DIAMINE OXIDASE: KINETIC STUDIES AND USE IN ORGANIC SYNTHESIS.

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

by

Angela Mary Equi

Department of Chemistry
The University
Glasgow

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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Finally I would like to dedicate this thesis to Sean.

ABBREVIATIONS

DAO	Diamine oxidase
PQQ	Pyrroloquinoline quinone
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
DFMO	α-Difluoromethylornithine
ODC	Ornithine decarboxylase
SDS	Sodium dodecyl sulphate
EPR	Electron paramagnetic resonance
FAD	Flavin adenine dinucleotide
DNPH	2,4-Dinitrophenylhydrazine
HPLC	High performance liquid chromatography
NMR	Nuclear magnetic resonance
UV	Ultraviolet
Vis	Visible
CID	Collision induced dissociation
MBTH	3-Methyl-2-benzothiazolinone
DMAB	3-(Dimethylamino)benzoic acid
BSA	Bovine serum albumin
E	Enzyme
S	Substrate
V	Reaction rate
ES	Enzyme-substrate complex
$[E_T]$	Total enzyme concentration
V_{max}	Maximum rate
K_M	Michaelis Menten constant
I	Inhibitor
K_i	Dissociation constant of enzyme-inhibitor complex

SUMMARY

The subject of this thesis is the enzyme diamine oxidase and four main topics are discussed: (1) oxidation of diamines using diamine oxidase; (2) inhibition of diamine oxidase; (3) stereochemistry and regiochemistry of the reactions catalysed by diamine oxidase; and (4) applications of diamine oxidase.

(1) Oxidation of diamines using diamine oxidase. Diamine oxidase catalyses the oxidative deamination of diamines to their corresponding aminoaldehydes (Scheme A).

$$H_2N(CH_2)_nNH_2 + H_2O + O_2$$

$$\downarrow \text{ Diamine Oxidase}$$

$$H_2N(CH_2)_{n-1}CHO + H_2O_2 + NH_3$$

Scheme A n = 4 (i) n = 5 (ii)

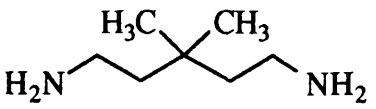
Putrescine (i) and cadaverine (ii) are the best substrates of diamine oxidase. *N*-Alkylputrescines and *C*-alkylcadaverines were synthesised and tested as substrates of diamine oxidase using an improved spectrophotometric assay. The assay involves the measurement of the hydrogen peroxide produced as a by-product of the enzymic reaction. From this assay K_M and V_{max} values were obtained for the oxidation of these substrates using diamine oxidase. The K_M is a measure of the strength of the enzyme-substrate complex and determines the binding efficiency of the substrate to the enzyme. The V_{max} is the maximal rate and is related to the turnover

number of an enzyme. Analysis of these results provided information on the steric constraints of the active site.

Also studied were α,ω-diamines with chain lengths varying from two to twelve. Analysis of these results showed that the best binding (lowest K_M value) was observed with diamines with chain lengths from five to seven and the highest V_{max} value was obtained with cadaverine (ii) as the substrate (chain length five). These results suggest that formation of a cyclic diamine intermediate with the enzyme is essential for recognition and catalysis.

(2) Inhibition of 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 should have a considerable effect on the polyamine metabolism and therefore on cell growth.

Substrate analogues [*eg* 3,3-dimethylcadaverine (iii)] were examined as inhibitors of diamine oxidase. Compounds resembling substrates although with no primary amine groups present [*eg* 1,6-*bis*(*N*-piperidyl)hexane (iv)] were also tested as inhibitors. These tests were carried out using the same spectrophotometric assay as before. Most of the compounds did inhibit the diamine oxidase catalysed reaction and were shown to be competitive inhibitors. K_i values were obtained for the compounds tested as inhibitors.



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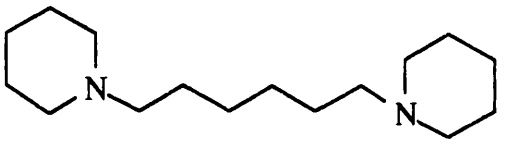

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(3) Stereochemistry and regiochemistry of the reaction catalysed by diamine oxidase. The stereochemistry and regiochemistry of most enzymic reactions are controlled. As not a great deal is known about the stereochemistry or regiochemistry of the oxidative deamination of diamines catalysed by diamine oxidase studies were carried out to find out more.

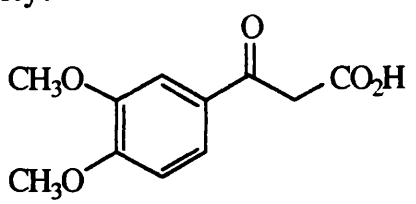
Analysis of the products of the reaction with *C*-alkylcadaverines was performed to determine any selectivity from the reactions. Isolation of the products was achieved by trapping the imines from the enzymic reaction using 3,4-dimethoxybenzoylacetic acid (v).

The compounds examined for selectivity in the diamine oxidase catalysed oxidation were (a) 3-methylcadaverine; (b) 2-methylcadaverine; and (c) 3-phenylcadaverine. With the first two selectivity does occur although it is not yet clear if this is due to the enzyme catalysed reaction or the second reaction involving the coupling with the β-keto acid (v). Surprisingly, no product was obtained with 3-phenylcadaverine.

(4) Applications of diamine oxidase. Enzymes can often be used to catalyse reactions which are difficult to carry out by other methods. The main advantages of using enzymes in synthesis are the mildness of the reaction conditions and also the possible control of the stereochemistry and regiochemistry.

Diamine oxidase catalyses the oxidation of diamines to their corresponding aminoaldehydes for which there are no chemically convenient methods. Therefore the use of this enzyme in synthesis could be very favourable.

The alkaloid, cryptopleurine (vi), has been synthesised using diamine oxidase in a key step. Cryptopleurine (vi) has known anti-cancer activity. Oxidation of cadaverine using diamine oxidase and subsequent coupling to 3,4-dimethoxybenzoylacetic acid (v) formed an intermediate in the synthesis of cryptopleurine (vi). Using *C*-alkylcadaverines and following the same procedure, a number of intermediate analogues of cryptopleurine were formed. These alkaloid analogues when made may also possess interesting biological activity.



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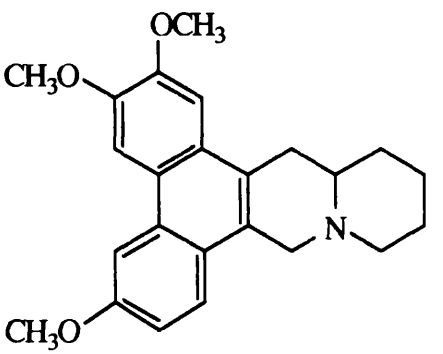
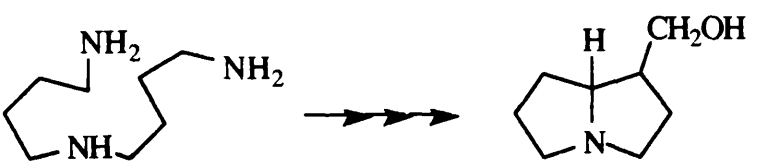


(vi)

The pyrrolizidine alkaloid, trachelanthamidine (viii), was also synthesised using diamine oxidase. Oxidative deamination of homospermidine (vii) and subsequent reduction of the likely product, 1-formylpyrrolizidine produced the alkaloid (viii).



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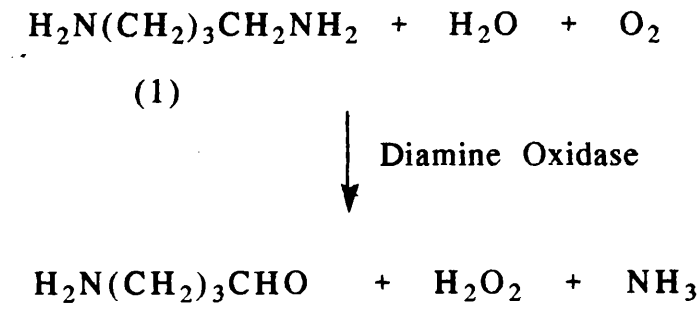
CHAPTER 1

INTRODUCTION

1.1 The Importance of Enzymes in Organic Synthesis

The use of enzymes in organic synthesis is of increasing importance. The main advantages of using enzymes in organic synthesis are the mildness of reaction conditions together with the possible control of stereochemistry and regiochemistry. Use of enzymes can provide methods for the preparation of optically active compounds. This is of particular importance in drug manufacture where only one enantiomeric form of a chiral drug usually possesses the desired biological activity. Work in the past has mainly been concerned with hydrolases and oxidoreductases,[1] but other enzymes catalyse transformations that are difficult to achieve by known synthetic methods. Diamine oxidases (DAO, EC 1.4.3.6) catalyse the oxidative deamination of a range of primary diamines to the corresponding aminoaldehydes (Scheme 1.1).



Scheme 1.1

The mechanism for this process is not fully understood but the literature evidence will be discussed in Chapter 2.

1.2 Diamine Oxidase

Enzymes which oxidise diamines are present in a wide variety of biological tissues, although two sources are particularly convenient. Pig kidney diamine oxidase is commercially available and pea seedling diamine oxidase is readily extracted from 10 day old pea seedlings.[2]

Diamine oxidases are copper containing proteins. It was shown that removal of the copper by dialysis against chelating agents caused consequent deactivation and that activity could be restored by the addition of Cu^{2+}.[3] The identity of the organic co-factor in amine oxidases has been a long standing problem. This was recently thought to have been solved by the development of the hydrazine method[4] which enabled identification of the co-factor as pyrroloquinoline quinone (PQQ). As a result of this method it was reported that a number of enzymes previously thought to have metal ions or pyridoxal phosphate as the sole co-factor contain PQQ. Published information on diamine oxidases and their possible co-factors will be reviewed in Chapter 2 as new evidence suggests that PQQ may not be the co-factor.

1.3 Polyamines and Cell Growth

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

Although the discovery of polyamines was made five hundred years ago, the study of polyamines has trailed behind that of many other biological areas. This is surprising as all

animals, plants and micro-organisms contain at least one polyamine, for example putrescine (1), spermidine (2) or spermine (3).

$H_2N(CH_2)_3NH(CH_2)_4NH_2$ $H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$

(2) (3)

Polyamines appear to have many key physiological roles but it is their involvement in cell growth and replication that is most important. The connection between polyamines and cell growth stems from the ability of the polyamines to undergo ionic interactions with the nucleic acids. At physiological pH, the protonated form of the polyamines can interact strongly with the phosphate anions of the nucleic acids. Therefore polyamines stabilise DNA and RNA and also speed up every step in the transcription-translation sequence, that is the process whereby information coded by genes is used in the manufacture of proteins.[5]

1.4 Polyamine Metabolism

There are two major pathways by which polyamines are metabolised: the interconversion pathway and terminal polyamine catabolism.

The interconversion pathway is a cyclic process which controls polyamine turnover. It regulates intracellular polyamine levels. Putrescine (1), the precursor of spermidine (2) and spermine (3), is formed by decarboxylation of ornithine. Spermidine synthase forms spermidine from

putrescine, and spermine synthase forms spermine from spermidine, by transfer of aminopropyl residues from decarboxylated *S*-adenosylmethionine. In the catabolic branch of the interconversion cycle, spermine is degraded to spermidine and spermidine to putrescine. The first step in the catabolism is *N'*-acetylation followed by oxidative cleavage of the acetylated polyamines.

Terminal polyamine catabolism is catalysed by Cu^{2+} dependent amine oxidases, of which only diamine oxidase has been well defined. By the oxidative deamination of a primary amino group, each diamine intermediate can be converted into the corresponding aminoaldehyde and further oxidised to the amino acid or γ-lactam. These products of terminal catabolism and the acetylated polyamines are urinary excretory products.

1.5 Inhibitors

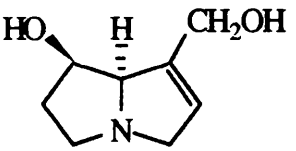
A good approach to discover what roles compounds play in cell physiology is to examine what happens to the system when the concentration of the compound is reduced or depleted.

Work began on synthesising inhibitors of polyamine biosynthesis in the early 1970's. α-Difluoromethylornithine (DFMO) was synthesised by Merrell-Dow Pharmaceuticals. They showed that DFMO inhibited growth in cells. This suggested that polyamines were involved in cell growth and replication and also proved to have other benefits. DMFO possessed very interesting anti-tumour activity as a result of binding irreversibly to ornithine decarboxylase (ODC). Tumour cells proliferate rapidly and have a higher demand for

polyamines. Therefore inhibition of polyamine biosynthesis has a greater effect on tumour cells. Inhibitors of diamine oxidase may also have possible roles in cancer chemotherapy.

1.6 Alkaloid Biosynthesis

Pyrrolizidine alkaloids are widespread in plants. Many of these alkaloids contain (+)-retronecine (4) as the base portion and are hepatotoxic. This is due to the unsaturated ring of the base portion being oxidised by liver oxidase enzymes to form pyrrole derivatives, which are bifunctional alkylating agents.



(4)

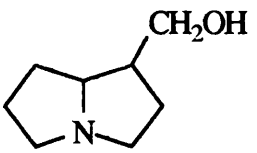
Homospermidine (5) is thought to be a key intermediate in pyrrolizidine alkaloid biosynthesis. Using ^{13}C-^{15}N doubly labelled putrescine, it was shown that two putrescine molecules combine to form retronecine (4).[6] These experiments confirmed that a symmetrical C_4-N-C_4 intermediate was involved.

$$H_2N(CH_2)_4NH(CH_2)_4NH_2$$

(5)

Homospermidine (5) was first isolated from a sandalwood tree, *Santalum album*,[7] and can be synthesised from 4-

bromobutanenitrile.[8] The initial steps in the conversion of homospermidine (5) into pyrrolizidine alkaloids plausibly involve oxidation of the primary amine groups. It has been shown that by incubating homospermidine (5) with diamine oxidase and subsequent reduction of the likely product, 1-formylpyrrolizidine, the pyrrolizidine alkaloid trachelanthamidine (6) is formed.[9]

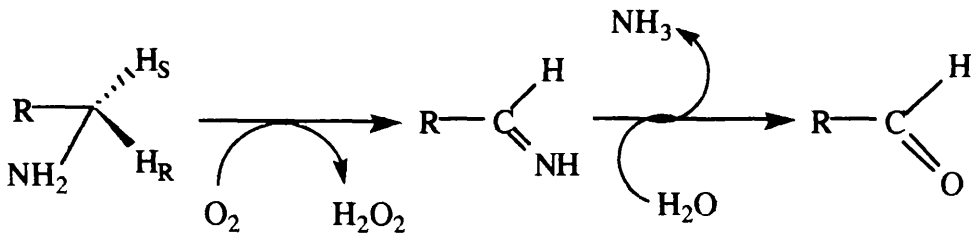


(6)

This evidence with isolated enzymes suggests that such transformations are involved in pyrrolizidine alkaloid biosynthesis. This use of isolated enzymes may prove to be a convenient method for the formation of pyrrolizidine alkaloid analogues.

1.7 Stereochemistry and Regiochemistry in Reactions with Diamine Oxidase

Diamine oxidases are of low substrate specificity, acting upon a broad range of amines (Scheme 1.2).



Scheme 1.2

The absolute stereochemistry of the removal of a hydrogen atom from the prochiral methylene group adjacent to the nitrogen has been determined by various methods. A wide range of substrates has been examined, using substrates enantiomerically labelled with deuterium or tritium whose absolute stereochemistry had been determined by correlation with compounds of known absolute configuration.[10] The consistency throughout the range of substrates examined showed that the reaction of diamines catalysed by diamine oxidase is accompanied by loss of the pro-*S* hydrogen.

The regioselectivity of diamine oxidase has also been studied. Santaniello *et al.*[11] showed that the diamine oxidase from pea seedlings catalyses the oxidation of 2-methylputrescine regioselectively oxidising both (*R*)- and (*S*)-2-methylputrescine at the less hindered C-4 position, whereas with pig kidney diamine oxidase the dependence relies more on the stereochemistry of the substrate. Oxidation of the (*R*)-isomer occurs at C-1 and the (*S*)-isomer is oxidised at the less hindered C-4 position.

1.8 Aims of Project

N-Alkylputrescines were tested as substrates for pea seedling and pig kidney diamine oxidase using a *bis*-hydrazone assay reported by Frydman *et al.*[12] They reported that *N*-methylputrescine was not a substrate for either enzyme although *N*-ethylputrescine and *N*-propylputrescine appeared to be oxidised easily. This was an unusual and unexpected result. We initially set out to check this result using the same assay system as Frydman,[13] then used an improved spectrophotometric assay.[14]

Cadaverine (7) is also a substrate of diamine oxidase. Cadaverine and analogues will be synthesised to be tested as substrates for both sources of diamine oxidase. All substrates will be tested using the improved assay system.

$$H_2N(CH_2)_5NH_2$$

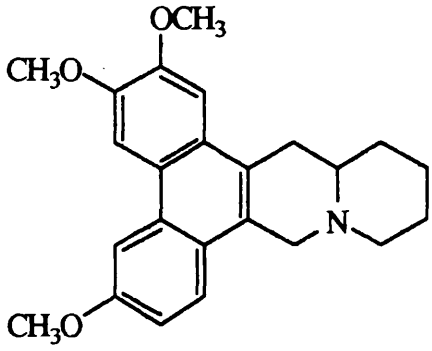
(7)

It was thought that information on the constraints of the active site could be obtained on analysis of the reaction with substrates of different carbon chain length. Chain lengths of up to twelve carbons will be tested as substrates of diamine oxidase to enable us to find out more about the steric requirements at the active site of the enzyme.

After analysis of results of enzymic oxidation of diamines, some compounds will be tested as inhibitors of diamine oxidase, inhibiting the oxidative deamination of putrescine and/or cadaverine. This will be carried out using

the improved spectrophotometric assay[14] as before with the addition of a constant concentration of potential inhibitor.

To determine the regio- and/or stereo-chemistry of the reaction catalysed by diamine oxidase we require to examine the products formed. The aminoaldehydes produced from the reaction with putrescine and cadaverine trimerise readily and are also difficult to extract. By coupling the imines with 3',4'-dimethoxybenzoylacetic acid, the products can be isolated and analysed. This has an extra benefit as the products formed can be used in the synthesis of alkaloids, such as cryptopleurine (8), tylophorine (9) and analogues.[15] These known alkaloids exhibit interesting biological activity including anti-cancer action.



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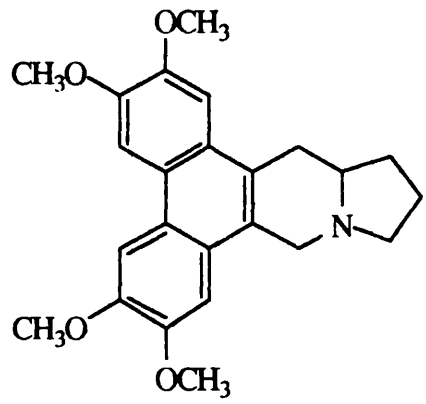


(9)

The reaction with homospermidine (5) and diamine oxidase produces the alkaloid trachelanthamidine (6).[9] It was thought that by using analogues of homospermidine, for example *N,N-bis*(5-aminopentyl)amine (10) other (quinolizidine) alkaloids might be formed using this enzymic process.

$$H_2N(CH_2)_5NH(CH_2)_5NH_2$$

(10)

CHAPTER 2

REVIEW OF THE ENZYME - DIAMINE OXIDASE

2.1 Purification of Diamine Oxidase (DAO)

Diamine oxidases (diamine: oxygen oxidoreductase: deaminating: copper containing EC 1.3.4.6) are enzymes which are widely distributed among living organisms. The main sources of diamine oxidase are from pig kidneys and peas. The latter source is by extraction from young pea seedlings. The diamine oxidase content of pea seedlings is at a maximum over the period between seven and sixteen days after germination.[16] Varieties differ slightly in their initial diamine oxidase activity, and in the ease with which the enzyme can be purified.

Hill[2] developed a procedure for purification of diamine oxidase which depends on removing much of the unwanted material from the crude extract by precipitation with a mixture of chloroform and ethanol. Then the enzyme is precipitated first with ammonium sulphate and second at pH 5 by the method of Tabor.[17] The enzyme can be finally purified by chromatography on hydroxyapatite DEAE-cellulose columns. The chloroform-ethanol step is unusual but important as if it is omitted difficulties arise in later stages.

Diamine oxidase was purified from pea epicotyls[18] to homogeneity by the criterion of polyacrylamide gel electrophoresis. The diamine oxidase was first purified by column chromatography on phosphocellulose and MGBG-

Sepharose. The procedures were developed using diamine oxidase which had been treated with 5% protamine and then concentrated with ammonium sulphate of 65% saturation.

The molecular weight of the diamine oxidase estimated by gel filtration was *ca.* 180,000. Sodium dodecylsulphate (SDS) gel electrophoresis yielded a single band at a molecular weight of 85,000. These results suggest that the enzyme consists of two identical subunits.

2.2 The Involvement of Copper

Diamine oxidase is a copper containing enzyme. Purified diamine oxidase was found to contain 0.08-0.09% copper. It was reported that diamine oxidase is inhibited by chelating ligands and this therefore suggested that it was a metalloprotein.[19] Hill and Mann[20] showed that when the enzyme was dialysed against chelating ligands such as sodium diethyldithiocarbamate, copper was removed. The copper-free protein was found to be inactive but it could be reactivated by Cu(II) ions. Zn(II) was found to be present in trace amounts in diamine oxidase[20] although addition of Zn(II) ions did not reactivate the copper-free enzyme. Other chelating ligands that caused inhibition of diamine oxidase include 8-hydroxyquinoline and 1,10-phenanthroline. Many metal ions prevented the inhibition of most chelating agents; in particular Ni(II) and Co(II) were the most effective.

It was also found by using the biquinolyl estimation method[21] that no copper in the Cu(I) form was present in diamine oxidase. At this stage it was concluded that diamine

oxidase is a metallo-enzyme containing copper only in the Cu(II) form. At the time this conclusion was thought to be correct, although it has been subsequently questioned. Electron paramagnetic resonance (EPR) experiments[22] were inconclusive in detecting any changes in the copper oxidation state in the presence of amine substrates. This inconclusive evidence has led to proposals that Cu(II) acts as a Lewis acid;[23] that it has an indirect role in catalysis;[24] or that it serves a structural role.[25] New evidence has recently been presented of the presence of a Cu(I)-state structure and this will be discussed later.[26]

2.3 Pyridoxal Phosphate and Flavin Adenine Dinucleotide (FAD) as Cofactors

It has been shown that diamine oxidase is strongly inhibited by reagents which form derivatives with carbonyl groups. Evidence was reported by Yamada[27] of the complete inhibition by hydroxylamine (at 1 x 10^{-5} M) and semicarbazide (at 1 x 10^{-6} M). This suggested the presence of a carbonyl group at the active site of the enzyme. Mann[19] suggested that the copper is present in the enzyme as a complex with a carbonyl compound and that this complex forms the prosthetic group.

Much evidence was then produced purporting to show that the prosthetic groups of both animal diamine oxidase and the plant form contain pyridoxal phosphate as the cofactor.[28]

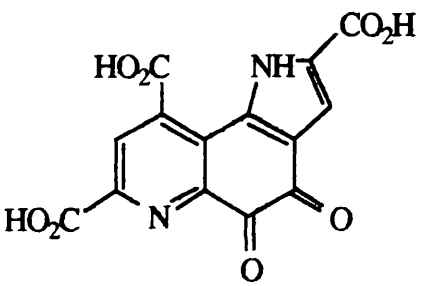
As hydrogen peroxide is a by-product of these reactions catalysed by amine oxidases it was proposed that these enzymes were flavoproteins. Kapeller-Alder[29] and

Gorychenkova[30] reported evidence that flavin adenine dinucleotide (FAD) forms part of the prosthetic group of the diamine oxidase from both animal and plant sources. As recently as 1984 FAD was reported to be present in diamine oxidase from rice embryos.[31]

The bias on the cofactor structure changed significantly in 1984, when two independent groups reported that bovine plasma amine oxidase. may contain pyrroloquinoline quinone (PQQ) as the cofactor.[4,32]

2.4 The History of Pyrroloquinoline Quinone (PQQ)

The first discovery of PQQ (11) as a cofactor came from studies on bacterial glucose dehydrogenase by Hauge in 1964.[33] He observed the presence of a dissociable cofactor which was not a nicotinamide or a flavin. Using extensive spectral characterisations Hauge identified the cofactor as a 'suitably substituted 1,4-naphthoquinone'. Also at this time Anthony and Zatman[34] were studying methanol dehydrogenase from *Hyphomicrobium X* and they discovered an organic cofactor which could be released on denaturation which was thought to be a pteridine.



(11)

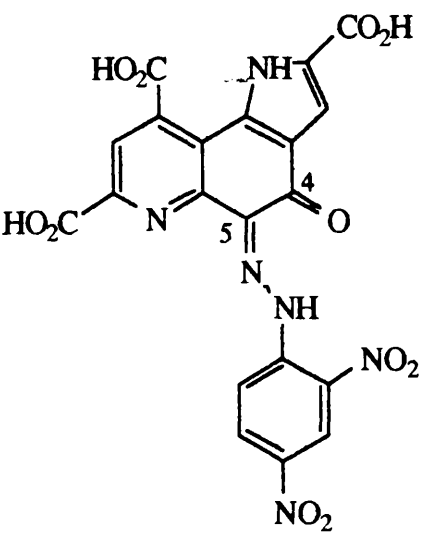
Duine and co-workers applied EPR spectroscopy and observed a hyperfine structure for the cofactor which was incompatible with the presence of a pterin cofactor.[35] They then proposed that the cofactor was a quinone compound containing two nitrogens. Further spectroscopic studies on the isolated cofactor by Duine supported this proposal. By 1979 an X-ray characterisation of the acetone adduct of the cofactor confirmed its structure to be pyrroloquinoline quinone (PQQ) (11).[36]

Accompanying the isolation and characterisation of PQQ in *Hyphomicrobium X*, an increasing number of alcohol dehydrogenases were shown to contain this cofactor. One example is glucose dehydrogenase found in *Acinetobacter calcoaceticus*.[37]

2.5 Pyrroloquinoline Quinone (PQQ) in Copper Amine Oxidases

As mentioned previously, in 1984 two independent reports produced evidence in support of PQQ as a cofactor in copper amine oxidases. These studies were carried out by Lobenstein-Verbeek *et al.*[4] and by Ameyama *et al.*[32] The former group concentrated on derivatisation of the bound prosthetic group with 2,4-dinitrophenylhydrazine (DNPH) before hydrolysing the protein. Although Lobenstein-Verbeek *et al.* found that the isolated product was identical to the hydrazone prepared from authentic PQQ and DNPH, the yield was very low (6%). This was later explained to be due to the destructive nature of the pronase (a specific enzyme used in the hydrolysis of the

peptide chain for the detachment of PQQ-DNPH) step in the proteolysis. Despite the low yield Lobenstein-Verbeek *et al.* considered that derivatisation with DNPH was the only feasible procedure. They found that direct hydrolysis produced many unidentifiable products because PQQ reacted with some of the amino acids from the protein. The derivatised product was shown to be homogeneous by HPLC and had similar retention time and absorption spectrum to that of the model PQQ-DNPH product. This in fact provided evidence against pyridoxal phosphate as the cofactor as the absorption spectrum was quite different from that obtained by incubating pyridoxal phosphate with 2,4-dinitrophenylhydrazine. The proton NMR spectrum of the product formed from derivatising the enzyme, bovine serum amine oxidase with DNPH and then proteolysis indicated that the mono-DNP derivative (12) was formed at C-5.[38]



(12)

Lobenstein-Verbeek *et al.* also formed a fluorescing product which was obtained by degradation of the isolated product (12) in sodium hydroxide solution. The same HPLC retention times were obtained with the fluorescing product obtained from the authentic PQQ sample and the isolated adduct (12).[4]

The approach taken by Ameyama and co-workers[32] was to use native enzyme and obtain flourescence spectra of the degradative adduct from the enzyme. They also observed stimulation of bacterial growth by the acid hydrolysate obtained. Duine and co-workers in 1986 attempted to reproduce results by Ameyama *et al.*[32] but were unable to detect any PQQ under the same circumstances.[39]

Since these first findings[4,32] the majority of investigations of mammalian oxidases have centred around the characterisation of a chemically derivatised cofactor. Duine and co-workers appear confident with their approach involving derivatisation with dinitrophenylhydrazine, followed by proteolytic degradation and chromatographic analysis.[4,39] They have used this approach to show that many proteins contain PQQ. However they have not discussed pea seedling diamine oxidase.

Glatz *et al.* have demonstrated the ability of pea seedling diamine oxidase to form derivatives with a range of reagents known to interact with PQQ. They also performed acid hydrolysis on native enzyme, identifying HPLC peaks as PQQ based on their absorbance and flourescence properties.[40] Although in the light of the work by Duine and co-workers this may not be conclusive evidence.[39]

2.6 Alternative Methods for Detecting Pyrroloquinoline Quinone (PQQ) as the Cofactor

Other approaches have since been employed in an attempt to establish PQQ as the organic cofactor in mammalian amine oxidases. Dooley and co-workers[41] carried out the first detailed structural characterisation of the dinitrophenylhydrazones. They used Raman spectroscopy of derivatives of either PQQ or pyridoxal phosphate from various enzymes, including porcine diamine oxidase. They observed frequencies and relative intensities for these derivatives indicating a close correspondence between proteins derivatised with 2,4-dinitrophenylhydrazine and the 2,4-dinitrophenylhydrazone of PQQ. These studies therefore rule out the possibility of pyridoxal phosphate as the cofactor and provide more evidence that PQQ or a derivative is the organic cofactor.

Due to the widespread nature of PQQ as a cofactor, Citro *et al.*[42] realised there was a need for new, fast analytical methods allowing detection and quantitative analysis of PQQ in biological samples in place of the usual chromatographic techniques previously used. They used antibodies which could react with free and protein bound PQQ and produced a specific antibody allowing detection of PQQ in lentil seedling diamine oxidase.

Gallop[43] stated that direct methods for detection of PQQ with carbonyl reagents are qualitative, insensitive and unreliable when applied to quinoproteins even after proteolysis. He and co-workers have developed highly

sensitive colorimetric assays for the detection of PQQ in cell extracts. They use glycine (as reductant) and nitroblue tetrazolium (as oxidant) to detect PQQ in the nanomolar range.

However this method has been criticized because of the reactivity of the PQQ with certain amino acids. This leads to decarboxylation of the amino acid and then to unreactive oxazole condensation products. Therefore it is questionable whether the method can be quantitative in the case of quinoproteins where proteolysis is required to detach the cofactor.[44]

2.7 Isolation of a Peptide Containing Pyrroloquinoline Quinone (PQQ) from Pig Kidney Diamine Oxidase

In 1989 Duine and co-workers[38] determined the primary structure of a peptide which contained PQQ which was isolated from pig kidney diamine oxidase. The protein was derivatised with DNPH and then subjected to proteolysis with trypsin. The hydrosylate contained a peptide with hydrazone formation at the C-5 position of PQQ. The peptide was purified to homogeneity and the amino acid sequence was determined. The peptide containing PQQ-DNPH again showed a certain spectral and chromatographic similarity to model PQQ-DNPH systems.

The peptide appeared to consist of eleven amino acids with PQQ attached to number eight which was found to be lysine.

The amino acid sequence was shown to be as follows.

1 8 11

His-Ser-Asp-Ala-Val-Phe-Thr-Lys-Asn-Tyr-Arg

PQQ

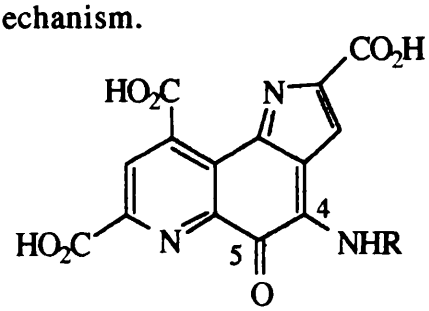
The PQQ was assumed to be attached to the lysine and not inserted in the peptide chain as the latter situation would have resulted in breakdown under the degradative conditions. The suggestion of dual crossslinking was discussed but was considered to be very unlikely since only one protein band was observed on electrophoresis of (dimeric) pig kidney diamine oxidase (containing one PQQ) in a SDS system.

2.8 Mechanism of Pyrroloquinoline Quinone (PQQ)

Initial efforts to establish the reaction mechanism in a PQQ-containing enzyme were focussed on methanol dehydrogenase.

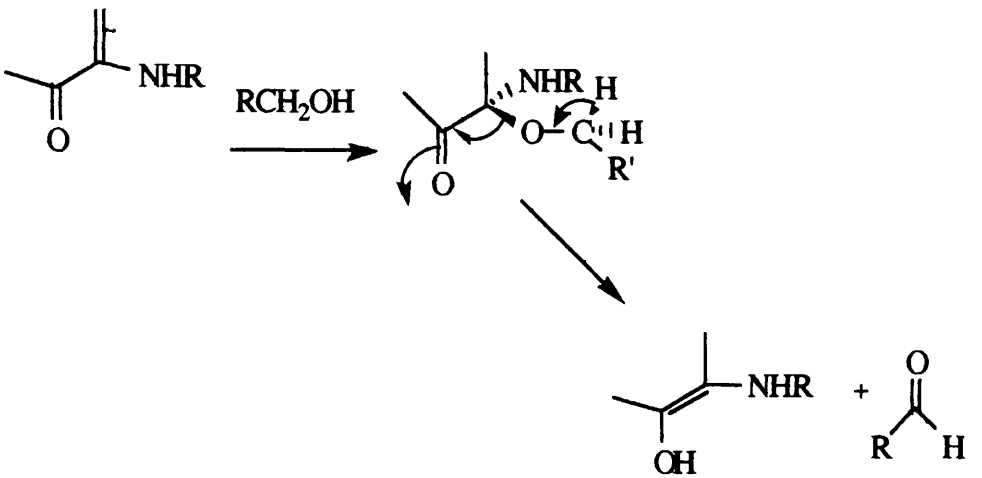
The concept of covalent adducts has appeared consistently in discussions of the mechanism of substrate oxidation by PQQ. Several investigators reported activation of PQQ-dependent enzymes by either ammonia or primary amines.[45,46]

Compound (13) was proposed[47] to be formed and to play a role in the mechanism.



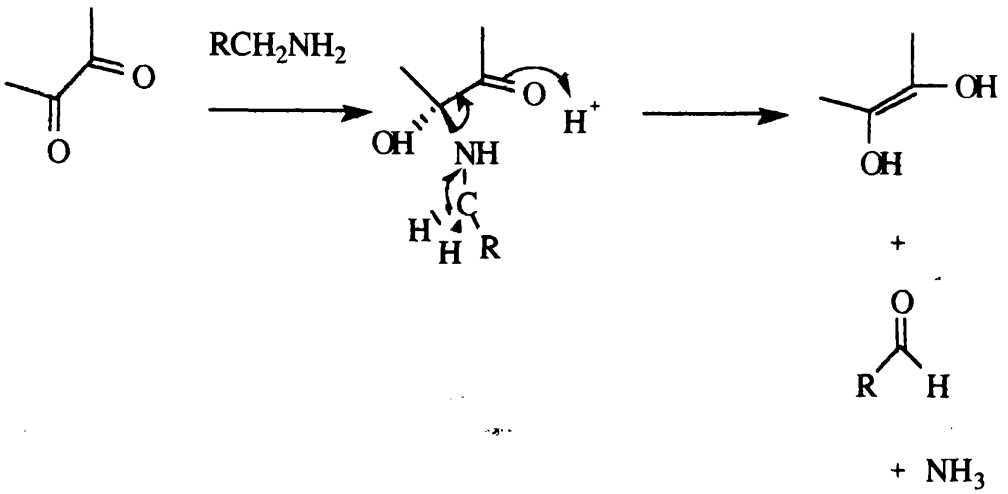
(13)

Using this species (13) as a reactive intermediate, subsequent attack of alcohol at C-4 would lead to a carbinolamine derivative intermediate which on oxidation of the substrate would produce an aminoquinol containing nitrogen at C-4 as the reduced form of the cofactor (Scheme 2.1).



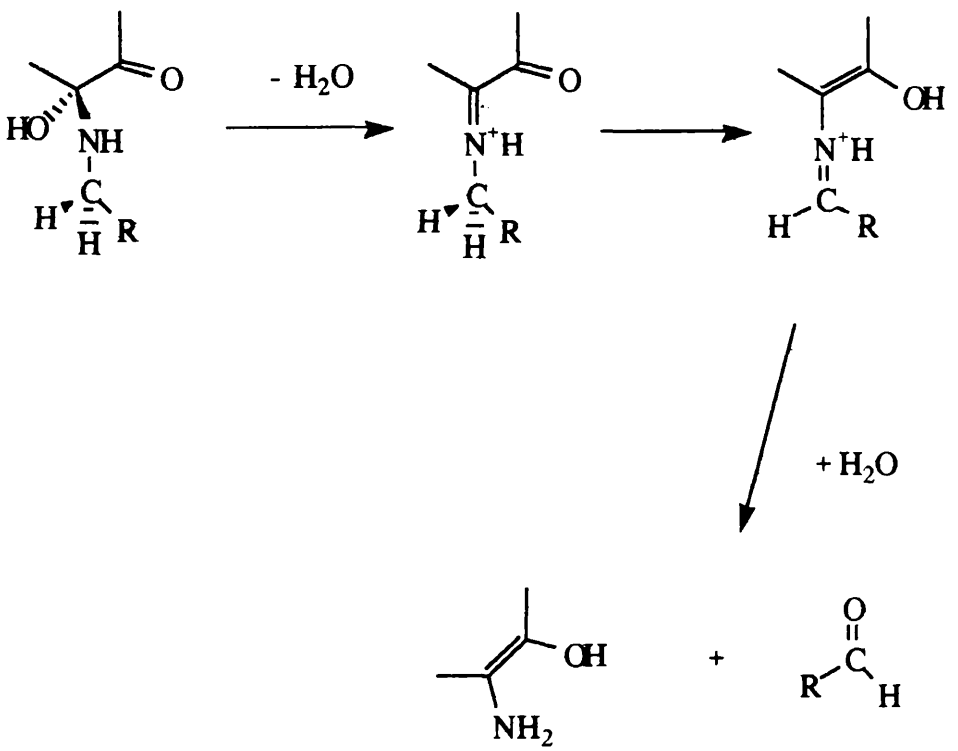
Scheme 2.1

This reaction mechanism has one major difficulty. In this case the C-4 position is the electrophilic centre whereas all existing literature on PQQ states that the C-5 position has an unusually high reactivity toward nucleophilic attack.[48] With this in mind a relatively straightforward mechanism can be written involving initial formation of a hemi-acetal at C-5 followed by substrate oxidation to yield directly the reduced quinol of PQQ. An analogous mechanism has been proposed for amine oxidation because the quinol form of reduced PQQ is a major product in anaerobic conditions (Scheme 2.2).



Scheme 2.2

Amines however can generate stable Schiff base intermediates with PQQ. Bruice and co-workers[49] reported a minor pathway for amine oxidation (Scheme 2.3).

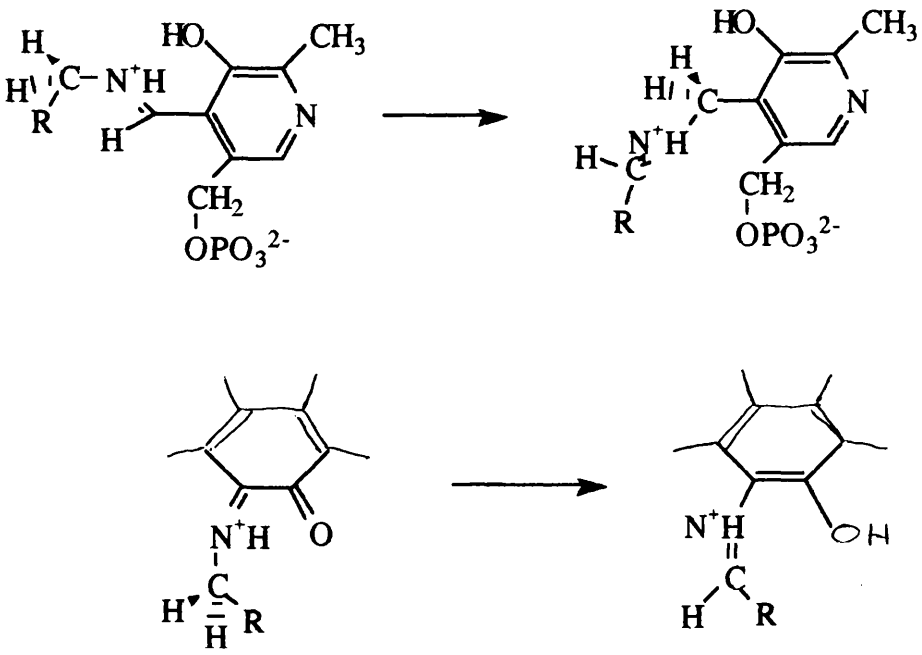


Scheme 2.3

To enable these workers to determine whether the Schiff base complexes are formed, examination of the nitrogen transfer from substrate to cofactor in the course of cofactor reduction was required. It was reported by Taylor *et al.*[50] that under anaerobic conditions, oxidation using porcine plasma amine oxidase produced a burst of ammonia. This result appeared to rule out the latter suggestion of the amino transferase mechanism. However further experiments included an elegant experiment by Ruis *et al.*[51] Using quenched-flow studies and a sensitive assay for the determination of ammonia they found that the time course for ammonia release correlated with enzyme reoxidation *ie.* favouring the last mechanism.

2.9 Further Studies on the Chemistry of Pyrroloquinoline Quinone (PQQ)

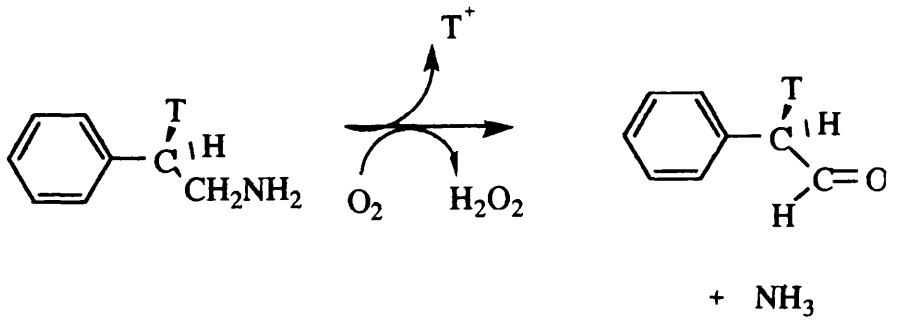
The proposed interaction of amine substrates with the bound cofactor of copper amine oxidases is very similar to that of pyridoxal phosphate chemistry. The Schiff base is formed and the proton is removed from C-1 of substrate by a base catalysed reaction. A possible difference may be in the fate of the α-hydrogen of the substrate (Scheme 2.4).



Scheme 2.4

With pyridoxal phosphate it undergoes a 1,3-prototropic shift whereas with PQQ direct transfer to the carbonyl oxygen at C-4 may occur.

Experiments carried out to investigate this point focussed on the unusual side reaction catalysed by bovine plasma amine oxidase. Lovenberg and Beaven[52] showed that plasma amine oxidases catalyse an exchange of the β-hydrogen from phenethylamines in the course of catalytic oxidation.



