AN IMMUNOCHEMICAL ANALYSIS OF MONOAMINE OXIDASE IN HEALTH AND DISEASE

CHERYL CHRISTINE FINCH

A thesis submitted in partial fulfilment of the requirements of The Nottingham Trent University for the degree of Doctor of Philosophy

June 1999

Funded by a Wellcome Trust prize studentship

To Pat

DECLARATION

This work has not been accepted for any other degree and is not concurrently being submitted for any other degree.

We certify that the work submitted was carried out by the author. Due acknowledgement has been made of any assistance received.

Signed..... (Candidate)

Signed..... (Director of Studies)

Our revels now are ended. These our actors,
As I foretold you, were all spirits and
Are melted into air, into thin air:
And, like the baseless fabric of this vision,
The cloud-capp'd towers,the gorgeous palaces,
The solemn temples, the great globe itself,
Yea, all which it inherit, shall dissolve
And, like this insubstantial pageant faded,
Leave not a rack behind. We are such stuff
As dreams are made on, and our little life
Is rounded with a sleep.

William Shakespeare (The Tempest)

ACKNOWLEDGEMENTS

Firstly, my thanks go to the driving force behind this project, my supervisor Dr Ellen Billett for her help and encouragement. A special thank you to Jon for his constant and unquestioning support, and for helping me 'back onto the rails' when at times straying from them felt like the easier option.

Thank you to Professor Adrian Williams and Dr Leong Ho and their research teams for collecting platelet samples, and, of course to those patients and volunteers for bleeding.

Thanks to Mr G. M. Filshie, Dr Dan Hay, Dr Graham Robinson, Mr Richard Church, Dr Rob Layfield and Alan and Flash Brown for collecting the placental samples and to those 'New Mums' and babies for their placentae.

To Dr Mike Billett and Professor Jim Lowe for collecting the human liver samples.

To those brave 'normal'?? volunteers who bled for me so I could begin all this, especially to Alan and Debbie when often gave me 'nearly an armful'.

Technical support and ideas were always available from the technical staff from the Biochemistry, Physiology and Radiochemistry Departments, especially Jane, Debbie, Mick, Bev and Mandy. Thanks also to Maggie for all her help with the photography and for always having a cheerful word. Thanks also to Brian for providing the negative control antibody.

To friends and colleagues from Biochemistry research, past and present, Karen, Brian, Tim, Alan, Ghazi, Maxine, Nadine, Lucy, Gino, Nico, Shiva, Kev, Grey, Mandy, Vicky and the honorary biochemist David. A big thank you to Maxine and Nadine for being 'a shoulder to lean/cry on' and helping me through some really tough bits, and thank you to Helen and Karen for their encouragement and listening.

Finally, a special thank you to my parents and family for their endless support and encouragement.

ABSTRACT

Monoamine oxidase A (MAO-A) and monoamine oxidase B (MAO-B) molecular activities (catalytic activity per mole MAO protein) were measured in extracts of human placenta and platelets respectively. Specific competitive enzyme linked immunosorbant assay (ELISA) systems were used to measure MAO protein concentration and activity assays involved the use of phenylethylamine and dopamine (MAO-B) and 5-Hydroxytryptamine (5-HT, MAO-A) as substrates.

An assessment of the status of platelet MAO-B in *de novo* Parkinson's Disease (PD) has been conducted in both a Caucasian and Hong Kong Chinese population compared with age matched controls. In each population no significant differences in MAO status were observed between PD patients and controls irrespective of the substrate used (phenylethylamine or dopamine) and the sex of the subjects. Heterogeneity was observed in PD in both populations.

Platelet MAO-B concentration was significantly higher in Hong Kong Chinese patients compared with Caucasians in both the disease and control groups, suggesting a race difference.

MAO has been measured in human placenta using similar techniques. When placental extracts were analysed the competitive ELISAs estimated that MAO-A constitutes 90 % of the total MAO protein, MAO-B 10 %; the activity assays estimated 97 % MAO-A, 3 % MAO-B. However using immunohistochemical methods MAO-B is not detected in the placental tissue. These combined data suggest that the MAO-B detected in extracts is derived from blood elements (lymphocytes, platelets).

MAO-A has also been assessed in a preliminary study of pregnancy induced hypertension (pre-eclampsia) compared with normotensive controls. These preliminary results suggest that MAO-A molecular activity may be reduced in pre-eclampsia; this may contribute to the increase in maternal peripheral 5-HT observed in the disease.

