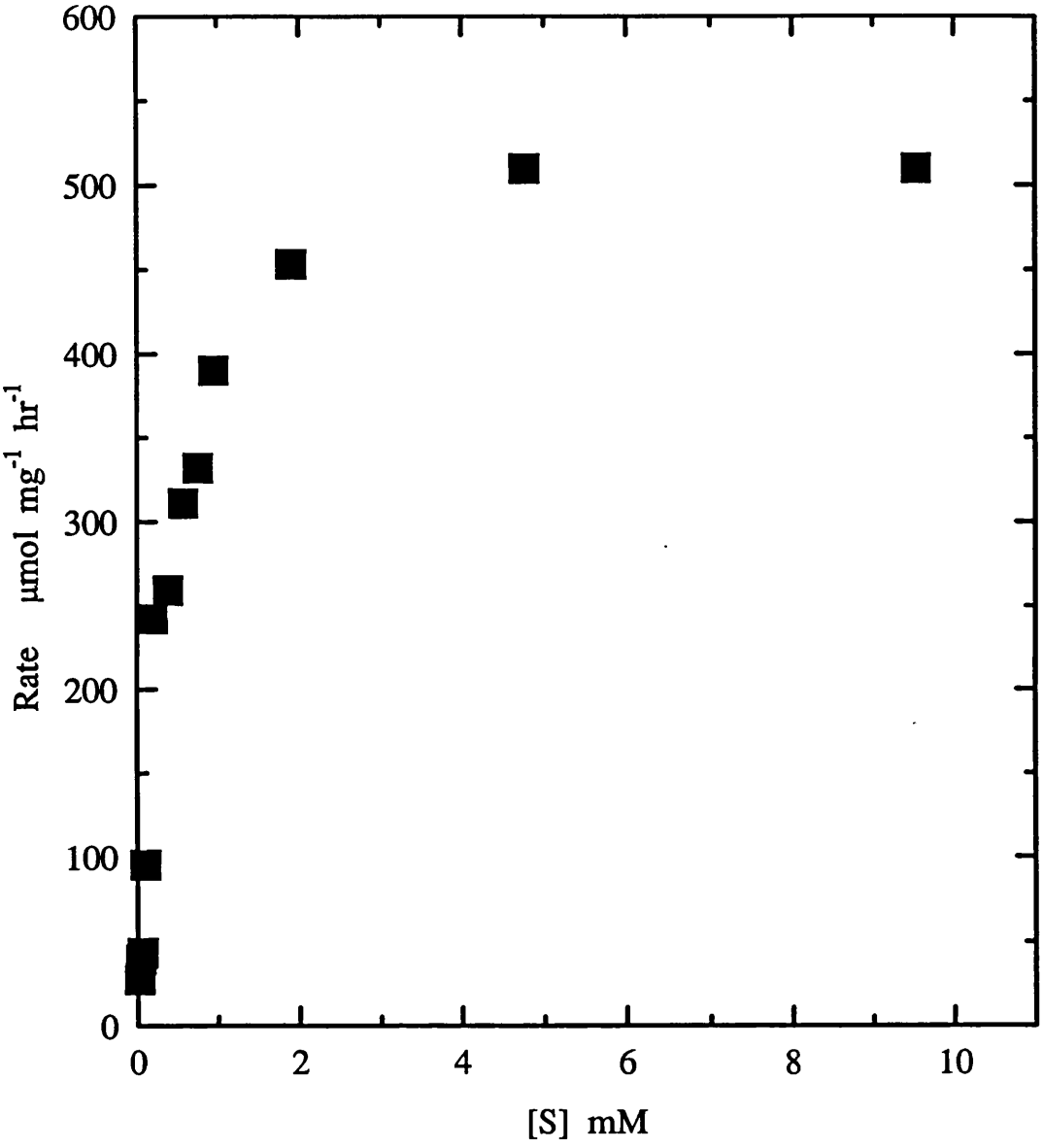
Substrate Concentration ($\times 10^{-4}$ M)	Rate (abs/sec $\times 10^{-3}$)	Rate (mmol/mg/hr)
9.54	5.63	510
4.77	5.63	510
1.91	5.00	453
0.95	4.31	390
0.76	3.67	332
0.57	3.44	311
0.38	2.86	259
0.19	2.67	242
0.10	1.05	95
0.06	0.48	43
0.04	0.43	39
0.02	0.30	27