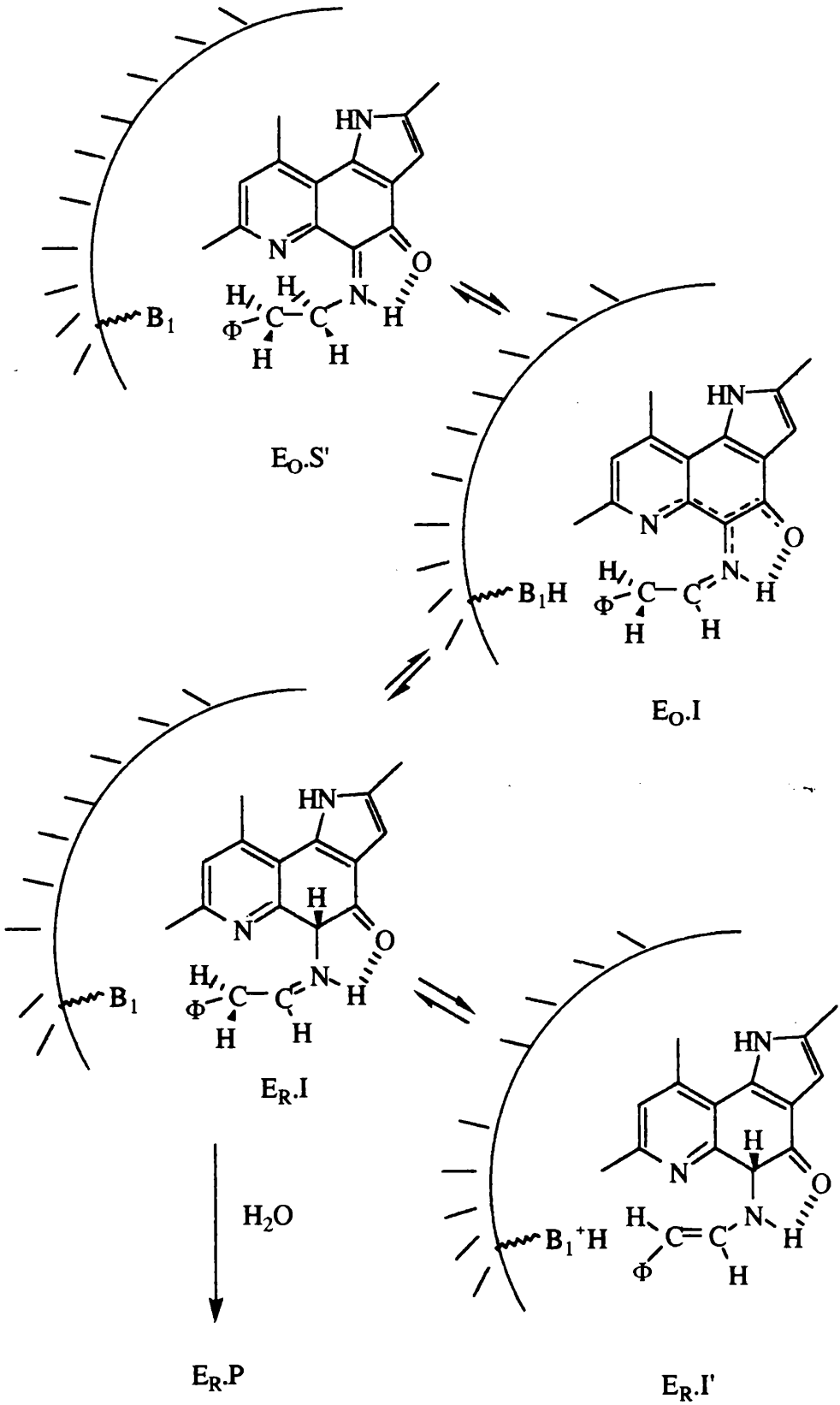
Scheme 2.5

Kinetic studies on this process showed that the transfer of reducing equivalents from substrate to cofactor requires to be reversible and, that a step subsequent to cofactor reduction is partially rate limiting.

As a consequence of this work bovine plasma amine oxidase was originally thought to function *via* a two base mechanism.

Further studies on pH dependencies of bovine plasma amine oxidase led to the discovery of identical pK_a values for exchange of the β-hydrogen and the substrate oxidation. This implies that the same residue performs both functions.[54] Strong support for this was provided by recent investigations into the stereochemistry of the reaction.[53] The data now available strongly suggest that it is a single catalytic residue that catalyses both oxidation of substrate and exchange of the β-hydrogen.

Scheme 2.6 incorporates an explanation of the role of PQQ in amine oxidation. It shows the formation of the Schiff base complex between amine substrate and PQQ; the oxidation of substrate *via* a proton abstraction mechanism; and also the transfer of both hydrogen and nitrogen from C-1 of the substrate to cofactor in the reductive half reaction.



Scheme 2.6 Proposed mechanism for bovine plasma amine oxidase.

Scheme 2.6 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; $E_R.I$ is the product Schiff base, involving 1,3 prototrophic shift from substrate to cofactor; $E_R.I'$ is the enamine formed in the course of hydrogen exchange from the β-carbon.[54]

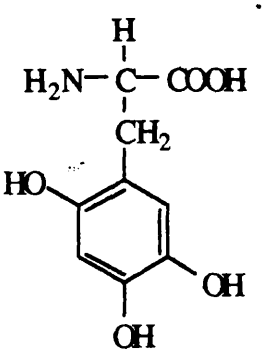
2.10 Arguments Against Pyrroloquinoline Quinone (PQQ) as the Cofactor

One major drawback throughout the studies on PQQ has been the lack of direct evidence for the presence of PQQ at the active site of a single mammalian protein. Although, as discussed earlier, an active site cofactor-containing peptide has been isolated from pig kidney diamine oxidase,[38] the yield reported was extremely low (0.1%). This work has been questioned, since the characterisation of the peptide was limited to sequencing. This was presumably due to insufficient material being available for further identification.

Recent X-ray studies have shown that there is no density corresponding to PQQ in some enzymes which were thought to contain it as the cofactor. Work on methylamine dehydrogenase from *Thiobacillus versutus* by Hol and co-workers[55] showed X-ray diffraction patterns which indicate an active site dicarbonyl which lacks the pyridine ring, present in PQQ. This contradicts the work of Duine on methylamine dehydrogenase.[56] Early in 1991 Ito *et al.*[57] reported the crystal

structure of galactose oxidase, previously claimed to contain PQQ.[58] The crystal structure work showed that all electron density observed could be accounted for by the known primary structure of the protein and by solvent and ions in the solution; no density corresponding to pyrroloquinoline quinone (PQQ) was evident.

Janes *et al.*[59] in 1990 isolated an active site cofactor-containing peptide from bovine serum amine oxidase. Due to the high yield (40%) and small size (five residues) of this peptide a complete structural characterisation was achieved. They were able to demonstrate that the cofactor is not PQQ and evidence shows it to be 6-hydroxydopa (topa) (14).



(14)

A derivatised peptide (phenylhydrazone and dihexyl derivative formed) was isolated by mild enzymatic proteolysis and purified by HPLC. The sequence of the purified peptide fraction was shown to be;

-Leu-Asn-X-Asp-Tyr-

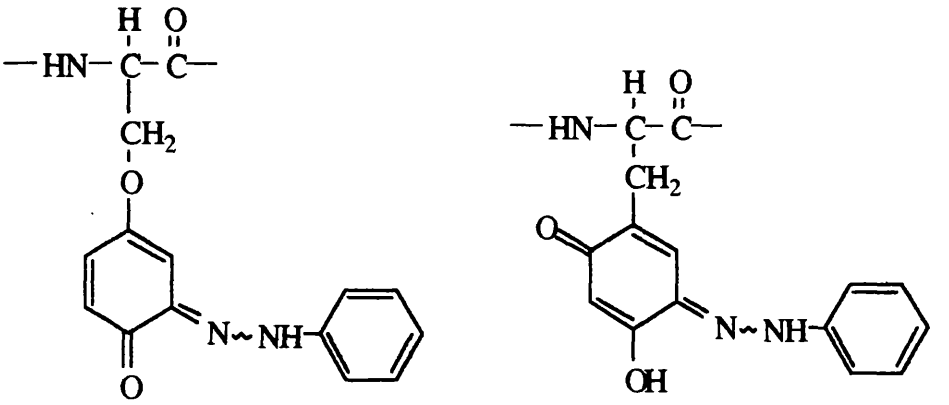
Because only one amino acid was detected at each round of peptide sequencing, Janes *et al.*[59] concluded that the cofactor had a single, stable point of attachment to the protein. The

sequence shown above has been verified in all subsequent peptide preparations and used for all the spectrometric characterisations. The spectrometric characterisations used included (1) mass spectrometry; (2) UV/Vis spectroscopy; and (3) proton NMR spectroscopy.

(1) Mass spectrometry: Studies on the dihexyl derivative of the pentapeptide led to an exact molecular mass measurement of 974.5123. Subtracting the two hexyl groups and the accurate masses of Leu, Asn, Asp and Tyr, a value for X of 283.0967 was obtained. This is not consistent with a PQQ type structure.

With the phenylhydrazone derivative, computer permutation of possible elemental compositions for X gave five possible empirical formulae within ± 5 ppm of the observed value. Only one of these was compatible with both UV/Vis absorbance properties of the active site cofactor and the presence of phenylhydrazone in X. Two structures (A) and (B) were consistent with the formula $C_{15}H_{13}N_3O_3$.

High energy collision induced dissociation (CID) mass spectroscopy was used in an effort to distinguish between (A) and (B). The observations from the spectra permitted a tentative assignment of the active site cofactor derivative in bovine serum amine oxidase to be (B), the phenylhydrazone of 6-hydroxydopa (quinone form).



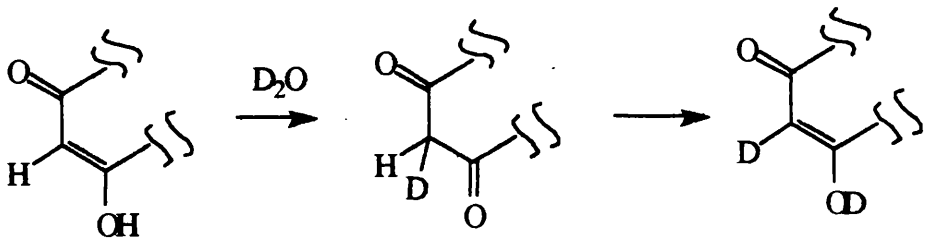
(A) (B)

Figure 2.1 Structures compatible with an empirical formula of $C_{15}H_{13}N_3O_3$ for residue X in the pentapeptide. (A) A serine residue, attached to the phenylhydrazone of a catechol *via* an ether linkage. (B) The phenylhydrazone of 6-hydroxydopa (shown as the quinone form).

(2) UV/Vis spectroscopy: Like many others Janes *et al.* observed λ_{max} at 448 nm for phenylhydrazone-derivatised bovine serum amine oxidase. This correlates with the value for the phenylhydrazone of PQQ. However Janes showed that there is a shift of *ca.* 14 nm for the λ_{max} of isolated peptides. Therefore removal of protein effects on electronic transitions leads to almost exact correspondence between λ_{max} values for phenylhydrazone-containing peptides and the phenylhydrazones of topa quinone.

(3) Proton NMR Spectroscopy: As final proof of the structure of the active site cofactor, proton NMR spectroscopy experiments were carried out. For the region above δ 6.0, two doublets at δ 6.8 and 7.1 integrate to four hydrogens and are

easily assigned to the tyrosine side chain (at position five of the peptide chain). Three other peaks at δ 7.5, 7.2 and 6.9 (integrating 4-5:1:1 resp.) were originally assigned to the five hydrogens in the phenylhydrazone ring (δ 7.5) and the two hydrogens in the cofactor (δ 7.2, 6.9). Decoupling experiments however showed the signal at δ 7.2 was due to a hydrogen on the phenylhydrazone ring. Only one of the two ring hydrogens of the cofactor could be detected because the experiments were carried out in D_2O. As shown the CH bond at position five of the cofactor ring lies between an enol and a ketone (Scheme 2.7). An acid-base-catalysed enol tautomerism is to be expected, which in the presence of D_2O would produce deuteriated cofactor. The NMR spectrum was eventually run in H_2O so that all proton signals could be observed.

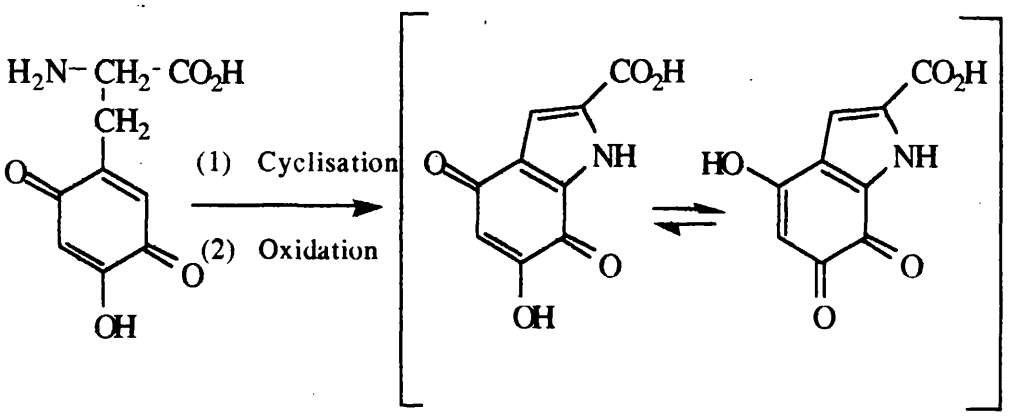


Scheme 2.7

Proof of the presence of PQQ as the cofactor has relied on the isolation of compounds from phenylhydrazine-inactivated enzymes that co-elute on HPLC with authentic samples of PQQ phenylhydrazones. It has been noted that retention times on HPLC represents a fairly tenuous form of structural proof.

It is possible that a derivative of the topa phenylhydrazone, formed from bovine serum amine oxidase during proteolysis may co-elute with the phenylhydrazone of

PQQ. Topa quinones undergo a rapid intramolecular cyclisation reaction shown in Scheme 2.8.



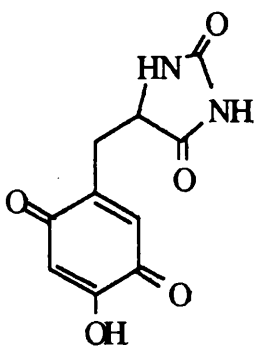
(15)

Scheme 2.8

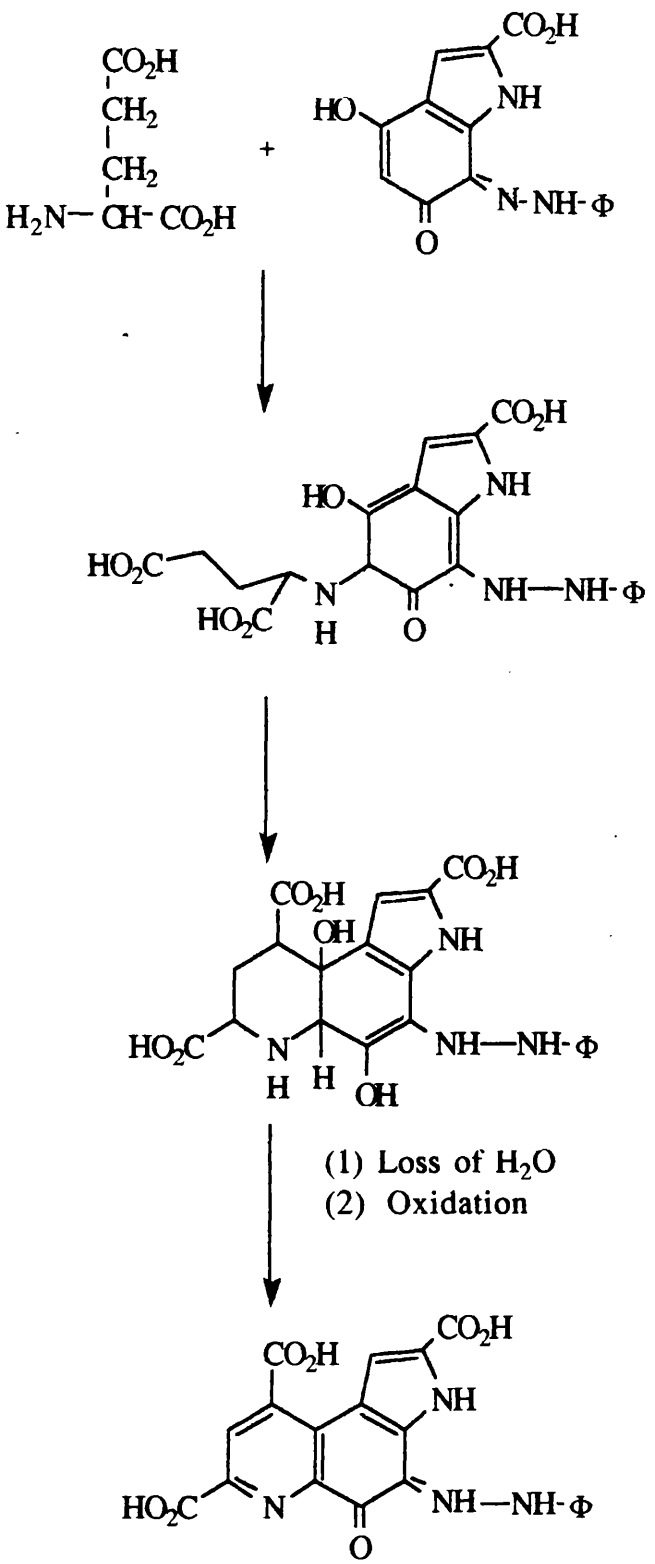
This compound formed bears a very close resemblance to PQQ. This may occur during pronase digestion. Compound (15) and its phenylhydrazone are highly reactive and are expected to form Michael adducts with a range of nucleophilic compounds. The derivative formed by the reaction of (15) with glutamate would co-elute with PQQ. Although many other amino acids have side chains more nucleophilic than glutamate, glutamate is expected to be accumulated after pronase digestion and the formation of this derivative may be favoured by high concentration of this specific amino acid. In addition the two nucleophilic positions in glutamate (at the α-amino group and the γ-carbon) may drive the condensation reaction *via* a favoured ring closure to form a stable six-membered ring (Scheme 2.9). Other amino acids such as serine and cysteine are incapable of the final oxidation step to form a stable aromatic species.

Although resonance Raman studies[41] on phenlhydrazone derivatives of bovine and procine serum amine oxidases revealed spectral properties that are similar to the phenylhydrazone of PQQ, they are not identical.

Using the highly sensitive assay for the detection of PQQ in cell extracts by Gallop,[43] mentioned earlier, Janes found that the glycine-nitroblue tetrazolium redox cyclising system of Gallop works nearly as well with topa hydantoin (shown in the oxidised form) (16) as with PQQ.



(16)



Scheme 2.9 Proposed mechanism for the generation of a PQQ-like product from the ring cyclised form of the topa quinone (phenylhydrazone derivative).

2.11 Is Topa the Cofactor?

The 6-hydroxy derivative of dopa has been recognised as being neurotoxic.[60] This is thought to be related to facile redox reactions carried out by this type of compound. It is therefore strange that the redox properties of 6-hydroxydopa have been harnessed in a constructive manner through its use as an active site, enzymatic cofactor. It is important to ascertain the full scope of topa as a redox cofactor. It is worthy to note that the X-ray studies of Vellieux and co-workers[55] on the bacterial methylamine dehydrogenase show a ring-cyclised isomer of topa, attached to the protein *via* a glutamate side chain.

In bovine serum amine oxidase a reasonable hypothesis for topa formation occurs *via* a post-translational process involving oxidation of an active site tyrosine. However, since topa is a naturally occurring amino acid, the possibility of its direct incorporation into the growing protein chain, *via* special transfer RNA, cannot be ignored.

These notes raise the possibility of a role for topa-like molecules either through the covalent attachment to active side chain (methylamine dehydrogenase) or through their direct incorporation into the protein backbone (bovine serum amine oxidase).

In light of the evidence discussed for and against PQQ as the cofactor, purification and crystallisation of the protein is required and X-ray structure studies need to be carried out on the purified enzyme. Until then we cannot know for certain the exact structure of the active site cofactor in diamine oxidases.

2.12 Radical Copper Galactose Oxidase

The involvement of copper in amine oxidases was discussed in Section 2.2. In 1991 new evidence was published on the involvement of copper in galactose oxidase.

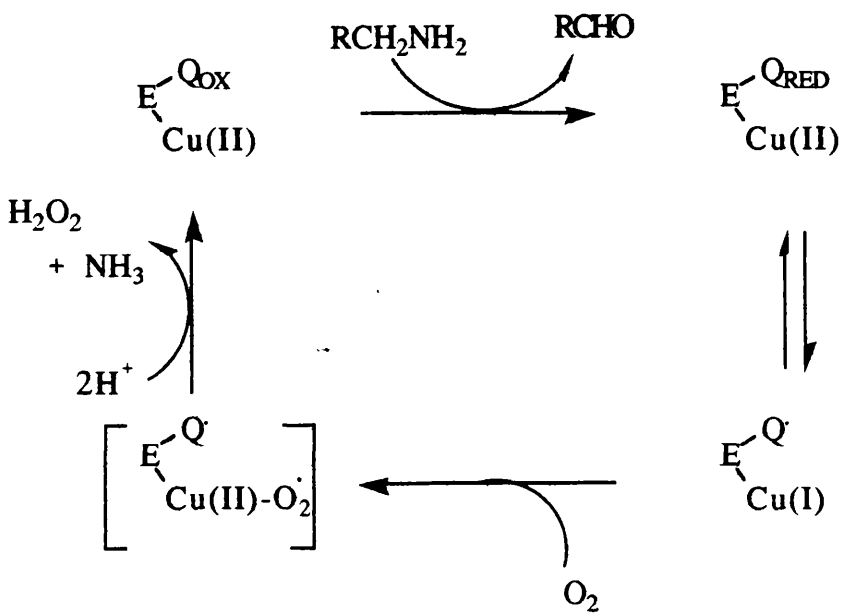
Oxidases containing a mononuclear copper site catalyse the two electron oxidation of substrates by dioxygen, which is thereby reduced to hydrogen peroxide. The mechanism of these enzymes has posed the question as to how a single copper centre capable of undergoing only a one electron change Cu(II)/Cu(I) can catalyse a two electron transfer from an organic substrate.

It was reported by Thomson[61] in 1991 that spectroscopic evidence indicates that the active site of galactose oxidase can store two oxidising equivalents, one as the Cu(II) ion and the other as a radical cation. As a result of the recent X-ray data on galactose oxidase[57] the radical cation has been identified as that of a modified tyrosine side chain.

2.13 A Cu(II)-Semiquinone State Exists in Substrate Reduced Amine Oxidases

Also in 1991 evidence was presented for the generation of a Cu(I)-semiquinone state by substrate reduction of amine oxidases under anaerobic conditions.[26] It was also suggested that the Cu(I)-semiquinone may be the catalyst intermediate that reacts directly with dioxygen. EPR spectral changes accompanying the addition of appropriate amine to various amine oxidases (including pea seedling and pig kidney diamine

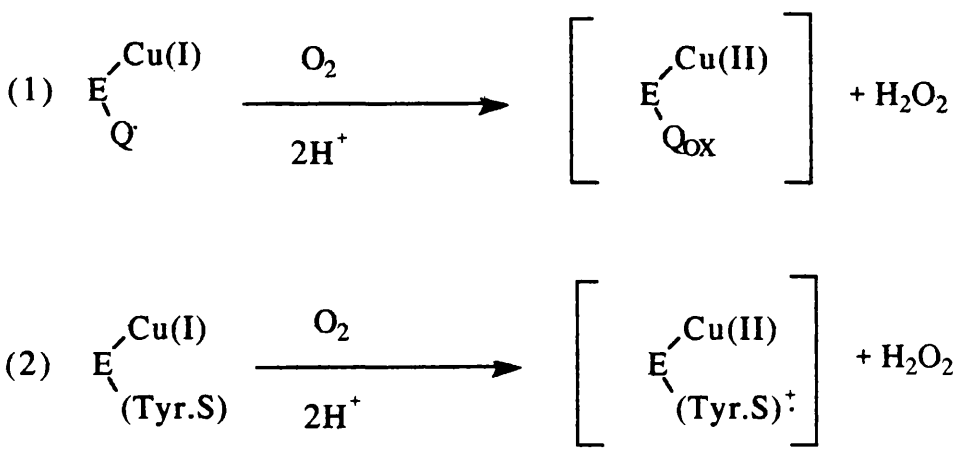
oxidase) were recorded and shown to be similar. Because the radical EPR spectrum is independent of the source of diamine oxidase and the substrate used, the radical must be associated with a moiety that is conserved among the amine oxidases examined. Cyanide or *t*-butylisocyanide were used to trap the semiquinone form by stabilising Cu(I) and enhancing the radical signal. These EPR data allow the development of a possible mechanism (Scheme 2.10) featuring well-precedented roles for both copper and the quinone, which negates the problem associated with two-electron reductions of dioxygen.



Scheme 2.10 Possible catalytic cycle of copper containing amine oxidases. The species in brackets is a hypothetical intermediate, shown here to emphasize the possibility of sequential one-electron step in the reduction of O_2. Q_{OX} is oxidised quinone; Q is semiquinone; Q_{RED} is two electron reduced quinone.

The Cu(I)-semiquinone state of amine oxidase has not been detected before because this state is apparently in equilibrium with Cu(II)-reduced quinone. Internal electron transfer from copper to semiquinone is favoured by low temperatures, and since previous work used low temperature EPR spectroscopy to study the copper centre, the semiquinone form was missed.

A comparison has been made between the structures of the catalytic intermediates of galactose oxidase and amine oxidases generated by dioxygen (Scheme 2.11)



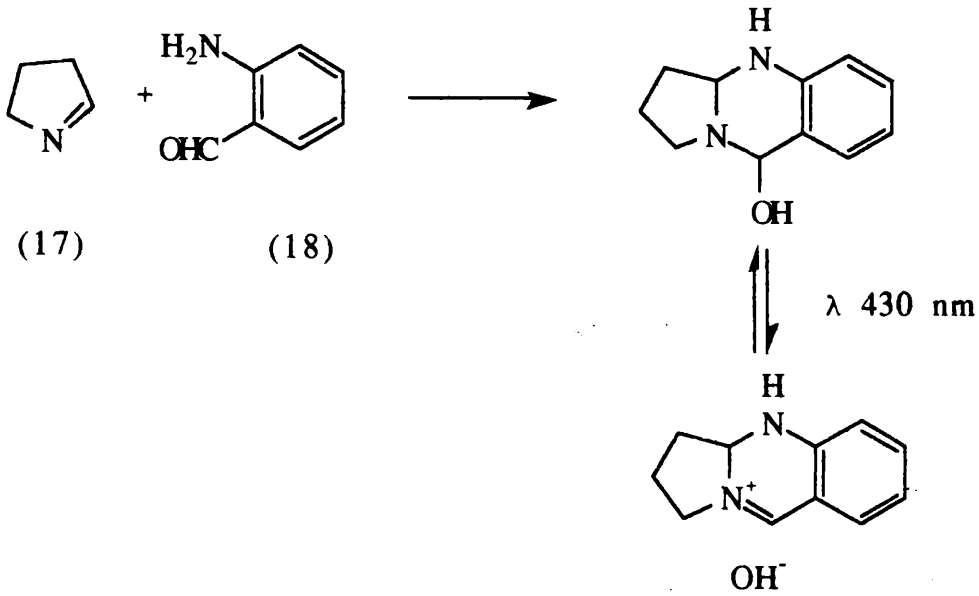
Scheme 2.11 The nature of the catalytic intermediate generated by dioxygen in (1) amine oxidases; Q is 6-hydroxydopa quinone. (2) galactose oxidase; Tyr-S is Tyrosine covalently linked to the thiol side chain of cysteine.

It is clear from the X-ray structure that, in the case of galactose oxidase, the tyrosine radical forms a ligand to the copper ion and therefore may function as a two electron oxidant. It is not yet known in the case of amine oxidases

whether the quinone is bonded to copper or if the substrates interact at the metal site.

2.14 Different Assay Systems Used for the Determination of Diamine Oxidase Activity

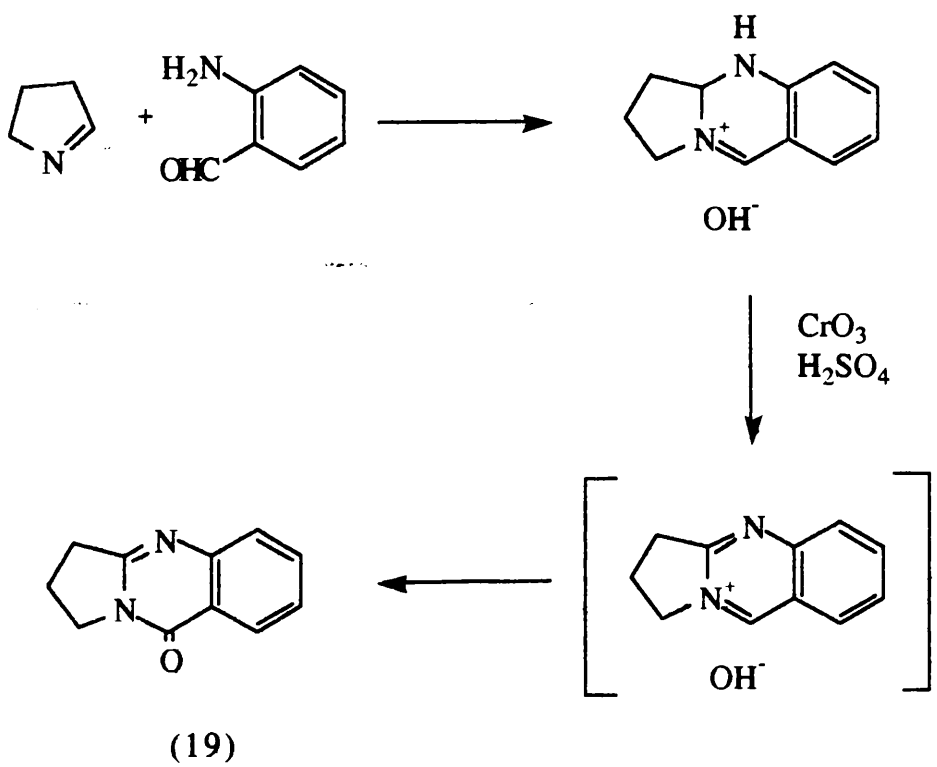
The technique used in early studies on the determination of diamine oxidase activity was the manometric measurement of oxygen uptake.[62,63] Several other methods have been developed. Holmstedt and Tham[64] used the production of 3,4-dihydro-2*H*-pyrrole (1-pyrroline) (17) from the reaction mixture of putrescine (1) and diamine oxidase (the initial oxidation product, γ-aminobutanal, cyclises to give 1-pyrroline) to determine the activity of diamine oxidase. With the addition of *o*-aminobenzaldehyde (18), 1-pyrroline (17) forms a yellow compound which can be determined spectrophotometrically (Scheme 2.12). Holmstedt *et al.*[65] calibrated this reaction using known concentrations of the acetal of γ-aminobutanal. This acetal was hydrolysed and the product was treated with *o*-aminobenzaldehyde. This procedure enabled them to obtain the activity of diamine oxidase in micromoles of diamine per mg of enzyme per hour. This allowed comparisons to be made with other methods and these results compared well with those obtained from the manometric measurements of oxygen uptake.



(17) (18)

Scheme 2.12

Sakamato[66] described a quantitative conversion of 1-pyrroline into (19) by oxidation as shown in Scheme 2.13.

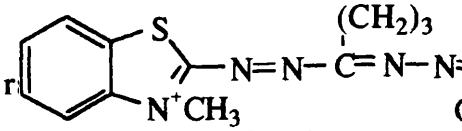


(19)

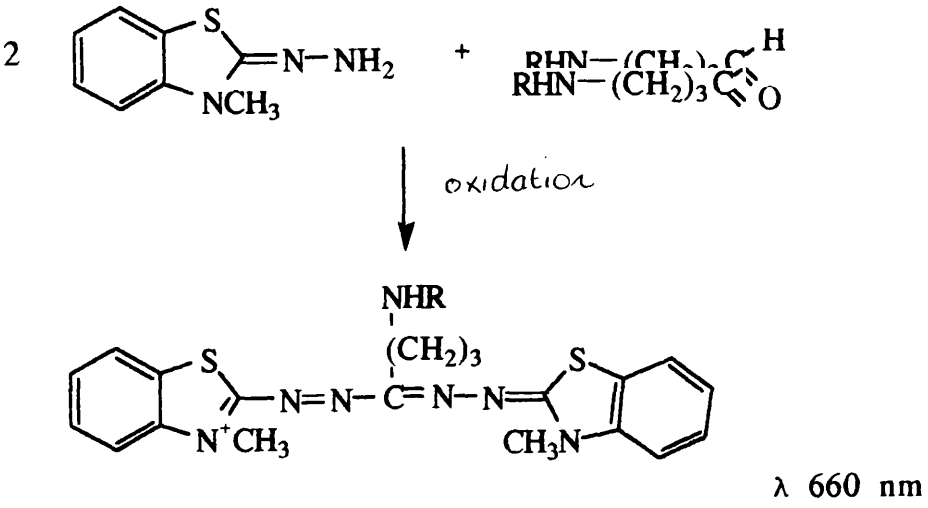
Scheme 2.13

A new colorimetric method for the assay of diamine oxidase was described by Naik *et al.*[67] in 1981. This involves the reaction of 1-pyrroline (17) with ninhydrin reagent in acidic medium to form a coloured complex with λ_{max} 510 nm.

The problem with the methods described using the production of 1-pyrroline (17) for the determination of diamine oxidase activity is that it is limited to the determination of the activity using only putrescine as the substrate.



Frydman *et al.*[12] determined the r
substrates of diamine oxidase by trapping the oxidation products (*ie.* aminoaldehydes) with 3-methyl-2-benzothiazolinone (MBTH) (20) and then measuring the absorbance of the resulting bishydrazone at 660 nm (Scheme 2.14).



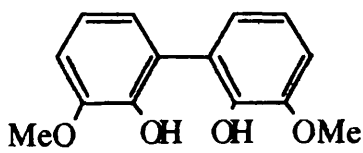
Scheme 2.14

Frydman *et al.*[12] used this method to determine the rates of the oxidation of *N*-alkylputrescines by diamine oxidase.

However the methods which involve the determination of the oxygen uptake are still generally used as they can be carried out for many substrates.[62,63]

Procedures have been developed for the measurement of hydrogen peroxide produced as this is a common product in all diamine oxidase reactions.

Booth and Saunders[68] developed a peroxidase coupling reaction. Using hydrogen peroxide, in the presence of peroxidase, guaiacol was rapidly oxidised to a brown-red solid from which 2,2'-dihydroxy-3,3'-dimethylbiphenyl (21) was isolated.



(21)

Smith[69] used this reaction to determine the activity of polyamine oxidases and diamine oxidases. He used a colorimetric procedure utilising the peroxidase/guaiacol assay which had been adapted to determine the hydrogen peroxide formed in the course of amine oxidation. The guaiacol oxidation products however include certain quinones[68] which are generally highly reactive. These may combine with other compounds in the reaction mixture modifying the chromogen, enzyme or substrate. It is also known that the 1-pyrroline formed from putrescine in the presence of diamine oxidase

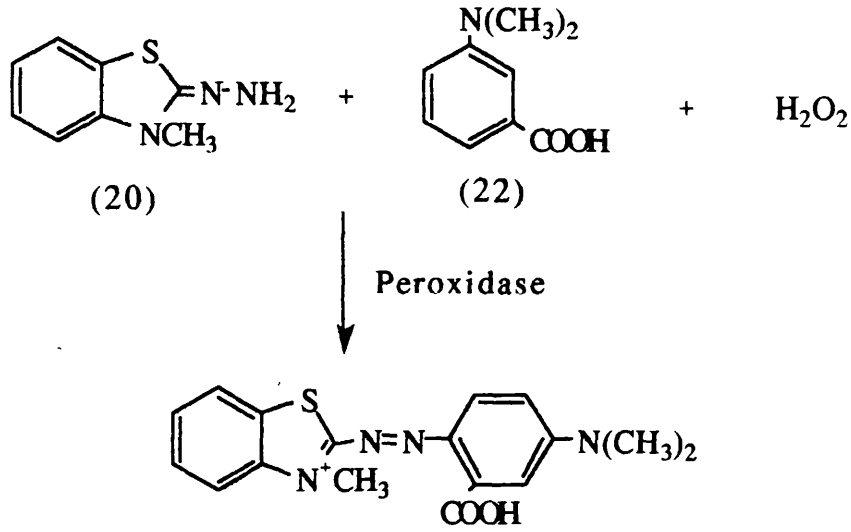
may be oxidised further on addition of peroxidase. Despite the potential complications however the stoichiometry obtained with the various substrates suggests that the method provides a reliable estimate of enzyme activity.

In 1985 Stoner[14] reported an improved spectrophotometric assay for the measurement of diamine oxidase activity. This was again based on the amount of hydrogen peroxide produced to determine the enzymatic reaction rate. It involves a coupled reaction with 3-methyl-2-benzothiazolinone hydrazone (MBTH) (20) with an appropriate acceptor such as 3-(dimethylamino)benzoic acid (DMAB) (22). The coupled reaction depends on the generation of hydrogen peroxide by diamine oxidase. In the presence of hydrogen peroxide and peroxidase, the chromogen MBTH is oxidatively coupled to DMAB, forming a purple indamine dye having an adsorption maximum at 595 nm (Scheme 2.15).

1. $H_2N-(CH_2)_4-NH_2$ —Diamine oxidase→ $H_2N-(CH_2)_3-C(=O)H$

$+ H_2O + O_2$ $\qquad$ $+ H_2O_2 + NH_3$

2.



λ_{max} 595 nm

Scheme 2.15

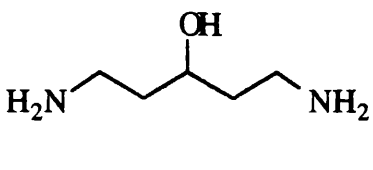
It has been shown that solutions of MBTH and DMAB will react slowly to form the coloured product although blanks have been used over the time period of the reaction to overcome that problem. Stoner showed that this assay system was a rapid and sensitive method of measuring diamine oxidase activity with histamine as the substrate, though it has since been shown by us to be a particularly reliable and convenient method for determining the activity with a wide range of substrates.[70,71]

2.15 Substrate Specificity and the Active Site

In order to gain information about the active site of diamine oxidases, it is necessary to examine the substrate specificity of diamine oxidase. Extracts of pea seedlings and partially purified diamine oxidase preparations made therefrom, catalyse the oxidation of many aliphatic diamines. Other substrates include histamine, agmatine, aliphatic monoamines, phenylalkylamines and the dibasic amino acids lysine and ornithine. Mann[72] concluded that the oxidation of all these compounds was catalysed by one enzyme of wide substrate specificity. These observations were confirmed when it was shown in 1961 that many of these compounds were oxidised by highly purified diamine oxidase.[73] This enzyme from pea seedlings resembles in substrate specificity the diamine oxidase of animal tissues which catalyses the oxidation of both mono- and di-amines. Monoamines however are less readily oxidised than diamines. This indicates the importance of the second amino group of the substrate in the active site of diamine oxidase. Although 1,4- and 1,5-diamines were readily oxidised, no activity was observed with 1,2- and 1,3-diamines with pea seedling diamine oxidase. The oxidation of 1,3-diaminopropane however was found to be catalysed by the animal enzyme.[62] This implies that the active sites of the enzymes from plant and animal sources are in some way different.

2.16 Differences in Substrate Specificity of Pea Seedling and Pig Kidney Diamine Oxidase

Macholan used three hydroxydiamines, 3-hydroxypentane-1,5-diamine (23), 2-hydroxybutane-1,4-diamine (24) and 2-hydroxypentane-1,5-diamine (25) to show different behaviour of plant and animal diamine oxidases.[74]



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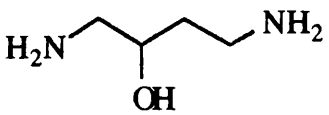

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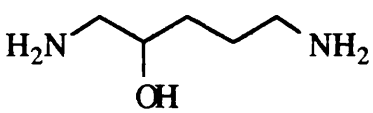


(25)

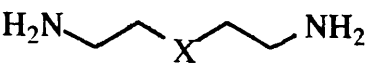
He found that 3-hydroxypentane-1,5-diamine (23) binds with the lowest affinity to pea seedling diamine oxidase whereas with pig kidney diamine oxidase it has the highest affinity. Macholan also reported that pea seedling and pig kidney diamine oxidase showed different behaviour towards C_4 and C_5 hydroxy-diamines. This demonstrates that certain differences in the structure of the active sites of these enzymes may exist.

Frydman *et al.*[12] studied the oxidation of *N*-alkylputrescines and *C*-alkylputrescines catalysed by diamine oxidase from both the animal and plant sources. *N*-Methyl-, *N*-ethyl-, *N*-propyl- and *N*-butylputrescine were assayed as

substrates of diamine oxidases. With the exception of *N*-methylputrescine they were found to be oxidised to their corresponding aminoaldehydes. This was a strange result and probably an error since we have since shown that *N*-methylputrescine is a good substrate of diamine oxidases.[70] Frydman also showed that *C*-alkylputrescines, namely 1-methyl-, 2-methyl-, 1-ethyl- and 1-propylputrescine were oxidised by diamine oxidase though at lower rates than the *N*-alkyl-derivatives. 1,4-Dimethylputrescine was found not to be a substrate of diamine oxidase. This implies that secondary amines are not oxidised by diamine oxidase to the corresponding ketones. 1-Propylputrescine and 2-methylputrescine were oxidised at very different rates by the mammalian and plant oxidases, indicating again that although both enzymes have similar catalytic functions they may differ in active site geometries. Therefore diamine oxidases have been shown to have a wide substrate specificity. However differences in the specificities of pea seedling and pig kidney diamine oxidase exist, indicating differences in the enzyme active sites.

2.17 Analogues of Diamines Containing Group VI Atoms (O, S, Se)

Thiodiamines, for example lanthionamine (26) and the oxygen analogue (27) were oxidised with pea seedling diamine oxidase.[75]

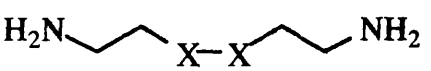


X = S (26)

X = 0 (27)

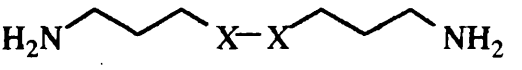
X = Se (32)

Other thiodiamines, like cystamine (28), homocystamine (29) and homolanthionamine (30) were shown to be substrates of pig kidney diamine oxidase. In the course of studies on seleno-analogues[76] of these sulphur amines it was observed that like cystamine (28) and lanthionamine (26), the selenocystamine (31) and selenolanthionamine (32) were also oxidatively deaminated by pig kidney diamine oxidase.



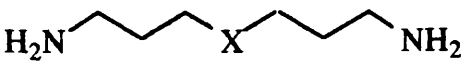
X = S (28)

X = Se (31)



X = S (29)

X = Se (33)



X = S (30)

X = Se (34)

Seleno-homocystamine (33) and seleno-homolanthionamine (34) were also shown to be substrates of pig kidney diamine oxidase, although they were not oxidised as rapidly as seleno-lanthionamine (32).

These results together with the work discussed in 2.15 and 2.16 emphasise the broad substrate tolerance of diamine oxidases.

2.18 The Requirement of Polyamines for Growth and Replication

Polyamines are required for optimal growth in all tested living cells. Many studies have shown that rapidly growing cells have higher levels of polyamines than slowly growing or inactive cells. It is also known that the polyamine content in the cells increases before an increase in DNA, RNA or protein content.

After administration of inhibitors of polyamine biosynthesis, the levels of putrescine (1) and spermidine (2) fall rapidly. This decrease is noticed especially in rapidly replicating cells where there is a dramatic inhibition of growth and replication.

It is clear therefore that polyamines are important to growth and replication of all living cells and that inhibitors of the polyamine biosynthesis can have a dramatic effect on the concentration of polyamines in rapidly proliferating cells.[77,5]

2.19 Inhibitors of Diamine Oxidase

The inhibition of enzymatic activity by specific compounds is important because it serves as a major control mechanism in many metabolic pathways. Many drugs act by inhibiting enzymatic reactions. Inhibition can also give an insight into

the mechanism of enzyme action: residues critical for catalysis can often be identified by using specific inhibitors.

There are two types of enzyme inhibition, reversible and irreversible. An irreversible inhibitor dissociates very slowly from its target enzyme because it becomes very tightly bound to the enzyme. In contrast, reversible inhibition is characterised by a rapid dissociation of the enzyme-inhibitor complex. Within reversible inhibition there are two main types; competitive and noncompetitive inhibition. In competitive inhibition the enzyme can bind the substrate or the inhibitor but not both at the same time. The inhibitor competes for the same site on the enzyme as the substrate. Many competitive inhibitors are therefore analogues of substrates. A competitive inhibitor slows down the rate of reaction by competing for the same site on the enzyme as the natural substrate.

In noncompetitive inhibition the substrate and inhibitor can bind to the enzyme at the same time. A noncompetitive inhibitor acts (in the simplest case, at least) by decreasing the turnover number of an enzyme rather than the proportion of enzyme molecules that are bound to substrate (Figure 2.2).