CONTENTS

CHAPTER THREE

ANALYSIS OF PLATELET SAMPLES FROM PARKINSON'S DISEASE PATIENTS AND AGE MATCHED CONTROLS

CHAPTER FOUR

A STUDY OF THE STATUS OF MAO-A IN HUMAN PLACENTA

FIGURES

CHAPTER THREE

CHAPTER FOUR

TABLES

CHAPTER 2

CHAPTER 3

CHAPTER 4

ABBREVIATIONS

ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
BCIP	5-Bromo-4-Chloro-3-indolyl phosphate, p-toluidine salt
cDNA	Complementary deoxyribonucleic Acid
CNS	Central nervous system
Da	Dalton
DAB	3,3' Diaminobenzidine
DEAE	Diethyl aminoethyl
DMF	Dimethyl formamide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleuc acid
DPM	Disintegrations per minute
EDTA	Ethylenediamine - tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FAD	Flavin adenine dinucleotide
HPLC	High performance liquid chromatography
5-HT	5-hydroxytryptamine
5-HIAA	5-hydroxyindoleacetic acid
Ig	Immunoglobulin
L-DOPA	Levo 3,4-dihydroxyphenylalanine
mRNA	Messenger ribonucleic acid
MAO	Monoamine oxidase
MIM	Mitochondrial inner membranes
MM	Mitochondrial membranes
MOM	Mitochondrial outer membranes
MPTP	1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine
MPP^+	1-methyl-4-phenyl pyridine
NBT	Nitro blue tetrazolium
PBS	Phosphate buffered saline
PD	Parkinson's Disease
PEA	Phenylethylamine
PFA	Paraformaldehyde
PMSF	Phenylmethylsulphonyl fluoride
PRP	Platelet rich plasma
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
TEMED	N,N,N,N' Tetramethylethylenediamine
TBS	Tris buffered saline
TMB	3,3,5,5 tetramethylbenzidine
Tween 20	Polyoxyethylene sorbitan monolaurate

CHAPTER ONE

GENERAL INTRODUCTION

CHAPTER 1

GENERAL INTRODUCTION

1.1 GENERAL PROPERTIES OF MONOAMINE OXIDASE

1.1.1 Reaction mechanism

The enzyme monoamine oxidase (MAO) was first discovered in liver over sixty years ago and named tyramine oxidase (Hare, 1928). Later Blaschko *et al* (1937) introduced the term amine oxidase. The enzyme name was finally modified to monoamine oxidase to reflect its substrate selectivity. Monoamine oxidase (amine : oxygen oxidoreductase [deaminating][flavin containing] EC 1.4.3.4) is now well documented and is known to be found on the outer mitochondrial membrane (Schnaitman *et al*, 1967). It is involved in the oxidative metabolism and homeostasis of endogenous neurotransmitters and exogenous biogenic amines including dietary amines and drugs. The enzyme readily oxidises primary aliphatic monoamines; however, secondary and tertiary monoamines are also oxidised but to a lower extent. These substrates are oxidatively deaminated to form the corresponding aldehyde product with the co-production of ammonia (Kopin, 1964), refer to Figure 1.1. MAO consists of two identical protein subunits with one FAD group per subunit (Weyler, 1989).

1.1.2 Isoenzyme classification

1.1.2.1 *Inhibitor and substrate studies*

At least two functionally and structurally distinct isoenzymes of MAO are known to exist, initially identified and differentiated by their substrate specificities and inhibitor sensitivities (Gorkin, 1963). MAO-A was described as being sensitive to inhibition by low concentrations of clorgyline whilst MAO-B was relatively insensitive (Johnston, 1968). However, it has since been discovered that this inhibition is concentration dependent, that is, clorgyline will inhibit MAO-B activity if present in sufficient concentration, and similarly deprenyl (initially thought to be specific to MAO-B) will