Using the following parameters :

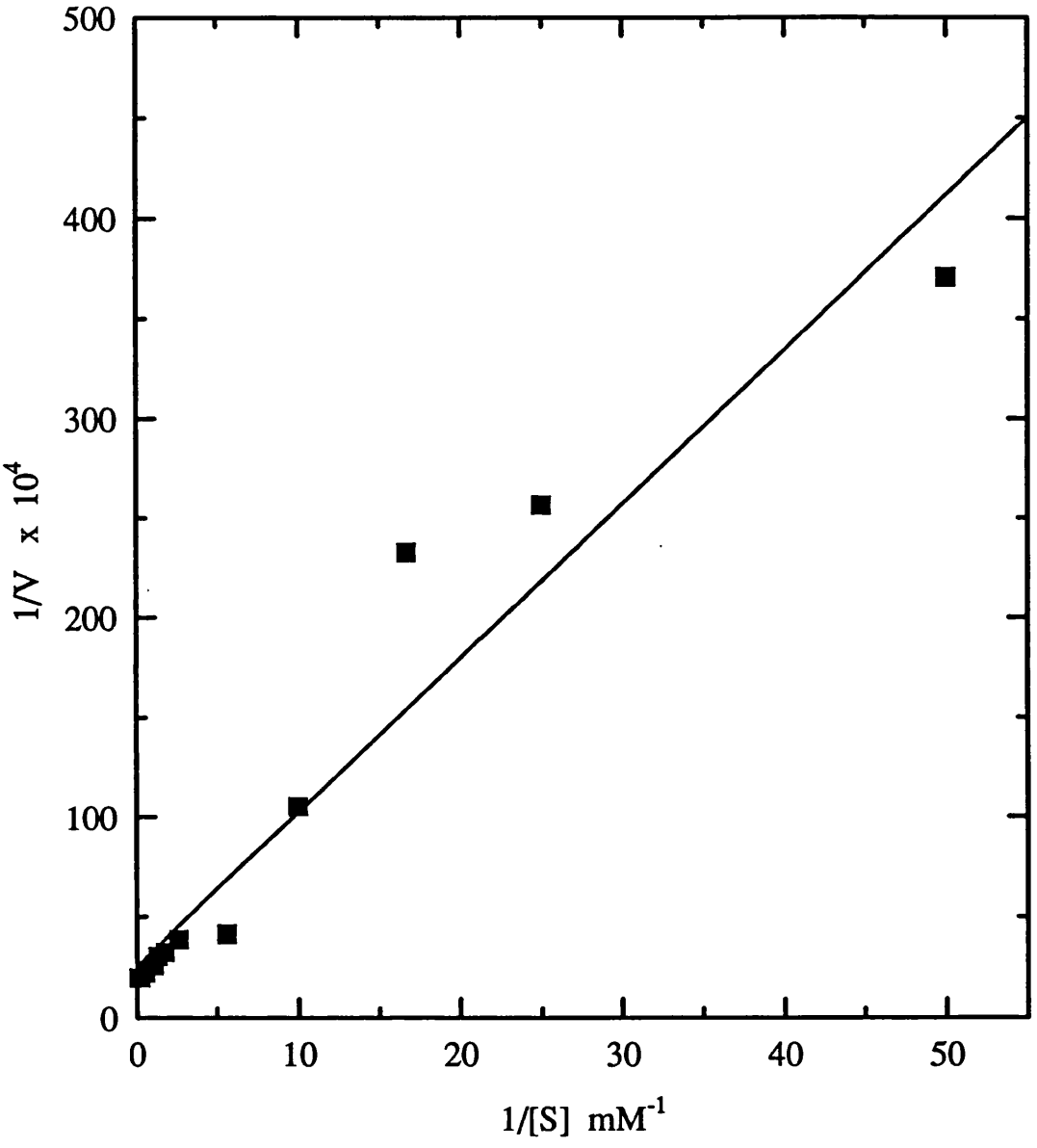
Extinction Coefficient : e = 2.6335×10^4 (Ref. 20)