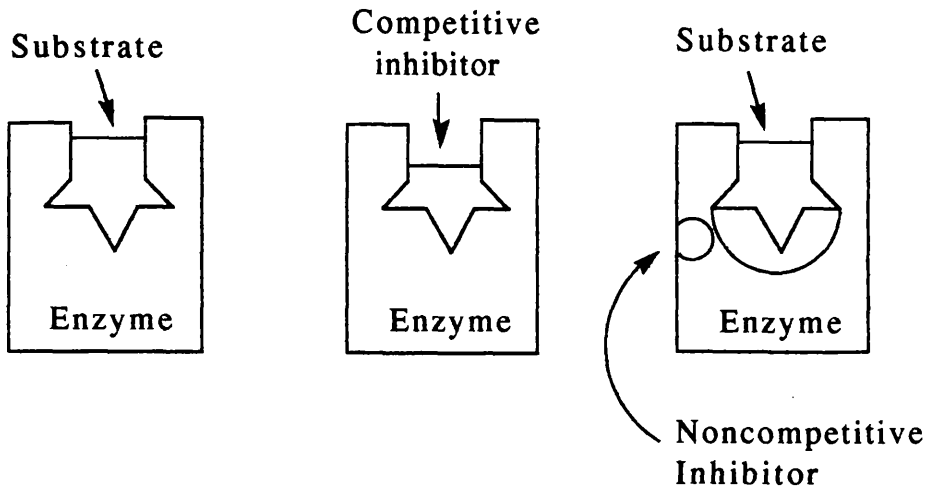


Figure 2.2

A study of inhibitors of diamine oxidase could shed light on the physiological role of the enzyme and could also indicate possible candidates for use as drugs.

Compounds that inhibit diamine oxidase can be classified into six different types:

(1) enzyme inactivators;
(2) copper chelating agents;
(3) substrate analogues;
(4) substrate inhibitors;
(5) product inhibitors; and
(6) suicide substrates.

The first three are the most common and will be discussed in more detail.

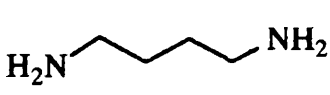
(1) Enzyme inactivators. Enzyme inactivators lead to partial or total loss of enzyme activity. Phenylhydrazine, hydroxylamine, semicarbazide and aminoguanidine are known enzyme

inactivators, reacting with the carbonyl function on the enzyme at the active site.

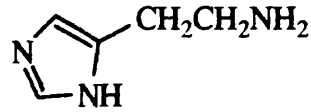
(2) Copper chelating agents. 1,10-Phenanthroline, 8-hydroxyquinoline and 2,2-bipyridyl act as inhibitors by chelating to the Cu(II) present in the enzyme. Azide and cyanide can also interact with the Cu(II). It must be noted however that this involves removing something from the enzyme and cannot be defined using Figure 2.2.

(3) Substrate analogues. Very good inhibitors are often found to be compounds that resemble the structure of the substrates, for example putrescine. Comparisons of structure *vs.* activity relationships between substrates and inhibitors of diamine oxidase and other enzymes have been made. Often a particular change in structure has been seen to bring out major changes in reactivity with the enzyme. Substrate analogues can act as competitive inhibitors of diamine oxidase. The rate at which the substrate analogue is enzymically converted into product is useful in predicting the possible inhibitor activity.

Bieganski *et al.*[78] explored the structural relationships between diamine oxidase inhibitors and two substrates, putrescine (1) and histamine (35), with respect to enzymes of different origins.



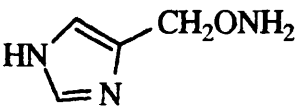
(1)



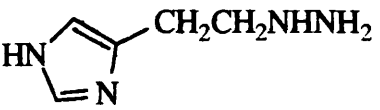
(35)

It was thought as a result of earlier investigations that imidazole and its derivatives inhibited mammalian diamine

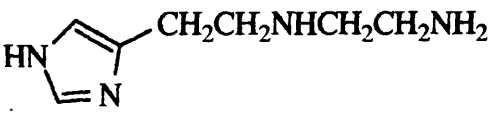
oxidase and not pea seedling diamine oxidase. By contrast the aliphatic diamine 1,5-diaminopentan-3-one was reported as a better inhibitor of diamine oxidase from pea seedlings than from the mammalian source. Amongst the compounds tested by Bieganski *et al.*[78] were two classes of compounds, (a) those with structures resembling histamine together with a reactive amino group, for example compounds (36) and (37); and (b) compounds combining histamine and the aliphatic diamine structures, for example compound (38).



(36)



(37)



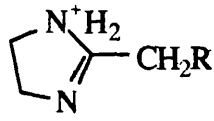
(38)

Compounds (36) and (37) were found to be potent inhibitors of diamine oxidase from both the mammalian and plant sources. The result showed that the presence of the imidazole ring could still lead to inhibition of the pea seedling diamine oxidase.

Compound (38) showed inhibition with the pea seedling diamine oxidase selectively. This may be due to the presence of the aliphatic diamine on the side chain of the ring.

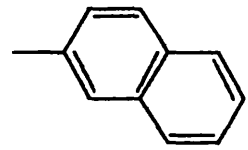
Pec and Hlidkova[79] reported the use of 4,5-dihydroimidazole derivatives as inhibitors of pig kidney diamine oxidase. The compounds tested included 2-methyl-

4,5-dihydroimidazole (39), natazolin (40), fentolamin (41) and anatazolin (42).

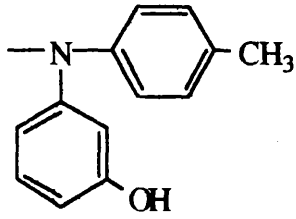


R = —H

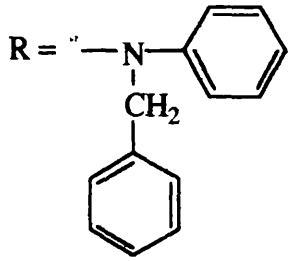
(39)

R = 

(40)

R = 

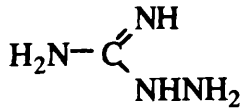
(41)

R = 

(42)

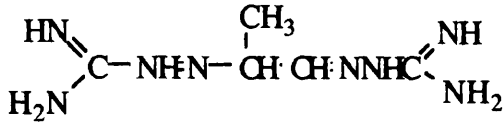
All the compounds tested with the exception of (39) were shown to be noncompetitive inhibitors of pig kidney diamine oxidase. This means that the inhibition could not be prevented by addition of excess substrate. The compounds studied are used in human medicine as adrenergics (40), antiadrenergics (41) and antihistamines (42).

Another group of compounds that are particularly potent inhibitors of diamine oxidase are the amidines, especially aminoguanidine (43).[80]



(43)

Other amidines shown to be inhibitors of diamine oxidase include MGBG (44) and pentamidine (45).[81]



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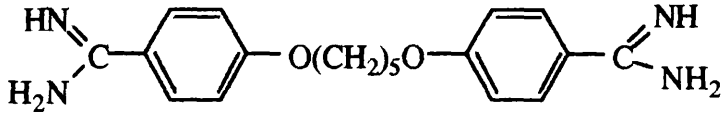


(45)

It is now thought that amidines are more useful as inhibitors than their corresponding amines although further investigations are required.

2.20 Stereochemistry of Reactions Catalysed by Diamine Oxidase

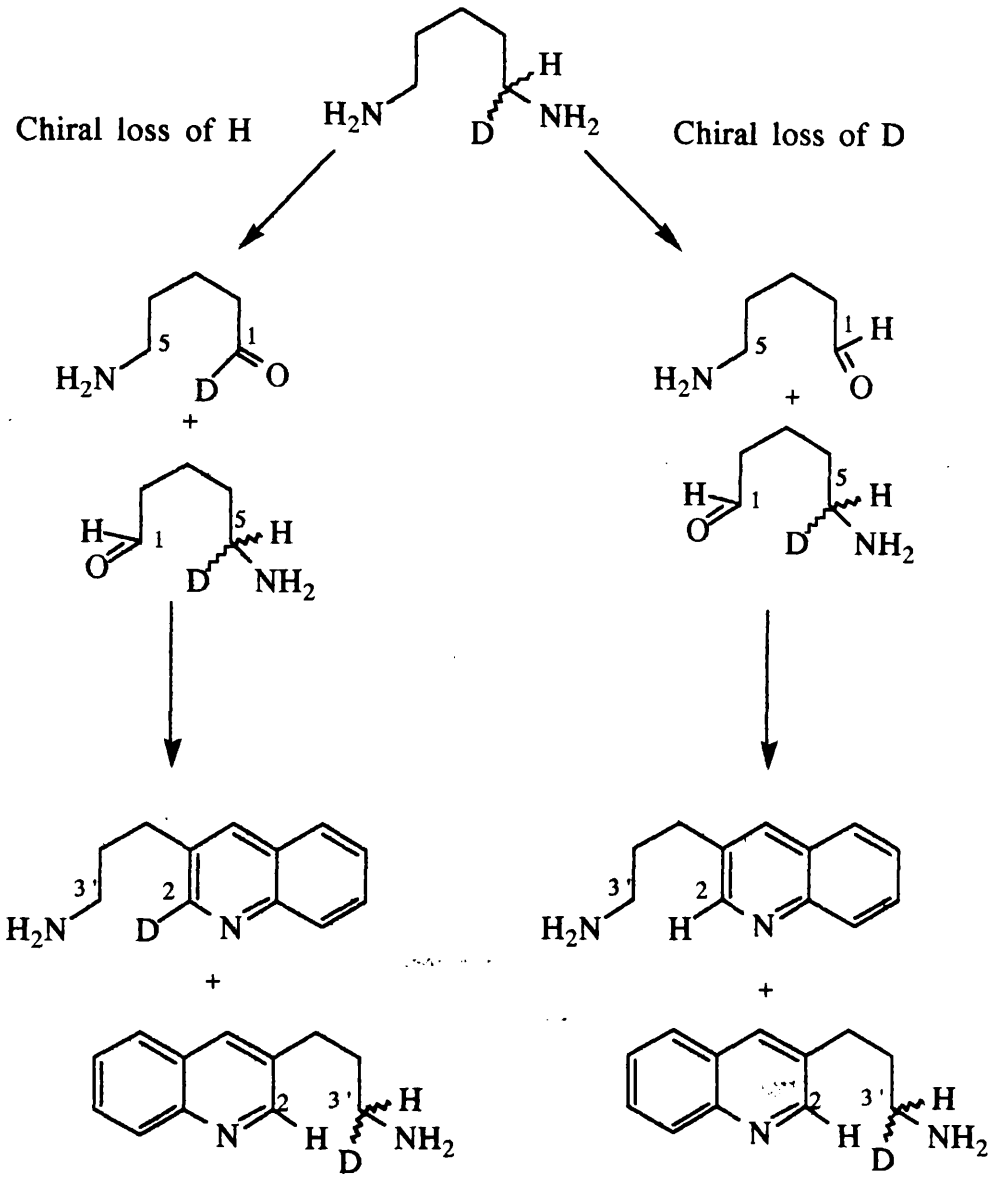
The oxidation of the methylene group adjacent to the nitrogen atom is of vital importance in the metabolism of primary amines in a wide range of organisms. The process is also a key step in the biosynthesis of many alkaloids. It is therefore important to understand the stereochemistry of the transformation.

Battersby *et al.*[10] in 1974, synthesised (*R*)- and (*S*)-[methylene-$^3H_1$]benzylamines with configurational purities greater than 95%. They then proved that pea seedling diamine oxidase abstracts the *pro-S* hydrogen from the methylene group of benzylamine by observing retention or loss of the

tritium label. Since then many groups have set out to understand the stereochemistry of the oxidation of diamines catalysed by diamine oxidase.

Richards and Spenser[10] investigated the stereochemistry of the deamination of cadaverine catalysed by pig kidney diamine oxidase. They employed ^2H NMR spectroscopy to show that it is the *pro-S* hydrogen that is removed by pig kidney diamine oxidase. The enzyme reaction mixture contained *o*-aminobenzaldehyde (18) which trapped the product of the enzymic oxidation, 5-aminopentanal, to yield 3-(3'-aminopropyl)quinoline (46) (Scheme 2.16).

^2H-NMR spectroscopy was carried out on the products obtained from enzyme catalysed oxidations using (1) (*S*)-[1-$^2H_1$]cadaverine, (2) (*R*)-[1-$^2H_1$]cadaverine and (3) [1,1-$^2H_2$]cadaverine to locate the deuterium in the samples. From the spectroscopic studies it was clear that the *pro-S* hydrogen from C-1 of cadaverine was lost in the oxidative deamination. This stereospecificity corresponds to that of the oxidative deamination of benzylamine to benzaldehyde, catalysed by pea seedling diamine oxidase.



(46)

Scheme 2.16

Battersby *et al.*[82] also studied the stereochemistry of cadaverine oxidation using pea seedling diamine oxidase. They converted 1-tritium labelled samples of cadaverine into the aminoaldehyde, 5-aminopentanal, using pea seedling diamine oxidase. Also in the reaction mixture was an alcohol

dehydrogenase and ethanol. The aldehyde formed was therefore immediately converted into the more chemically stable amino alcohol, 5-aminopentan-1-ol. An added bonus to this method was that the amino alcohol produced was also a substrate of diamine oxidase and was oxidatively deaminated under the incubation conditions to form eventually the diol, pentane-1,5-diol. In practise a mixture of amino alcohol and diol was produced though these were separated by t.l.c. This resolved the problem arising with the symmetry of the molecule which means that either of the two amino groups can be oxidised initially by the diamine oxidase enzyme. Deamination of both amino groups allowed Battersby *et al.* to establish complete loss or retention of the tritium labels. When the isotope occupied the *S* -position of C-1 very little tritium was observed. The complementary result was obtained when the isotope occupied the *R* -position of the C-1 of cadaverine. The latter method for proving the stereochemistry of the oxidative deamination of cadaverine by pea seedling diamine oxidase combined with the result by Richards and Spenser[10] using ^{2}H NMR spectroscopic experiments and pig kidney diamine oxidase proved that it is indeed the *pro*-S hydrogen which is removed during the process.

The *pro-S* hydrogen has also been shown to be lost in the course of the oxidation, catalysed by pea seedling diamine oxidase, of (*S*)-[α-^{3}H]- and (*R*)-[α-^{3}H]-3'-*o*-methyldopamine,[10] (*R*)-[α-^{3}H]-histamine[83] and (*S*)-1-amino[1-^{3}H]-heptane.[84]

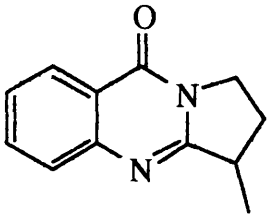
The stereochemical consistency observed with such a wide range of substrates would appear to confirm that in the

reaction catalysed by diamine oxidase, oxidation of the substrates is accompanied by the loss of the *pro-S* hydrogen.

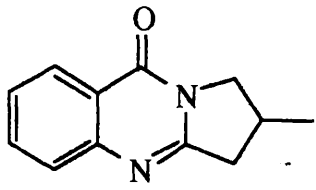
2.21 Regioselectivity and Stereoselectivity in the Oxidative Deamination of 2-Methyl-1,4-diaminobutane (2-Methyl-putrescine) Catalysed by Diamine Oxidases

Santaniello and co-workers studied the regioselectivity in the diamine oxidase catalysed oxidative deamination of 2-methylputrescine using diamine oxidase from different sources. The two main sources used were pea seedling and pig kidney diamine oxidase. As discussed earlier, Macholan[74] observed differences in the active sites of the enzymes from both these sources. Santaniello and co-workers aimed to throw more light on the catalytic action of these enzymes, giving new information about their regioselectivities.

In 1982 they reported that diamine oxidase from pea seedlings catalysed the oxidation of 2-methylputrescine in a regioselective manner, whereas diamine oxidase from pig kidney showed no regioselectivity for the same reaction.[85] They observed these differences by condensing the substituted 1-pyrrolines (3,4-dihydro-2*H*-pyrroles) with *o*-aminobenzaldehyde. Subsequent oxidation of the formed quinazolinium salt afforded 1'- or 2'-methyl-2,3-trimethylene-4(3*H*)-quinazolone (47) or (48).



(47)



(48)

^{1}H NMR spectroscopy of the methylquinazolones obtained from products of the oxidations, catalysed by pea seedling and pig kidney diamine oxidase were recorded. Two doublets were observed at δ 1.24 and 1.46 of the same intensity with the product from the pig kidney diamine oxidase reaction. With the reaction using pea seedling diamine oxidase only one doublet at δ 1.24 accompanied by a minor amount (<10%) of the signal due to the other methyl was observed. At this point Santaniello and co-workers reported that pea seedling diamine oxidase oxidises 2-methylputrescine in a regioselective manner, whereas the enzyme from pig kidney diamine oxidase lacks this regioselectivity.

Santaniello and co-workers then studied the stereospecificity of the reaction, using (*R*)- and (*S*)-2-methylputrescines as substrates and diamine oxidases from the same sources as in earlier work.[11] The (*R*)- and (*S*)-2-methylputrescines were synthesised from (*R*)- and (*S*)-3-methyladipic acid respectively.[86] The compounds were incubated as before and quantitative analyses of the products of oxidative deamination and coupling with *o*-aminobenzaldehyde were performed by HPLC.

The results obtained showed that pea seedling diamine oxidase catalyses the deamination of 2-methylputrescine at the

less hindered amino group, independent of the configuration of the substrate used, since (48) is preferentially formed by oxidation of both (*R*)- and (*S*)- 2-methylputrescine (85% and 95% respectively). With pig kidney diamine oxidase however, the oxidation has two different patterns depending upon the configuration of the substrate. With the (*R*)-isomer it is the C-1 primary amine that is converted into the corresponding aldehyde (95%) whereas with the (*S*)-isomer it is the less hindered position that is oxidised. This suggests that for the enzyme from pig kidney, the deaminating site is more sensitive to the configuration of the substrate and in fact this enzyme is stereoselective.

It is possible therefore to conclude that in their structural requirements, the active sites of diamine oxidases from plant and animal sources are certainly different.

It is clear that this is an area which requires much further work to understand completely the regioselectivity and stereoselectivity of diamine oxidases.

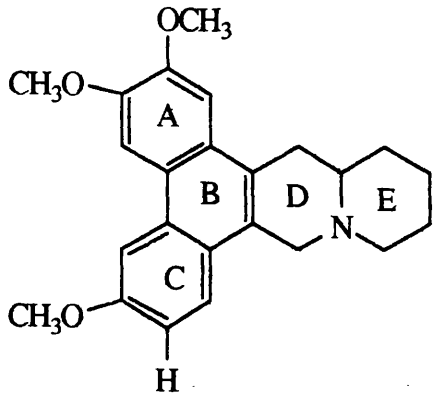
2.22 Applications of Diamine Oxidase

Alkaloids represented by cryptopleurine (8) and typhorine (9) exhibit various interesting biological activities, including anti-cancer action. Analogues of these compounds might also possess this useful activity.

A convenient synthesis of these alkaloids was described by Cragg and Herbert.[15] 3',4'-Dimethoxy-2-(2-piperidyl)acetophenone (49) was found in *Boehmeria platyphylla* and *B. cylindrica*. It was easily synthesised using

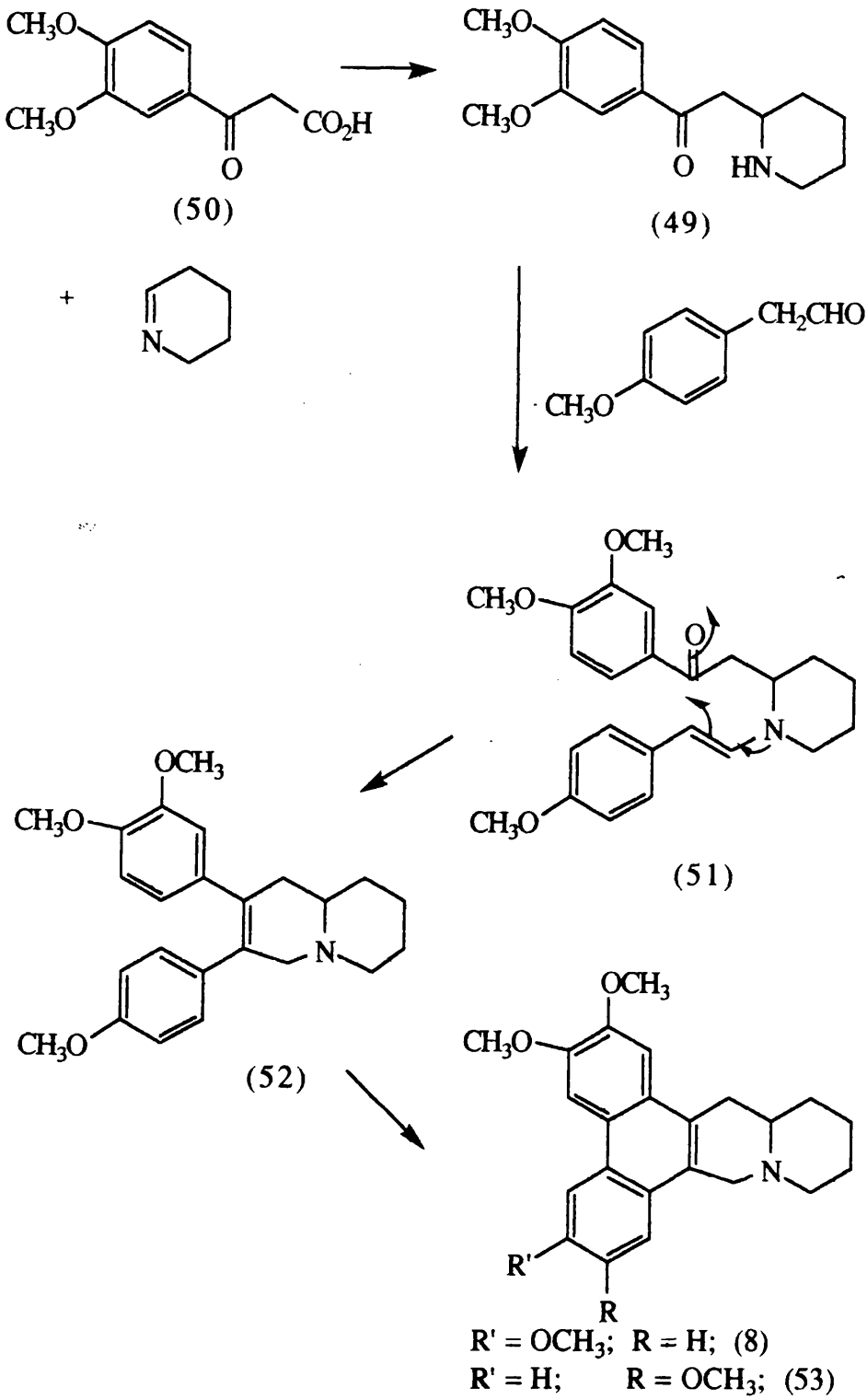
diamine oxidase and it served as a key intermediate in the synthesis of cryptopleurine (8). Condensation of 3,4-dimethoxybenzoylacetic acid (50)[87] with piperidine, generated *in situ* from cadaverine using pea seedling diamine oxidase,[2] gave the alkaloid (49) in high yield. Rapid condensation of compound (49) with substituted phenylacetaldehydes in benzene at room temperature produced the enamine (51). The enamine function then underwent ring closure with the keto group using selected Lewis acid catalysts, for example titanium(IV) chloride and silicon(IV) chloride. Compound (52) was formed after dehydration and reduction. Thallium(III) trifluoroacetate was used in effecting the linkage of the two aromatic rings to form cryptopleurine (8). A minor product was also identified as a cryptopleurine isomer (53) (Scheme 2.17).

The diamine oxidase catalysed reaction is a key step in this alkaloid synthesis. Analogues with alternative substituents on ring E (shown in diagram below) might also be easily synthesised by using substituted cadaverine analogues in the first step. The size of ring E might also be altered by using diamines of various carbon chain lengths, for example putrescine and 1,6-hexanediamine.



(8)

This small alteration in the synthetic route might open the way for the formation of many different analogues which might have useful biological activity.



Scheme 2.17 Synthesis of cryptopleurine.

CHAPTER 3

OXIDATION OF DIAMINES BY DIAMINE OXIDASE

3.1 Enzyme Preparation

3.1a Extraction and Partial Purification of Diamine Oxidase from Pea Seedlings

Pea seedling diamine oxidase was extracted and purified using the method described by Hill.[2] Pea seedlings from the 'Fillbasket' variety were grown for *ca.* 10 days. The extraction method depends on removing much of the unwanted material from the crude extract by precipitation with a 2:1 mixture of chloroform and ethanol. The enzyme was then precipitated with ammonium sulphate. The solid separated after about 1 h. At this point Hill siphoned and discarded the lower liquid. A clean separation was not obtained by us after the 1 h of standing and therefore it was decided to leave the mixture to separate overnight to achieve maximum separation. The procedure was then carried out as by Hill including further ammonium sulphate precipitation and dialysis. Partial purification was obtained at this stage and the further chromatographic preparations described by Hill were excluded as the protein obtained at this stage was adequate for our purposes. The protein obtained was taken up in phosphate buffer (pH 7) and stored in 0.5 ml aliquots in the freezer at *ca.* -20 ^{o}C. The enzyme was found to be stable for several months

with very little loss of activity (For more details of the method see the Experimental section).

3.1b Determination of Protein Concentration

The determination of protein concentration was carried out using the method of Sedmak and Grossberg.[88] This depends on the conversion of Coomassie brilliant blue G in dilute acid from a brownish-orange into an intense blue colour with the addition of protein. The absorbance of the mixture was measured immediately after the protein was added although the colour was found to be stable for about 1 h at room temperature. The method was carried out using bovine serum albumin (BSA) as the protein standard (1 mg of BSA is equivalent to 1 mg of protein) (For more details of the method see the Experimental section).

The absorbances of the mixture at A_{620} (blue) and A_{465} (brownish-orange) were recorded for various concentrations of BSA. A standard graph was obtained by plotting A_{620}/A_{465} *vs.* protein concentration. This graph was used to determine the concentration of protein samples by measuring the A_{620}/A_{465} ratio (Appendix 1).

This assay for the determination of protein concentration was highly reproducible and has been shown to detect less than 1 μg of albumin.

3.1c Slab Gel Electrophoresis

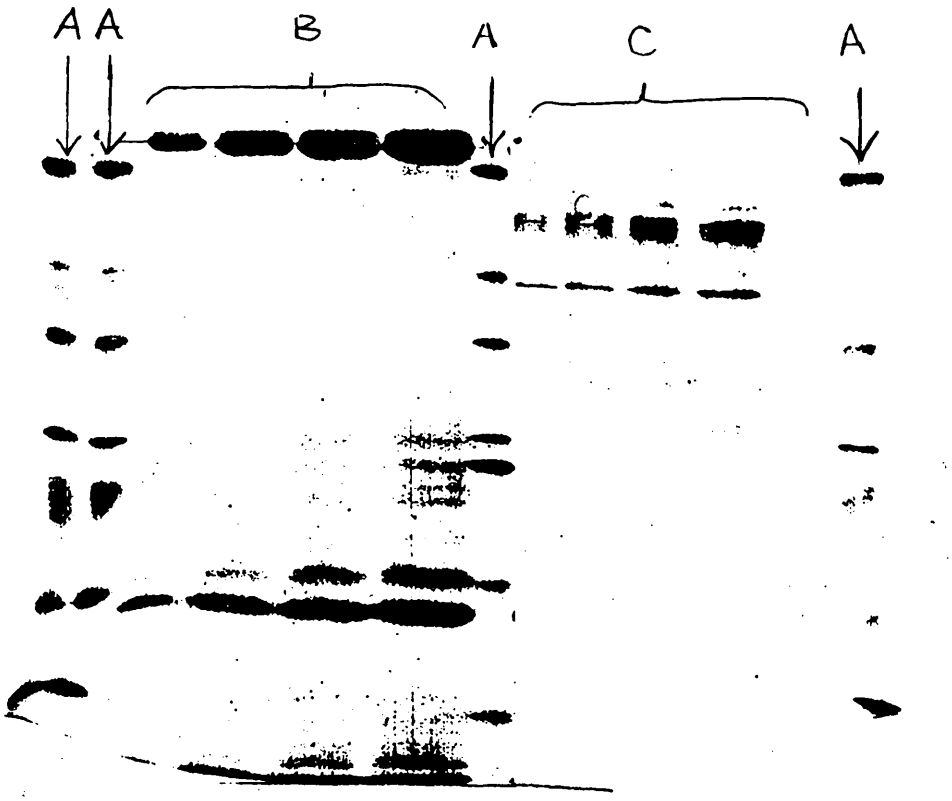
Slab gel electrophoresis was carried out using the modified Laemmli gel system[89] on pig kidney and pea seedling diamine oxidase.

The protein samples were prepared using 2-mercaptoethanol and were heated in boiling water for 2 min.

The gel was stained using Coomassie blue dye and destaining was carried out using 10% glacial acetic acid[90] (For more details of the method see the Experimental section).

The results from the gel are shown in Figure 3.1. This shows that both enzymes are heterogeneous consisting of mainly two bands. Therefore both enzymes had impurities and no attempt to purify these enzymes was made.

Molecular weights of the bands were found to be *ca.* 70,000 and 20,000 (pea seedling diamine oxidase) and *ca.* 47,000 and 45,000 (pig kidney diamine oxidase. It is unclear whether all these bands are diamine oxidase or are due to impurties.



A: Standard protein markers
(Sigma Dalton Mark VII-L)

B: Various concentrations of pea seedling diamine oxidase

C: Various concentrations of pig kidney diamine oxidase

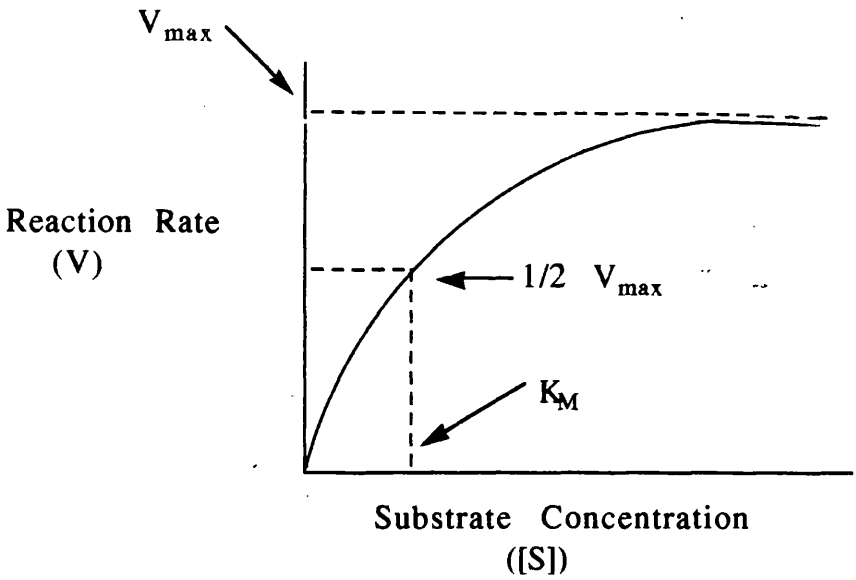
Figure 3.1 Slab gel electrophoroseis

3.2 Enzyme Kinetics

3.2a Michaelis-Menten Kinetics

For most enzymes, the rate of catalysis (V) varies with the substrate concentration ([S]) as shown in Graph 3.1. The initial rate of product formation by the enzyme is directly

proportional to the substrate concentration. The rate then approaches saturation with respect to the substrate concentration.



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

Michaelis and Menten in 1913[91] proposed a simple model to account for these kinetic characteristics. The main feature is that a enzyme-substrate (ES) complex is a necessary intermediate in the reaction. The model proposed is shown in Scheme 3.1.

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

Scheme 3.1

As shown, once the ES complex is formed it can either go on and form the product (k_{cat}) or dissociate to give the enzyme and substrate (k_2).

An expression to relate the rate of catalysis to the concentrations of substrate and enzyme and the rates of the individual steps was required. Starting with equation (3.1),

$$V = k_{cat}[ES] \tag{3.1}$$

[ES] needs to be expressed in terms of known quantities. The rates of formation and breakdown of ES are given by,

$$\text{Rate of formation} = k_1[E][S] \tag{3.2}$$

$$\text{Rate of breakdown} = (k_2 + k_{cat})[ES] \tag{3.3}$$

In this model the catalytic rate is assumed to be under steady-state conditions. In steady state, the concentration of the intermediates remain the same while the concentrations of the starting materials and products change. This occurs when the rates of formation and breakdown of the ES complex are equal,

$$k_1[E][S] = (k_2 + k_{cat})[ES] \tag{3.4}$$

Rearranging equation (3.4) gives,

$$[ES] = [E][S]/\{(k_2 + k_{cat})/k_1\} \tag{3.5}$$

By defining a new constant, K_M (Michaelis constant),

$$K_M = (k_2 + k_{cat})/(k_1) \quad (3.6)$$

equation (3.5) can be simplified to,

$$[ES] = [E][S]/K_M \quad (3.7)$$

The concentration of uncombined substrate, [S], is very nearly equal to the total concentration of substrate. This is of course provided that the concentration of enzyme is much smaller than the substrate concentration. The concentration of uncombined enzyme, [E] is equal to the total enzyme concentration, $[E_T]$, minus the concentration of the ES complex [ES].

$$[E] = [E_T] - [ES] \quad (3.8)$$

Substituting equation (3.8) into equation (3.7) gives,

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

This implies that,

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

Substituting the expression for [ES] into equation (3.1) gives,

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

The maximal rate, V_{max}, is attained when the enzyme sites are saturated with substrate, that is when [S] is much greater than K_M so,

$$[S]/([S] + K_M) \simeq 1 \qquad (3.12)$$

This then gives equation (3.13),

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

and substituting equation (3.13) into equation (3.11) gives,

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

This equation now explains the shape of the curve in Graph 3.1. At low substrate concentration, that is when [S] is much smaller than K_M, equation (3.14) becomes,

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

Therefore the rate is directly proportional to the substrate concentration. However at high substrate concentration, that is when [S] is much greater than K_M, equation (3.14) becomes,

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

The rate is therefore maximal and independent of substrate concentration. From equation (3.14) we get a definition of K_M. When the substrate concentration is equal to K_M then,

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

Therefore, K_M is equal to the substrate concentration at which the reaction rate is half of its maximal value.

3.2b Determination of the V_{max} and K_M by Varying the Substrate Concentration

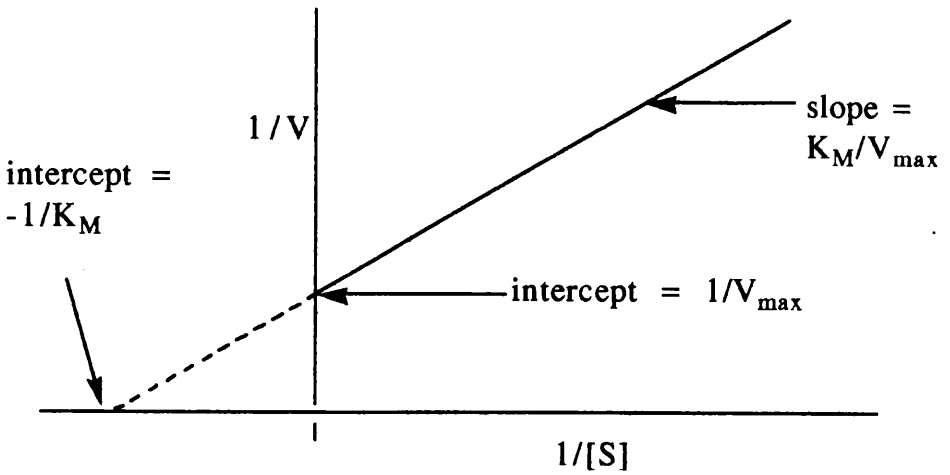
The Michaelis constant, K_M, and the maximal rate, V_{max}, can be derived from the rates of catalysis measured at different substrate concentrations if the enzyme obeys the kinetics shown in Scheme 3.1.

The graph of the Michaelis-Menten equation, reaction rate (V) against substrate concentration [S] is not entirely satisfactory for the determination of V_{max} and K_M. If there are not three consistent points on the plateau of the curve at different substrate concentrations then an accurate value of V_{max} and hence K_M cannot be obtained. Also as the graph is a curve, it cannot be accurately extrapolated upwards from non-saturating values of substrate concentrations. Lineweaver and Burk in 1934[92] overcame this problem, not by making fresh assumptions but by inverting the original Michaelis-Menten equation. Therefore equation (3.14) becomes,

$$(1/V) = K_M/V_{max}(1/[S]) + 1/Vmax \qquad (3.18)$$

This is of the form $y = mx + c$, which means that a plot of y against x has a slope m and intercept on the y axis of c.

Therefore a plot of 1/V against 1/[S] allows the accurate determination of V_{max} and K_M (Graph 3.2).



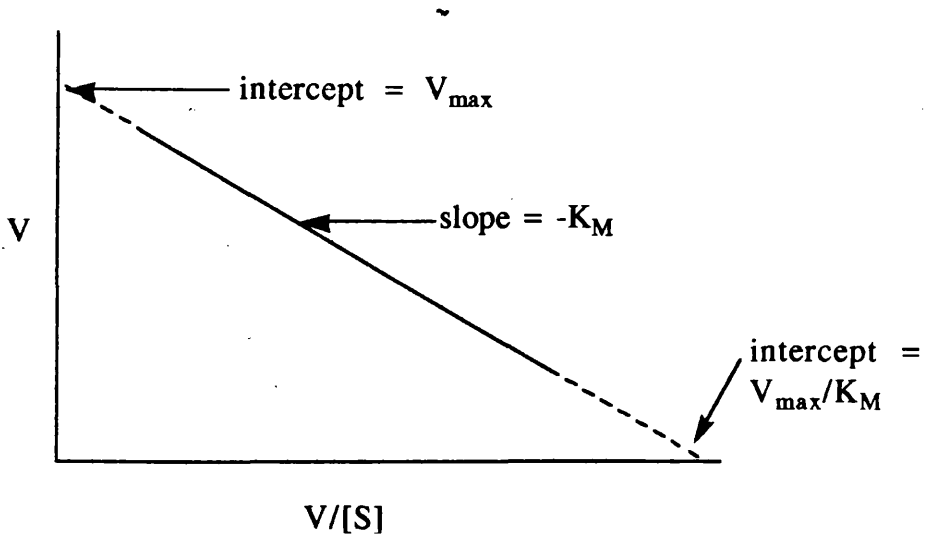
Graph 3.2 Lineweaver-Burk Plot.

However the accuracy of the Lineweaver-Burk plot has been criticised. A simple point is the fact that graphs often have to be redrawn due to the sometimes unexpected long extrapolation. More importantly it is said to give undue weight to low substrate concentrations and these values are not as accurate. Another criticism of the Lineweaver-Burk plot is that departures from linearity are less obvious than from other plots, particularly the Eadie-Hofstee plot.[93,94] This plots starts with the Lineweaver-Burk equation and both sides of the equation are multiplied by $V.V_{max}$.

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

$$V = -K_M\,(V/[S]) + V_{max} \qquad (3.19)$$

Again it is an equation of a straight line. Plotting V against V/[S] gives the slope, $-K_M$ and the intercept on the y-axis, V_{max} (Graph 3.3).



Graph 3.3 Eadie-Hofstee Plot.

The Lineweaver-Burk plot is still widely used by enzymologists in general. Using the Lineweaver-Burk plot together with the Eadie-Hofstee plot enables the best values for V_{max} and K_M to be obtained. The most important point is to obtain good data covering a wide range of substrate concentrations which then spread evenly over the plot used.

3.2c The Significance of K_M and V_{max} Values

The Michaelis constant, K_M, is related to the rate constants in the individual steps in Scheme 3.1. Consider a rate limiting case in which k_2 is much greater than k_{cat}.

Under these conditions,

$$K_M = k_2/k_1 \qquad (3.20)$$

and the dissociation constant of the ES complex is given by,

$$K_{ES} = [E][S]/[ES] = k_2/k_1 \qquad (3.21)$$

Therefore, K_M is equal to the dissociation constant of the ES complex if $k_2 >> k_{cat}$. When this condition is met the K_M is a measure of the strength of the ES complex. A high K_M indicates weak binding and a low K_M indicates strong binding.

The V_{max} is the maximal rate and is related to the turnover number of an enzyme (kinetic constant k_{cat}) in equation (3.22),

$$V_{max} = k_{cat}[E_T] \qquad (3.22)$$

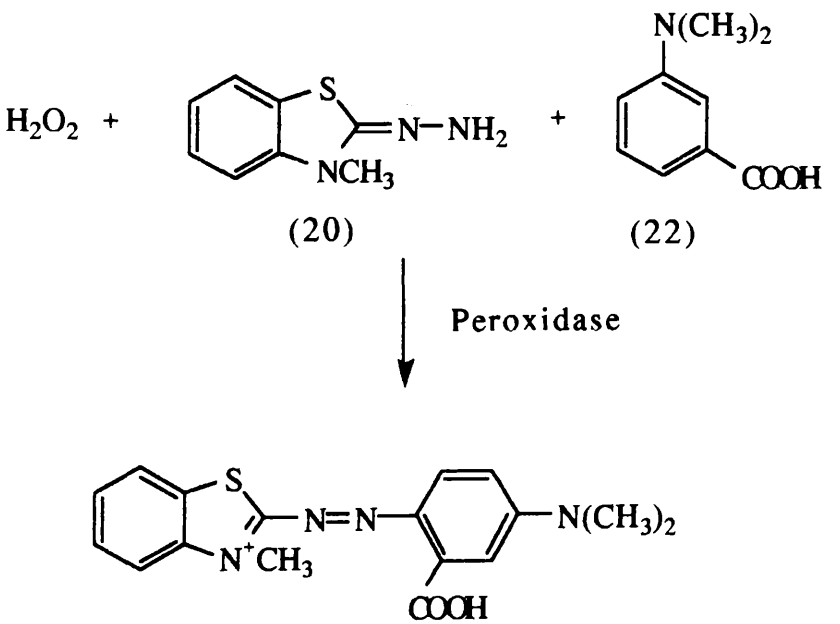
where $[E_T]$ = the total enzyme concentration

3.3 The Assay Procedure

As discussed in Chapter 2, 2.16 there are many assay procedures for the measurement of diamine oxidase activity. For this work it was necessary to be able to determine the

rates of reaction for many substrates. The assay used therefore must involve the measurement of a common factor from the enzyme reaction. The spectrophotometric assay by Stoner[14] determines the amount of hydrogen peroxide produced from the catalytic oxidative deamination of diamines using diamine oxidase. This was the main assay system used throughout this work as it proved to be a very reliable and convenient method.

The assay involves a coupled reaction with peroxidase and 3-methyl-2-benzothiazolinone hydrazone (MBTH) (20) with the acceptor, 3-(dimethylamino)benzoic acid (DMAB) (22). In the presence of hydrogen peroxide and peroxidase, the chromogen MBTH is oxidatively coupled to DMAB, forming a purple indamine dye having an adsorption maximum at 595 nm (Scheme 3.2).



Scheme 3.2 The Spectrophotometric Assay[14]

The rates of reaction were determined directly from the spectrophotometer. The formation of the dye was initially calibrated using standard solutions of hydrogen peroxide.

Stoner discussed the possibility of MBTH as an inhibitor of diamine oxidase. He kept this inhibition to a minimum by controlling the concentration of MBTH. In addition, it was found that initiating the reaction by the addition of enzyme solution to the thermally equilibrated reaction mixture, followed immediately with the addition of substrate decreased the possibility of inhibiting effects (For more details of the assay see the Experimental section and Appendices 2 and 3).

To be sure of the validity of the assay system certain control experiments were required. To confirm the accuracy of the method described, it was necessary to carry out the test with histamine and pig kidney diamine oxidase as used in the original studies by Stoner.[14] A K_M value of 2.64×10^{-5} M was observed compared with 2.90×10^{-5} M, the result obtained by Stoner.

It has been assumed that the O_2 content of the assay system is not a limiting factor and so the oxygen concentration in our reaction vessel was not controlled. Experiments showed that when samples degassed to 25% O_2 saturation were used, no difference was observed on the measured rates. This confirmed that the O_2 level in the reaction vessel was not a limiting factor in the rate of reaction.

The reaction with cadaverine as the substrate was also carried out using an oxygen electrode to measure the rates. This method is widely used but it was found not to be as convenient as the peroxidase-coupled assay. However the

method was used to show that both assays produced the same results. Similar rate data and K_M values were obtained as with the spectrophotometric assay. This confirms the validity of the latter assay (For more details of the method see the Experimental section and Appendix 4).

Using the spectrophotometric assay system in all cases Michaelis-Menten behaviour[91] was observed. Rate data were analysed for V_{max} and K_M by least squares fitting of Lineweaver-Burk (1/V *vs.* 1/[S])[92] and Eadie-Hofstee plots (V *vs.* V/[S]).[93,94] The experiments were carried out three times with each substrate and data are quoted as an average of the three determinations.