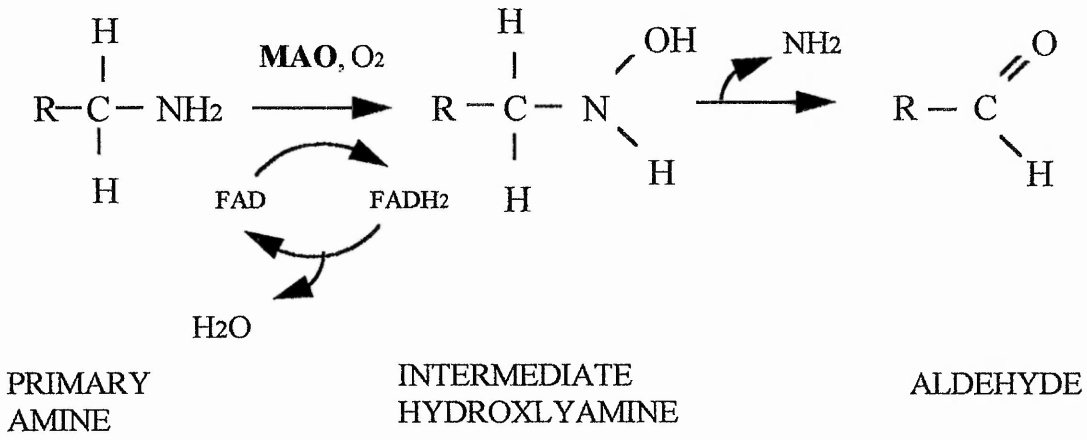


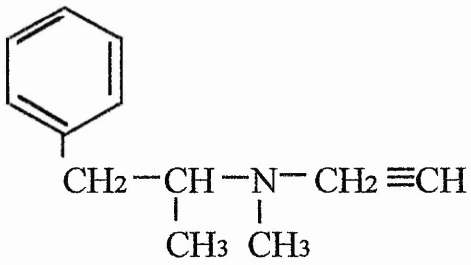
Figure 1.1. The general mechanism of action of monoamine oxidase. The reaction may proceed via the initial formation of a schiffs base, imine or hydroxylamine intermediate, which then quickly dissociates forming an aldehyde product.

inhibit MAO-A at high enough concentration. The structures of clorgyline and deprenyl are shown in Figure 1.2. The introduction of (-) deprenyl, the first selective MAO-B inhibitor (Knoll and Magyar, 1972) was deemed an important breakthrough in the treatment of central nervous system diseases. Other earlier MAO inhibitors although having their desired effect of increasing the levels of brain noradrenaline and 5-hydroxytryptamine also produced an unpleasant side-effect termed the "cheese effect", i.e. they potentiated the sympathomimetic action of indirectly acting amines (e.g. tyramine in certain cheeses and foods) resulting in an acute hypertensive crisis. Consequently the use of MAO inhibition was discontinued as a treatment of depression. The use of (-) deprenyl as an inhibitor of MAO-B without the "cheese reaction" together with the abundance of MAO-B in human basal ganglia led to investigation of the clinical importance of (-) deprenyl therapy in Parkinson's Disease (therapy for Parkinson's disease is discussed in section 1.2).

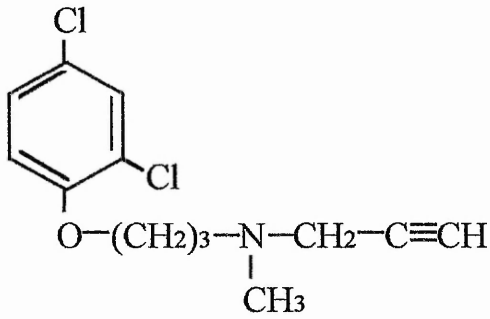
Other evidence important in the determination of the existence of two different forms of the enzyme occurred as a result of MAO purification from various tissue sources, i.e placenta (MAO-A) and platelets (MAO-B). A number of groups produced evidence for the presence of the two forms of MAO based on their having different substrate specificities and inhibitor sensitivities (Tipton *et al*, 1982). It is now generally accepted that substrate specificity is dependent on the concentrations involved with so called MAO-A specific substrates becoming MAO-B substrates at high enough concentrations and vice versa. Therefore only within defined limits is phenylethylamine a specific substrate for MAO-B and 5-hydroxytryptamine specific for MAO-A, with dopamine and tyramine being substrates for both isoenzymes regardless of concentration. The important MAO substrates are shown in Figure 1.3.

Recent literature has provided evidence indicating that in rat liver MAO there is a key amino acid responsible for substrate specificity (Tsugeno and Ito, 1997). This work showed that an amino acid residue at position 208 on MAO-A and the corresponding position 199 on MAO-B is important in conferring substrate specificity.