Protein Concentration : 25 ml of enyme (1/10 dilute) contains 4.75×10^{-3} mg of protein

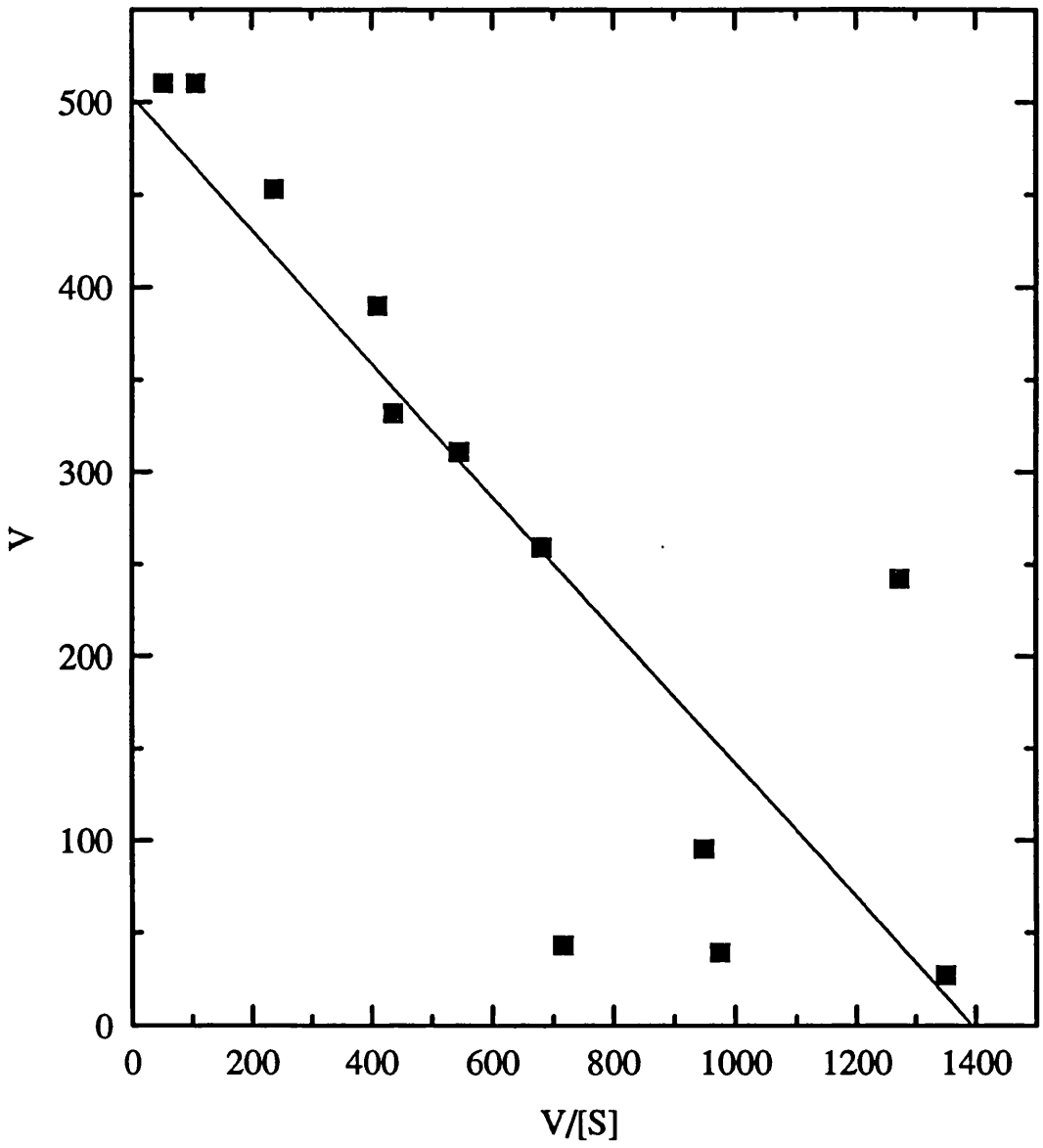
the units of rate were converted from abs/sec to mmol/mg/hr.