3.4 Oxidation of Putrescine and *N*-Alkylputrescines by Diamine Oxidases

3.4a Introduction

Frydman *et al.*[12] recently reported studies on the oxidation of putrescine (1) and the *N*-alkylputrescines (54) - (56) catalysed by both pea seedling and pig kidney diamine oxidase

$H_2N(CH_2)_4NHR$

(1) R = H;

(54) R = CH_3:

(55) R = C_2H_5;

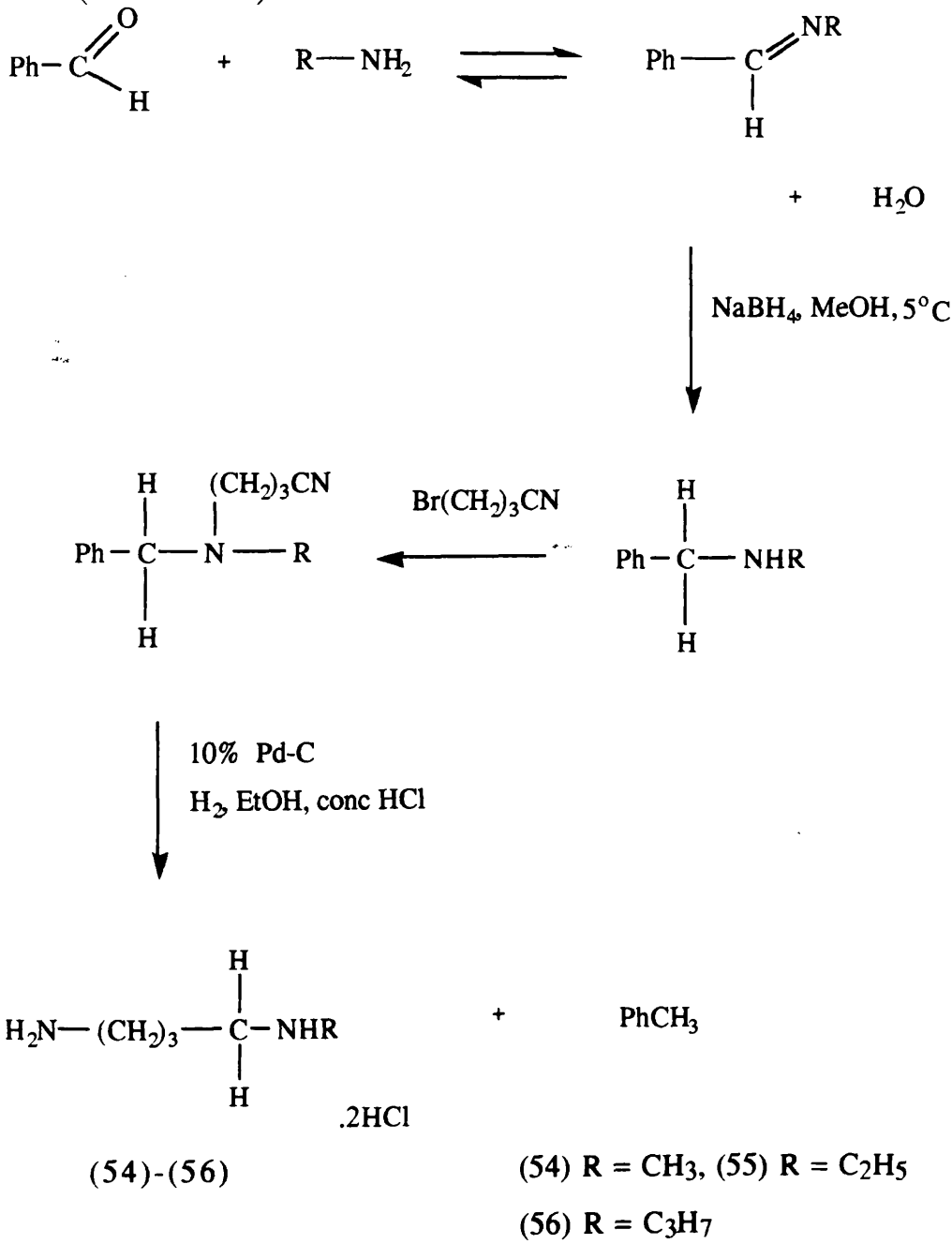
(56) R = C_3H_7;

They obtained an unusual and unexpected result for which no explanation was given. *N*-Methylputrescine (54) showed little or no apparent substrate activity with either enzyme, whereas *N*-ethylputrescine (55) and other higher homologues were oxidised reasonably efficiently to the corresponding aminoaldehydes. This was a surprising result due to the known susceptibility to oxidation of *N*-methylputrescine in a number of plants including *Nicotiana tabacum*, *Datura stramonium* and *Atropa belladonna*.[95] These experiments by Frydman were repeated by Ner[70] using the same assay as Frydman. She found that *N*-methylputrescine is a reasonable substrate for both forms of diamine oxidase.

To confirm this result it was decided that an alternative assay system should be used. The bishydrazone method used by Frydman and by Ner produces results that may not be accurate as the rate is recorded after the first thirty minutes of the reaction. After this period of time the reaction is essentially complete for good substrates and the same amounts of bishydrazone are recorded in these cases, illustrating the inadequate nature of this assay. Therefore these *N*-alkylputrescines (54)-(56) were assayed as substrates of both forms of diamine oxidases (pea seedling and pig kidney) using the peroxidase coupled assay[14] which concentrates on recording the initial rates of reaction over a shorter period of time.

3.4b Synthesis of *N*-Alkylputrescines

The *N*-alkylputrescine dihydrochlorides were prepared by A. B. Watson as described,[96] and purified by recrystallisation of the dihydrochlorides to give literature m.p.s and correct analytical data (Scheme 3.3).



(54)-(56)

(54) R = CH_3, (55) R = C_2H_5

(56) R = C_3H_7

Scheme 3.3 Synthesis of *N*-alkylputrescine dihydrochlorides.[96]

3.4c Results and Discussion

It was verified by the peroxidase-coupled assay (Table 3.1), that the oxidation of *N*-methylputrescine is catalysed by both enzymes. This again contradicts the results obtained by Frydman.[12] The conflict of results is difficult to explain, but could be due to the purity of the *N*-methylputrescine used by Frydman *et al.* There may have been an inhibitory impurity in the original *N*-methylputrescine preparation causing no oxidation to be observed.

Analysis of the kinetic parameters (K_M and V_{max}) for the pea seedling diamine oxidase reaction (Table 3.1) shows that the continuing decrease in the catalytic efficiency with increasing bulk in subsistuents arises from changes in catalytic rate constant (V_{max}) rather than binding efficiency (K_M). There is a rapid decrease in V_{max} as the size of *N*-alkyl group is increased, whereas the K_M values stay constant at about 1 mM throughout the group of substrates (Table 3.1).

Substrate	Pea Seedling DAO		Pig Kidney DAO	
	K_M	V_{max}	K_M	V_{max}
Putrescine (1)	1.18 (±0.37)	1170 (±220)	0.43 (±0.17)	0.030 (±0.005)
N-Methyl-putrescine (54)	1.12 (±0.14)	211 (±24)	1.34 (±0.28)	0.011 (±0.002)
N-Ethyl-putrescine (55)	1.17 (±0.40	3.1 (±0.3)	-	<0.002
N-Propyl-putrescine (56)	0.68 (±0.22)	0.26 (±0.06)	-	<0.002

Units K_M : mM

V_{max} : μmol/mg/hr

Table 3.1 K_M and V_{max} values for oxidation of putrescine and *N*-alkylputrescines catalysed by diamine oxidase from pea seedlings and pig kidney at 25 °C and pH 6.3.

These results suggest that the active site of pea seedling diamine oxidase is relatively uncrowded, at least at the distal end of the substrate. As the binding affinity does not vary significantly within the group of *N*-alkylputrescines tested it is thought that the substrate selectivity in the enzyme exists in the catalytic mechanism rather than the binding affinity. The decrease in V_{max} values observed as the size of *N*-alkyl subsistuent increases may be due to inhibition of important conformational changes in the catalytic step of the enzyme by the bulky substituents. Also, due to their bulk, the

substituents may limit the access of other reactants to the active site of the enzyme.

It was not possible to obtain complete kinetic data for all the substrates listed in Table 3.1 with pig kidney diamine oxidase. This was due to the very low specific activity of this enzyme. It is not known whether this is due to the impure state of the enzyme preparation or an underlying property of the enzyme itself.

The K_M values from both enzyme sources should be unaffected by protein impurities and it was noted that the K_M values were very similar for substrates which could be studied for both enzymes. However the difference in the reactivities of putrescine and *N*-methylputrescine is not as significant with the pig kidney diamine oxidase and arises from a combination of changes in both binding affinity and catalytic rate.

From the results obtained on the oxidation of *N*-alklputrescines it appears possible that the two enzymes from quite different sources may employ alternative kinetic strategies for molecular recognition of diamine substrates.

This work has been published in preliminary form[70] and as a full paper.[71]

3.5 Oxidation of Cadaverine and Analogues by Diamine Oxidase

3.5a Introduction

As discussed earlier (Chapter 2, 2.1) Frydman and co-workers[12] also examined the oxidation rates of *C*-

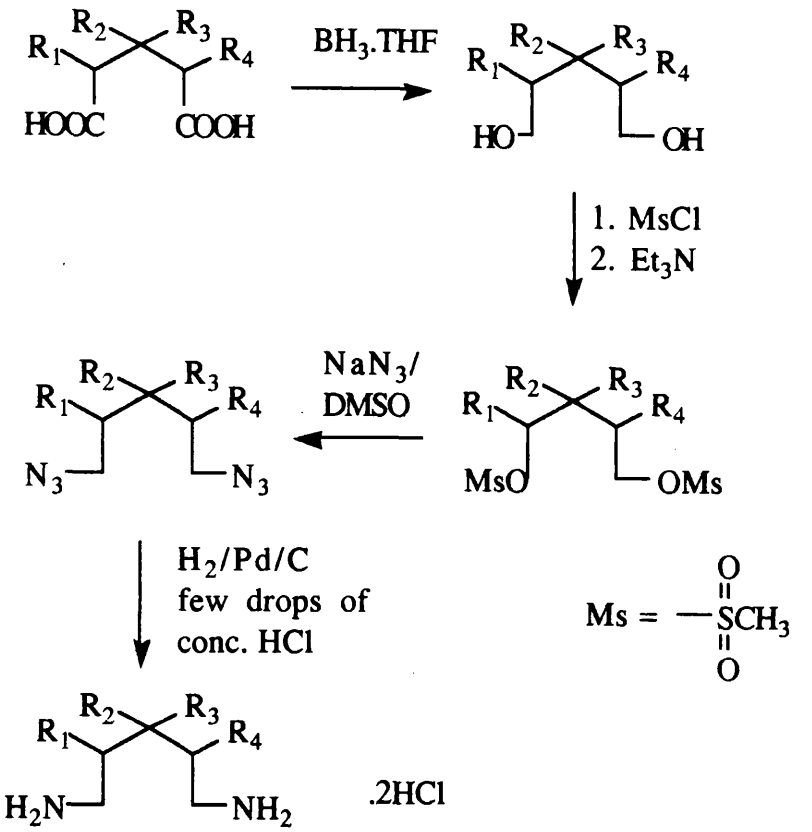
alkylputrescine derivatives. These putrescine analogues were reported to be oxidised by diamine oxidases at lower rates than the *N*-alkylputrescines.

It was decided to synthesise *C*-alkylcadaverine analogues for testing as substrates of diamine oxidase. Cadaverine is also an important substrate of diamine oxidase in polyamine metabolism.

Analysis of these results should provide further information on the active sites of the enzyme from both sources.

3.5b Synthesis of Cadaverine and Analogues

Cadaverine analogues (57)-(60) were made from substituted glutaric diacids or anhydrides. The first step involved reduction to the corresponding diols using diborane in tetrahydrofuran (Scheme 3.4). The diols were then purified by distillation. The dimethanesulphonates of the diols were formed using methanesulphonyl chloride and triethylamine. The dimethanesulphonates were purified by crystallisation from diethyl ether. The diazides were then formed *via* nucleophilic substitution by the azide ion from sodium azide. The diazides formed were essentially pure by t.l.c. Without further purification the diazides were hydrogenated in the presence of palladium on carbon and a few drops of concentrated hydrochloric acid. The diamine dihydrochlorides were formed and were recrystallised from aqueous ethanol and acetone (Scheme 3.4).

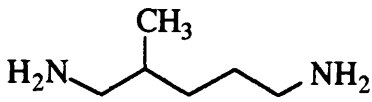


(7) $R_1 = R_2 = R_3 = R_4 = H$;
(57) $R_1 = R_2 = R_4 = H$; $R_3 = CH_3$;
(58) $R_1 = R_4 = H$; $R_2 = R_3 = CH_3$;
(59) $R_1 = R_4 = H$; $R_2 = OH$; $R_3 = CH_3$;
(60) $R_1 = R_4 = CH_3$; $R_2 = R_3 = H$;

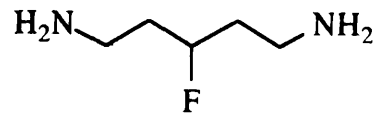
Scheme 3.4 Synthesis of Cadaverine and Substituted Cadaverines.

3-Phenylcadaverine dihydrochloride (61) was synthesised using an alternative method. 3-Phenylpentane-1,5-diol was formed by reduction of the corresponding glutaric acid. Conversion of the diol into the diamine dihydrochloride was carried out by the procedure of Golding and co-workers[97] *via* the diazides and purified in a similar manner as before.

2-Methylcadaverine (62) was available from Aldrich and converted into the dihydrochloride and purified by recrystallisation as before. 3-Fluorocadaverine (63) dihydrochloride was prepared for our use by A. M. Brown.[98]



(62)



(63)

Correct analytical data were obtained for all the diamine dihydrochlorides (57)-(63) prepared.

3.5c Results and Discussion

Using the peroxidase-coupled spectrophotometric assay kinetic parameters for each substrate with pea seedling diamine oxidase were obtained (Table 3.2). However only a few substrate systems could be investigated for the pig kidney enzyme because of the poor specific activity of the commercial product.

Within the range of cadaverine analogues the K_M values were slightly smaller than those of putrescine but again did not vary much within the series. Differences in the rates in the cadaverine series were observed with pea seedling diamine oxidase. Oxidation of cadaverine (7) was ten times faster than 3-methylcadaverine (57) which in turn was ten-fold faster than 3,3-dimethylcadaverine (58). However 3-hydroxy-3-methylcadaverine (59) gave a rate three times that of 3-

methylcadaverine (57). 3-Fluorocadaverine (63) and 3-phenylcadaverine (61) gave rates similar to the 3-hydroxy-3-methylcadaverine (59).

2,4-Dimethylcadaverine (60) appeared to be a better substrate than 3,3-dimethylcadaverine (58) (Table 3.2).

From the results it is clear that the substrate activity of this enzyme is particularly sensitive to substitution of the central 3-position.

This work has been published along with the work carried out with putrescine (1) and the *N*-alkylputrescines (54)-(56).[71]

	Pea Seedling DAO		Pig Kidney DAO	
Substrate	K_M	V_{max}	K_M	V_{max}
Cadaverine (7)	0.24 (±0.07)	2680 (±410)	0.91 (±0.35)	0.18 (±0.07)
3-Methyl-cadaverine (57)	0.60 (±0.02)	260 (±70)	-	<0.008
3,3-Dimethyl-cadaverine (58)	0.61 (±0.06)	38 (±4)	-	<0.004
3-Hydroxy-3-methyl-cadaverine (59)	0.23 (±0.03)	770 (±150)	-	-
3-Fluoro-cadaverine (63)	0.15 (±0.06)	754 (±135)	-	-
3-Phenyl-cadaverine (61)	0.36 (±0.06)	1144 (±130)	-	-
2-Methyl-cadaverine (62)	0.26 (±0.03)	1250 (±130)	-	<0.008
2,4-Dimethyl-cadaverine (60)	0.30 (±0.05)	300 (±30)	-	<0.004

Units K_M : mM

V_{max} : μmol/mg/hr

Table 3.2 K_M and V_{max} values for oxidation of cadaverine and substituted cadaverines catalysed by diamine oxidases from pea seedlings and pig kidney at 25 °C and pH 6.3.

3.6 Oxidation of Diamines with Carbon Chain Length from 2 to 12 by Pea Seedling Diamine Oxidase

3.6a Introduction

The best substrates for diamine oxidase are known to be putrescine (1) and cadaverine (7). Ethane-1,2-diamine (64) and propane-1,3-diamine (65) have been assayed as potential substrates of diamine oxidase from pea seedlings.[62] By measuring the uptake of O_2 Hill and Mann observed no O_2 uptake with ethane-1,2-diamine (64) and propane-1,3-diamine (65). Hexane-1,6-diamine (66) was also tested and it was shown to be a reasonable substrate of diamine oxidase. This was an interesting result and further investigation in this field was necessary to determine the effect of chain length of diamines on K_M and V_{max} values with pea seedling diamine oxidase.

Using the sensitive peroxidase-coupled assay system, data were collected by us for a range of α,ω-diamines with carbon chain lengths of two to twelve. Using this more sensitive assay the substrates which previously showed no oxygen uptake were examined more closely. The kinetic parameters of the enzyme catalysed reactions with diamines of larger chain lengths (66)-(71) when analysed should provide more information on the steric constraints of the active site.

3.6b Preparation of Diamine Dihydrochlorides

The diamines were available from Aldrich, and they were converted into the dihydrochloride salts and purified by recrystallisation. Appropriate analytical data were obtained for all diamine (64)-(71) dihydrochlorides used as substrates in the assay.

3.6c Results

Michaelis-Menten kinetics were obtained for each substrate [(64)-(71), (1) and (7)] with pea seedling diamine oxidase.

$$H_2N(CH_2)_nNH_2$$

n = 2 (64); n = 3 (65); n = 4 (1); n = 5 (7); n = 6 (66);
n = 7 (67); n = 8 (68); n = 9 (69); n = 10 (70); n = 12 (71);

The results are shown in Table 3.3 and are clearly represented in Graph 3.4 - K_M *vs.* Chain length of substrate and V_{max} *vs.* Chain length for substrate.

The first point to note from Table 3.3 is that ethane-1,2-diamine (64) and propane-1,3-diamine (65) have similar K_M values and both are substrates, although with low activity. From graph 3.4, the plot of K_M *vs.* chain length values shows that the binding efficiency increases rapidly as the chain length is increased from three and levels off with the best binding observed for chain lengths five to seven. Apparent substrate affinities then decrease to a minimum at nine and then

increase again slightly. The rates also show strong dependence on chain length with the maximum rate observed with C_5 (cadaverine). This trend resembles that with the difficulty encountered in forming medium sized rings, particularly ring sizes eight and nine.[99] Both probability (entropy) and energy (strain) factors influence the ease with which these rings are formed. Rings with five or six carbons are relatively easy to form and longer chains experience higher entropy barriers and steric hindrance.[100] This may explain the similar difficulty observed when diamines bind to diamine oxidase. It suggests that the mechanism for the diamine oxidase recognition process involves binding of both substrate amine groups (by imine formation or non-covalent interactions) to the enzyme forming a cyclic structure.

As both K_M and V_{max} values show similar chain length dependence, it suggests a two stage equilibrium model for the process (Scheme 3.5)

$$E + S \underset{}{\overset{K_1}{\rightleftharpoons}} ES \overset{K_2}{\rightleftharpoons} ES^* \xrightarrow{k_{cat}} E + P$$

Scheme 3.5 where $K_1 = [E][S]/[ES]$ and $K_2 = [ES]/[ES^*]$ and are the equilibrium constants for substrate binding and intermediate formation, and k_{cat} is the rate constant for the (irreversible) catalytic step.

The first step in Scheme 3.5 may represent the initial binding of one of the amino groups of the substrate to the enzyme.

This is unlikely to be dependent on carbon chain length. The second step may therefore involve cyclisation and the binding of the second amino group to the enzyme. This would explain the results obtained. The second step would be very difficult for small chain lengths (*eg.* 2 and 3), and optimal with chain lengths 5 and 6.

For this model we can show that:

$$K_M = K_1K_2/(1 + K_2) \qquad \text{and} \quad V_{max} = k_{cat}/(1 + K_2)$$

Therefore both K_M and V_{max} depend on K_2. If the second step is difficult (cyclisation) *ie.* K_2 is large then V_{max} will be small and K_M would be *ca.* K_1. This represents the weak binding in the first stage. However if the cyclisation is easy then the V_{max} will be large and the K_M is smaller as K_2 is small.

The model agrees with the results obtained shown in Table 3.3 and Graph 3.4.

This work has been submitted for publication.[101]

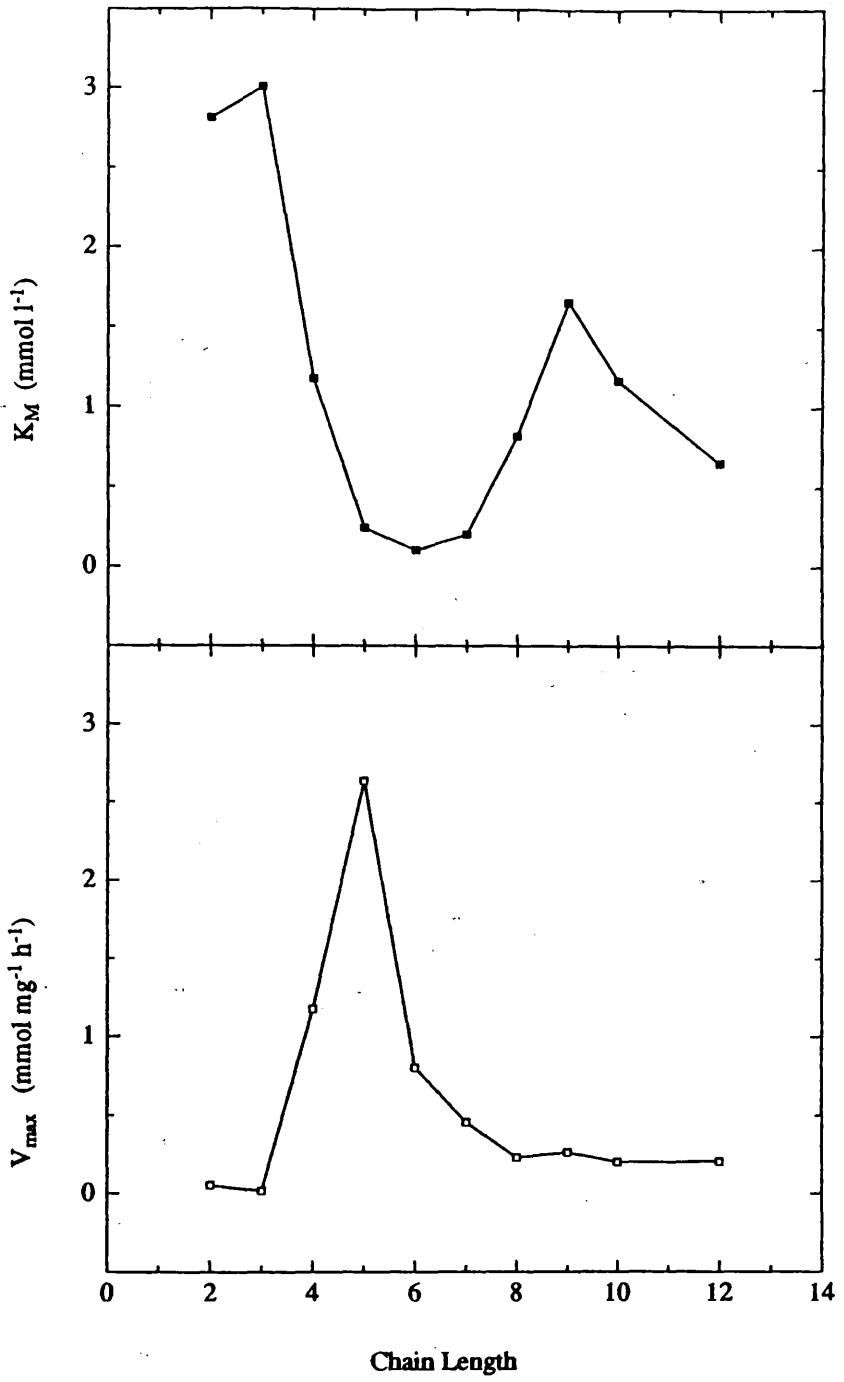
Substrate	K_M	V_{max}
Ethane-1,2-diamine (64)	2.81	55
	(±0.64)	(+3)
Propane-1,3-diamine (65)	3.01	16
	(+0.44)	(+1)
Putrescine (1)	1.18	1174
	(+0.37)	(+219)
Cadaverine (7)	0.24	2636
	(+0.06)	(+336)
Hexane-1,6-diamine (66)	0.10	800
	(+0.04)	(+77)
Heptane-1,7-diamine (67)	0.20	455
	(+0.10)	(+74)
Octane-1,8-diamine (68)	0.82	229
	(+0.35)	(+61)
Nonane-1,9-diamine (69)	1.66	262
	(+0.95)	(+61)
Decane-1,10-diamine (70)	1.17	200
	(+0.30)	(+32)
Dodecane-1,12-diamine (71)	0.65	205
	(+0.26)	(+52)

Units K_M: mM

V_{max} : μmol/mg/hr

Standard deviations in parentheses

Table 3.3 K_M and V_{max} values for oxidation of diamines with carbon chain length 2-12 catalysed by diamine oxidase from pea seedlings at 25 °C and pH 6.3.



Graph 3.4 K_M *vs.* Chain length of substrate and V_{max} *vs.* Chain length for substrate.

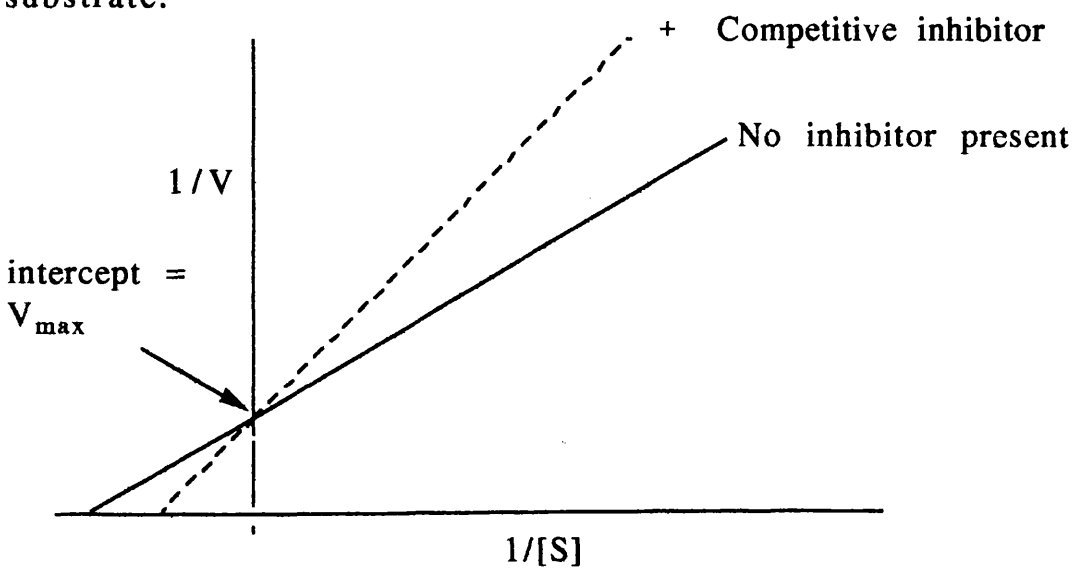
CHAPTER 4

INHIBITORS OF DIAMINE OXIDASE

4.1 Enzyme Kinetics

Measurements of the rate of catalysis at different concentrations of substrate and inhibitor with an enzyme provide information to distinguish between competitive and non-competitive inhibition.

In competitive inhibition the intercept on the y-axis of the plot of 1/V *vs.* 1/[S] (Lineweaver-Burk plot) is the same with or without inhibitor present. The intercept corresponds to the V_{max} and it is therefore not affected by a competitive inhibitor (Graph 4.1). This shows that competitive inhibition can be overcome by a high concentration of substrate because at this point, virtually all the active sites are filled by substrate.



Graph 4.1 Competitive Inhibition: Double reciprocal plot of enzyme kinetics

The difference in slope when the inhibitor is present indicates the difference in binding.

The Lineweaver-Burk equation;

$$1/V = 1/V_{max} + K_M/V_{max}(1/[S]) \qquad (4.1)$$

is replaced by;

$$1/V = 1/V_{max} + K_M/V_{max}(1 + [I]/K_i)1/[S] \qquad (4.2)$$

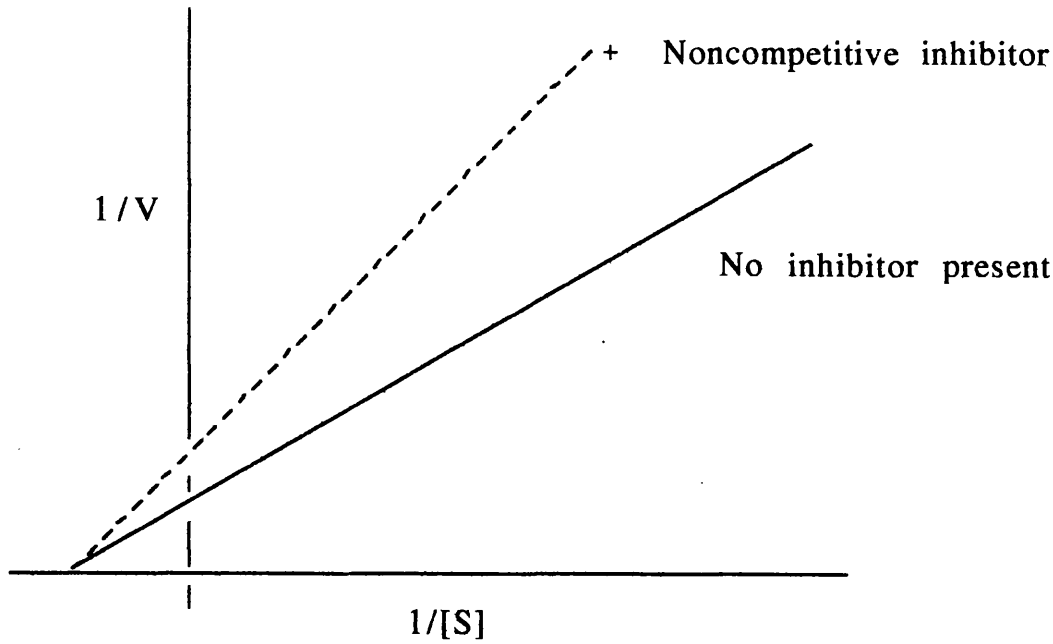
Where [I] is the inhibitor concentration and K_i is the dissociation constant of the enzyme-inhibitor complex. In other words,

$$(slope)^I/(slope) = 1 + [I]/K_i \qquad (4.3)$$

$(slope)^I$ = the slope of the line when competitive inhibitor is present.

(slope) = the slope of the line without inhibitor.

In non-competitive inhibition the V_{max} is decreased to V_{max}^I so the intercept on the y-axis is increased (Graph 4.2).



Graph 4.2 Non-competitive Inhibition: Double reciprocal plot of enzyme kinetics.

The slope which is equal to K_M/V_{max}^I is larger by the same factor. In this case K_M is not altered. The maximum velocity in the presence of a non-competitive inhibitor is given by,

$$V_{max}^I = V_{max}/(1 + [I]/k_i) \qquad (4.4)$$

Non-competitive inhibition cannot therefore be affected by a high substrate concentration.

4.2 The Assay Procedure for Inhibition Studies

Inhibition studies were performed using the same peroxidase-coupled spectrophotometric assay procedure as before.[14] The difference was that a constant amount of inhibitor was added

for each experiment. A typical inhibition study consisted of three or four experiments. For example;

(a) Substrate only

(b) Substrate + [I]

(c) Substrate + 2[I]

(d) Substrate + 4[I]

where [I] = substrate concentration.

Every inhibition experiment was accompanied by measurements with no inhibitor present to provide a standard for comparison as experimental values for each oxidation can vary slightly from day to day.

As discussed in 3.3 the reaction without inhibitor present was initiated with addition of enzyme immediately followed by substrate. In the case with inhibitor present the inhibitor was added after the addition of the enzyme and was immediately followed by addition of substrate (See Appendix 5). This was done to avoid the faster oxidation occurring before the inhibitor was present.

4.3 Inhibitor Studies with Putrescine and Cadaverine Analogues

4.3a Introduction

A number of putrescine and cadaverine analogues discussed earlier in 3.4 and 3.5 were shown to be substrates for diamine oxidase. For some of these analogues, although they bound well to the enzyme, the rate of the oxidation was much slower

than with the natural substrates. This condition is a good requirement for competitive inhibitors. Many competitive inhibitors are substrate analogues. It is useful to know the rate of oxidation of the substrate analogue when determining how well it will inhibit the enzyme activity.

4.3b Synthesis of Inhibitors

The putrescines and cadaverine analogues were synthesised as discussed in Chapter 3 (3.4b, 3.5b). Appropriate analytical data were obtained for all of the compounds tested.

4.3c Results and Discussion

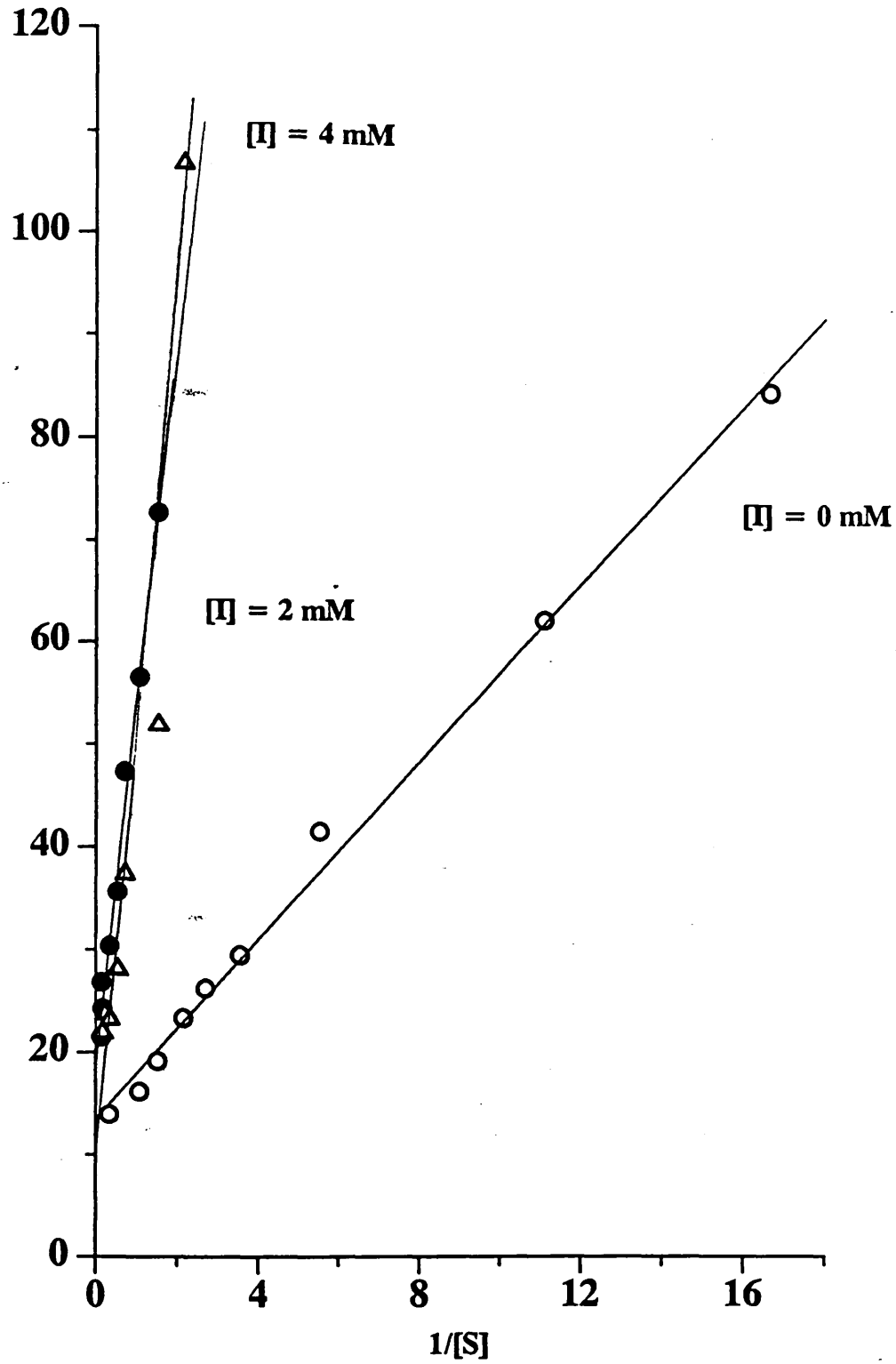
The compounds selected for testing as inhibitors of diamine oxidase from the range of putrescine and cadaverine analogues originally tested as substrates were the dihydrochlorides of *N*-propylputrescine (56) and 3,3-dimethylputrescine (58). These compounds were chosen as they both had binding affinities (K_M values) of similar magnitude to the natural substrate with the enzyme and they also had very poor rates of oxidation. Therefore they possessed the requirements for a competitive inhibitor.

(1) *N*-Propylputrescine (56). This was first tested as an inhibitor of pea seedling diamine oxidase with putrescine as the substrate. Results showed that with addition of up to 5 mM of *N*-propylputrescine the V_{max} showed no change. However the rate of oxidation did change with lower substrate concentrations but not in a systematic manner. This does

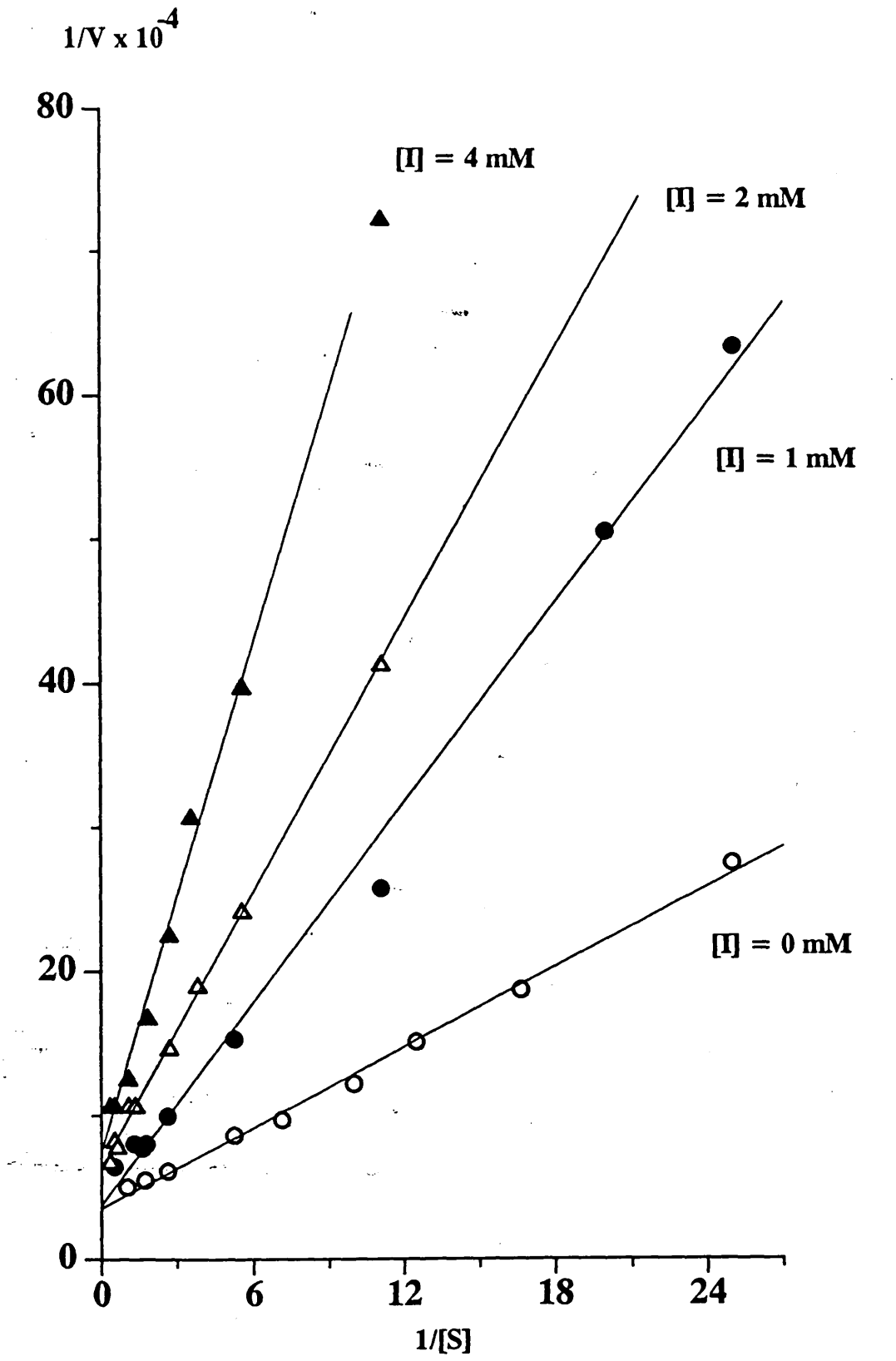
suggest however that *N*-propylputrescine (56) may be a competitive inhibitor of pea seedling diamine oxidase.

(2) 3,3-Dimethylcadaverine (58) 3,3-Dimethylcadaverine (58) was then tested first as an inhibitor of pea seedling diamine oxidase with putrescine as the substrate. The results obeyed Michaelis-Menten kinetics. They are represented in Graph 4.3 showing that 3,3-dimethylcadaverine (58) is a competitive inhibitor of diamine oxidase with putrescine as substrate. K_i values are shown in Table 4.1. 3,3-Dimethylcadaverine (58) was then tested as an inhibitor of pea seedling diamine oxidase with cadaverine as the substrate. Again 3,3-dimethylcadaverine (58) was shown to be a competitive inhibitor of diamine oxidase as the data obtained are consistent with what is expected for competitive inhibition (Graph 4.4).

The inhibition constants (K_i) obtained from such experiments are comparable to the K_M values of these compounds when assayed as substrates. For example with cadaverine (7) as substrate and 3,3-dimethylcadaverine (58) as inhibitor, the K_i value was 0.71 mM compared to the K_M value of 0.61 ($\pm$0.06) for 3,3-dimethylcadaverine (58) as a substrate.



Graph 4.3 Double reciprocal plot of enzyme kinetics. Inhibition of oxidative deamination of putrescine using pea seedling diamine oxidase with 3,3-dimethylcadaverine (58) as the inhibitor. [I] = inhibitor concentration.



Graph 4.4 Double reciprocal plot of enzyme kinetics. Inhibition of oxidative deamination of cadaverine using pea seedling diamine oxidase with 3,3-dimethylcadaverine (58) as the inhibitor. [I] = inhibitor concentration.

Substrate	Inhibitor Concentration	K_i value
Putrescine	2	0.41
	4	0.24
	1	0.69
Cadaverine	2	0.72
	4	0.73

Units: mM

Table 4.1 Inhibition Constants (K_i) for competive inhibitor, 3,3-dimethylcadaverine with pea seedling diamine oxidase.

An added benefit of using 3,3-dimethylcadaverine to inhibit diamine oxidase *in vivo* is that its susceptiblity to diamine oxidase oxidation will limit its active lifetime in the biological system. Due to this, the potentially harmful conditions caused by the addition of these compounds can be avoided in the more sensitive tissues.

4.4 *N*-Alkylated Componds as Inhibitors of Pea Seedling Diamine Oxidase

A. A. Denholm[102] synthesised a group of *N*-(aminoalkyl)-piperidines (72) and (73) and their corresponding piperidinium salts (74) and (75) and assayed them as substrates of pea seedling diamine oxidase using the peroxidase coupled assay system.[14]

It was clear from these results that all of the compounds tested were substrates of pea seedling diamine oxidase, although they showed low activity. It was also found from these results that this group of compounds actually bind as well as [*N*-(aminoalkyl)-piperidines (72)-(73)] or better [(*N*-(aminoalkyl)-piperidiniums (74)-(75)] than cadaverine or putrescine. These results are shown in Table 4.2.

All these compounds could prove to be very good inhibitors due to their low rates of oxidation and binding affinities (K_M).

N-(Aminopentyl)piperidine (73) was tested by us as an inhibitor of pea seedling diamine oxidase with cadaverine as the substrate. *N*-(Aminopentyl)piperidine (73) is comparable to cadaverine in chain length. The results obtained obeyed Michaelis-Menten kinetics with inhibitor concentrations of 0.4 mM and 0.8 mM and gave a K_i value of 1.21 mM. This inhibition is not as great as observed with 3,3-dimethylcadaverine although it is still significant.

The *N*-(aminoalkyl)piperidinium salts (74)-(75) were shown to bind more strongly to the enzyme than the natural substrates (Table 4.2).[102]

One explanation is that the introduction of the double bond produces changes in the pK_a and/or polarity of the substrate. Also the presence of the double bond will alter the conformation of the compounds and electron density around one of the nitrogens. Further inhibition studies are required on the *N*-(aminoalkyl)piperidinium salts as this significant strong binding could be the key to developing good inhibitors.