DEPRENYL



CLORGYLINE



PARGYLINE

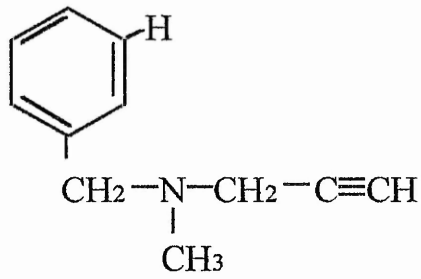
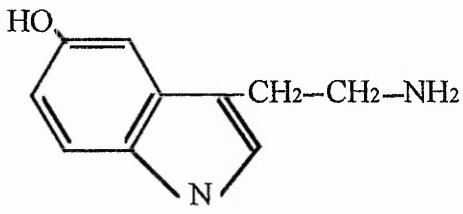
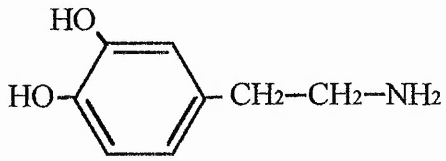


Figure 1.2. The structures of some important monoamine oxidase inhibitors.

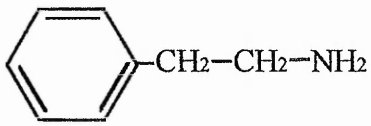
5 - HYDROXYTRYPTAMINE



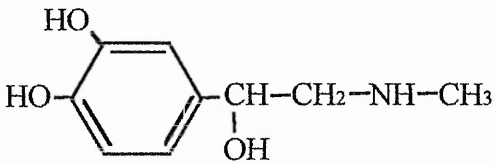
DOPAMINE



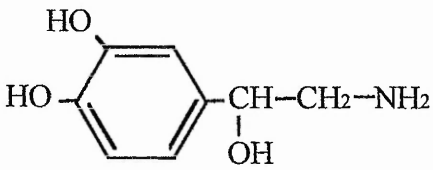
PHENYLETHYLAMINE



ADRENALINE



NORADRENALINE



TYRAMINE

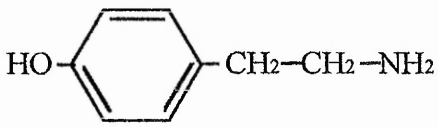


Figure 1.3. Substrates of monoamine oxidase

1.1.2.2 *Immunological studies*

Although the two mammalian forms of MAO show many similarities, evidence has now firmly established that the two enzyme forms are unique. Immunological evidence for the two forms of the enzyme has now been well documented although early reports suggested that the two forms were immunologically indistinguishable. For example in 1977 Dennick and Mayer reported that the enzyme activities recovered from extracts of mitochondrial preparations from both human and rat liver were associated with only one immunogenic species i.e. an inability of their polyclonal antisera to distinguish between the two enzymatic forms. Russell *et al*, (1979) recorded that the enzymes found in human liver, brain cortex, platelets and placenta were recognised by the same antisera and may be identical although functionally different as defined by pharmacological (Johnston, 1968) or kinetic studies (Houslay and Tipton, 1974). On the other hand other early studies (McCauley and Racker, 1973) reported that two forms of MAO isolated from extracts of bovine brain were both functionally different and immunologically unique. Studies by Powell and Craig, (1977) also provided evidence that the two enzyme forms could be distinguished by specific antisera and indicated that they were immunologically unique. Antisera raised against solubilised and partially purified placental (MAO-A) and platelet (MAO-B) forms of the enzyme indicated that, although the two forms are immunologically cross-reactive, the A type enzyme has antigenic determinants which appear to be different from the B enzyme (Brown *et al*, 1982). Other studies (Craig *et al*, 1982; Pintar *et al*, 1983) have supported these findings. The development of monoclonal antibodies specific for the two enzyme forms (Denney *et al*, 1982a and b; Denney *et al*, 1983; Kochersperger *et al*, 1985 and Billett and Mayer, 1986) have confirmed that the two forms are indeed different.