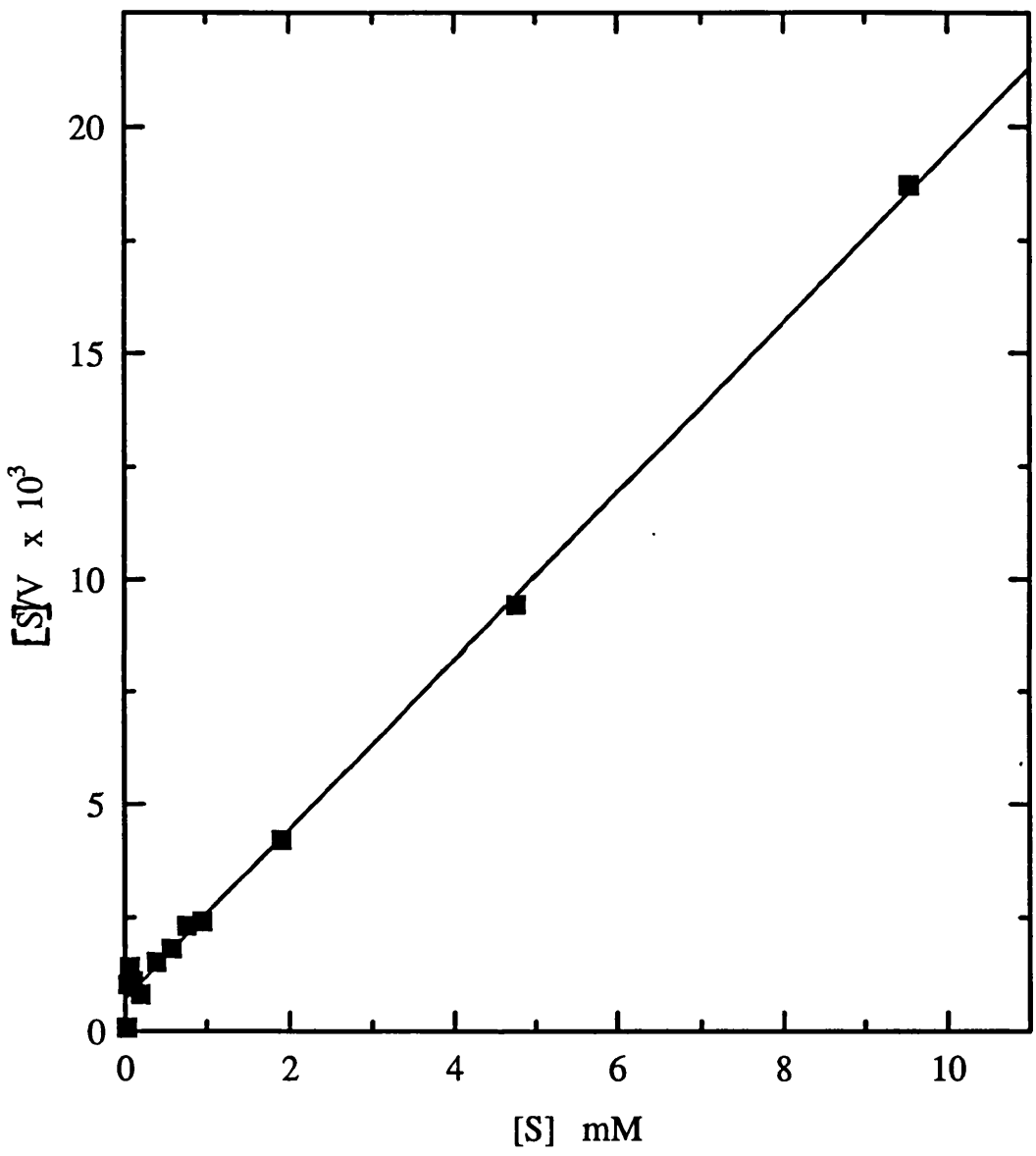
Graph 2A: Typical kinetic data, rate vs. substrate concentration [S], for the DAO-catalyzed hydrolysis of 2-bromo-1,4-xylylenediamine at 25°C.