Substrate	K_M	V_{max}
N-(aminobutyl)piperidine (72)	1.19	5.4
N-(aminopentyl)piperidine (73)	0.37	3.6
N-(aminobutyl)piperidinium (74)	0.056	7.7
N-(aminopentyl)piperidinium (75)	0.037	8.0

Units K_M : mM

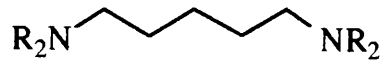
V_{max} : μmol/mg/hr

Table 4.2 K_M and V_{max} values for oxidation of *N*-alkylated compounds (72)-(75) catalysed by diamine oxidase from pea seedling diamine oxidase.[102]

4.5 Di-*N*-alkylated Diamines as Inhibitors of Diamine Oxidase

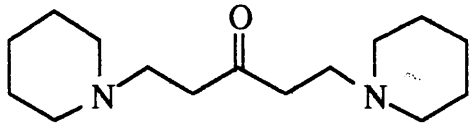
4.5a Introduction

It was thought that di-*N*-alkylated diamines (76) could be inhibitors of diamine oxidase. Although they are not substrates they are still substrate analogues and may be accepted into the site. No oxidation is likely to occur. The di-*N*-aklylated diamines (76) might bind well and prevent the natural substrate from entering the active site.

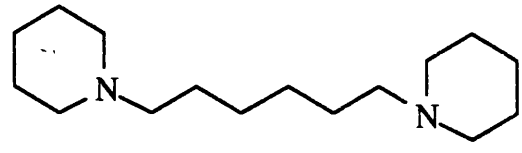


(76)

Following on from the inhibition studies on *N*-(aminopentyl)-piperidine (73), 1,5-*bis*(*N*-piperidyl)pentan-3-one (77) dihydrochloride and 1,6-*bis*(*N*-piperidyl)hexane (78) dihyhrobromide were synthesised to be tested as inhibitors of diamine oxidase from pea seedlings.



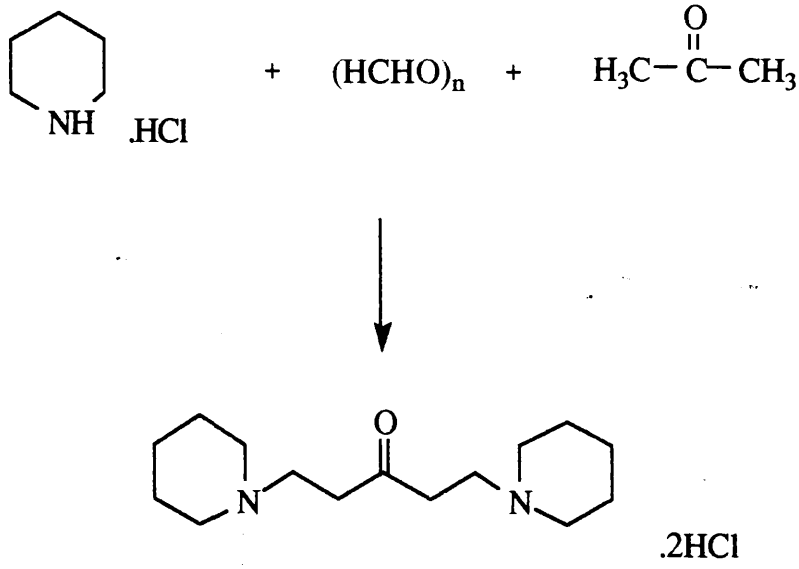
(77)



(78)

4.5b Synthesis

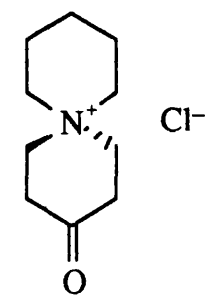
1,5-*bis*(*N*-piperidyl)pentan-3-one (77) dihydrochloride was synthesised by the method of van Tamelen *et al.* (Scheme 4.1).[103] The product was triturated with hot acetone followed by hot chloroform to remove any excess starting materials or side products.



(77)

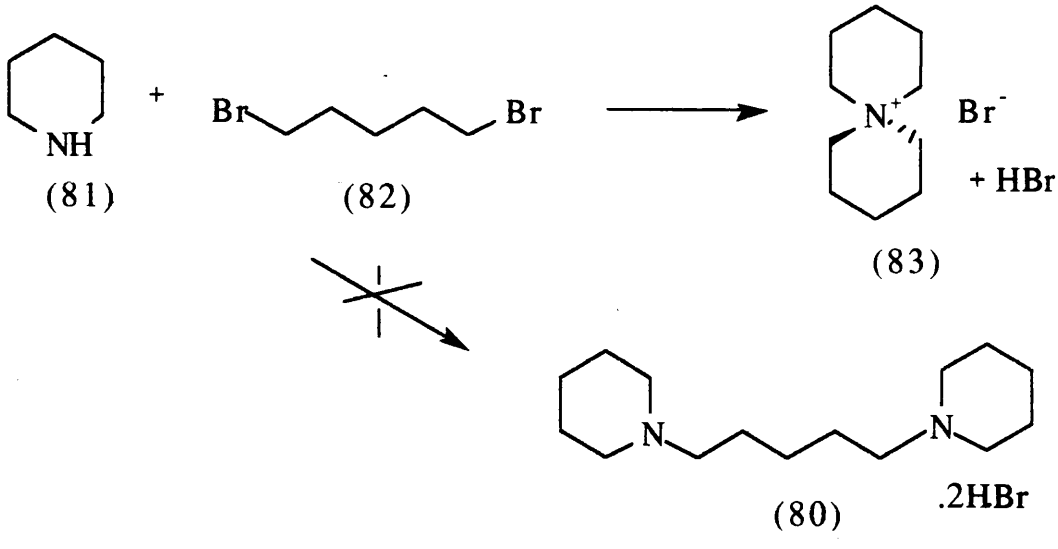
Scheme 4.1 Synthesis of 1,5-*bis*(*N*-piperidyl)pentan-3-one (77) dihydrochloride

The problem with this method was that the yield was very low (15%). This may be due to the formation of the quaternary ammonium product (79).



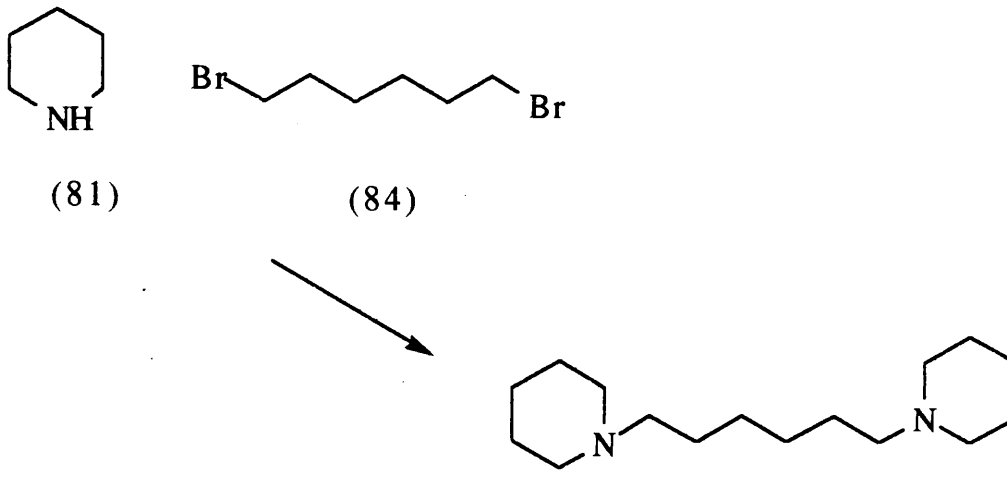
(79)

This problem also arose when the synthesis of 1,5-*bis*(*N*-piperidyl)pentane (80) was attempted using piperidine (81) and 1,5-dibromopentane (82).[104] Under various conditions the product was not formed. This was due to the preferential formation of the quaternary ammonium salt (83) (Scheme 4.2).



Scheme 4.2 Attempted Synthesis of 1,5-*bis*(*N*-piperidyl)pentane (80) dihydrobromide.

This problem was not observed however in the formation of 1,6-*bis*(*N*-piperidyl)-hexane (78) dihydrobromide using 1,6-dibromohexane (84) as the formation of the quaternary ammonium salt would involve the formation of a less preferred seven-membered ring.[104] Therefore the major product is the 1,6-*bis*(*N*-piperidyl)-hexane (78) dihydrobromide (Scheme 4.3).



(81) (84)

(78) .2HBr

Scheme 4.3 Synthesis of 1,6-*bis*(*N*-piperidyl)-hexane (78) dihydrobromide.

4.5c Results and Discussion

Both compounds (77) and (78) were tested as substrates using the peroxidase-coupled spectrophotometric assay system.[14] No oxidation was observed in either case.

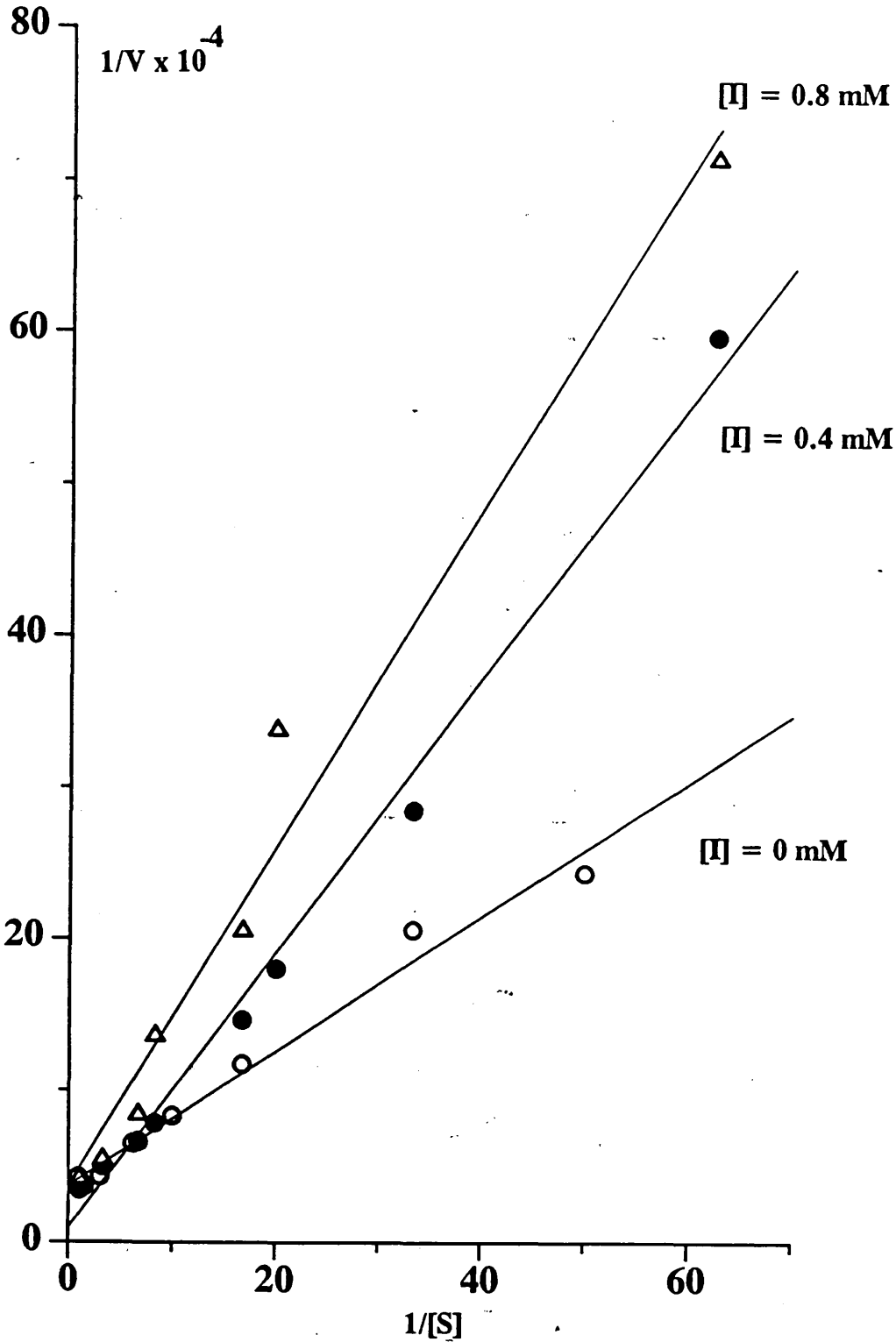
The compounds (77) and (78) were examined as inhibitors of diamine oxidase from pea seedlings with cadaverine as the substrate. Inhibitor concentrations of 0.4 mM and 0.8 mM were used and the results are shown in Table 4.3.

Inhibitor	Inhibitor Concentration	K_i
1,5-*bis*(*N*-piperidyl)-pentan-3-one-dihydrochloride (77)	0.4	0.36
	0.8	0.49
1,6-*bis*(*N*-piperidyl)-hexane-dihydrobromide (78)	0.5	1.11
	1.0	1.61

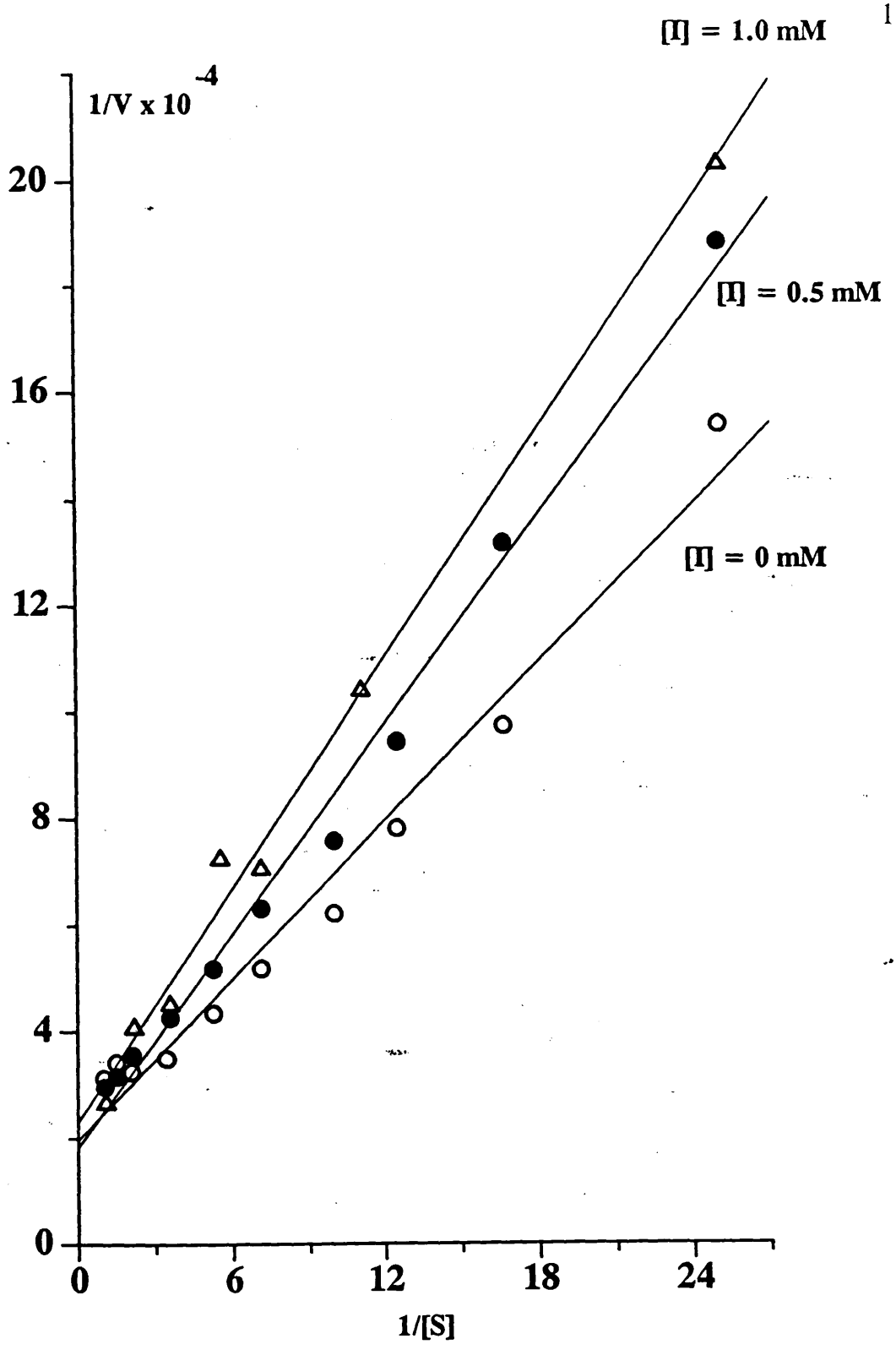
Units: mM

Table 4.3

Assays in the presensce of both compounds obeyed Michaelis-Menten kinetics and were characteristic of competitive inhibition. *ie.* V_{max} remains unchanged while the K_M differs (Graph 4.5 and Graph 4.6). These results show that 1,5-*bis*(*N*-piperidyl)pentan-3-one (77) dihydrochloride is the better inhibitor of the two. This could be due to the presence of the carbonyl in (77) or due to the size of the chain length also differing in (77) and (78). The carbonyl possibly interacts through hydrogen bonding with residues at the active site. The presence of the carbonyl also reduces the degrees of freedom in the chain.



Graph 4.5 Double reciprocal plot of enzyme kinetics. Inhibition of oxidative deamination of cadaverine using pea seedling diamine oxidase with 1,5-*bis*(*N*-piperidyl)pentan-3-one (77) as the inhibitor. [I] = inhibitor concentration.

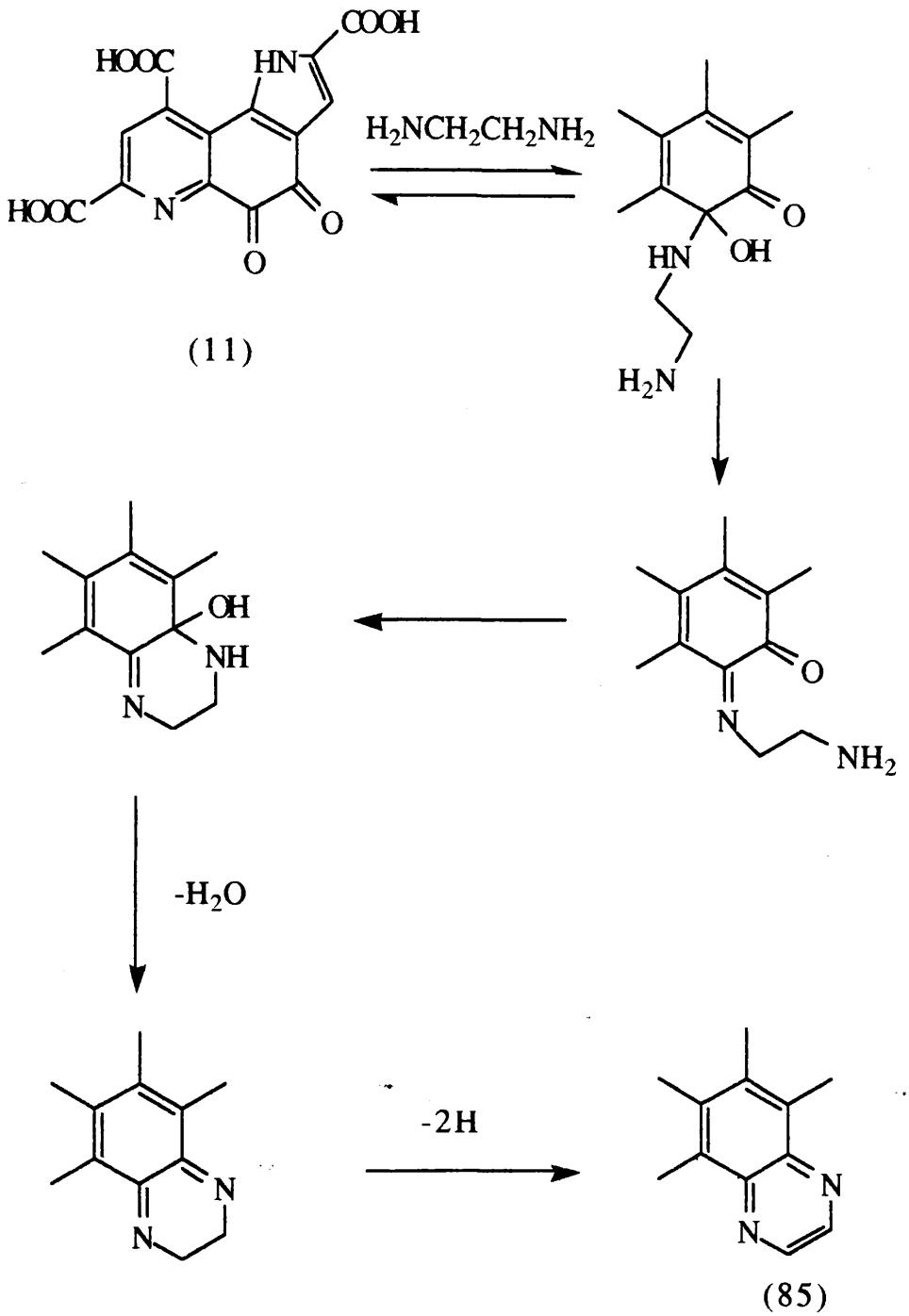


Graph 4.6 Double reciprocal plot of enzyme kinetics. Inhibition of oxidative deamination of cadaverine using pea seedling diamine oxidase with 1,6-*bis*-(*N*-piperidyl)hexane (78) as the inhibitor. [I] = inhibitor concentration.

4.6 1,2-Diamines as Inhibitors of Diamine Oxidase

4.6a Introduction

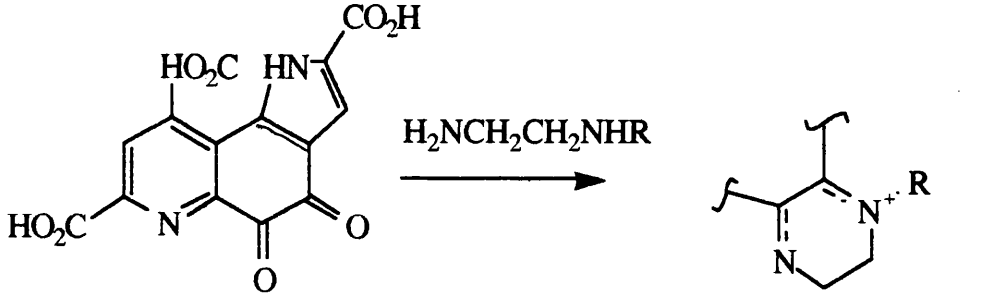
Ethane-1,2-diamine (64) is thought to be a inhibitor of diamine oxidase.[62] In 1989 Mure *et al.*[105] described the behaviour of ethane-1,2-diamine (64) with the proposed cofactor of diamine oxidase pyrroloquinoline quinone (PQQ) (11). Under acidic conditions the pyrazine derivative (85) was formed (Scheme 4.4).



Scheme 4.4 Formation of the Pyrazine Derivative (85) of PQQ

However it should be noted that they found that ethane-1,2-diamine at neutral pH acts both as a substrate and an inhibitor.

Cragg and Herbert[75] reported in 1990 that when two nitrogens are present and are separated by a chain of two carbon atoms little or no oxidation was observed. Tetramethylene triamine (86) was also shown not to be oxidised with pea seedling diamine oxidase. The explanation for this inhibition was similar to that described by Mure (Scheme 4.5).[105]



R = $CH_2CH_2NH_2$

Scheme 4.5 Reaction of PQQ with Tetramethylene Triamine

These explanations both relied on PQQ as the cofactor. However in the last year this has been in doubt (Chapter 2, 2.10).

Ethane-1,2-diamine and tetramethylene triamine were tested as inhibitors of pea seedling diamine oxidase using the sensitive peroxidase-coupled assay and the results are discussed below.

4.6b Synthesis

Ethane-1,2-diamine (64) and tetramethylene triamine (86) were available from Aldrich, and were converted into the

dihydrochloride/trihydrochloride salts and purified by recrystallisation. Appropriate analytical data were obtained for both compounds.

4.6c Results and Discussion

Both ethane-1,2-diamine (64) and tetramethylene triamine (86) were first tested as substrates of diamine oxidase. The results are shown in Table 4.4.

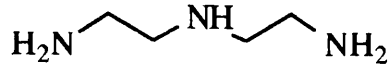
Substrate	K_M	V_{max}
Ethane-1,2-diamine (64)	2.81	55
Tetramethylene triamine (86)	0.13	166

Units K_M : mM

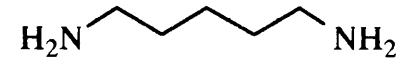
V_{max} : µmol/mg/hr

Table 4.4 K_M and V_{max} values for oxidation of the substrates catalysed by diamine oxidases from pea seedlings at 25 °C and pH 6.3.

The results contradict work by Cragg *et al.*[75] They show that tetramethylene triamine (86) is in fact oxidised by pea seedling diamine oxidase. The K_M value of 0.13 mM shows that the triamine (86) binds very well to the enzyme. The value is similar to that with cadaverine (7). This is probably due to the similar distance between the two terminal amino groups.



(86)



(7)

Therefore the triamine acts as a cadaverine analogue rather than an ethane-1,2-diamine analogue with respect to binding.

The fact that some reporters[62,75] have found it difficult to observe the oxidation of these di/tri-amines may be due to the lack of sensitivity of their assay systems. In most cases measurement of O_2 uptake was the method used.

No orderly inhibition was observed when the two compounds (64) and (86) were added to enzymic reactions with putrescine (1). With the triamine (86) as the possible inhibitor no orderly inhibition was observed because at low concentrations of substrate the rate of oxidation of the constant concentration of tetramethylene triamine (86) was greater than that of the natural substrate itself and therefore a constant rate was observed. Some inhibition may have been present in this case but accurate values could not be obtained due to the inhibitor also being a competing substrate for the enzyme.

Therefore, when using most assay systems, if a substrate analogue is to be tested as an inhibitor the rate of oxidation of the analogue must be low enough not to interfere with the kinetic behaviour of the enzymic reaction.

CHAPTER 5

5.1 Stereoselectivity and Regioselectivity of the Oxidative Deamination Catalysed by Pea Seedling Diamine Oxidase

5.1a Introduction

The regioselectivity of the oxidative deamination of 2-methylputrescine using diamine oxidase by Santaniello and co-workers[11] is reviewed in Chapter 2, 2.21. As mentioned earlier, little work has been carried out in this field.

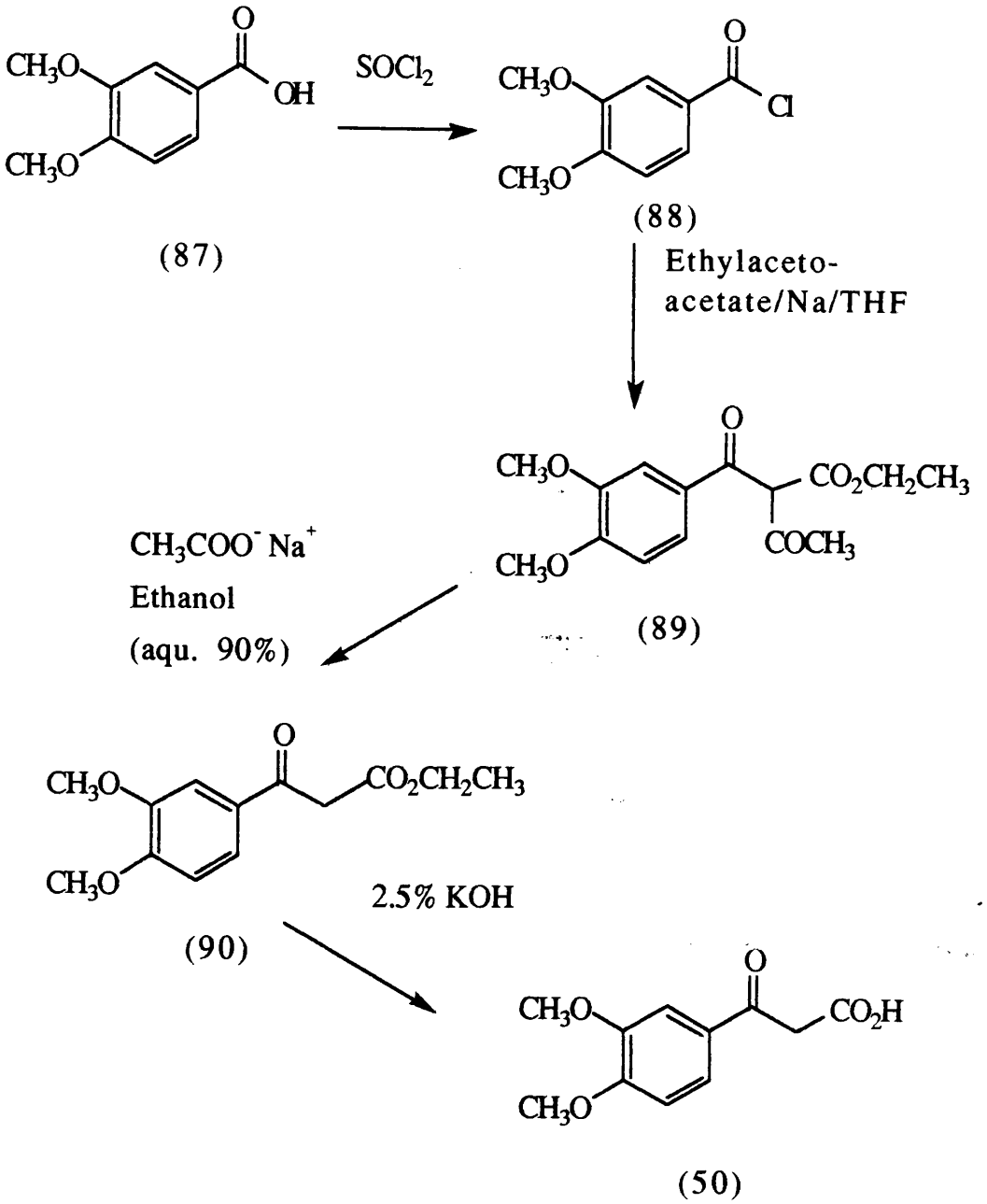
It was therefore decided to use the cadaverine analogues as substrates and analyse the products of the catalytic oxidative deamination. However the aminoaldehydes are unstable and readily form cyclic imines. These cyclic imines can then trimerise. This made extraction and characterisation of the products of the oxidation extremely difficult. A coupling reagent was required to trap the initial imine formed. 3,4-Dimethoxybenzoylacetic acid (50) has been used for this purpose.[15] The 3',4'-dimethoxy-2'-[2-(alkyl)-piperidyl]acetophenones were formed when *C*-alkylcadaverines were incubated with diamine oxidase, catalase and 3,4-dimethoxybenzoylacetic acid (50) in phosphate buffer (pH 7) at 25 °C. Catalase was required to remove the inhibitory hydrogen peroxide. These compounds were easily extracted and were analysed by NMR spectroscopy and HPLC.

The compounds examined for any selectivity in the diamine oxidase catalysed oxidation were (a) 3-methylcadaverine (57), (b) 3-phenylcadaverine (61) and (c) 2-methylcadaverine (62).

5.1b Synthesis of 3,4-Dimethoxybenzoylacetic acid (50) and Cadaverine Analogues

3,4-Dimethoxybenzoylacetic acid (50) was synthesised from 3,4-dimethoxybenzoic acid (87) (Scheme 5.1). The acid (87) was converted into the acid chloride (88) by stirring with excess thionyl chloride. The acid chloride formed was unstable and used immediately in the formation of ethyl 3,4-dimethoxybenzoylacetoacetate (89), with ethylacetoacetate and sodium. Ethyl 3,4-dimethoxybenzoylacetate (90) was formed by selective removal of the acetyl group using a catalytic amount of sodium acetate. The β-keto ester (90) formed was then hydrolysed using 2.5% potassium hydroxide. The β-keto acid (50) formed decarboxylates readily above 25 °C. Therefore care was taken to prevent decarboxylation occurring. The β-keto acid (50) was stored in the freezer. To minimise the amount of β-keto acid lost by decarboxylation, the final step was not carried out until the reagent was required.

The cadaverine analogues (57), (61)-(62) used were synthesised as described in Chapter 3, 3.5. Correct analytical data were obtained for all the analogues formed. The coupling reactions were carried out at pH 7 and 25 °C for 24 h. The conditions were kept the same for each reaction. The products were purified by flash column chromatograpy.

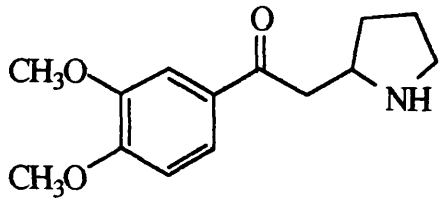


Scheme 5.1 Synthesis of 3,4-Dimethoxybenzoylacetic acid (50).

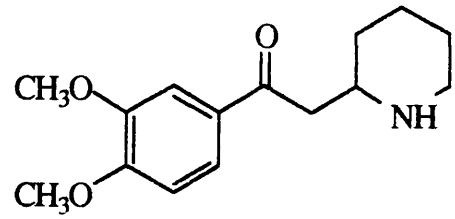
5.1c Results and Discussion

The diamine oxidase catalysed oxidation and subsequent coupling reaction was carried out firstly using putrescine (1)

dihydrochloride and cadaverine (7) dihydrochloride as substrates. Products (91) and (49) were formed respectively in reasonable yields.

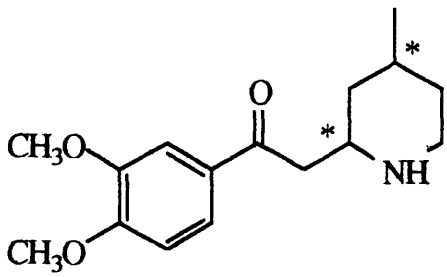


(91)



(49)

3-Methylcadaverine was then used as the substrate and incubated under the same conditions. In this case the product (92) has two chiral centres and four possible stereoisomers could be formed.

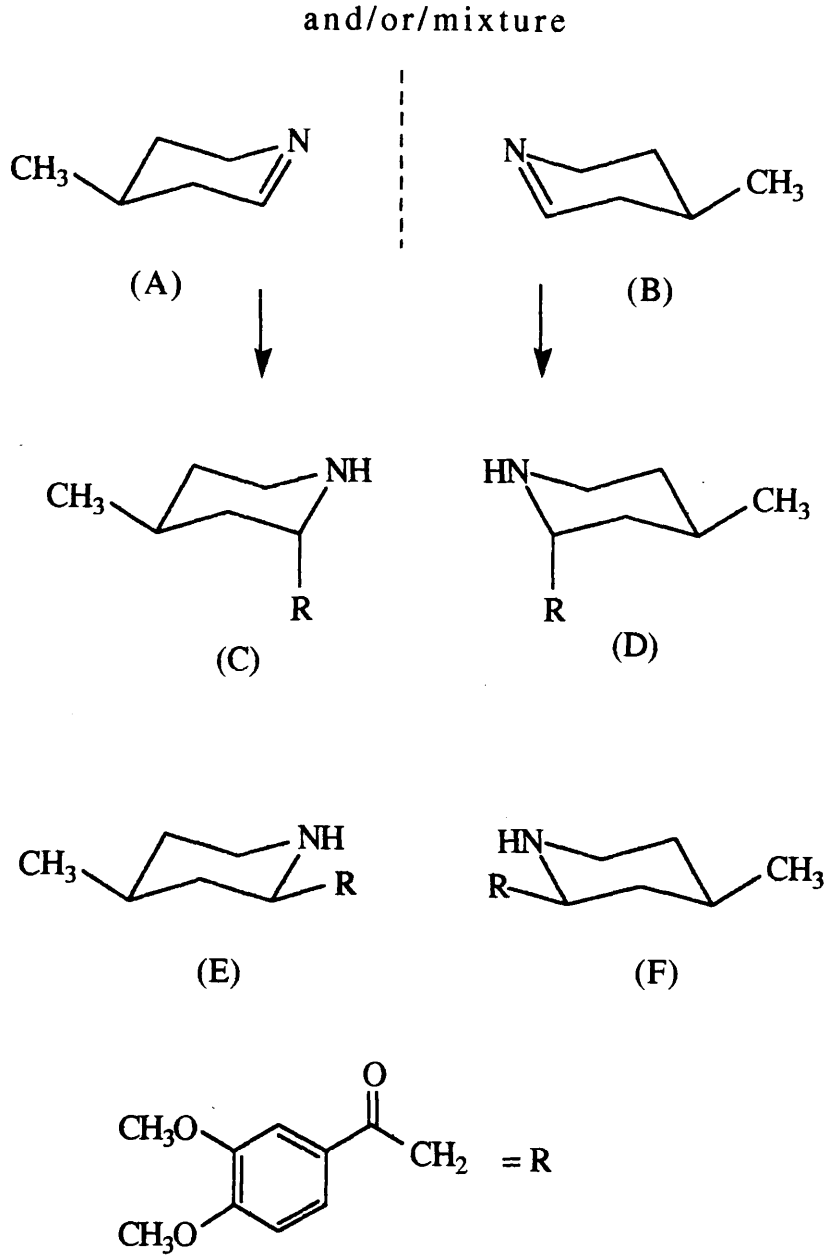


* Chiral Centres

(92)

The cyclic imines (A) and (B) (Scheme 5.2) could be formed from the catalytic oxidative deamination of 3-methylcadaverine using pea seedling diamine oxidase. One may be formed selectively or both formed as a mixture.

The second step (coupling of the 3,4-dimethoxybenzoylacetic acid) then could produce a further two products from both (A) and (B) (Scheme 5.2).



Scheme 5.2 Possible products from the oxidation of 3-methylcadaverine using diamine oxidase and subsequent coupling with 3,4-dimethoxybenzoylacetic acid.

The ^{13}C NMR spectrum of 3',4'-dimethoxy-2-[2'-(4-methyl)-piperidyl]-acetophenone (92) showed sixteen carbon signals. These could be assigned to sixteen carbons on (92). This implies that one product is formed. The proton NMR

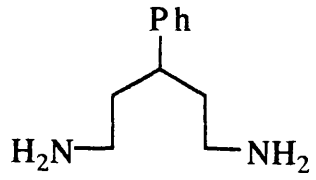
spectrum of (92) showed a doublet at δ 0.81 which integrated as three protons. This represents the methyl group on the piperidyl ring. The proton spectrum was however very broad and complex.

Elution with methanol and water (50:50) showed one major peak on reverse phase column HPLC. This peak had a retention time of 1.51 min and an intensity of 85.6% of the total mixture. Another peak was observed at 4.42 min with an intensity of 8.7% of the total mixture. This may be another stereoisomer of the product. The ratio of 10:1 is significant showing that one isomer is formed preferentially. For one product to be observed selectivity must occur in the second step. Therefore (C), (D), (±)-(CD) or a mixture are produced or (E), (F), (±)-(EF) or a mixture are produced. The ^{13}C NMR spectrum rules out the possibility of (C) and (E) [or (F)] or (D) and (F) [or (E)] as products as this would have shown two sets of carbon signals. This was not observed.

Measurement of the optical activity could rule out (±)-(CD) or (±)-(EF). No optical activity was observed; however the readings were recorded at low concentrations.

Therefore selectivity does appear to occur at the second step however we are not yet sure if there is also selectivity in the first step.

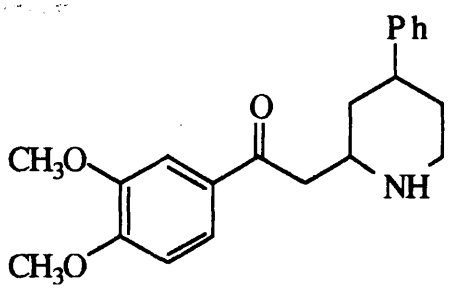
Using 3-phenylcadaverine (61) dihydrochloride as the substrate and incubating this as before with 3,4-dimethoxybenzoylacetic acid (50) did not prove fruitful. Several attempts yielded a product which was identified as the starting diamine, 3-phenylcadaverine.



.2HCl

(61)

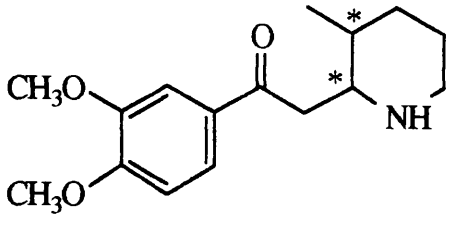
This was an unusual result as 3-phenylcadaverine (61) dihydrochloride was shown to be a good substrate for pea seedling diamine oxidase (Chapter 3, 3.5). The reaction was carried out with ample enzyme over a large period of time. No 3',4'-dimethoxy-2-[2'-(4-phenyl)piperidyl]acetophenone (93) was formed from several attempts.

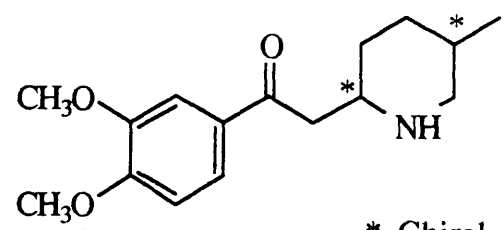


(93)

This was an unexpected result and is difficult to explain.

An additional problem with 2-methylcadaverine was that attack on either amino group could occur and would result in two different structural isomers (94) and (95) being formed. As before with 3-methylcadaverine the products formed have two chiral centres and each structural isomer has four possible stereoisomers.





* Chiral centre

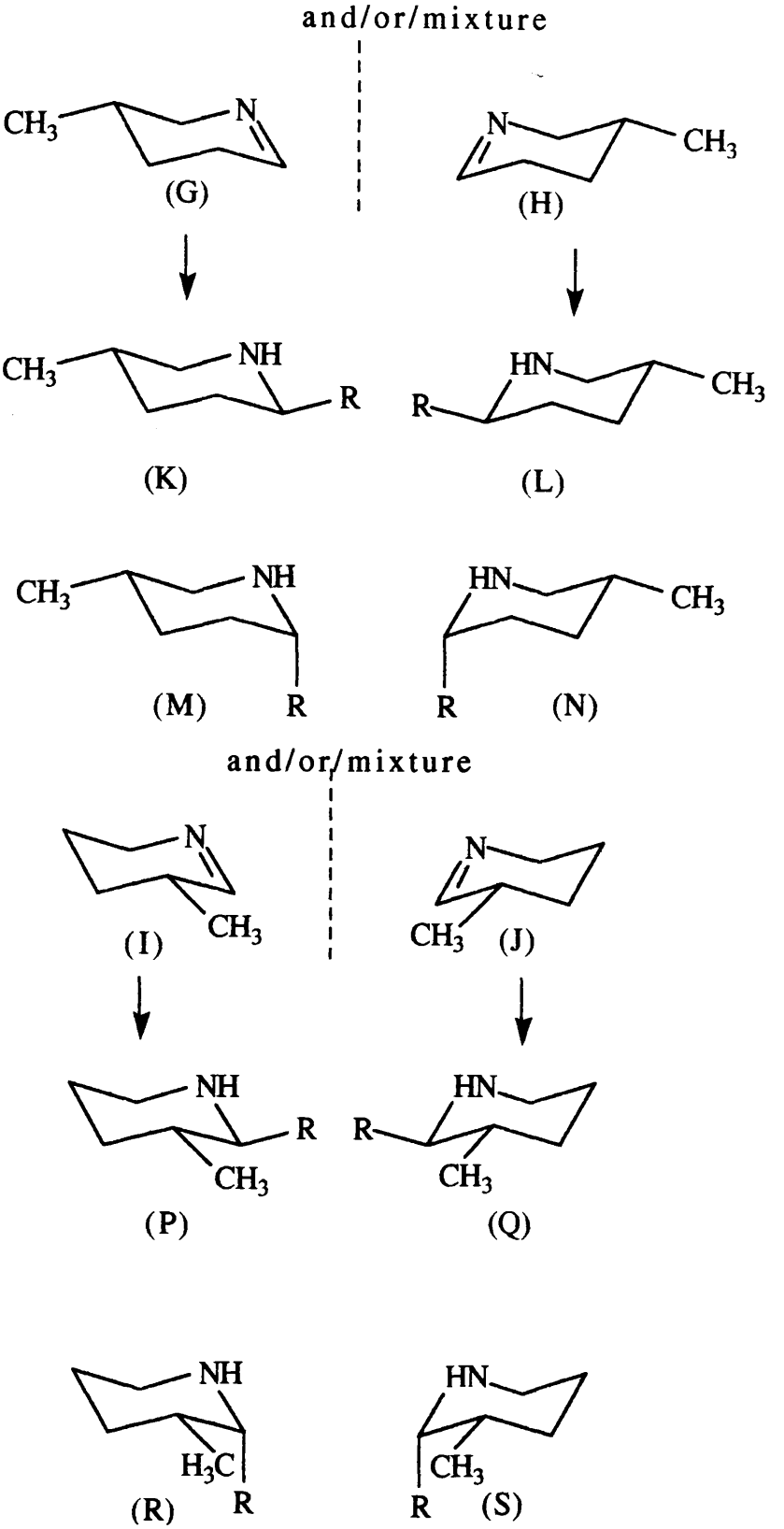
(94) (95)

Therefore four cyclic imines can be formed from catalytic oxidative deamination of 2-methylcadaverine using pea seedling diamine oxidase.

The second step (coupling of the 3,4-dimethoxybenzoylacetic acid) then could again produce a further two products from each of the cyclic imines (Scheme 5.3).

It is clear from the ^{13}C-NMR spectrum that again one major product is formed. There is however evidence for a minor product. The proton NMR spectrum showed a doublet at δ 0.79 and a minor doublet at δ 0.86 with a ratio of 3:1.

On HPLC analysis, elution with methanol and water (50:50) showed one major peak at 4.51 min with an intensity of 66.2% of the total mixture and another peak at 1.40 min with an intensity of 25.4%. Prep. HPLC was carried out and mass spectroscopy showed the parent ions of both peaks to be the same (M^+ 277). These peaks elute with similar retention times to the products with 3-methylcadaverine.



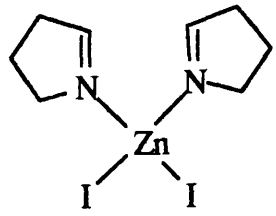
Scheme 5.3 Possible products from the oxidation of 2-methylcadaverine using diamine oxidase and subsequent coupling with 3,4-dimethoxybenzoylacetic acid.

The 3.4-dimethoxybenzoylacetic acid coupling procedure is not a very convenient method for characterisation of the products of the oxidative deamination of diamines with pea seedling diamine oxidase as the coupling reaction provides added complications due to the addition of a second chiral centre.

5.2 Stabilisation of Cyclic Imines Formed in the Reaction Involving Diamine Oxidase by Complexation With Zinc Iodide

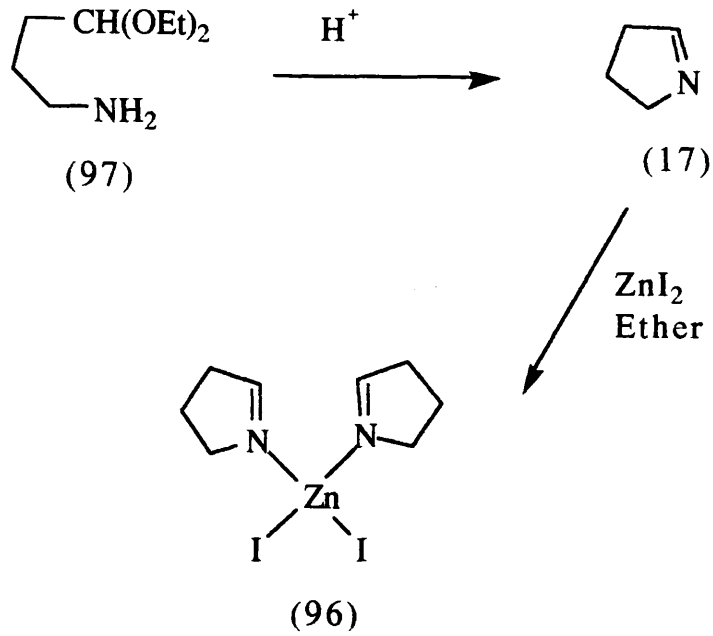
The identification and characterisation of the cyclic imines formed in the oxidative deamination of diamines has proved to be very difficult. This is due to their volatility and tendency to trimerise in neutral or basic solution.[106] A simple method for the stabilisation of these imines which does not involve formation of another chiral centre is required to allow complete characterisation of the products of the reactions with diamine oxidase.

Earlier in 1991 it was reported that 1-pyrroline (3,4-dihydro-2*H*-pyrrole) could be stabilised by complexing with zinc iodide to form a stable crystalline complex (96).[107] This complex could also be regenerated for further use in synthesis.



(96)

The 1-pyrroline (17) was generated by acid hydrolysis of 4-aminobutanal diethylacetal (97) followed by extraction of the basified solution with ether (Scheme 5.4).



(97) (17) (96)

Scheme 5.4 Formation of the ZnI_2 complex with pyrroline.

The formation of a stable ZnI_2 complex was therefore attempted with the product of the oxidative deamination of putrescine (butane-1,4-diamine) with diamine oxidase. The original reaction was carried out at 0 °C in acid and a two phase solvent system of buffer and diethyl ether. The enzyme diamine oxidase is inactive at 0 °C. Therefore it was not possible for the reaction to be carried out under the same conditions as previously reported.[107] The reaction was unsuccessful under various conditions (Table 5.1)

Reaction Time (h)	Solvent	Temp. °C	pH
24	Buffer	25	7
1	Buffer	25	7
1	Buffer	5	7
1	Buffer/ Diethyl ether	5	6

Table 5.1 Conditions used for the ZnI_2 complexation reaction.

Under the usual conditions of phosphate buffer (pH 7) and 25 °C no complex with ZnI_2 was formed. Lowering the temperature to 5 °C and carrying the reaction out in slightly acidic solution did not prove successful and again no complex with ZnI_2 was formed.

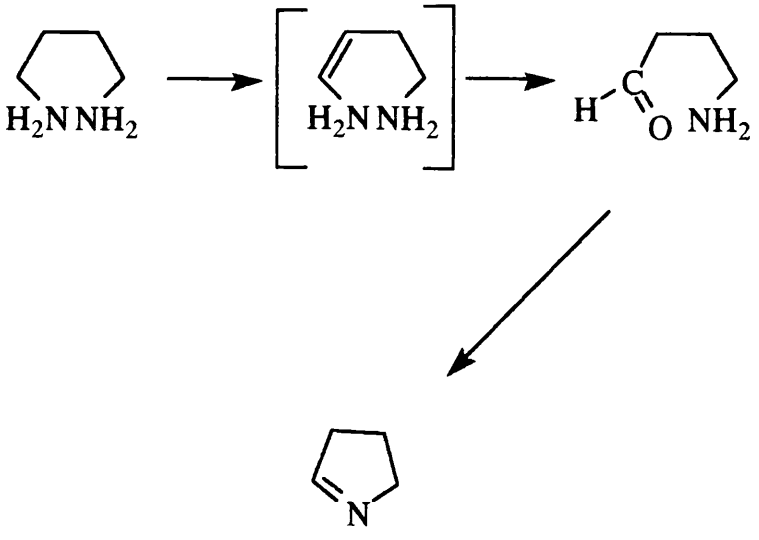
Under the conditions of the enzyme reaction the imine formed tends to trimise rapidly. This is probably the reason why no complex with ZnI_2 is formed under these conditions and at 0 °C the enzyme, diamine oxidase is inactive.

Therefore so far conditions have not been found for making the complex with ZnI_2 with imines generated from enzyme reactions.

5.3 The Mechanism of Formation of 1-Pyrroline from the Oxidative Deamination of Putrecsine

5.3a Introduction

It has been suggested that the oxidation of putrescine by diamine oxidase might go through an enamine intermediate (Scheme 5.5).[12]



(17)

Scheme 5.5 Proposed mechanism of putrescine oxidation by diamine oxidase.

We carried out experiments using [2,3-$^2H_4$]putrescine dihydrochloride to determine whether any deuterium is lost from these labelled positions during the oxidation (Note: imine-enamine tautomerism of 1-pyrroline (17) would also lead to loss of deuterium).

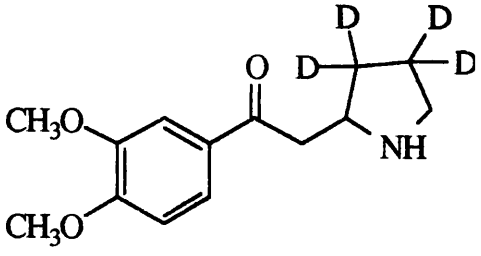
5.3b Synthesis

Synthesis of [2,3-$^2H_4$]putrescine dihydrochloride with *ca.* 90% $^2H_4$ species was carried out by A. B. Watson.[108] 3,4-Dimethoxybenzoylacetic acid was formed as described in 5.1. They were incubated together with diamine oxidase and catalase in phosphate buffer for 24 h, also as described in 5.1.

The product was then purified by flash column chromatography and fully characterised.

5.3c Results and Discussion

The major product of the oxidative deamination of [2,3-$^2H_4$]-putrescine dihydrochloride using diamine oxidase was found to be the tetradeuteriated product (98).



(98)

Initial evidence for this structure came from the mass spectrum of the product. The parent ion was at *m/z* 253 with an accurate mass of 253.1619 where 253.1616 is expected for $C_{14}H_{15}D_4NO_3$. Furthermore 50-MHz ^{13}C NMR spectrum then showed only twelve signals. The signals for carbon 3 and 4 were not visible. This was due to the deuterium attached.

When the 600 MHz proton NMR spectrum was run with the aid of homonuclear decoupled spectra key protons were identified. The signal for H-2 is a triplet at *ca.* δ 4.1 and the two protons bonded to C-6 are at δ 3.46 and δ 3.86 and are partly obscured by other resonances. Decoupling at δ 3.46 perturbs both the other signals as does decoupling at δ 4.1. This latter experiment also collapses a small doublet at δ 2.28. This is also clear in the decoupling difference spectrum which reveals a similar effect at *ca.* δ 1.80. These two signals may correspond

to molecules where there is one proton in α or β positions on C-3. An exact ratio could not be worked out but it is clear that partial loss of one deuterium atom does occur. The formation of the compound containing three deuterium atoms could be due to imine-enamine tautomerism of 1-pyrroline (17).

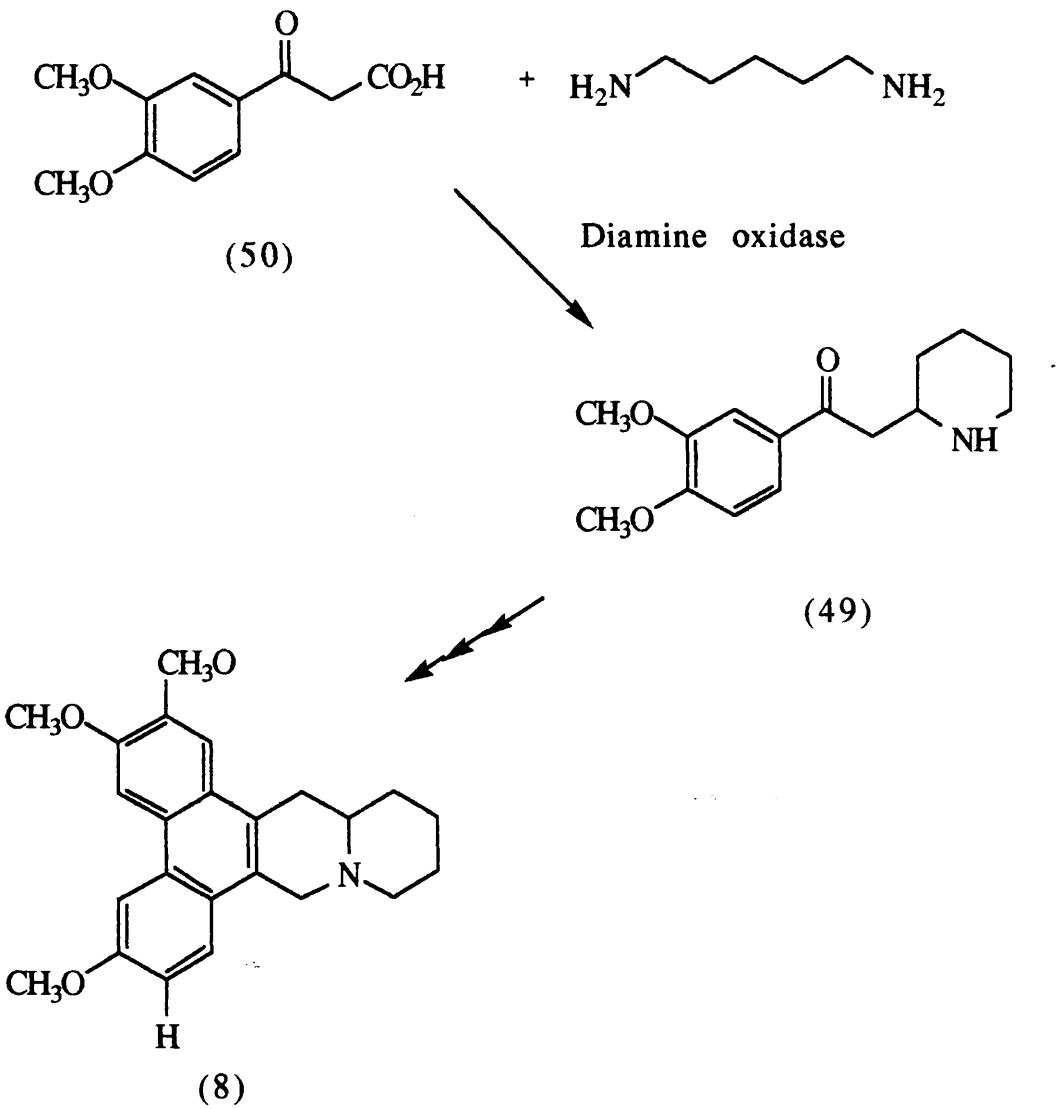
Assumptions regarding the formation of an enamine intermediate in the oxidative deamination of putrescine appear to be incorrect as we have shown that the major product of the reaction with [2,3-$^{2}H_4$]-putrescine dihydrochloride is in fact compound (98), containing four deuterium atoms.

CHAPTER 6

APPLICATIONS OF DIAMINE OXIDASE

6.1 Synthesis of Cryptopleurine Analogues: Possible Anti-cancer Agents

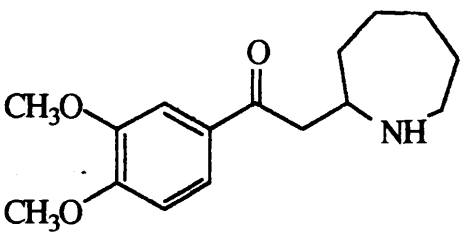
Cryptopleurine (8) and tylophorine (9) exhibit interesting biological activities, including anti-cancer action. Herbert and co-workers established a synthesis of cryptopluerine (8).[15] A key step in this synthesis involved the use of diamine oxidase (Scheme 6.1).



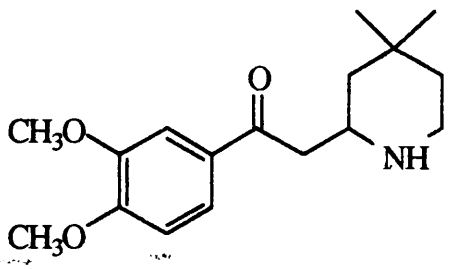
Scheme 6.1 Synthesis of cryptopleurine (8).

Using cadaverine dihydrochloride as the substrate for diamine oxidase cryptopleurine (8) was formed after further steps. Analogues of cryptopleurine (8) may also have useful biological activity. The use of substituted cadaverines and other diamines could lead to the formation of many different alkaloid analogues.

Tylophorine (9) can be synthesised using putrescine dihydrochloride as the substrate in the initial step. Compounds (99) and (100) were synthesised by the method described in Chapter 5, 5.1b using hexane-1,6-diamine and 3,3-dimethylcadaverine as substrates.



(99)



(100)

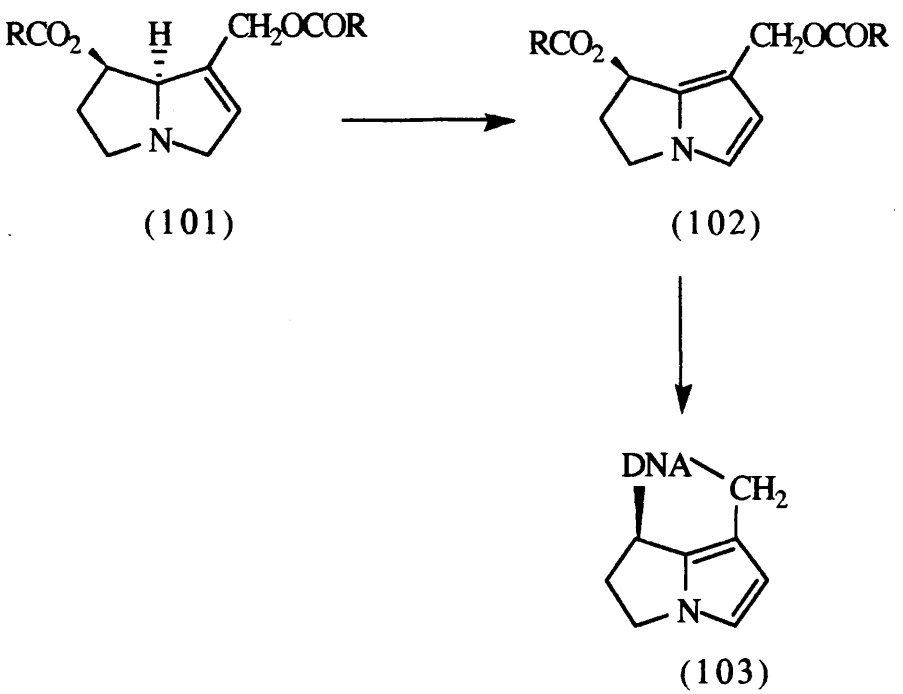
Due to time constraints these compounds were not further reacted to form the analogues of cryptopleurine (8). However no problems could be foreseen in completing the synthesis of the analogues. This shows that the diamine oxidase step could be of benefit in making alkaloid analogues with potentially useful biological activity.

6.2 Formation of Pyrrolizidine Alkaloids Using Diamine Oxidase

6.2a Introduction

Another application of diamine oxidase is in the synthesis of pyrrolizidine alkaloids. They are widespread in plants and many of them are hepatotoxic.[109] This is due to the unsaturated ring of the base portion (101) which can be oxidised by liver oxidase enzymes to form pyrrole derivatives (102).

These pyrrole derivatives can act as bifunctional alkylating agents and become bound to DNA (103) (Scheme 6.2).



(101) (102) (103)

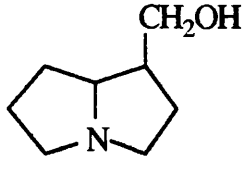
Scheme 6.2

Homospermidine (5) is thought to be a key intermediate in the biosynthesis of these alkaloids. ^{13}C-^{15}N Labelling experiments confirmed that a C_4-N-C_4 intermediate is involved.[6]

$H_2N(CH_2)_4NH(CH_2)_4NH_2$

(5)

It has been shown that incubation of homospermidine with pea seedling diamine oxidase and subsequent reduction of the likely product, 1-formylpyrrolizidine, produces the pyrrolizidine alkaloid, trachelanthamidine (6).[9]



(6)

It was decided to attempt to improve the yield and purity of the product of this reaction and to form analogues of the pyrrolizidine alkaloids using analogues of homospermidine as the substrates for diamine oxidase.

6.2b Preparation of Pyrrolizidine Alkaloids

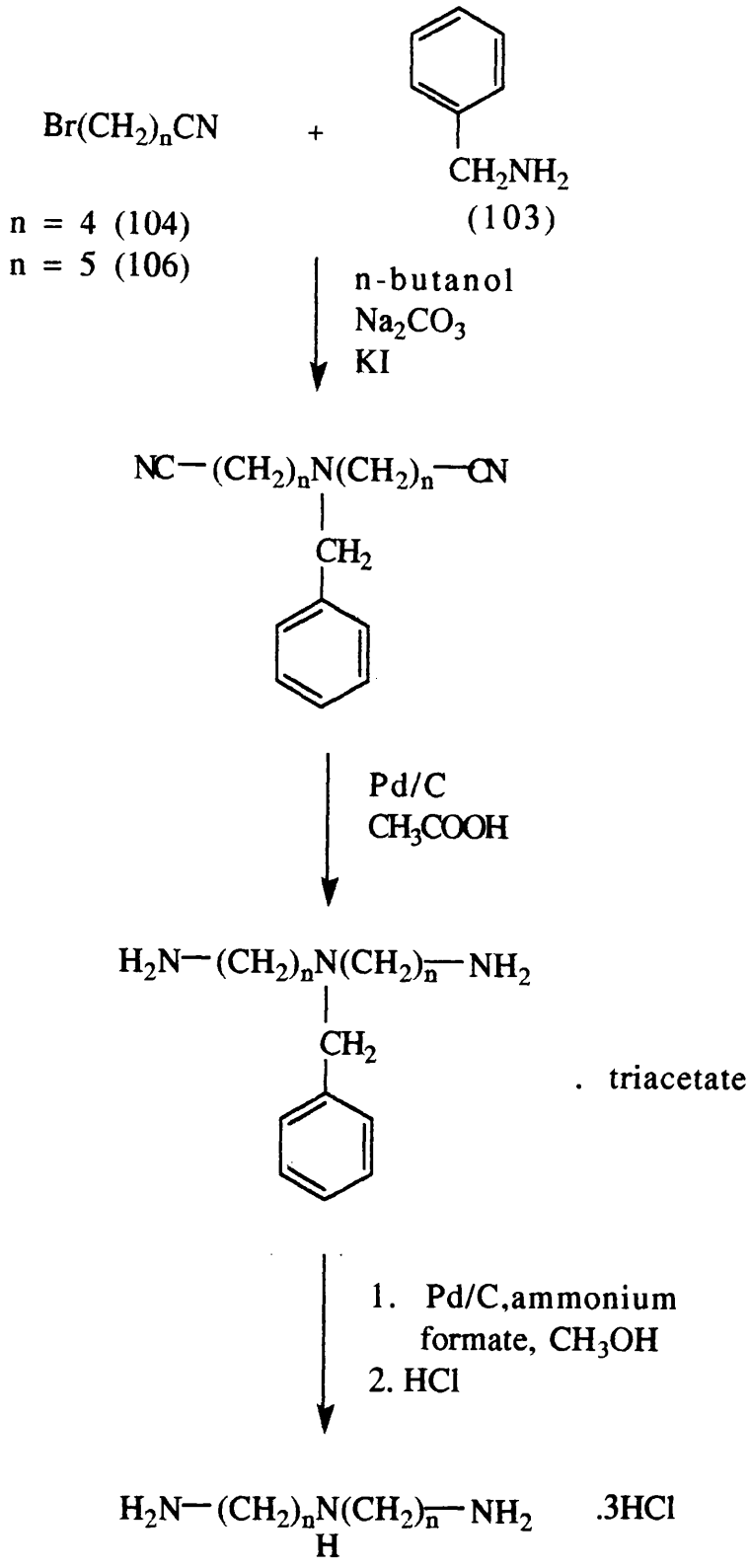
Homospermidine (5) trihydrochloride was synthesised from two moles of 4-bromobutanenitrile (104) and benzylamine (105) (Scheme 6.3). *N,N-bis*-(3-cyanopropyl)benzylamine was obtained as a clear oil after distillation. Bergeron *et al.*[8] reported that under hydrogenation conditions the nitriles

produced were reduced to the corresponding amines and the benzyl group was removed. We found that when the dinitrile was hydrogenated at 1 atmosphere for *ca.* 18 h only the nitrile groups were reduced. The benzyl group remained. Stronger conditions were then employed to remove the benzyl group. This was achieved by catalytic transfer hydrogenation[110] using ammonium formate and palladium on charcoal in methanol at reflux conditions. The reaction was monitored by t.l.c. and the catalyst was filtered off when the reaction was complete. Homospermidine (5) trihydrochloride was formed when the product was extracted with hydrochloric acid.

N,N-bis-(5-Aminopentyl)amine (10) trihydrochloride was prepared in the same manner using two moles of 5-bromopentanenitrile (106) (Scheme 6.3).

Homospermidine (5) trihydrochloride was then incubated with pea seedling diamine oxidase in phosphate buffer for two days at 25 °C. Catalase was added to remove the inhibitory hydrogen peroxide. The mixture was basified and the product was extracted with chloroform. The likely is product 1-formylpyrrolizidine (107). This was immediately reduced with sodium borohydride in methanol. Unfortunately, with several attempts, a complex mixture of products was formed and could not be identified.

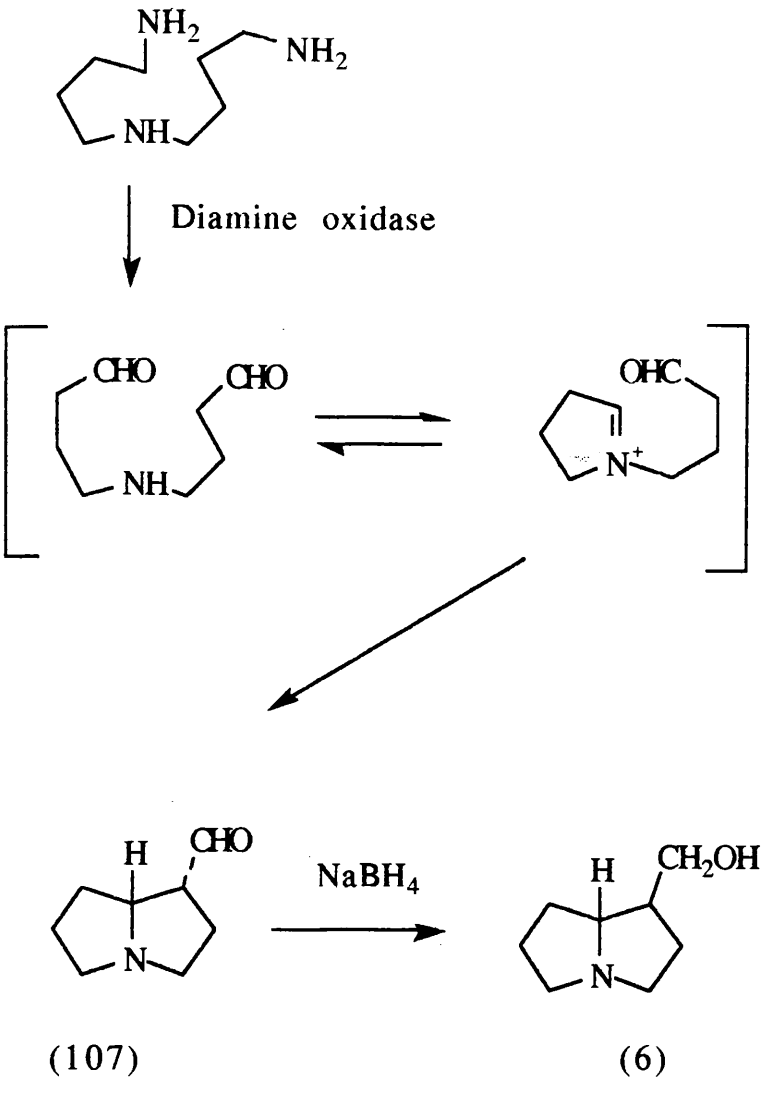
A second method was used. This involved carrying out the reduction with a large excess of sodium borohydride added after two days. The final isolation procedure was carried out as for the first method. This method produced 1-hydroxy-methylpyrrolizidine (6) in a yield of 10%. This yield could not be improved after several attempts (Scheme 6.4).



n = 4 Homospermidine (5) .3HCl

n = 5 *N,N-bis*-(5-Aminopentyl)amine (10)..3HCl

Scheme 6.3 Synthesis of Homospermidine (5) trihydrochloride and *N,N-bis*-(5-Aminopentyl)amine (10).trihydrochloride



(107) (6)

Scheme 6.4 Synthesis of trachelanthamidine (6).

6.2c Conclusion

Due to the poor results obtained with homospermidine and time constraints the enzyme reaction was not carried out using *N,N-bis*-(5-aminopentyl)amine (10) trihydrochloride.

We have shown that this enzymic oxidation of homospermidine does lead to 1-hydroxymethylpyrrolizidine (6) after a reduction step. Much more work is required to allow this sequence to be used as a convenient method for the formation of analogues of pyrrolizidine alkaloids.

CHAPTER 7

EXPERIMENTAL

7.1 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 with a Perkin Emler R32 spectrophotometer operating at 90 MHz, or a Bruker WP200-SY spectrophotometer operating at 200 MHz (δ_H), 50.3 (δ_C), or 30.72 MHz (δ_D). 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.

T.l.c. 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) and HPLC using a reverse phase Silica C 18 column and solvent system of methanol and water (50:50).

All solvents were purified by standard techniques.[111] Tetrahydrofuran (THF) and diethyl ether were dried by distillation from sodium-benzophenone under argon immediately before use.

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

7.2 Experimental to Chapter 3 and Chapter 4

Extraction of Diamine Oxidase (EC 1.4.3.6) from Pea Seedlings[2]

Step 1

Pea seeds (500 g), variety 'Fillbasket', were soaked in tap water for 24 h. 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 d until the shoots were 5-10 cm tall. Note: the Perlite was kept moist throughout but not too wet as this reduced 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 °C.

Step 2

Ethanol/chloroform (2:1 v/v, 30 ml per 100 ml of extract) was cooled to -10 °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 °C during this addition. The mixture was allowed to stand for *ca.* 1 h 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.* 2 h. The solution was stirred for 1.5 h 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 1.5 h 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, 20 ml). The solution was dialysed in a 30 cm tube (diameter 15 mm) for 2-3 h 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 1 h 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 1 h 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.

Enzyme activity was *ca.* 1200 units per mg of solid (at 25 °C), with a yield of *ca.* 30 mg per kg of seedlings. [See Appendix 3 for method of calculation.]

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

Sodium Dodecylsulphate (SDS) Slab Gel Electrophoresis modified Laemmli Gel System[89]

Slab gel electrophoresis was carried out as described by Laemmli.[89]

Stacking Buffer

0.4% SDS in 0.5 M TRIS

pH adjusted to 6.8 with 6 N hydrochloric acid

x2 Sample Buffer

10% SDS (30 ml)

Stacking buffer (12.5 ml)

Glycerol (10 ml)

pH adjusted to 6.8 with 5 M hydrochloric acid

2-Mercaptoethanol Solution

x2 Sample buffer (900 µl)

2-Mercaptoethanol (100 µl)

Bromophenol Blue Solution

1% Bromophenol blue (200 µl)

2-Mercaptoethanol (200 µl)

Sample Preparation

Samples were prepared using 2-mercaptoethanol solution (100 µl) and protein (100 µl). The samples were heated in boiling water for 2 min. Bromophenol blue solution (5 µl) was added to a protein sample (50 µl).

Standard Protein Solution

Sigma Dalton Mark VII-L was dissolved in distilled water (2 mg/ml). This was then treated in the same way as the protein samples and used as the standard.

Staining Solution

The gel was stained using Coomassie Blue Dye.

0.1% Coomassie Blue R250, C.I. 42660 was dissolved in glacial acetic acid, methanol and distilled water (2:5:5).

Destaining Solution

10% Glacial acetic acid

Determination of Protein Concentration[88]

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. The stock dye was diluted to A_{465} 1.3-1.5 (the absorbance maximum for the leuko form of the dye). The 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

x = 50 to 5 µl.

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

Spectrophotometric Assay[14]

The kinetics of DAO-catalysed oxidation of putative substrates were determined by the procedure of Stoner.[14] 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	0.34 mg/ml phosphate buffer pH 6.3 (150-200 units per mg solid. One unit will form 1.0 mg of purpurogallin from pyrogallol in 20 s at pH 6 at 20 °C.)
Pea Seedling DAO	0.03-0.06 mg/ml phosphate buffer pH 6.3

Pig Kidney DAO (From Sigma)	2 mg/ml phosphate buffer pH 6.3 (0.06 units per mg solid. One unit will oxidise 1 μmole of putrscine per h at pH 7.2 at 37 °C.)

A typical reaction mixture in a 1 cm pathlength cuvette comprised:

1. With Pea Seedling DAO (psDAO)

2.5 ml	Phosphate buffer pH 6.3
100 μl	MBTH
170 μl	DMAB
50 μl	peroxidase
25 μl	psDAO
300 μl	substrate (concentration range up to 3 mM)

2. With Pig Kidney DAO (pkDAO)

2.5 ml	pkDAO 2 mg/ml phosphate buffer pH 6.3
100 μl	MBTH
170 μl	DMAB
50 μl	peroxidase
300 μl	substrate (concentration range up to 3 mM)

The production of the indamine dye was calibrated using standard solutions of hydrogen peroxide. The hydrogen peroxide was standardised using potassium permanganate.[112]

The reaction was initiated by addition of standard enzyme solution to the thermally equilibrated reaction mixture, followed immediately by substrate addition, therefore minimizing the possibly inhibiting effects of extensive preincubation of DAO with the chromogenic agents.[14] Initial rates were determined over a range of substrate concentrations from the linear absorbance changes during the first minute of reaction. Michaelis-Menten kinetic behaviour was observed in all cases. Rate data were analysed for k_M and V_{max} by least squares fitting of Eadie-Hofstee[93,94] (V *vs.* V/[S]) and Lineweaver-Burk[92] (1/V *vs.* 1/[S]) plots. [See Appendices 2 and 3]

All experiments were carried out at least three times with each respective substrate. Therefore all data quoted are the means of three determinations.

Control Experiments

1. Experiments were carried out using histamine as substrate. This was the substrate used in the original studies by Stoner.[14] The K_M value was comparable. (K_M 2.64 x 10^{-5} M compared with 2.90 x 10^{-5} M.)

2. No special precautions were taken to control oxygen concentrations in the reaction mixtures, but experiments using partially degassed buffers showed that reduction of O_2 to as low as 25% saturation (oxygen electrode) had no effect on measured rates. This confirmed that the reaction kinetics are not limited by O_2 levels under normal circumstances.

3. As a check on the validity of this assay procedure, reaction rates were measured under the same conditions by monitoring the O_2 uptake as a function of time in a thermostatted reaction vessel fitted with a calibrated oxygen electrode. Similar rate data were obtained. [See Appendix 4.]

4. Control experiments showed that at the low concentrations used in this assay, bishydrazone formation by reaction with MBTH was negligible.

Inhibition Studies

Inhibition studies were performed by the same method as above and by incorporating various concentrations of putative inhibitor or co-substrate into the initial reaction mixture. A separate experiment was carried out for each concentration of putative inhibitor or co-substrate. The potential inhibitor was added to the reaction mixture after DAO addition but before the addition of substrate. [See Appendix 5]

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

Diacid or anhydride (20.5 mmol) in anhydrous THF (20 ml) was brought to reflux under a dry nitrogen atmosphere. 1 M Borane.THF (41 ml, 41 mmol) was added dropwise *via* syringe. The reaction mixture was heated at reflux for *ca.* 18 h. After cooling, water (20 ml) was added *via* syringe. The aqueous layer was then saturated with K_2CO_3 (*ca.* 4 g). Diethyl ether (10 ml) was added to the reaction mixture. The mixture was extracted with diethyl ether (4 x 20 ml). The organic layer was dried (Na_2SO_4) and evaporated to dryness under reduced pressure to leave the product.

3-Methylpentane-1,5-diol (108)

3-Methylglutaric acid (109) was reduced to the corresponding diol (108) using general procedure (A). A clear oil was obtained after distillation (75%); b.p. 160 °C at 1 mm Hg (lit.,[71] b.p. 160 °C); υ_{max} (thin film) 3500-3150, 2960, 1460 and 1055 cm^{-1}; δ_H ($CDCl_3$) 1.05 (3H, d), 1.30-2.00 (5H, complex), 3.80 (4H, t), and 4.08 (2H, br s exchangeable with D_2O, OH); *m/z* 101, 88 and 41 (100%).

3,3-Dimethylpentane-1,5-diol (110)

3,3-Dimethylglutaric acid (111) was reduced to the corresponding diol (110) using general procedure (A). A clear oil was obtained after distillation (75%); b.p. 165 °C at 1 mm Hg; υ_{max} (thin film) 3600-3100, 2960, 1470 and 1035 cm^{-1}; δ_H

($CDCl_3$) 0.88 (6H, s), 1.40-1.70 (5H, comlex), 3.52 (2H, br s, OH), and 3.65 (4H, t); *m/z* 113 and 69 (100%).

2,4-Dimethylpentane-1,5-diol (112)

2,4-Dimethylglutaric anhydride (113) was reduced to the corresponding diol (112) using general procedure (A). A clear oil was obtained after distillation (75%); b.p. 165 °C at 1 mm Hg (lit.,[71] b.p. 165 °C); υ_{max} (thin film) 3550-3120, 2960, 1465 and 1075 cm^{-1}; δ_H ($CDCl_3$) 0.80-1.00 (6H, complex), 1.35-1.85 (2H, complex), 2.95 (2H, br s, OH) and 3.43 (4H, d); *m/z* 130, 67 and 55 (100%).

2,2-Dimethylpentane-1,5-diol (114)

2,2-Dimethylglutaric anhydride (115) was reduced to the corresponding diol (114) using general procedure (A). A clear oil was obtained after distillation (88%); b.p. 175 °C at 1 mm Hg (lit.,[71] b.p. 180 °C); υ_{max} (thin film) 3600-3100, 2950 and 1060 cm^{-1}; δ_H ($CDCl_3$) 0.90 (6H, s), 1.10-1.70 (4H, complex), 3.25 (4H, br s, OH) and 3.60 (2H, t); *m/z* 115 and 55 (100%).

3-Methylpentane-1,3,5-triol (116)

3-Hydroxy-3-methylglutaric acid (117) was reduced to the corresponding triol (116) using general procedure (A). The product was obtained as a yellow oil, (62%); υ_{max} (thin film) 3600-3100, 2950 and 1070 cm^{-1}; δ_H ($CDCl_3$) 0.96 (3H, s), 1.40 (4H, complex), 1.68 (2H, br s, OH), and 3.68 (4H, t); *m/z* 133 and 43 (100%).

General Procedure (B) for Reduction of a Diacid to a Diol

Diacid (7.21 mmol) was dissolved in anhydrous THF (20 ml) under a dry nitrogen atmosphere and cooled in an ice bath to 0 ^{o}C. 1 M Borane.THF (26.5 ml, 26.5 mmol) was then added slowly, dropwise over a period of 10-15 min. The solution was stirred at room temperature for a further 1 h. Excess borane was destroyed by slow addition of water. The mixture was separated by salting out the organic layer using K_2CO_3. The aqueous layer was extracted with ether (3 x 20 ml). The organic extracts were dried (Na_2SO_4) and evaporated to dryness under reduced pressure to yield the product.

3-Phenylpentane-1,5-diol (118)

3-Phenylglutaric acid (119) was reduced to the corresponding diol (118) using general procedure (B). The product was obtained as a clear oil, (70%); υ_{max} (thin film) 3500-3300, 2940, 1600, 1590 and 1050 cm^{-1}; δ_H ($CDCl_3$) 1.60-1.90 (5H, complex), 3.20-3.70 (4H, complex) and 7.00-7.30 (5H, complex); *m/z* 180 (M^+), 162 and 105 (100%).

2-Phenylbutane-1,4-diol (120)

Phenylsuccinic acid (121) was reduced to the corresponding diol (120) using general procedure (B). The procduct was obtained as a clear oil, (68%); υ_{max} (thin film) 3600-3100, 2940, 1600, 1590, 1335 and 1050 cm^{-1}; δ_H ($CDCl_3$) 1.70-2.10 (2H, complex), 2.85 (1H, m), 3.30 (2H, t), 3.40-3.80 (4H, complex) and 7.00-7.40 (5H, complex); *m/z* 166 (M^+), 105 (100%) and 91.

General Procedure (C) for 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 (4 ml, 52 mmol) was added with stirring and triethylamine (5.25 g, 52 mmol) was added slowly over 5 min. The mixture was allowed to reach room temperature overnight. The mixture was then poured into ice 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 was crystallised from diethyl ether.

Pentane-1,5-diyl dimethanesulphonate (122)

Using pentane-1,5-diol (123), pentane-1,5-diyl dimethanesulphonate (122) was obtained as a white crystalline solid (65%); m.p. 35 °C; υ_{max} (KBr disc) 2940, 1175 and 1040 cm^{-1}; δ_H ($CDCl_3$) 1.75 (6H, m), 3.00 (6H, s) and 4.25 (4H, t); *m/z* 261 (M^+ + 1), 165 and 68 (100%).

3-Methylpentane-1,5-diyl dimethanesulphonate (124)

Using 3-methylpentane-1,5-diol (108), 3-methylpentane-1,5-diyl dimethanesulphonate (124) was obtained as a white crystalline solid (63%); m.p. 59-60 °C; υ_{max} (KBr disc) 2960, 1350, 1175 and 1040 cm^{-1}; δ_H ($CDCl_3$) 1.00 (3H, s), 1.50-2.00 (5H, m), 3.00 (6H, s) and 4.27 (4H, t); *m/z* 205, 82 (100%) and 79; (Found: C, 35.08; H, 6.65; S, 23.59. $C_8H_{18}O_6S_2$ requires C, 35.04; H, 6.57; S, 23.36%).

3,3-Dimethylpentane-1,5-diyl dimethanesulphonate (125)

Using 3,3-dimethylpentane-1,5-diol (110), 3,3-dimethylpentane-1,5-diyl dimethanesulphonate (125) was obtained as a white crystalline solid (60%); m.p. 63 °C; υ_{max} (KBr disc) 2940 and 1175 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 0.97 (6H, s), 1.70 (4H, t), 2.97 (6H, s) and 4.24 (4H, t); δ_C ($CDCl_3$) 27.22 (q, $C\underline{C}H_3$), 31.62 (s, $\underline{C}CH_3$), 37.25 (q, $O_2S\underline{C}H_3$), 40.05 (t, $\underline{C}H_2CH_2$) and 62.75 (t, $O\underline{C}H_2$); *m/z* 289 (M^+ + 1) 193 and 69 (100%); (Found: C, 37.53; H, 6.91; S, 22.14. $C_9H_{20}O_6S_2$ requires C, 37.50; H, 6.94; S, 22.22%).

2,4-Dimethylpentane-1,5-diyl dimethanesulphonate (126)

Using 2,4-dimethylpentane-1,5-diol (112), 2,4-dimethylpentane-1,5-diyl dimethanesulponate (126) was obtained as a white crystalline solid (59%); υ_{max} (KBr disc) 2940, 1350 and 1175cm^{-1}; δ_H (200 MHz, $CDCl_3$) 0.80-1.10 (6H, m), 1.60-2.00 (4H, m), 2.95 (6H, s) and 4.00 (4H, d); δ_C ($CDCl_3$) mixture of diastereoisomers: major isomer 15.75 (q, $C\underline{C}H_3$), 30.13 (d, $\underline{C}HCH_3$), 35.68 (t, $\underline{C}H_2CH$), 37.07 (q, $S\underline{C}H_3$) and 74.56 (t, $\underline{C}H_2O$); minor isomer 17.02 (q, $C\underline{C}H_3$), 30.28 (d, $\underline{C}HCH_3$), 36.06 (t, $\underline{C}H_2CH$), 37.07 (q, $S\underline{C}H_3$) and 73.64 (t, $\underline{C}H_2$); *m/z* 179 and 83 (100%); (Found: C, 37.35; H, 6.94; S, 22.39. $C_9H_{20}O_6S_2$ requires C, 37.50; H, 6.94; S, 22.22%).

2,2-Dimethylpentane-1,5-diyl dimethanesulphonate (127)

Using 2,2-dimethylpentane-1,5-diol (114), 2,2-dimethylpentane-1,5-diyl dimethanesulponate (127) was obtained as a viscous oil (62%); υ_{max} (KBr disc) 2940, 1370 and 1175cm^{-1}; δ_H ($CDCl_3$) 1.00 (6H, s), 1.20-1.90 (4H, m), 3.00 (6H, s), 3.88 (2H, s) and 4.20 (2H, t).

3-Hydroxy-3-methylpentane-1,5-diyl 1,5-dimethanesulphonate (128)

Using 3-methylpentane-1,3,5-triol (116), 3-hydroxy-3-methylpentane-1,5-diyl 1,5-dimethanesulphonate (128) was obtained as a brown crystalline solid (48%); m.p. 90-95 ^{o}C; υ_{max} (KBr disc) 3600-3300, 2920, 1350 and 1170cm^{-1}; δ_H ($CDCl_3$) 1.25 (3H, m), 1.70-2.00 (4H, m), 3.00 (6H, s) and 4.20 (4H, m); *m/z* 205, 95, 79 and 15 (100%).

General Procedure (D) for Converting Dimethanesulphonates into Diazides

The dimethanesulphonate (1.81 mmol) in distilled DMSO (2 ml) was added to a solution of NaN_3 (0.284 g, 4.37 mmol) in distilled DMSO (8 ml) under a dry nitrogen atmosphere. The mixture was then stirred at room temperature and monitored by t.l.c. 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 layers was repeated to increase the yield.