Graph 2B: Typical kinetic data from Graph 2A for the DAO-catalyzed hydrolysis of 2-bromo-1,4-xylylenediamine at 25°C, displayed as a Lineweaver-Burk plot. The line shows the least-squares regression fit for these data.



Graph 2C: Typical kinetic data from Graph 2A for the DAO-catalyzed hydrolysis of 2-bromo-1,4-xylylenediamine at 25°C, displayed as a Eadie-Hofstee plot. The line shows the least-squares regression fit for these data.



Graph 2D: Typical kinetic data from Graph 2A for the DAO-catalyzed hydrolysis of 2-bromo-1,4-xylylenediamine at 25°C, displayed as a Hanes plot. The line shows the least-squares regression fit for these data.

Calculation of Results

Table 2 (b): Kinetic Parameters Obtained from Lineweaver Burk, Eadie Hofstee and Hanes Plots

	Lineweaver Burk	Eadie Hofstee	Hanes
K_M (mM)	0.03	0.04	0.04
V_{max} (mmol/mg/hr)	398	503	538

In practise three experiments were carried out with the kinetic parameters being reported as an average of nine determinations.

Appendix 3

Inhibition of Diamine Oxidase

For Example: Putrescine as Substrate, (2'-pyridyl)methylamine dihydrochloride (119) as Competetive Inhibitor with Pea Seedling Diamine Oxidase

Assay:

2500 ml	Phosphate Buffer
170 ml	DMAB
100 ml	MBTH
50 ml	Peroxidase
25 ml	Pea Seedling DAO
300 ml	Putrescine (varying concentration)
100 ml	(2'-pyridyl)methylamine dihydrochloride

See Experimental Section for more details of the method.

Appendix 3 (contd)

Table 3 (a) Substrate Concentration and Rate Readings with Inhibitor Concentration of 0 mM

Substrate (mmol/mg/hr)	Rate Concentration (mM)	Rate (abs/sec x 10^{-3})
4.77	5.50	910
3.82	4.67	771
1.91	5.09	840
0.95	4.67	771
0.76	3.88	640
0.57	3.13	518
0.38	2.30	379
0.19	1.87	309
0.10	0.88	144
0.08	0.64	105
0.06	0.41	70

Appendix 3 (contd)

Table 3 (b) Substrate Concentration and Rate Readings with Inhibitor Concentration of 0.15 mM

Substrate (mmol/mg/hr)	Rate Concentration (mM)	Rate (abs/sec x 10^{-3})
4.77	5.33	880
3.82	5.50	910
2.86	5.50	910
1.91	4.92	814
0.95	3.33	549
0.76	2.94	488
0.57	2.53	418
0.38	1.58	261
0.19	0.96	157
0.10	0.53	87
0.08	0.44	74
0.06	0.37	61

Appendix 3 (contd)

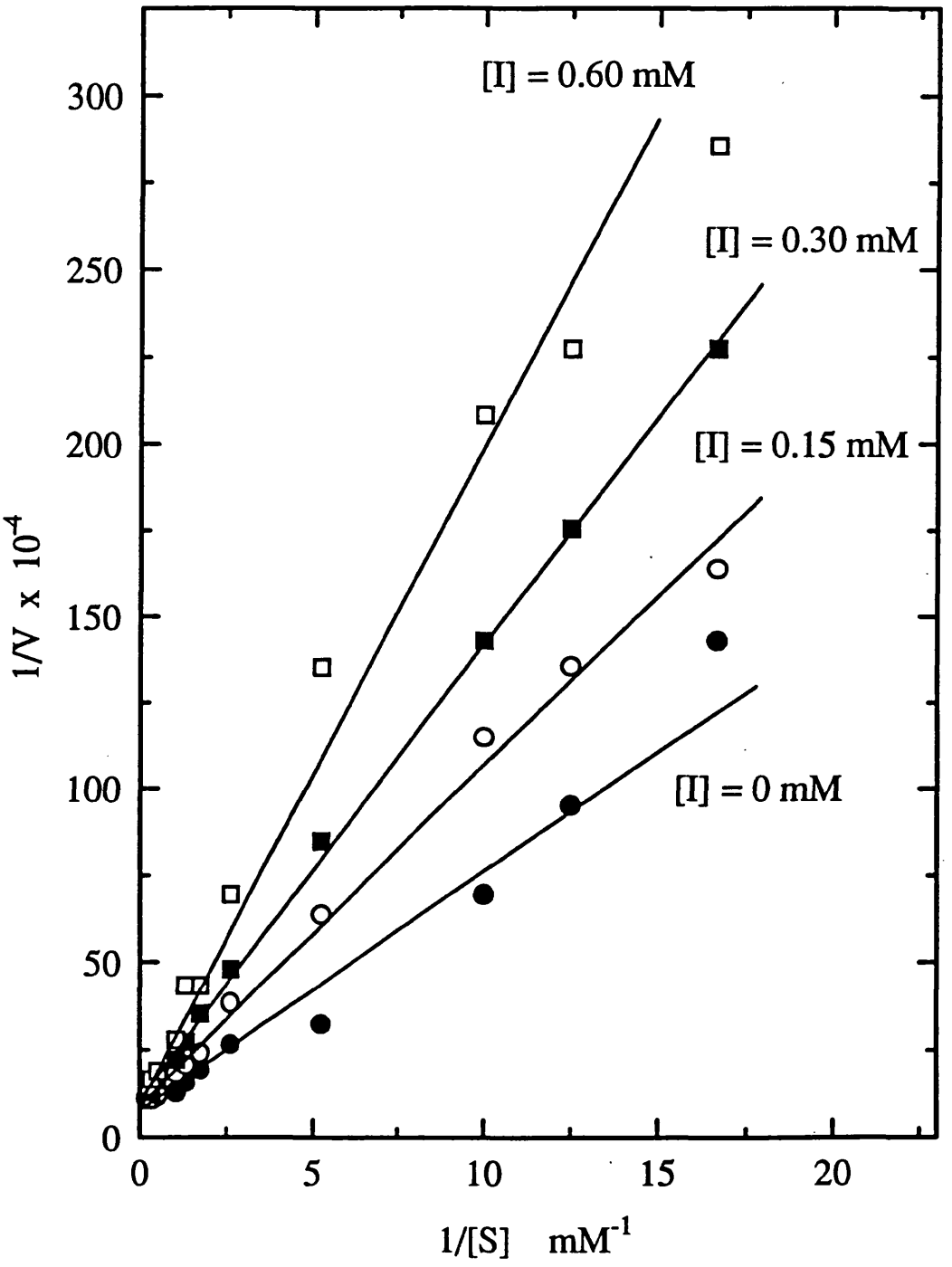
Table 3 (c) Substrate Concentration and Rate Readings with Inhibitor Concentration of 0.30 mM

Substrate (mmol/mg/hr)	Rate Concentration (mM)	Rate (abs/sec x 10^{-3})
4.77	4.27	705
3.82	4.44	736
2.86	4.72	779
1.91	3.73	618
0.95	2.75	453
0.76	2.23	370
0.57	1.72	283
0.38	1.27	209
0.19	0.71	118
0.10	0.42	70
0.08	0.33	57
0.06	0.26	44

Appendix 3 (contd)

Table 3 (d) Substrate Concentration and Rate Readings with Inhibitor Concentration of 0.60 mM

Substrate (mmol/mg/hr)	Rate Concentration (mM)	Rate (abs/sec x 10^{-3})
4.77	4.50	745
3.82	4.33	714
2.86	3.67	605
1.91	3.20	531
0.95	2.18	361
0.76	1.40	231
0.57	1.40	231
0.38	0.87	144
0.19	0.44	74
0.10	0.29	48
0.08	0.27	44
0.06	0.22	35



Graph 6.1: Double reciprocal plot of enzyme kinetics. Inhibition of oxidative deamination of putrescine by pea seedling diamine oxidase with (2'-pyridyl)methylamine as inhibitor. [I] = inhibitor concentration.

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