Note: Due to the potentially explosive nature of the diazide produced, no mass spectrum could be produced.

1,5-Diazidopentane (129)

Using pentane-1,5-diyl dimethanesulphonate (122) and after stirring for 1 d, 1,5-diazidopentane (129) was obtained as a yellow oil (71%); Rf = 0.78 (chloroform); υ_{max} (thin film) 2940, 2840, 2100 (s) and 1375 cm^{-1}; δ_H ($CDCl_3$) 1.55 (6H, m) and 3.25 (4H, t).

1,5-Diazido-3-methylpentane (130)

Using 3-methylpentane-1,5-diyl dimethanesulphonate (124) and stirring for 1 d, 1,5-diazido-3-methylpentane (130) was obtained as a yellow oil (72%); Rf = 0.79 (chloroform); υ_{max} (thin film) 2940, 2100 (s), 1460 and 1275 cm^{-1}; δ_H ($CDCl_3$) 0.95 (3H, s), 1.20-1.90 (5H, complex) and 3.30 (4H, t).

1,5-Diazido-3,3-dimethylpentane (131)

Using 3,3-dimethylpentane-1,5-diyl dimethanesulphonate (125) and stirring for 1 d, 1,5-diazido-3,3-dimethylpentane (131) was obtained as a yellow oil (68%); Rf = 0.79 (chloroform); υ_{max} (thin film) 2960, 2870, 2100 (s), 1475 and 1275 cm^{-1}; δ_H ($CDCl_3$) 0.90 (6H, s), 1.55 (4H, m) and 3.27 (4H, t);

1,5-diazido-2,4-dimethylpentane (132)

Using 2,4-dimethylpentane-1,5-diyl dimethanesulphonate (126) and stirring for 4 d, 1,5-diazido-2,4-dimethylpentane (132) was obtained as a yellow oil (63%); Rf = 0.65 (chloroform); υ_{max} (thin film) 2960, 2945, 2870, 2100 (s), 1460, 1370 and 1275 cm^{-1}; δ_H ($CDCl_3$) 0.09-1.10 (6H, m), 1.00-1.48 (2H, m), 1.50-2.00 (2H, m) and 3.15 (4H, d).

5-Azido-2,2-dimethyl-pentan-1-yl methanesulphonate (133)

Using 2,2-dimethylpentane-1,5-diyl dimethanesulphonate (127) and stirring for 7 d, 5-azido-2,2-dimethyl-pentan-1-yl methane-sulphonate (133) was obtained as a yellow oil (48%); υ_{max} (thin film) 2960, 2880, 2100 (s), 1475, 1350 and 1180 cm^{-1}; δ_H ($CDCl_3$) 0.95 (6H, s), 1.20-1.70 (4H, complex), 3.00 (3H, s), 3.25 (2H, t) and 3.90 (2H, s).

1,5-Diazido-3-methylpentan-3-ol (134)

Using 3-hydroxy-3-methylpentane-1,5-diyl 1,5-dimethane-sulphonate (128) and stirring for 3 d, 1,5-diazido-3-methylpentan-3-ol (134) was obtained as a yellow oil (60%); υ_{max} (thin film) 3500-3300, 3200-3100, 2930, 2875, 2100(s), 1460, 1350, 1270 and 1175 cm^{-1}; δ_H ($CDCl_3$) 1.20 (3H, m), 1.50-2.60 (4H, complex) and 3.20-3.50 (4H, complex).

General Procedure (E) for the Reduction of Diazide to Diamine Dihydrochloride

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.* 18 h. The catalyst was filtered through Celite and the Celite was washed thoroughly with absolute alcohol and 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.

Pentane-1,5-diamine (Cadaverine) (7) dihydrochloride

1,5-Diazido-pentane (129) was converted into cadaverine (7) dihydrochloride using general procedure (E). The product was obtained as a white crystalline solid, (98%); m.p. 265 °C; υ_{max} (KBr disc) 3600-3400, 3300-2800, 2030, 1600 and 1475 cm^{-1}; δ_H (200 MHz, D_2O) 1.35 (2H, m), 1.60 (4H, m) and 2.90 (4H, t); δ_C (D_2O with dioxan as the reference at 67.4 ppm) 23.50 (t, $N(CH_2)_2\underline{C}H_2$), 27.70 (t, $NCH_2\underline{C}H_2$) and 40.04 (t, $N\underline{C}H_2$); *m/z* 103 (M^+ + 1), 86 and 30 (100%); (Found: C, 34.09; H, 9.04; N, 16.07; Cl, 40.41. $C_5H_{16}N_2Cl_2$ requires C, 34.28; H, 9.14; N, 16.00; Cl, 40.51%).

3-Methylcadaverine (57) dihydrochloride

1,5-Diazido-3-methylpentane (130) was converted into 3-methylcadaverine (57) dihydrochloride using general procedure (E). The product was obtained as a white crystalline solid, (97%); m.p. >270 °C (lit.,[71] 267-268 °C); υ_{max} (KBr disc) 3600-3300, 3200-2800, 2500, 2400 and 1475 cm^{-1}; δ_H (200 MHz, D_2O) 0.74 (3H, d), 1.44 (5H, m) and 2.83 (4H, m); δ_C (D_2O with dioxan as the reference at 67.4 ppm) 18.62 (q, $CH\underline{C}H_3$), 28.34 (d, $\underline{C}HCH_3$), 34.16 (t, $\underline{C}H_2CH_2N$) and 38.30 (t, $CH_2\underline{C}H_2N$); *m/z* 112, 70 and 30 (100%).

3,3-Dimethylcadaverine (58) dihydrochloride

1,5-Diazido-3,3-dimethylpentane (131) was converted into 3,3-dimethylcadaverine (57) dihydrochloride using procedure (E). The product was obtained as a white crystalline solid, (96%); m.p. 268 °C (lit.,[71] 247-249 °C); υ_{max} (KBr disc) 3600-3400,

2940, 2520, 2450 and 1510 cm^{-1}; δ_H (200 MHz, D_2O) 0.63 (6H, s), 1.47 (4H, m) and 2.89 (4H, m); δ_C (D_2O with dioxan as the reference at 67.4 ppm) 26.21 (q, $C\underline{C}H_3$), 31.68 (s, $\underline{C}CH_3$), 36.66 (t, $\underline{C}H_2CH_2N$) and 39.11 (t, $\underline{C}H_2N$); (Found: C, 41.24; H, 10.00; N, 13.61; Cl, 35.12. $C_7H_{20}N_2Cl_2$ requires C, 41.38; H, 9.85; N, 13.79; Cl, 34.97%).

2,4-Dimethylcadaverine (60) dihydrochloride

1,5-Diazido-2,4-dimethylpentane (132) was converted into *meso*-2,4-dimethylcadaverine (60) dihydrochloride using general procedure (E). The product was obtained as a white crystalline solid, (80%); m.p. 220-225 °C (lit.,[71] 223-224 °C); υ_{max} (KBr disc) 3500-3300, 3200-2800, 2380, 1495 and 1400 cm^{-1}; δ_H (200 MHz, D_2O) 0.76 (3H, d), 0.80 (3H, d), 0.97-1.20 (2H, complex), 1.65-1.83 (2H, complex) and 2.46-2.85 (4H, complex); δ_C (D_2O with dioxan as the reference at 67.4 ppm) Mixture of isomers: major isomer 16.00 (q, $CH\underline{C}H_3$), 29.08 (d, $\underline{C}HCH_3$), 38.20 (t, $\underline{C}H_2CH_2N$) and 46.30 (t, $\underline{C}H_2N$); minor isomer 17.00 (q, $CH\underline{C}H_3$), 29.03 (d, $\underline{C}HCH_3$), 38.90 (t, $\underline{C}H_2CH_2N$) and 45.40 (t, $\underline{C}H_2N$); *m/z* 131 (M^+ + 1), 113, 98 and 30 (100%).

3-Hydroxy-3-methylcadaverine (59) dihydrochloride

1,5-Diazido-3-methylpentan-3-ol (134) was converted into 3-hydroxy-3-methylcadaverine (59) dihydrochloride using general procedure (E). The product was obtained as a white crystalline solid after recrystallisation, (60%); m.p. 250 °C (lit.,[71] 249 °C); υ_{max} (KBr disc) 3500-3300, 2975, 2560, 2330, 2030, 1450 and 1120 cm^{-1}; δ_H (D_2O) 3.00 (4H, m), 1.70 (4H, m) and 0.90 (3H, s); *m/z* 135, 71 and 30 (100%).

3-Phenylcadaverine (61) dihydrochloride

Step1[97]

Preparation of Hydrazoic Acid

Sodium azide (32.5 g, 0.5 mol) and water (32.5 ml) were mixed into a paste, and benzene (200 ml) was added with stirring, and the whole reaction mixture was cooled to below 10 °C. Conc. sulphuric acid was added dropwise (1 mole of acid to 2 moles sodium azide), and the temperature of the mixture was maintained below 10 °C. The mixture was cooled to 0 °C and the organic layer was decanted and dried (Na_2SO_4), filtered and titrated with 1 M sodium hydroxide solution.

Step2

Reaction of Hydrazoic Acid with the Diol

A solution of hydrazoic acid in benzene (1.1 M, 9.85 ml, 10.84 mmol), was added to 3-phenylpentane-1,5-diol (118) (0.812 g, 4.51 mmol) in anhydrous THF (10 ml). A solution of diisopropylazodicarboxylate (1.98 g, 9.92 mmol) in anhydrous THF (8 ml) was added with stirring. To this mixture was added triphenylphosphine (5.21 g, 19.2 mmol) in anhydrous THF (30 ml). [The reaction temperature, which depends on the rate of addition, must be kept at 40 °C.] The reaction mixture was stirred for 1 h at room temperature, then was heated at 50 °C for 3 h. Water (2 ml) was added and the solution stirred for a further 3 h. The solvents were removed *in vacuo* and the

residue was partitioned between 1 M hydrochloric acid (60 ml) and dichloromethane (60 ml). The aqueous layer was further extracted with dichloromethane (2 x 60 ml). The aqueous layer was evaporated to dryness under reduced pressure to yield 3-phenylcadaverine dihydrochloride which was recrystallised using 95% aqueous ethanol and acetone, (45%); m.p. 238-243 ^{o}C; υ_{max} (KBr disc) 3600-3300, 3100-2900, 2300, 1600 and 1500 cm^{-1}; δ_H (200 MHz, D_2O) 1.80-1.90 (4H, complex), 2.48-2.66 (5H, complex) and 7.10-7.25 (5H, complex); δ_C (D_2O with dioxan as the reference at 67.4 ppm) 34.22 (t, $\underline{C}H_2CH_2N$), 38.59 (t, $\underline{C}H_2N$), 41.12 (d, $\underline{C}HPh$), 128.30 (d, Ar-C), 128.44 (d, Ar-C), 130.00 (d, Ar-C) and 142.54 (d, Ar-C); *m/z* 179 (M^+ + 1), 178 (M^+), 135, 101, 91 and 30 (100%); (Found: C, 51.70; H, 7.77; N, 11.29. $C_{11}H_{20}N_2Cl_2$ requires C, 52.59; H, 7.97; N, 11.16%).

First Attempted Synthesis of 2-Methylputrescine (135) Dihydrochloride[86]

3-Methyladipic diamide dihydrate (136)

3-Methyladipic acid (137) (3.07 g, 19.2 mmol) was disolved in ethanol (100 ml) at -40 ^{o}C with stirring. Dry ammonia (200 ml) was distilled from sodium and bubbled through the reaction mixture over 2 h at -40 ^{o}C. A white precipitate formed immediately. The reaction temperature was allowed to warm to room temperature overnight. The mixture was evaporated to dryness under reduced pressure and the resultant 3-methyladipic diamide dihydrate was obtained as a white crystalline solid which was recrystallised using water, (75%); m.p. 186-189 ^{o}C (Lit.[86] 191 ^{o}C); υ_{max} (KBr disc) 3600-3300,

3100-2800, 2200, 1975, 1560 and 1400 cm^{-1}; δ_H (D_2O) 1.05 (3H, d), 1.50-1.80 (3H, complex) and 1.90-2.50 (4H, complex); *m/z* 143, 142, 114, 100, 72.5, 59 and 55 (100%); (Found: C, 43.30; H, 9.28; N, 14.43. $C_7H_{18}O_4N_2$ requires C, 43.36; H, 9.28; N, 14.54%).

2-Methylputrescine dibenzoyl derivative (138)

Bromine (1.02 g, 6.38 mmol) was added dropwise to a stirring solution of sodium hydroxide (1.42 g, 33.5 mmol) in water (2.8 ml) and ice (4 g). 3-Methyladipic diamide dihydrate (0.5 g, 2.58 mmol) was added cautiously in small portions to the reaction mixture, which was then stirred overnight at 40 °C. The reaction mixture was then cooled, filtered and shaken gently with benzoyl chloride (1.2 g, 8.54 mmol). The resultant solid was filtered and recrystallised from 95% aqueous ethanol, m.p. >230 °C; δ_H ($CDCl_3$) 7.50-8.50 (complex); *m/z* 122, 121 and 105 (100%).

Second Attempted Synthesis of 2-Methylputrescine (135) Dihydrochloride

1,2-Dicyanopropane (139)

A solution of dibromopropane (2 g, 9.9 mmol) in distilled DMSO (10 ml) was added dropwise to a stirred mixture of sodium cyanide (1.08 g, 22 mmol) and distilled DMSO (20 ml) under a dry nitrogen atmosphere at 80 °C. The mixture was stirred at this temperature overnight. The resultant mixture was diluted with dichloromethane (50 ml) and washed with brine (5 x 50 ml). The organic extract was dried (Na_2SO_4), filtered and

evaporated to dryness under reduced pressure to yield 1,2-dicyanopropane (139), (50%); υ_{max} (thin film) 3200, 2980, 2950, 2250 (s), 1630, 1470, 1425 cm^{-1}; δ_H ($CDCl_3$) 1.55 (3H, d), 2.75 (2H, d) and 2.90-3.20 (1H, m); *m/z* 95 (M^+ + 1), 68, 54, 41 (100%) and 28.

2-Methylputrescine (135) dihydrochloride

1,2-Dicyanopropane (139) (0.214 g, 2.28 mmol) was added to a suspension of Adams' catalyst (15% w/w) in distilled acetic anhydride (25 ml). The mixture was hydrogenated at 1 atmosphere for *ca.* 18 h. The mixture was filtered through Florisil and washed with glacial acetic acid (25 ml). Conc. hydrochloric acid was added and the solution was evaporated to dryness to yield 2-methylputrescine, (15%); υ_{max} (KBr disc) 3600-3100 (b), 2360, 2330, 2310, 1635, 1570 and 1400 cm^{-1}; δ_H (D_2O) 0.90 (3H, d), 1.10-1.90 (3H, complex) and 2.98-3.30 (4H, complex); *m/z* 102 (M^+), 86, 72, 58, 43 (100%) and 30.

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

Diamine (2 mmol) was partitioned between dichloromethane (80 ml) and 1 M hydrochloric acid (80 ml). The aqueous layer was further extracted 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 (1) dihydrochloride

Using butane-1,4-diamine, the corresponding dihydrochloride was obtained as a white crystalline solid after recrystallisation, (81%); m.p. >270 °C; υ_{max} (KBr disc) 3400-3300, 3100-2900, 2560, 2040, 1470 and 1450 cm^{-1}; δ_H (D_2O) 1.60-1.90 (2H, complex) and 2.95-3.10 (2H, m); *m/z* 89 (M^+ + 1), 88 (M^+), 72 and 30 (100%); (Found: M^+ 88.0990; C, 29.84; H, 8.89; N, 17.52. $C_4H_{14}N_2Cl_2$ requires M^+ 88.1000; C, 29.81; H, 8.70; N, 17.39%).

2-Methylcadaverine (62) dihydrochloride

Using 2-methylpentane-1,5-diamine, the corresponding dihydrochloride was obtained as a white crystalline solid after recrystallisation, (75%); m.p. 165-167 °C; υ_{max} (KBr disc) 3500-3400, 3100-2900, 2400 and 2040 cm^{-1}; δ_H (D_2O) 1.00 (3H, d), 1.20-2.10 (5H, complex), 2.95 (2H, t) and 3.05 (2H, d); *m/z* 117 (M^+ + 1), 100, 56 and 30 (100%); (Found: C, 38.01; H, 9.49; N, 14.73. $C_6H_{18}N_2Cl_2$ requires C, 38.09; H, 9.52; N, 14.81%).

Ethane-1,2-diamine (64) dihydrochloride

Using ethane-1,2-diamine, the corresponding dihydrochloride was obtained as a white crystalline solid, (70%); υ_{max} (KBr disc) 3500-3200, 2920, 2680, 2500, 1510 and 1035 cm^{-1}; δ_H (D_2O) 3.30 (4H, m); *m/z* 61 (M^+ + 1) and 30 (100%); (Found: C, 18.27; H, 7.62; N, 21.14. $C_2H_{10}N_2Cl_2$ requires C, 18.04; H, 7.52; N, 21.05%).

Propane-1,3-diamine (65) dihydrochloride

Using propane-1,3-diamine, the corresponding dihydrochloride was obtained as a white crystalline solid, (70%); υ_{max} (KBr disc)

3600-3400, 3000, 2695, 2500, 2420, 1480 and 1465 cm^{-1}; δ_H (D_2O) 1.90-2.20 (2H, complex) and 2.95-3.20 (4H, complex); *m/z* 75, 57, 44 and 30 (100%); (Found: C, 24.67; H, 7.94; N, 18.96. $C_3H_{12}N_2Cl_2$ requires C, 24.49; H, 8.16; N, 19.05%).

Hexane-1,6-diamine (66) dihydrochloride

Using hexane-1,6-diamine, the corresponding dihydrochloride was obtained as a white crystalline solid, (75%); υ_{max} (KBr disc) 3500-3300, 2940, 2500, 2430, 2040, 1500 and 1480 cm^{-1}; δ_H (D_2O) 1.32-1.92 (8H, complex) and 3.05 (4H, t); *m/z* 117 (M^+ + 1), 101, 87, 56 and 30 (100%); (Found: C, 37.96; H, 9.68; N, 14.70. $C_6H_{18}N_2Cl_2$ requires C, 38.09; H, 9.52; N, 14.81%).

Heptane-1,7-diamine (67) dihydrochloride

Using heptane-1,7-diamine, the corresponding dihydrochloride was obtained as a white crystalline solid, (75%); υ_{max} (KBr disc) 3500-3300, 2940, 2500, 2000, 1500 and 1470 cm^{-1}; δ_H (D_2O) 1.50-1.80 (6H, complex), 1.82-2.00 (4H, complex) and 3.20 (4H, t); *m/z* 131 (M^+ + 1), 115, 56 and 30 (100%); (Found: C, 41.24; H, 10.18; N, 13.83. $C_7H_{20}N_2Cl_2$ requires C, 41.37; H, 9.85; N, 13.79%).

Octane-1,8-diamine (68) dihydrochloride

Using octane-1,8-diamine, the corresponding dihydrochloride was obtained as a white crystalline solid, (75%); υ_{max} (KBr disc) 3500-3300, 2930, 2330, 2050, 1505 and 1480 cm^{-1}; δ_H (D_2O) 1.40-1.60 (8H, m), 1.60-1.90 (4H, m) and 3.05 (4H, t); *m/z* 145 (M^+ + 1), 115, 72, 44 and 30 (100%); (Found: C, 44.12; H, 10.36; N, 12.91. $C_8H_{22}N_2Cl_2$ requires C, 44.24; H, 10.14; N, 12.90%).

Nonane-1,9-diamine (69) dihydrochloride

Using nonane-1,9-diamine, the corresponding dihdrochloride was obtained as a white crystalline solid, (70%); υ_{max} (KBr disc) 3500-3300, 2930, 2860, 2050, 1595 and 1465 cm^{-1}; δ_H (D_2O) 1.40-1.60 (10H, m), 1.60-1.90 (4H, m) and 3.10 (4H, t); *m/z* 160, 129, 86, 44 and 30 (100%); (Found: C, 45.40; H, 10.45; N, 11.70. $C_9H_{24}N_2Cl_2$ requires C, 46.75; H, 10.39; N, 12.12%).

Decane-1,10-diamine (70) dihydrochloride

Using decane-1,10-diamine, the corresponding dihydrochloride was obtained as a white crystalline solid, (70%); υ_{max} (KBr disc) 3500-3300, 2920, 2850, 2500, 2320, 2050 and 1510 cm^{-1}; δ_H (D_2O) 1.37-1.58 (10H, complex), 1.60-2.10 (6H, complex) and 3.10 (4H, t); *m/z* 173 (M^+ + 1), 156, 44 and 30 (100%); (Found: C, 49.00; H, 10.67; N, 11.36. $C_{10}H_{26}N_2Cl_2$ requires C, 48.98; H, 10.61; N, 11.43%).

Dodecane-1,12-diamine (71) dihydrochloride

Using dodecane-1,12-diamine, the corresponding dihydrochloride was obtained as a white crystalline solid, (70%); υ_{max} (KBr disc) 3500-3300, 2910, 2850, 2500, 2320, 2050, 1510 and 1470 cm^{-1}; δ_H (D_2O) 1.10-1.35 (16H, m), 1.40-1.70 (4H, m) and 2.95 (4H, t); *m/z* 201 (M^+ + 1), 185, 100 and 30 (100%); (Found: C, 52.77; H, 11.07; N, 10.22. $C_{12}H_{30}N_2Cl_2$ requires C, 52.75; H, 10.99; N, 10.26%).

Tetramethylene Triamine (86) Trihydrochloride

By employing the general procedure described for the conversion of diamines into dihydrochlorides but using 2 M

hydrochloric acid, tetramethylene triamine was converted into the corresponding trihydrochloride. This was obtained as a white crystalline solid, (75%); υ_{max} (KBr disc) 3600-3350, 3250, 3000, 2910, 2690, 2590, 2540, 1560 and 1490 cm^{-1}; δ_H (200 MHz, D_2O) 3.14-3.41 (8H, complex); δ_C (D_2O with dioxan as the reference at 67.4 ppm) 37.70 (t, $\underline{C}H_2N$) and 45.81 (t, $CH_2N\underline{C}H_2CH_2N$); *m/z* 104 ($M^+$ + 1), 73, 44 (100%) and 30.

1,5-bis*(*N*-Piperidyl)pentan-3-one (77) dihydrochloride* [103]

Acetone (0.90 g, 15.51 mmol) was added to a solution of piperidine dihydrochloride (3.63 g, 29.88 mmol), para-formaldehyde (0.90 g, 29.88 mmol) and glacial acetic acid (6 ml) at 110 °C. The solution was heated at reflux for 2.5 h. The solvent was removed under reduced pressure. The resultant solid was triturated with hot acetone and then with hot chloroform. 1,5-*bis*(*N*-piperidyl)pentan-3-one (77) dihydrochloride was obtained as a white crystalline solid which was recrystallised using methanol, (15%); m.p. 218-220 °C (lit.,[103] 212-214 °C); υ_{max} (KBr disc) 3600-3200, 2650, 2525, 1730, 1500 and 1400 cm^{-1}; δ_H (200 MHz, D_2O) 1.15-1.80 (12H, complex), 2.74 (4H, m), 2.92 (4H, m), 3.16 (4H, m) and 3.29 (4H, m); δ_C (D_2O with dioxan as the reference at 67.4 ppm) 21.80 (t, $\underline{C}H_2(CH_2)_2N$), 23.56 (t, $\underline{C}H_2CH_2N$), 37.11 (t, $\underline{C}H_2CO$), 51.76 (t, $\underline{C}H_2N$) and 54.37 (t, $N\underline{C}H_2CH_2CO$); *m/z* 168, 167, 98 (100%) and 84.

1,6-bis*(*N*-Piperidyl)hexane (78) dihydrobromide* [104]

Piperidine (2.92 g, 34.8 mmol) was added to water (30 ml). To this solution 1,6-dibromohexane (84) (2.12 g, 8.7 mmol) was

added with stirring. The mixture was heated at reflux and monitored by t.l.c. When the reaction was complete (*ca.* 6 h) the mixture was evaporated to dryness under reduced pressure to yield 1,6-*bis*(*N*-piperidyl)hexane *(78)* dihydrobromide as a white crystalline solid. The product was recrystallised using methanol, (55%); m.p. 281-283 ^{o}C; υ_{max} (KBr disc) 3600-3300, 2960, 2940, 2640, 2540 and 1450 cm^{-1}; δ_H (200 MHz, D_2O) 1.10-1.85 (20H, complex), 2.70 (4H, m), 2.86 (4H, m) and 3.26 (4H, m); δ_C (D_2O with dioxan as the reference at 67.4 ppm) 21.99 (t, C-9, 10), 23.65 (t, C-4, 16), 24.04 (t, C-8, 12), 26.15 (t, C-3, 5, 15, 17), 53.65 (t, C-7, 12) and 57.55 (t, C-2, 6, 14, 18); *m/z* 252 (M^+), 168, 154, 98 (100%) and 84.

Attempted Synthesis of 1,5-*bis*(*N*-Piperidyl)pentane (80) dihydrobromide

Method 1[104]

Piperidine (2.95 g, 34.8 mmol) was added to water (30 ml). To this solution 1,5-dibromopentane (82) (2 0 g, 8.7 mmol) was added with stirring. The reaction was monitored by t.l.c. and when it was complete (*ca.* 7 h) the mixture was evaporated to dryness to yield a mixture of products.

Method 2

A solution of the 1,5-dibromopentane (82) (0.07 g, 3 mmol) in dry *n*-butanol (4 ml) was added to a stirred mixture of piperidine (0.51 g, 7.2 mmol), anhydrous sodium carbonate (0.76 g, 7.2 mmol) and potassium iodide (0.83 g, 0.5 mmol),

and the mixture was stirred for *ca.* 18 h at 90 °C. The solution was cooled and the solid was filtered and washed with diethyl ether (50 ml). The washings and filtrate were combined and extracted with 4 M hydrochloric acid (3 x 50 ml). The aqueous layers were washed with diethyl ether (2 x 100 ml), basified with sodium carbonate and extracted with diethyl ether (3 x 100 ml). The ethereal extracts were dried (Na_2SO_4), filtered and evaporated to dryness to yield a complex mixture of products in poor yield.

7.3 Experimental to Chapter 5 and 6

Synthesis of 3,4-Dimethoxybenzoylacetic acid (50)[15,113]

3,4-Dimethoxybenzoyl chloride (88)

3,4-Dimethoxybenzoic acid (87) (5 g, 0.027 mol) in thionyl chloride (5 ml) was heated at reflux overnight. The excess thionyl chloride was removed by concentration under reduced pressure leaving the acid chloride in quantitative yield. The acid chloride was used immediately in the next stage. (The distilled thionyl chloride was quenched with $NaHCO_3$.) m.p. 73-75 °C; υ_{max} (KBr disc) 3080, 2980, 1760, 1590, 1510, 1270, 1135 and 1015 cm^{-1}; δ_H ($CDCl_3$) 3.90 (3H, s), 6.90 (1H, d), 7.52 (1H, br s) and 7.82 (1H, d, with fine splitting).

Ethyl 3,4-dimethoxybenzoylacetoacetate (89)

To a suspension of ethyl acetoacetate (10.66 g, 0.0815 mol) and sodium (1.64 g, 0.0713 mol) in anhydrous THF (75 ml) under a nitrogen atmosphere, the acid chloride (88) (7.997 g, 0.0272 mol) in anhydrous THF (40 ml) was added dropwise with good mixing. The solution was heated at reflux for 5 h and left to stand overnight. The precipitate was filtered and washed with diethyl ether. The sodium salts were dissolved in cold water (1.5 l) and decomposed with 10% hydrochloric acid. The diketo ester separated as an oil which was extracted with diethyl ether (5 x 250 ml). The combined organic extracts were dried (Na_2SO_4), filtered, and evaporated to dryness under reduced pressure to yield the product (89) (80%); m.p. 79-81 °C; υ_{max} (KBr disc) 2980, 1720, 1710, 1680, 1595, 1525, 1460, 1280

and 1130 cm^{-1}; δ_H ($CDCl_3$) 1.15 (3H, t), 2.20 (3H, s), 3.80 (6H, s), 4.15 (2H, q), 5.20 (1H, s), 6.70 (1H, d), 7.35 (1H, br s) and 7.40 (1H, d, with fine splitting); *m/z* 295 (M^+ + 1), 294 (M^+), 252, 221 and 165 (100%).

Ethyl 3,4-dimethoxybenzoylacetate (90)

Ethyl 3,4-dimethoxybenzoylacetoacetate (89) (6.89 g, 0.023 mol) was added to aqueous ethanol (90%, 30 ml). To this mixture sodium acetate (96 mg, 1.17 mmol) was added and the mixture was heated at 100 °C for *ca.* 20 h. After a long period of cooling the product crystallised (82%); m.p. 44-45 °C; υ_{max} (KBr disc) 2940, 2840, 1735, 1675, 1595, 1510, 1420, 1275, 1150 and 1025 cm^{-1}; δ_H ($CDCl_3$) 1.25 (3H, t), 3.90 (6H, s), 4.18 (2H, q), 6.90 (1H, d), 7.50 (1H, br s) and 7.58 (1H, d, with fine splitting); *m/z* 253 (M^+ + 1), 252 (M^+), 165 (100%), 137 and 87; (Found: M^+ 252.0997; C, 61.90; H, 6.43. $C_{13}H_{16}O_5$ requires M^+ 252.0998; C, 61.90; H, 6.35%).

3,4-Dimethoxybenzoylacetic acid (50)

This stage was not carried out until 2 d before the acid was required for the final coupling stage. The β-keto acid decarboxylates readily.

A solution of the ester (90) (2.02 g, 8.0 mmol) in 2.5% potassium hydroxide solution (140 ml) was stirred for 48 h at room temperature. The solution was then washed with diethyl ether (3 x 40 ml), acidified at 10 °C with 2 M sulphuric acid and extracted with diethyl ether (5 x 50 ml). The combined organic extracts were dried (Na_2SO_4), filtered, and evaporated to

dryness under reduced pressure at room temperature to yield 3,4-dimethoxybenzoylacetic acid (50) which was stored <0 °C (75%); m.p. 30 °C (decarb.); υ_{max} (KBr disc) 3200-2800, 1740, 1675, 1590, 1515, 1420, 1270, 1150 and 1025 cm^{-1}; δ_H ($CDCl_3$) 3.92 (3H, s), 3.95 (3H, s), 4.02 (2H, s), 6.95 (1H, d), 7.52 (1H, br s) and 7.65 (1H, d, with fine splitting); *m/z* 181, 180, 137, 165 (100%) and 43.

General Procedure for Oxidative Deamination of Diamines using Diamine Oxidase and Subsquent Coupling with 3,4-Dimethoxybenzoylacetic acid

A solution of 3,4-dimethoxybenzoylacetic acid (50) (0.44 g, 1.96 mmol), diamine dihydrochloride (0.1 M aqueous solution, 20 ml) and 0.02 M sodium phosphate buffer (pH 7, 7 ml) was prepared. The pH was adjusted to 7 if necessary. Catalase (0.2 mg) and pea seedling DAO (300 µl, *ca.* 200 mg, enzyme activity 1200 units per mg) were added. The solution was incubated on a shaker at 25 °C for 24 h. The pH was monitored throughout the reaction and did not rise above pH 7. The solution was acidified with dilute sulphuric acid and extracted with diethyl ether (4 x 30 ml). The aqueous solution was basified with conc. ammonia and extracted with chloroform (3 x 30 ml). The combined organic extracts were dried (Na_2SO_4), filtered and evaporated to dryness under reduced pressure to yield the product.

3',4'-Dimethoxy-2'-(2-piperidyl)acetophenone (49) [15]

Using cadaverine (7) dihydrochloride as the substrate, 3',4'-dimethoxy-2'-(2-piperidyl)acetophenone (49) was obtained as a yellow solid after crystallisation from acetone (52%); m.p. 81-82 °C; (lit.,[15] 79-80.5 °C); υ_{max} (KBr disc) 3340, 3080, 2940, 2790, 1670, 1600, 1585, 1515, 1450, 1260, 1160 and 1025 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 1.00-1.90 (6H, complex), 2.50-2.70 (2H, complex), 2.70-3.10 (3H, complex), 3.95 (6H, s), 6.90 (1H, d), 7.50 (1H, s) and 7.60 (1H, d, with fine splitting); δ_C($CDCl_3$) 24.54 (t, $\underline{C}H_2(CH_2)_2N$), 25.75 (t, $\underline{C}H_2CH_2N$), 32.50 (t, $\underline{C}H_2(CH_2)_2CO$), 44.86 (t, $\underline{C}H_2CO$), 46.69 (t, $\underline{C}H_2N$), 53.02 (d, $\underline{C}HN$), 55.78 (q, $\underline{C}H_3O$), 55.90 (q, $\underline{C}H_3O$), 109.62 (d, Ar-C), 122.75 (d, Ar-C), 130.16 (s, Ar-C), 148.83 (s, Ar-C), 153.23 (s, Ar-C) and 197.69 (s, $\underline{C}O$); *m/z* 264 (M^+ + 1), 263 (M^+), 180, 165, 137, 98 and 55 (100%); (Found: M^+ 263.1521. $C_{15}H_{21}NO_3$ requires M^+ 263.1521).

3',4'-Dimethoxy-2'-(2-pyrrolidyl)acetophenone (91)

Using putrescine (1) dihydrochloride as the substrate, 3',4'-dimethoxy-2'-(2-pyrrolidyl)acetophenone (91) was obtained as a yellow oil (60%); υ_{max} (thin film) 3600-3200, 2920, 2850, 2360, 2340, 1670, 1640, 1600, 1515, 1215 and 1050 cm^{-1}; δ_H ($CDCl_3$) 1.30-1.50 (4H, complex), 2.20 (1H, br s), 3.30 (5H, complex), 3.95 (6H, s), 6.90 (1H, d), 7.52 (1H, s) and 7.60 (1H, d, with fine splitting); *m/z* 250 (M^+ + 1), 249 (M^+), 180, 165, 137, 84 and 28 (100%); (Found: M^+ 249.1355. $C_{14}H_{19}O_3N$ requires M^+ 249.1365).

3',4'-Dimethoxy-2'-[2-(4-methyl)-piperidyl]acetophenone(92)

Using 3-methylcadaverine (57) dihydrochloride as the substrate, 3',4'-dimethoxy-2'-[2-(4-methyl)-piperidyl]acetophenone (92) was obtained as a yellow oil (55%); HPLC (reverse phase column) 1.51 (85.6%) and 4.42 (8.7%) min; υ_{max} (thin film) 3600-3300, 2950, 2930, 2560, 2450, 2040, 1670, 1595, 1515, 1270 and 1020 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 0.81 (3H, d), 0.90-1.30 (2H, complex), 1.50-1.90 (4H, complex), 2.82-3.62 (5H, complex), 3.72 (3H, s), 3.75 (3H, s), 6.92 (1H, d), 7.33 (1H, s) and 7.52 (1H, d, with fine splitting); δ_C ($CDCl_3$) 21.78 (q, $\underline{C}H_3CH$), 29.87 (d, $\underline{C}HCH_3$), 31.06 (t, $\underline{C}H_2CH_2N$), 37.60 (t, $\underline{C}H_2CHCH_2CO$), 41.92 (d, $\underline{C}H_2CO$), 46.12 (t, $\underline{C}H_2N$), 54.39 (d, $\underline{C}HN$), 56.86 (q, $\underline{C}H_3O$), 57.05 (q, $\underline{C}H_3O$), 111.44 (d, Ar-C), 112.06 (d, Ar-C), 125.29 (d, Ar-C), 129.93 (d, Ar-C), 149.28 (d, Ar-C), 154.78 (d, Ar-C) and 199.65 (s, $\underline{C}O$); *m/z* 277 (M^+), 180, 165, 112, 98 (100%), 42 and 28; (Found: M^+ 277.1672. $C_{16}H_{23}NO_3$ requires 277.1678).

3',4-Dimethoxy-2'-[2-(5-methyl)-piperidyl]acetophenone (95)

Using 2-methylcadaverine (62) dihydrochloride as the substrate, 3',4'-dimethoxy-2'-[2-(5-methyl)-piperidyl]acetophenone (95) was obtained as a yellow oil (60%); HPLC (reverse phase column) 1.40 (25.42%) and 4.51 (66.20%) min; υ_{max} (thin film) 3350-3300, 3050, 2960, 2940, 2260, 1640, 1600, 1515, 1420, 1270 and 1060 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 0.79 (9/4H, d), 0.86 (1/4H, d), 1.10-1.78 (5H, complex), 2.27 (2H, m), 2.32-2.80 (3H, complex), 3.87 (3H, s), 3.88 (3H, s), 6.81 (1H, d, with fine splitting), 7.45 (1H, complex) and 7.50 (1H, complex); δ_C ($CDCl_3$) major product: 19.39 (q, $CH\underline{C}H_3$), 31.30 (d, $\underline{C}HCH_3$), 32.49 (t,

$\underline{C}H_2CHCH_3$), 33.33 (t, $\underline{C}H_2CHN$), 44.64 (t, $\underline{C}H_2CO$), 52.68 (d, $\underline{C}HN$), 54.25 (t, $\underline{C}H_2N$), 55.79 (q, $O\underline{C}H_3$), 55.92 (q, $O\underline{C}H_3$), 109.81 (d, Ar-C), 122.76 (d, Ar-C), 130.13 (d, Ar-C), 148.82 (d, Ar-C), 153.21 (d, Ar-C) and 179.91 (s, $\underline{C}O$): minor product 18.80, 26.17, 28.41, 29.55, 33.89, 35.79, 52.00; *m/z* 278(M^+ + 1), 277 (M^+), 180, 165, 111 and 98 (100%); (Found: M^+ 277.1684. $C_{16}H_{23}NO_3$ requires 277.1678).

3-Phenylcadaverine (61)

Using 3-phenylcadaverine (61) dihydrochloride as the substrate, no 3',4'-dimethoxy-2'-[2-(4-phenyl)-piperidyl]acetophenone (93) was obtained. The product was identified as 3-phenylcadaverine. υ_{max} (thin film) 3500-3200 (b), 3020, 2930, 2200-2100, 1575, 1490 and 1325 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 1.50-1.90 (5H, complex), 2.48-2.74 (4H, complex) and 7.00-7.30 (5H, complex); δ_C ($CDCl_3$) 39.88 (t, $\underline{C}H_2CH_2N$), 40.35 (t, $\underline{C}H_2N$), 40.92 (d, $\underline{C}HPh$), 126.07 (d, Ar-C), 127.31 (d, Ar-C), 128.31 (d, Ar-C) and 144.55 (d, Ar-C); *m/z* 148, 135, 91, 77 and 30 (100%).

3',4'-Dimethoxy-2'-(2-azacycloheptanyl)acetophenone (99)

Using hexane-1,6-diamine (66) dihydrochloride as the substrate, 3',4'-dimethoxy-2'-(2-azacycloheptanyl)acetophenone (99) was obtained as a yellow oil (50%); υ_{max} (thin film) 2940, 2860, 2340, 2100, 1640, 1600, 1515 and 1270 cm^{-1}; δ_H ($CDCl_3$) 1.20-1.80 (8H, complex), 1.96 (1H, br s), 2.50-3.20 (5H, complex), 3.85 (6H, s), 6.85 (1H, d), 7.48 (1H, br s) and 7.58 (2H, d, with fine splitting); *m/z* 277 (M^+), 180, 165 (100%) and 98.

3',4'-Dimethoxy-2'-[2-(4,4-dimethyl)-piperidyl]acetophenone (100)

Using 3,3-dimethylcadaverine (58) dihydrochloride as the substrate, 3',4'-dimethoxy-2'-[2-(4,4-dimethyl)-piperidyl]-acetophenone (100) was obtained as a yellow solid after crystallisation from acetone (51%); m.p. 56-58 °C; υ_{max} (KBr disc) 3600-3300, 2950, 2770, 2450, 2360, 2330, 1670, 1270, 1170 and 1040 cm^{-1}; δ_H ($CDCl_3$) 1.05 (6H, d), 1.30-2.00 (4H, complex), 3.00-3.20 (2H, complex), 3.30-3.80 (3H, complex), 3.89 (3H, s), 3.90 (3H, s), 6.75 (1H, d), 7.45 (1H, br. s), 7.56 (1H, d, with fine splitting) and 9.10 (1H, br s); *m/z* 291 (M^+), 180, 165, 137, 112 (100%) and 56; (Found: M^+ 291.1823. $C_{17}H_{25}O_3N$ requires M^+ 29.1834).

3',4'-Dimethoxy-2'-(2-[3,4-$^2H_4$]-pyrrolidyl)acetophenone (98)

Using [2,3-$^2H_4$]-putrescine dihydrochloride as the substrate, 3',4'-dimethoxy-2'-(2-[3,4-$^2H_4$]-pyrrolidyl)acetophenone (98) was obtained as a yellow oil (52%); υ_{max} (thin film) 3600-3300, 2940, 2850, 2360, 2340, 1670, 1630, 1600, 1510, 1210 and 1050 cm^{-1}; δ_H (600 MHz, $CDCl_3$) 3.38 (2H, br s), 3.46 (1H, m), 3.86 (1H, m), 3.88-3.94 (6H, complex m), 4.10 (1H, t), 6.80 (1H, d), 7.46 (1H, s) and 7.57 (1H, d, with fine splitting); δ_C ($CDCl_3$) 39.87 (t, $\underline{C}H_2CO$), 44..78 (t, $\underline{C}H_2N$), 56.03 (q, $\underline{C}H_3O$), 56.03 (d, $\underline{C}HN$), 109.97 (d, Ar-C), 123.18 (d, Ar-C), 128.99 (d, Ar-C), 148.97 (d, Ar-C), 153.74 (d, Ar-C) and 195.61 (s, $\underline{C}O$); *m/z* 254 (M^+ + 1), 253 (M^+), 252, 251, 180, 165, 137, 88 and 43 (100%); (Found: M^+ 253.1619. $C_{14}H_{15}D_4O_3N$ requires M^+ 253.1616).

Preparation of 1-Pyrroline (17)

Putrescine (1) dihydrochloride (1.8 mmol), a small amount of catalase (0.2 mg) and pea seedling diamine oxidase (300 µl, *ca.* 200 mg, enzyme activity 1200 units per mg) were added to 0.2 M phosphate buffer (20 ml, pH 7). The mixture was incubated at 25 °C for 24 h. Diethyl ether (10 ml) was added and the mixture was basified with potassium carbonate. The aqueous layer was extracted with pre-cooled diethyl ether (3 x 20 ml) and the combined organic extracts were cooled to 0 °C, dried ($MgSO_4$), filtered and used immediately to form the zinc complex.

Note: The solution must be kept below 5 °C to prevent the cyclic imine from trimerising.

Attempted Preparation of Di-iodo Zinc Complex (96)

The ethereal extracts containing the cyclic imine were cooled to 0 °C and the zinc iodide (0.574 g, 1 equiv.) was added with stirring. No precipitate was formed after stirring for 2 h at 0 °C.

The reaction was also carried out under the various conditions shown in the table below;

Reaction Time	Solvent	Temp. °C	pH
24	Buffer	25	7
1	Buffer	25	7
1	Buffer	5	7
1	Buffer/ Ether	5	6

No precipitate was obtained using any of the above conditions.

General Procedure (J) for the formation of cyclic imine trimers

Diamine dihydrochloride (0.525 g, 3 mmol), a small amount of catalase (0.2 mg) and pea seedling diamine oxidase (300 µl, *ca.* 200 mg, enzyme activity 1200 units per mg) were added to 0.2 M phosphate buffer (30 ml, pH 7). The mixture was incubated at 25 °C for 24 h. Ethyl acetate (20 ml) was added and the mixture was basified with potassium carbonate. The aqueous layer was extracted with ethyl acetate (3 x 20 ml), dried ($MgSO_4$), filtered and evaporated to dryness under reduced pressure to yield the product.

α-Tripiperideine (140)

Using general procedure (J) and cadaverine (7) dihydrochloride as the substrate, α-tripiperideine (140) was obtained as a yellow oil, (62 %); υ_{max} (thin film) 2940, 2860, 2500, 1650 and 1445 cm^{-1}; δ_H ($CDCl_3$) 1.10-2.20 (18H, complex), 2.50-2.80 (3H, complex), 3.00-3.30 (3H, complex) and 3.50-3.90 (3H, complex); *m/z* 250 (M^+ + 1), 249 (M^+), 165 and 84 (100%).

Synthesis of Homospermidine and Analogues

General Procedure (G) for *N*-Di-alkylation of Benzylamine (105) using 4-Bromobutanenitrile (104) or 5-Bromo-pentanenitrile (106)[8]

To a mixture of benzylamine (105) (1.37 g, 0.0128 mol), sodium carbonate (4.20 g, 0.0396 mol) and potassium iodide (0.77 g, 0.0046 mol) under a dry nitrogen atmosphere at 90 °C, 4-bromobutanenitrile (104) (3.79 g, 0.0256 mol) or 5-bromopentanenitrile (106) (4.15 g, 0.0256 mol) in anhydrous n-butanol (12 ml) was added dropwise. The mixture was left stirring at this temperature overnight. The solution was cooled to room temperature and the solid was filtered off and washed with diethyl ether. The filtrate and washings were extracted with 4 M hydrochloric acid (2 x 30 ml). The aqueous extracts were washed with diethyl ether (2 x 30 ml), basified with sodium carbonate, and extracted with diethyl ether (3 x 50 ml). The organic extracts were dried (Na_2SO_4), filtered and evaporated to dryness under reduced pressure to yield the product.

N,N-bis*(3-Cyanopropyl)benzylamine (141)*

Using the method described in general procedure (G), benzylamine was *N*-di-alkylated using 4-bromobutanenitrile (104). *N,N-bis*(3-Cyanopropyl)benzylamine (141) was obtained as a clear oil after distillation (62%); b.p. 170 °C at 1 mm Hg; υ_{max} (thin film) 2950, 2820, 2250 (s), 1600 and 1495 cm^{-1}; δ_H ($CDCl_3$) 1.60-2.00 (4H, complex), 2.35 (4H, t), 3.50 (2H, s) and 7.27 (5H, s); *m/z* 242 (M^+ + 1), 241 (M^+), 187, 91 (100%) and 68; (Found: M^+ 241.1590. $C_{15}H_{19}N_3$ requires 241.1579).

N,N-bis*(4-Cyanobutyl)benzylamine (142)*

Using the method described in general procedure (G), benzylamine was *N*-di-alkylated using 5-bromopentanenitrile (106). *N,N-bis*(4-Cyanobutyl)benzylamine (142) was obtained as a clear oil (62%); υ_{max} (thin film) 3040, 3030, 2940, 2250, 1600, 1495, 1130 and 1030 cm^{-1}; δ_H ($CDCl_3$) 1.40-1.90 (8H, complex), 2.10-2.60 (8H, complex), 3.50 (2H, s) and 7.30 (5H, s); *m/z* 270 (M^+ + 1), 269 (M^+), 201 and 91 (100%).

General Procedure (H) for Reduction of Dinitriles to Diamines by Catalytic Hydrogenation[8]

The dinitrile (2.07 mmol) was added to a suspension of Adams' catalyst (15% w/w) in glacial acetic acid (20 ml) and the mixture was hydrogenated at 1 atmosphere for *ca.* 18 h. The catalyst was filtered through Florisil and washed well with glacial acetic acid (30 ml). The filtrate and washings were

evaporated to dryness under reduced pressure to yield the product.

N,N-bis*(4-Aminobutyl)benzylamine (143) triacetate*

N,N bis(3-Cyanopropyl)benzylamine (141) was reduced using general procedure (H). *N,N-bis*(4-Aminobutyl)benzylamine (143) triacetate was obtained as a white crystalline solid (75%); υ_{max} (KBr disc) 3600-3300, 2950, 2360, 2340, 1710 and 1015 cm^{-1}; δ_H (D_2O) 1.80-2.20 (4H, complex), 2.30 (6H, s), 2.70-3.00 (2H, complex), 3.05-3.50 (2H, complex), 4.00 (2H, s) and 7.60 (5H, s); *m/z* 252, 251, 160, 91 (100%) and 72.

N,N-bis*(5-Aminopentyl)benzylamine (144) triacetate*

N,N-bis(4-Cyanobutyl)benzylamine (142) was reduced using general procedure (H). *N,N-bis*(5-Aminopentyl)benzylamine (144) triacetate was obtained as a white crystalline solid (76%); υ_{max} (KBr disc) 3550-3250, 2950, 2620, 2330, 1650 and 1450 cm^{-1}; δ_H (D_2O) 1.15-2.00 (10H, complex), 2.78-3.25 (10H, complex), 4.25 (2H, s) and 7.47 (5H, s); *m/z* 278 (M^+ + 1), 248, 205, 191 and 91 (100%).

General Procedure (I) for the Removal of *N*-Benzyl Groups by Catalytic Transfer Hydrogenation[110]

A mixture of *N*-benzyl derivative (0.72 mmol), ammonium formate (0.216 g, 3.43 mmol), 10% palladium on charcoal (0.50 g) and methanol (30 ml) was heated at reflux. The reaction was monitored by t.l.c. When the cleavage was complete the catalyst was filtered through Celite and washed with methanol.

The filtrate and washings were combined and conc. hydrochloric acid was added. The mixture was then evaporated to dryness under reduced pressure to yield the amine hydrochloride. This was recrystallised using 95% aqueous ethanol and acetone.

Homospermidine (5) trihydrochloride [7]

The *N*-benzyl group was cleaved from *N,N*-bis(4-aminobutyl)benzylamine (143) triacetate using general procedure (I). Homospermidine (5) trihydrochloride was obtained as a white crystalline solid (60%); m.p. >290 °C (lit.,[7] 283-285 °C); υ_{max} (KBr disc) 3600-3300, 2950, 2800 and 2430 cm^{-1}; δ_H (200 MHz, D_2O) 1.30-2.00 (8H, complex) and 2.60-3.30 (8H, complex); δ_C (D_2O with dioxan as the reference at 67.4 ppm) 23.64 (t, $CH_2\underline{C}H_2CH_2$), 24.77 (t, $CH_2\underline{C}H_2CH_2$), 39.64 (t, $\underline{C}H_2N$) and 47.72 (t, $\underline{C}H_2N$); *m/z* 158, 88, 43 and 35 (100%).

N,N-bis*(5-Aminopentyl)amine (10) trihydrochloride*

The *N*-benzyl group of *N,N-bis*(5-aminopentyl)benzylamine (144) triacetate was cleaved using general procedure (I). *N,N-bis*(5-aminopentyl)amine (10) trihydrochloride was obtained as a white crystalline solid (60%); m.p. 279-280 °C; υ_{max} (KBr disc) 3500-3300, 2940, 2800, 2540, 2450, 2400 and 1460 cm^{-1}; δ_H (200 MHz, D_2O) 1.10-1,38 (6H, complex), 1.40-1.65 (6H, complex) and 2.75-2.91 (8H, complex); δ_C (D_2O with dioxan as the reference at 67.4 ppm) 24.06 (t, $\underline{C}H_2(CH_2)_2N$), 26.25 (t, $\underline{C}H_2CH_2N$), 27.40 (t, $\underline{C}H_2CH_2NHCH_2$), 40.31 (t, $\underline{C}H_2NH_2$) and 48.38 (t, $\underline{C}H_2NHCH_2$); *m/z* 188 (M^+ + 1), 187 (M^+), 171, 157, 98

(100%) and 30; (Found: M^+ 187.2061. $C_{10}H_{25}N_3$ requires 187.2048).

1-Hydroxymethylpyrrolizidine (6)

Method 1

Homospermidine (5) trihydrochloride (0.10 g, 0.37 mmol) was incubated at 25 °C with pea seedling diamine oxidase (500 µl, *ca.* 350 mg, enzyme activity 1200 units per mg) and catalase (0.02 mg) in 0.2 M phosphate buffer (2 ml, pH 7). After 2 d, the mixture was basified using 1 M sodium hydroxide (10 ml) and extracted with chloroform (3 x 15 ml). The organic extracts were combined, dried (Na_2SO_4), filtered and evaporated to dryness to yield an oil. The presumed intermediates were reduced immediately with sodium borohydride (0.22 g, 4.70 mmol) in methanol (8 ml) at 0 °C for 2 h. 1 M Hydrochloric acid was added to quench the sodium borohydride. The mixture was then evaporated to dryness under reduced pressure and 1 M sodium hydroxide (15 ml) was added. The aqueous solution was extracted with chloroform (3 x 20 ml). The combined organic extracts were dried (Na_2SO_4), filtered and evaporated to dryness under reduced pressure to yield a complex mixture of products.

Method 2

Homospermidine (5) trihydrochloride (0.10 g, 0.37 mmol) was incubated as described in method 1. After 2 d the mixture was cooled to 0 °C and sodium borohydride (0.70 g, 18.50 mmol)

was added slowly with stirring. The mixture was left stirring at 0 °C for 2 h. 1 M Hydrochloric acid was again added to quench the sodium borohydride and the isolation procedure was the same as method 1. The product (6) was obtained as a yellow oil, (10%); υ_{max}(thin film) 3680, 3010, 2960, 2400, 1620, 1260 and 1220 cm^{-1}; δ_H ($CDCl_3$) 1.00-1.50 (4H, complex), 1.50-2.10 (5H, complex) and 3.00-3.70 (5H, complex); *m/z* 141 (M^+), 110, 83, 58 and 43 (100%). These spectra are consistent with previous data for 1-hydroxymethylpyrolizidine.

Appendix 1

Determination of Protein Concentration

See experimental section for method.

Table 1 (a): Protein Concentration and A_{465} and A_{620} Readings (1)

Protein (µg)	A_{465}	A_{620}	A_{465}/A_{620} - A_{465}/A_{620} (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.)

Determination of Protein Concentration

Table 1 (b): Protein Concentration and A_{465} and A_{620} Readings (2)

Protein (µg)	A_{465}	A_{620}	A_{465}/A_{620} - A_{465}/A_{620} (blank)
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

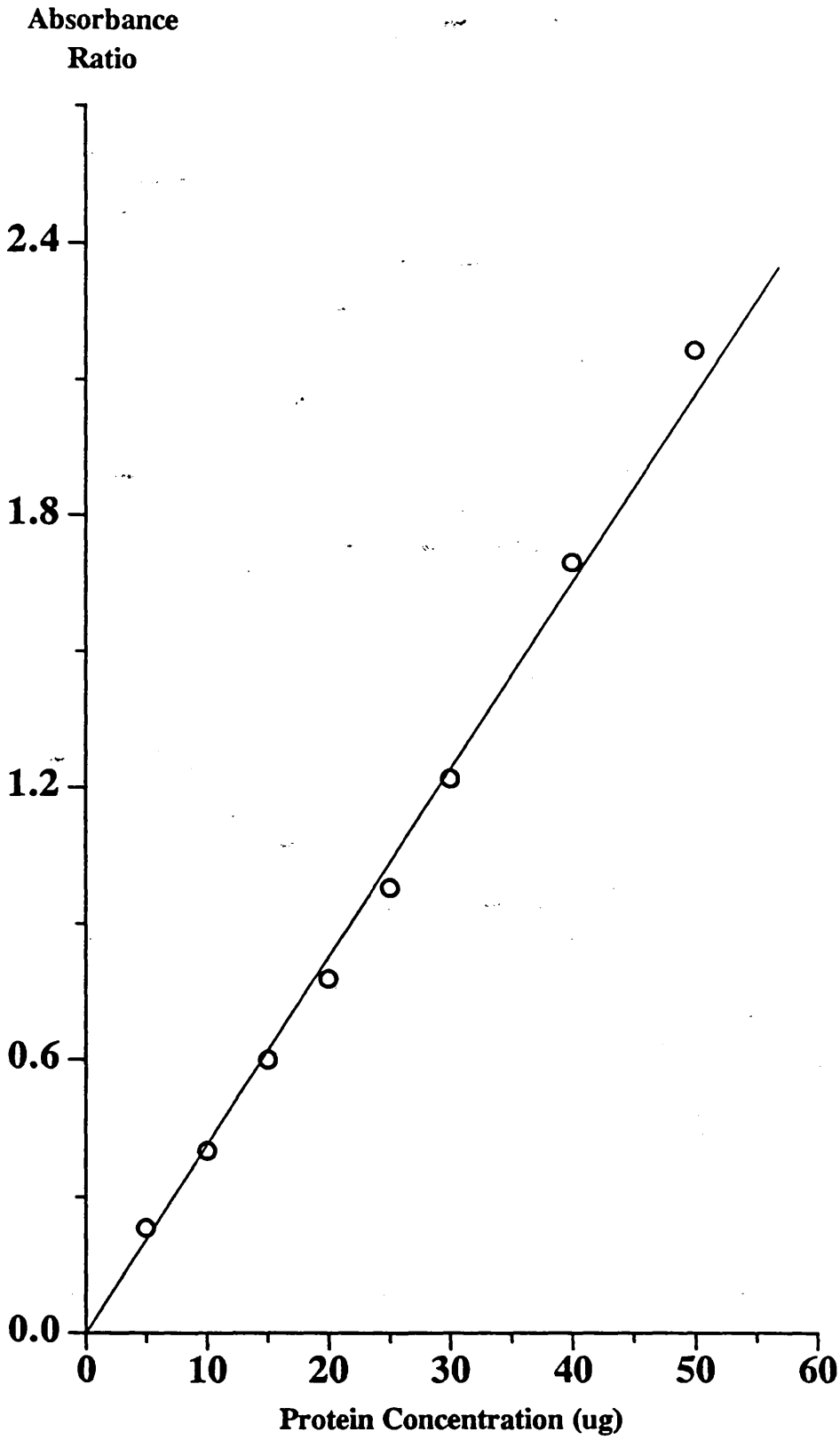
Appendix 1 (contd.)

Determination of Protein Concentraion

Table 1 (c): Average Readings from Table 1 (a) and Table 1 (c)

Protein (µg)	A_{465}/A_{620} - A_{465}/A_{620} (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 **Absorbance Ratio against Protein Concentration (ug)**



Appendix 2

Determination of the Extinction Coefficent for the Assay System

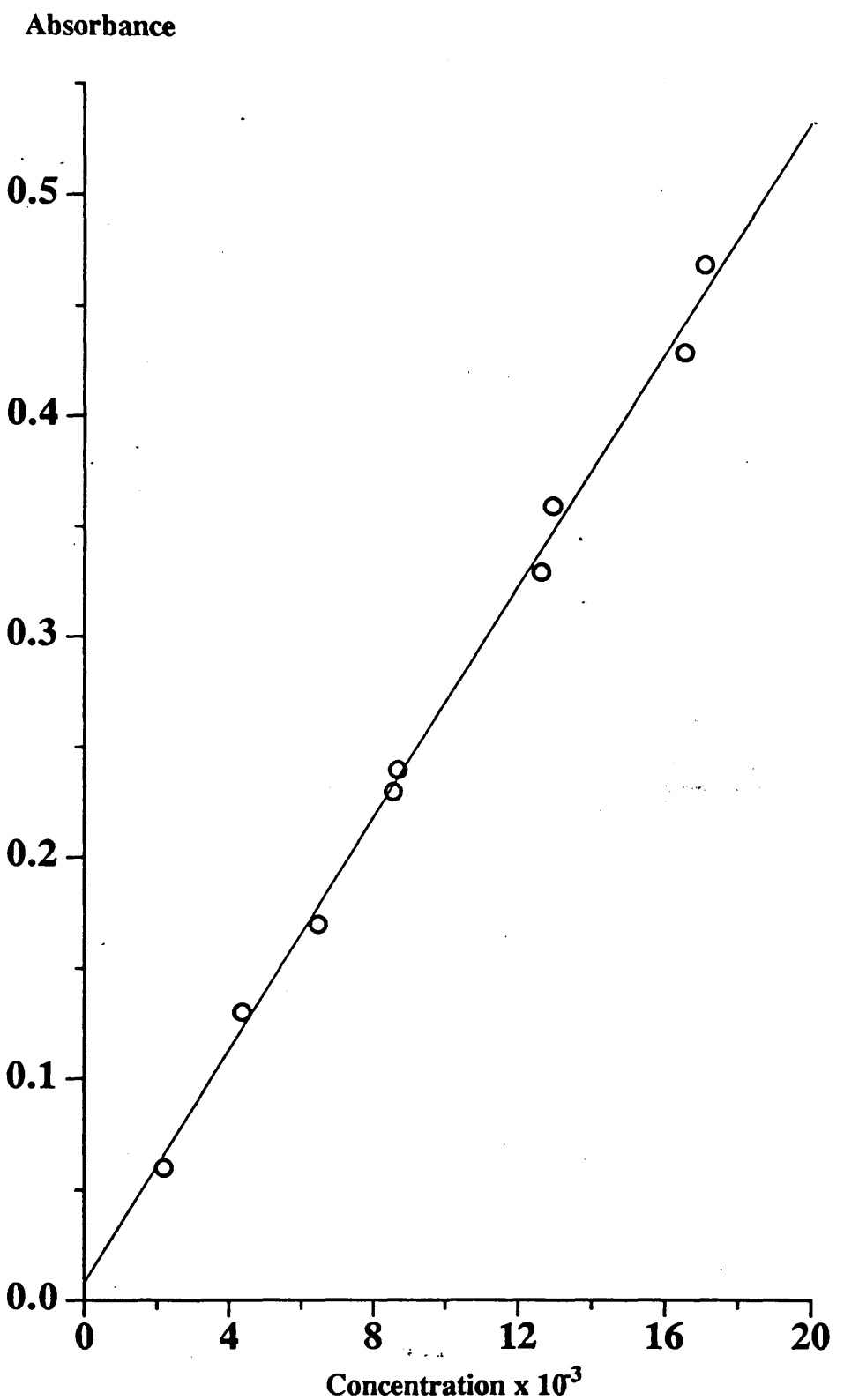
Assay:		
	2500 µl	Phosphate buffer pH 6.3
	170 µl	DMAB
	100 µl	MBTH
	50 µl	Peroxidase
	50 µl	Pea seedling DAO (psDAO)
	300 µl	Putrescine (varying concentration)

Concentration x 10^{-3} mM	Absorbance
2.20	0.06
4.36	0.13
4.39	0.13
6.48	0.17
8.56	0.23
8.70	0.24
12.63	0.33
12.95	0.36
16.56	0.43
17.12	0.47

ε = Alc path length, l = 1

From the slope of Graph 2A $\varepsilon = 2.6335 \times 10^4$.

Graph 2A **Absorbance against Concentration**



Appendix 3

Spectrophotometric Assay

Calculation of Results

For Example: Cadaverine as substrate with pea seedling DAO

Assay:	2500 µl	Phosphate buffer pH 6.3
	170 µl	DMAB
	100 µl	MBTH
	50 µl	Peroxidase
	25 µl	Pea seedling DAO (psDAO)
	300 µl	Cadaverine (varying concentration)

See experimental section for more details of the method.

Appendix 3 (contd.)

Spectrophotometric Assay

Calculation of Results

Table 3 (a): Substrate Concentration and Rate Readings

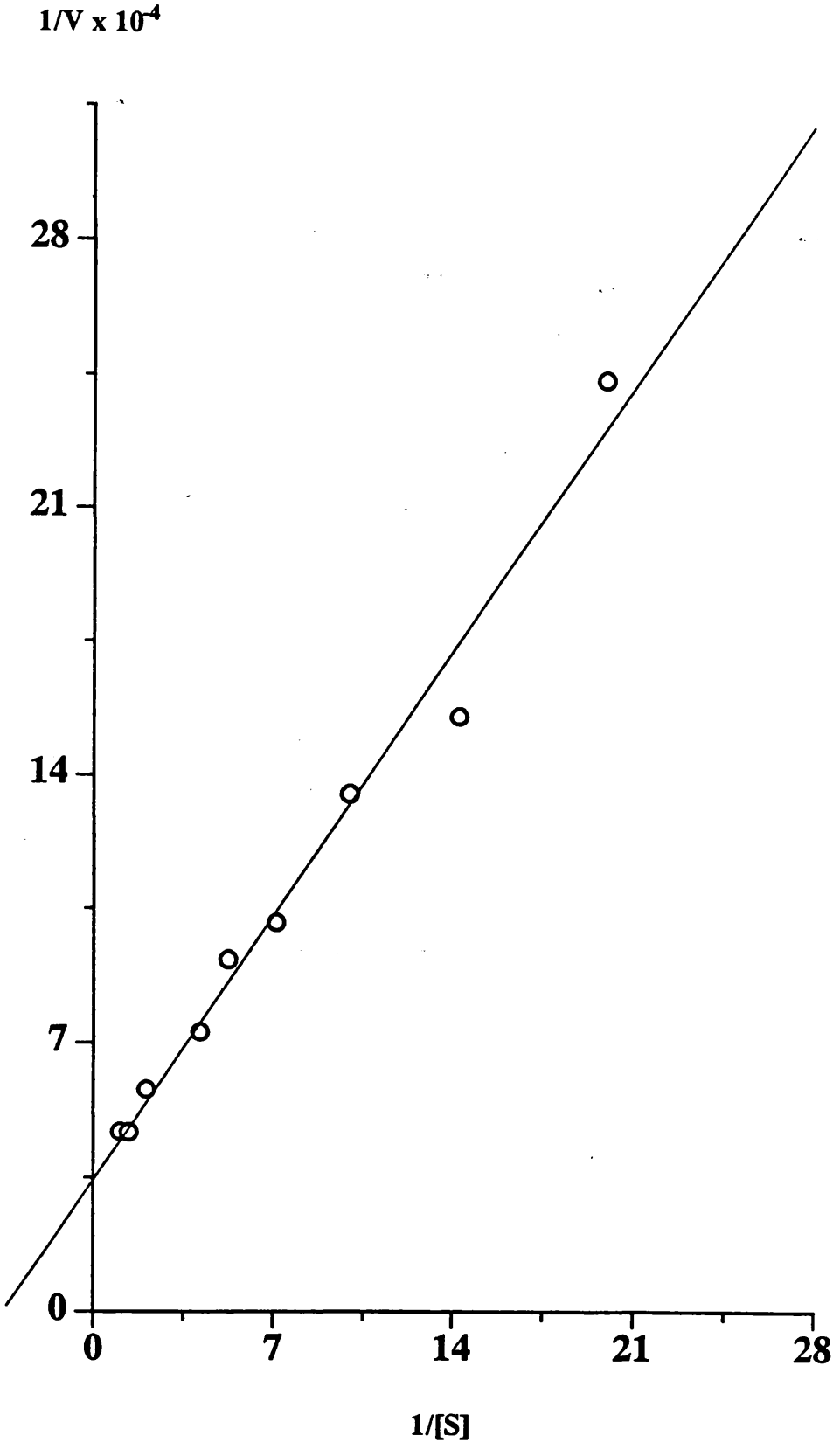
Substrate Concentration (mM)	Rate (abs/sec x 10^{-3})	Rate (µmol/mg/hr)
0.95	9.23	2130
0.72	9.28	2136
0.48	7.50	1726
0.24	5.95	1371
0.19	4.72	1089
0.14	4.28	985
0.10	3.21	740
0.07	2.78	643
0.05	1.78	410

Conversion of Rate from abs/sec to µmol/mg/hr

Using the extinction Coefficient (ε = 2.6335 x 10^4) the units of rate can be converted from abs/sec to µmol/mg/hr.

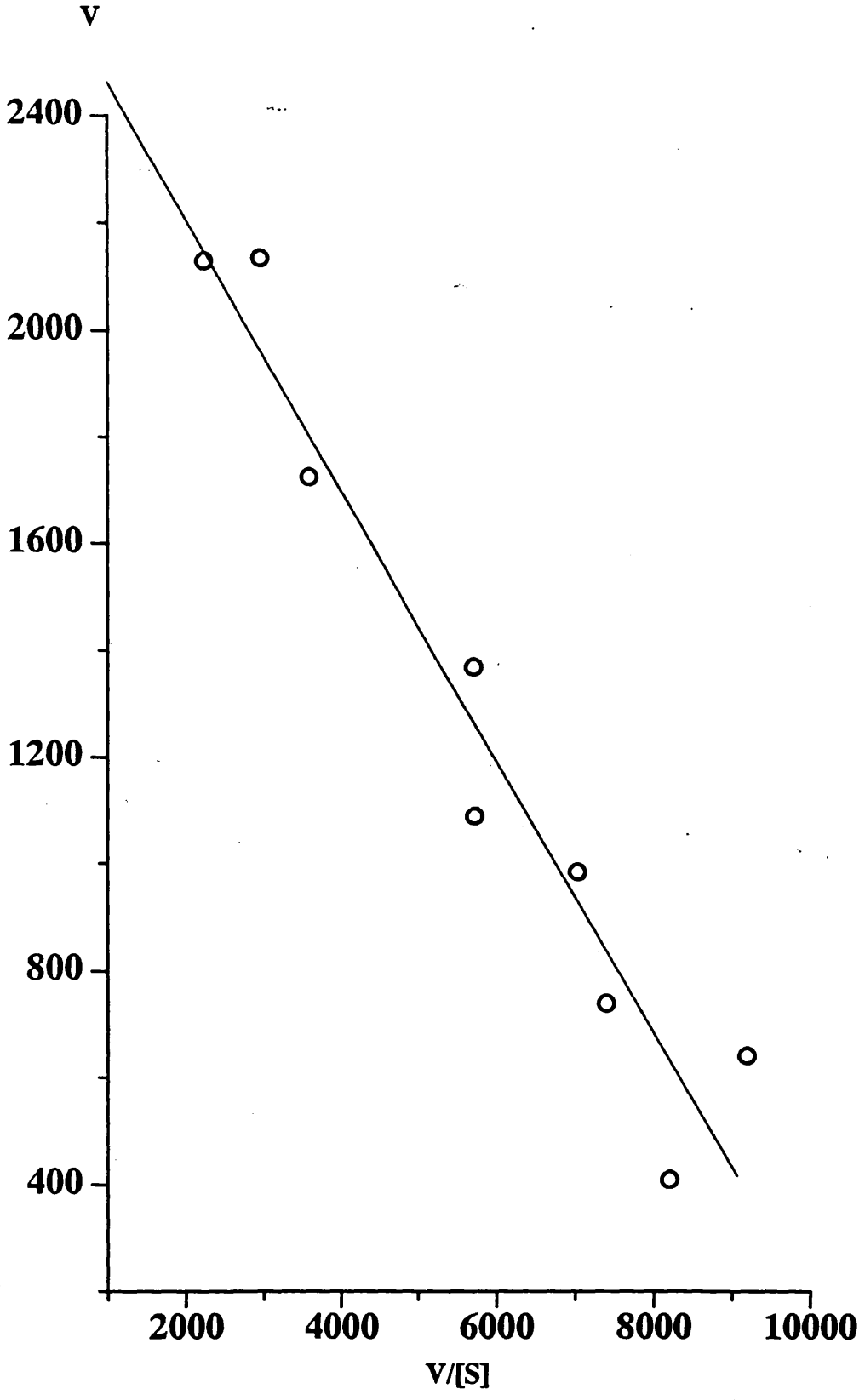
Graph 3A

Lineweaver-Burk Plot



Graph 3B

Eadie-Hofstee Plot



Appendix 3 (contd.)

Spectrophotometric Assay

Calculation of Results

Table 3 (b): Results from Lineweaver Burk and Eadie Hofstee Plots

	Lineweaver Burk Plot	Eadie Hofstee Plot
K_M (mM)	0.28	0.25
V_{max} (μmol/mg/hr)	2858	2729

Appendix 4

Determination of the Rates of Reaction and K_M Values Using the Dissolved Oxygen Meter (O_2 Electrode)

For Example: Cadaverine as substrate with pea seedling DAO

Reaction temperature 25 °C

See experimental section for more details of the method.

The reaction vessel contained:

3500 µl	Phosphate buffer pH 6.3
50 µl	Catalase (0.34 mg/ml)
50 µl	Pea seedling DAO (varying concentrations)
500 µl	Cadaverine (varying concentrations)

Appendix 4 (contd.)

Determination of the Rates of Reaction and K_M Values Using the dissolved Oxygen Meter (O_2 Electrode)

Table 4 (a)

Reaction 1:

Subst. Conc. 1.83 mM

Enzyme Conc. 1.90 x 10^{-2} mg

Time (sec)	Oxygen (mg/l)
0	9.2
5	8.9
10	8.5
15	8.0
20	7.6
25	7.1
30	6.7
35	6.2
40	5.8
45	5.4
50	5.1
55	4.8
60	4.5

Reaction 2:

Subst. Conc. 1.10 mM

Enzyme Conc. 1.90 x 10^{-2} mg

Time (sec)	Oxygen (mg/l)
0	7.8
5	7.5
10	7.2
15	6.8
20	6.4
25	6.1
30	5.7
35	5.4
40	5.0
45	4.7
50	4.4

Rate 1:

1990 µmol/mg/hr

Rate 2:

1690 µmol/mg/hr

Appendix 4 (contd.)

Determination of the Rates of Reaction and K_M Values Using the Dissolved Oxygen Meter (O_2 Electrode)

Table 4 (b)

Reaction 3:

Subst. Conc. 0.55 mM

Enzyme Conc. 1.90 x 10^{-2} mg

Time (sec)	Oxygen (mg/l)
0	7.9
5	7.7
10	7.2
15	6.8
20	6.4
25	6.1
30	5.6
35	5.2
40	4.9
45	4.5
50	4.2

Reaction 4:

Subst. Conc. 0.37 mM

Enzyme Conc. 1.90 x 10^{-2} mg

Time (sec)	Oxygen (mg/l)
0	8.7
5	8.5
10	8.1
15	7.8
20	7.5
25	7.2
30	6.8
35	6.5
40	6.3
45	6.0
50	5.8

Rate 3:

1862 µmol/mg/hr

Rate 4

1464µmol/mg/hr

Appendix 4 (contd.)

Determination of the Rates of Reaction and K_M Values Using the Dissolved Oxygen Meter (O_2 Electrode)

Table 4 (c)

Reaction 5:

Subst. Conc. 0.26 mM

Enzyme Conc. 1.90 x 10^{-2} mg

Time (sec)	Oxygen (mg/l)
0	8.9
5	8.7
10	8.4
15	8.1
20	7.8
25	7.6
30	7.3
35	7.1
40	6.9
45	6.7
50	6.5
55	6.4
60	6.2

Rate 5:

1192 µmol/mg/hr

Reaction 6:

Subst. Conc. 0.11 mM

Enzyme Conc. 3.80 x 10^{-2} mg

Time (sec)	Oxygen (mg/l)
0	8.6
5	8.4
10	8.1
15	7.8
20	7.6
25	7.4
30	7.3
35	7.1

Rate 6

529 µmol/mg/hr

Appendix 4 (contd.)

Determination of the Rates of Reaction and K_M Values Using the Dissolved Oxygen Meter (O_2 Electrode)

Table 4 (d)

Reaction 7:

Subst. Conc. 0.09 mM

Enzyme Conc. 3.80×10^{-2} mg

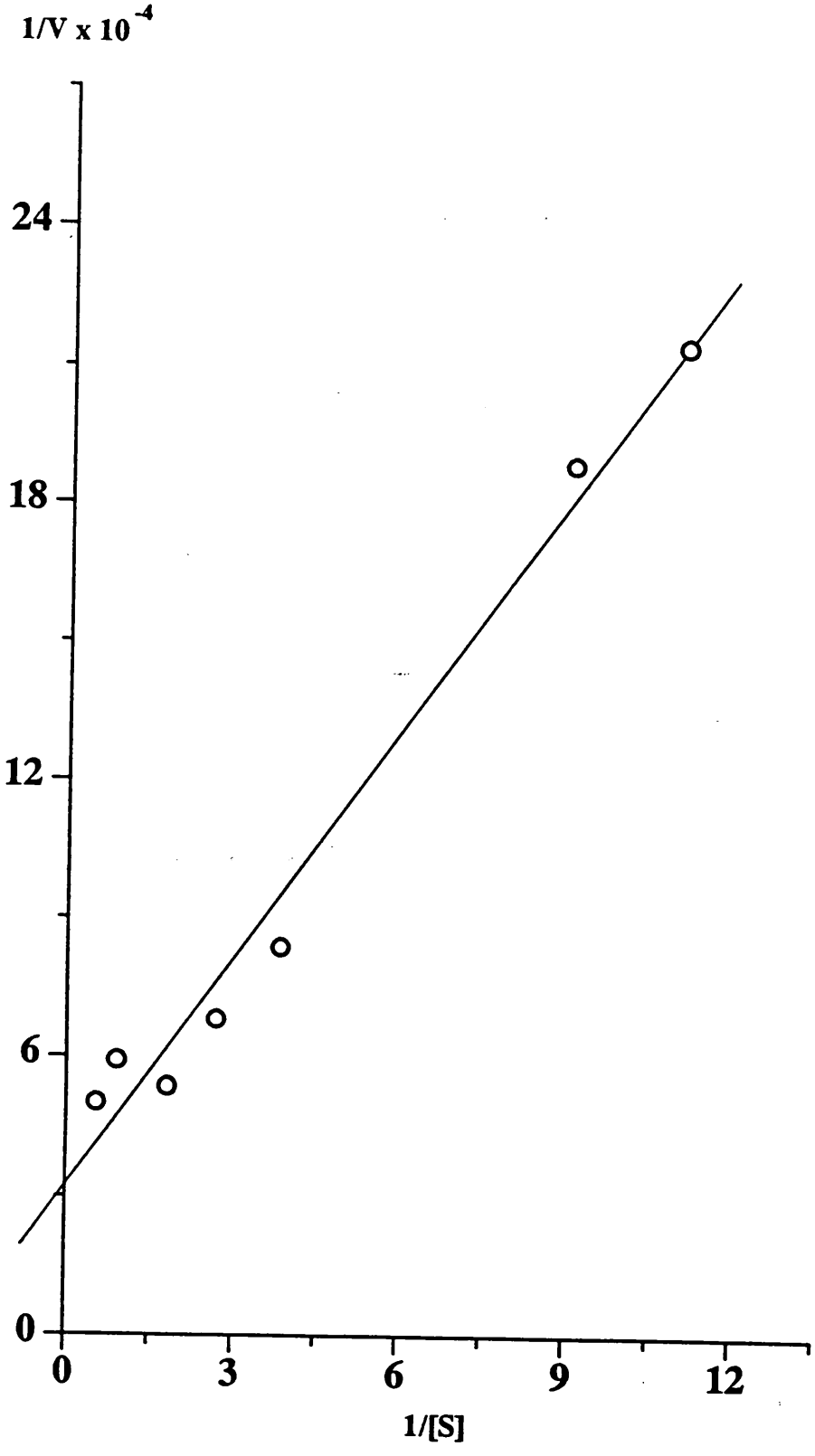
Time (sec)	Oxygen (mg/l)
0	8.8
5	8.6
10	8.4
15	8.2
20	8.0
25	7.8
30	7.6
35	7.5

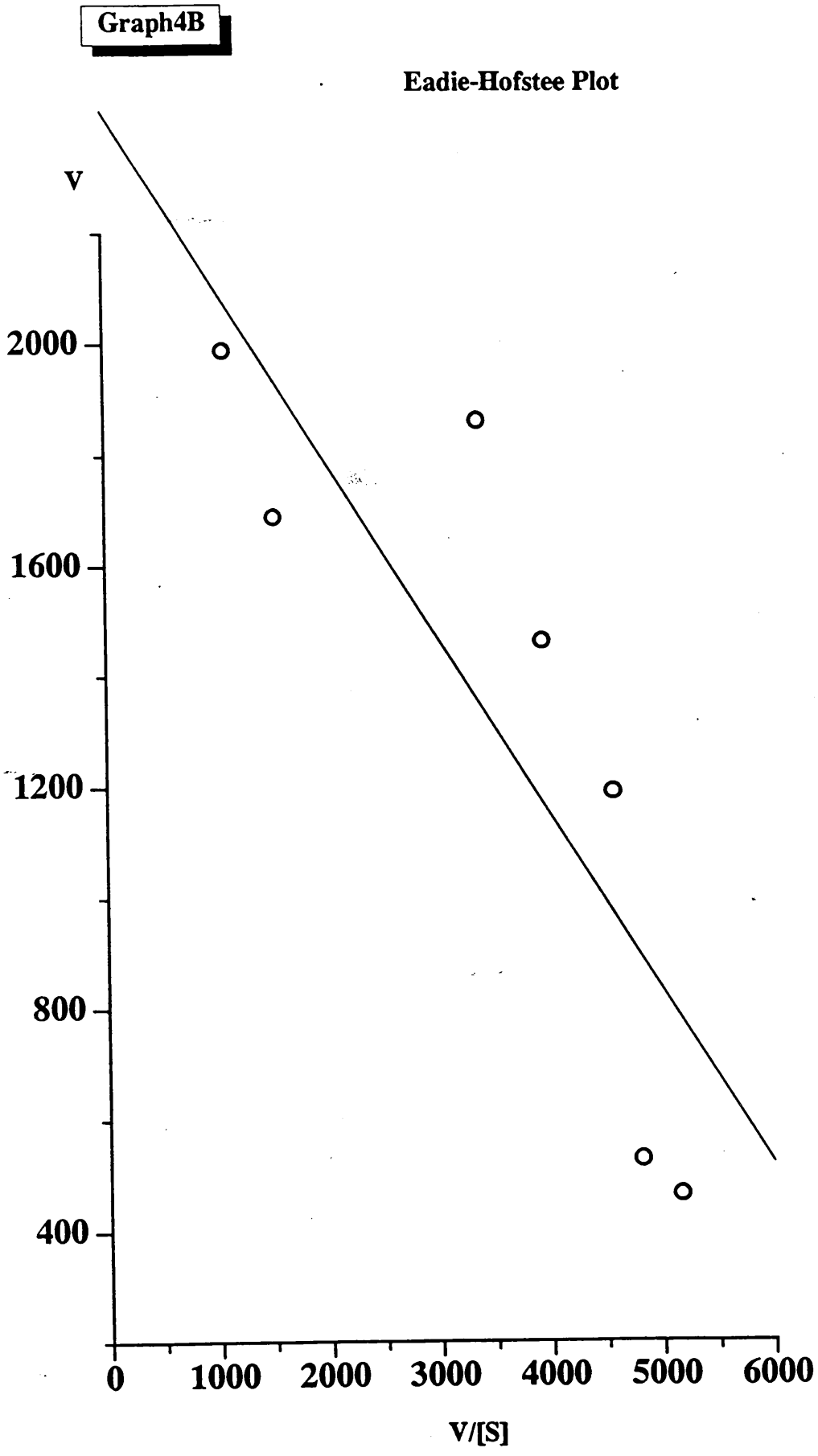
Rate 7:

465 µmol/mg/hr

Graph 4A

Lineweaver-Burk Plot




Graph4B
Eadie-Hofstee Plot
V
2000
1600
1200
800
400
0
1000
2000
3000
4000
5000
6000
V/[S]

Appendix 4 (contd.)

Determination of the Rates of Reaction and K_M Values Using the Dissolved Oxygen Meter (O_2 Electrode)

Table 4 (e): Results from Lineweaver Burk and Eadie Hofstee Plots

	Lineweaver Burk Plot	Eadie Hofstee Plot
K_M (mM)	0.47	0.31
V_{max} (µmol/mg/hr)	2984	2413

Appendix 5

Inhibition of Diamine Oxidase

Calculation of Results

For Example: Cadaverine as substrate, 3,3-dimethylcadaverine as competitive inhibitor with pea seedling diamine oxidase

Assay:		
	2500 µl	Phosphate buffer pH 6.3
	170 µl	DMAB
	100 µl	MBTH
	50 µl	Peroxidase
	25 µl	Pea seedling DAO (psDAO)
	300 µl	Cadaverine (varying concentration)
	100 µl	3,3-Dimethylcadaverine (Inhibitor)

See experimental section for more details of the method.

Appendix 5 (contd.)

Inhibition of Diamine Oxidase

Table5 (a) Substrate Concentration and Rate Readings with Inhibitor Concentration of 0 mM

Substrate Concentration (mM)	Rate (abs/sec x 10^{-3})	Rate (μmol/mg/hr)
0.95	10.64	1970
0.57	10.01	1812
0.38	9.01	1630
0.19	6.43	1163
0.14	5.71	1034
0.10	4.53	820
0.08	3.66	663
0.06	2.95	534
0.04	2.00	362

Appendix 5 (contd.)

Inhibition of Diamine Oxidase

Table5 (b) Substrate Concentration and Rate Readings with Inhibitor Concentration of 1 mM

Substrate Concentration (mM)	Rate (abs/sec x 10^{-3})	Rate (μmol/mg/hr)
1.88	8.32	1554
0.94	6.93	1295
0.75	6.66	1244
0.56	6.66	1244
0.38	5.37	1003
0.19	3.50	654
0.09	2.68	388
0.05	1.05	197
0.04	0.84	157

Appendix 5 (contd.)

Inhibition of Diamine Oxidase

Table5 (c) Substrate Concentration and Rate Readings with Inhibitor Concentration of 2 mM

Substrate Concentration (mM)	Rate (abs/sec x 10^{-3})	Rate (µmol/mg/hr)
2.77	7.85	1466
1.89	6.43	1200
1.54	6.79	1269
0.92	5.00	934
0.74	5.00	934
0.37	3.63	679
0.26	2.82	526
0.18	2.21	413
0.09	1.29	241

Appendix 5 (contd.)

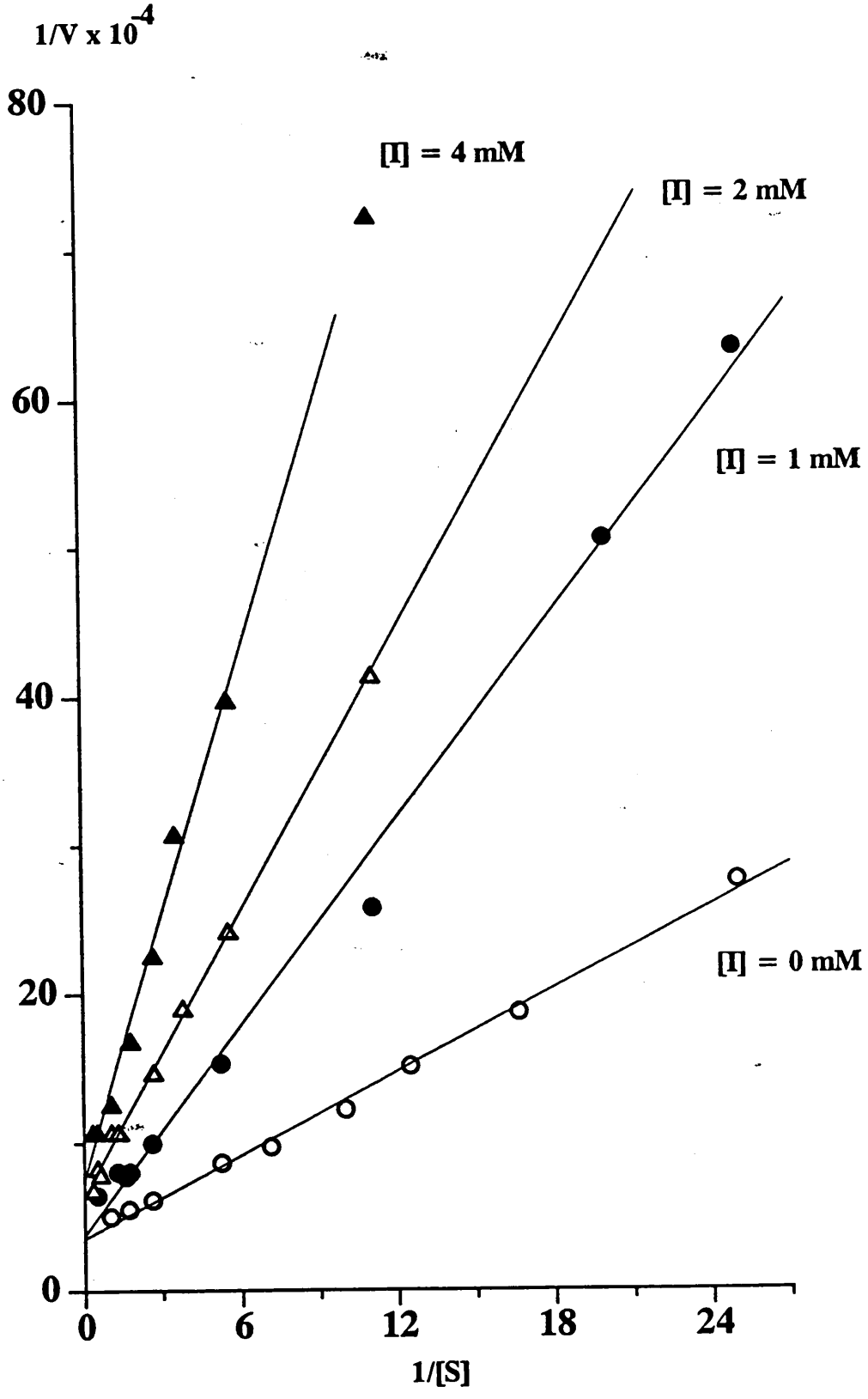
Inhibition of Diamine Oxidase

Table5 (d) Substrate Concentration and Rate Readings with Inhibitor Concentration of 4 mM

Substrate Concentration (mM)	Rate (abs/sec x 10^{-3})	Rate (μmol/mg/hr)
2.77	5.00	934
1.85	5.00	934
0.92	4.24	792
0.55	3.19	595
0.37	2.37	443
0.28	1.74	325
0.18	1.34	251
0.09	0.74	138

Graph 5A

Inhibition Studies



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