1.1.3 Molecular studies

Molecular studies providing evidence that MAO-A and MAO-B are distinct proteins were reported by Cawthon and Breakefield, 1979, when techniques involving limited proteolysis and peptide mapping revealed that the A and B forms of the enzyme may be coded for by separate genes and are consequently functionally different proteins. These initial findings were subsequently confirmed in a later study by Cawthon *et al* (1981)

which described that MAO-B from blood platelets and MAO-A from the placental trophoblast of the same individual produced different maps following proteolysis, indicating that distinct enzyme molecules were associated with the two types of MAO catalytic activity.

Subsequent work using electrophoretic analysis showed that labelled enzymes had different mobilities and hence different molecular weights. Smith *et al*, 1985 showed that the analysis of tritiated pargyline labelled rat liver MAO by polyacrylamide gel electrophoresis followed by fluorography revealed the presence of two radioactive proteins. Peptide maps were constructed using these proteins, revealing that monoamine oxidase A and B had different amino acid sequences.

The genes for both MAO-A and MAO-B are located on the X chromosome (Kochersperger *et al*, 1986; Ozelius *et al*, 1988; Lan *et al*, 1989). Results from cDNA cloning experiments indicated that the deduced amino acid sequences for human liver MAO-A and MAO-B have molecular weights of 59,700 and 58,800 Da respectively and have 70% sequence homology, but based on the analysis of the primary amino acid sequences, the two isoenzymes appear to be derived from different genes (Bach *et al*, 1988). The amino acid sequences of MAO-A and MAO-B in several other species have also shown that there is an overall similarity (approximately 68%) between sequences of MAO-A and MAO-B but they are encoded by distinct genes (Powell *et al*, 1989). Although different levels of MAO activity in different individuals have been found to be the result of polymorphisms on various alleles, these differences in activity are not a result of differing primary structures (Hotamisligil and Breakefield, 1991). The human MAO-A and MAO-B genes exhibit identical intron-exon organisation suggesting that the two genes were originally derived from duplication of a common ancestral gene (Grimsby *et al*, 1991). Chen *et al* (1993) reported that the deduced amino acid sequences of MAO-B from different human tissues, namely, platelet, liver and frontal cortex are identical. The results from this study validate the supposition that platelet MAO may be an important model for brain MAO-B.

To understand MAO gene expression it is important to have a clear understanding of the promoter region of both genes for MAO-A and MAO-B. In 1992 Zhu *et al* provided

evidence for the location of the promoter regions for both these genes. Differences in organisation of MAO-A and MAO-B promoters have been identified (Shih *et al*, 1994) these differences may account for differences in cell and tissue expression of the MAO enzymes.

1.2 MONOAMINE OXIDASE IN THE CENTRAL NERVOUS SYSTEM

1.2.1 Distribution of MAO in the brain

As MAO has been implicated in a number of neurological disorders (sections 1.2.2.3 and 1.4), the distribution of both forms of the enzyme in the human brain is deemed important for an understanding of some of these conditions.

The brain contains one of the highest activities of MAO-A and B in the body, but the distribution of the two enzyme types is not uniform giving rise to regional variations. The distribution of brain MAO has been studied using various techniques, including autoradiography (using labelled specific inhibitors) and immunohistochemistry.

Autoradiography using radiolabelled inhibitors has provided some evidence for MAO brain distribution. Jossan *et al*, 1991 have used radiolabelled L-deprenyl to map brain MAO-B distribution. High deprenyl binding was observed in the caudate nucleus, putamen, cingulate gyrus and insula cortex indicating a high concentration of MAO-B in these sites. Moderate to low binding was observed in the globus pallidus, temporal and parietal cortex and various thalamus nuclei. This radioligand data was substantiated using biochemical analyses to quantify MAO-B activity and were found to have a high positive correlation. Richards *et al* (1992) have used enzyme autoradiography coupled with *in situ* hybridisation to investigate both protein and mRNA expression. MAO-A protein and mRNA have been detected in the noradrenergic neurones of the locus coeruleus whereas MAO-B mRNA and protein were present in the serotonergic neurones of the raphe nuclei. Distribution of the mRNA for both enzymes correlated with the respective protein distribution but was not identical.

Monoclonal antibodies have been used to map the distribution of MAO in the human brainstem (Westlund *et al*, 1988). This study found that the neurones rich in the catecholamines (nucleus locus coeruleus, nucleus subcoeruleus and the medullary reticular formation) were positive for MAO-A. This included areas rich in dopamine neurones i.e the substantia nigra and periventricular region of the hypothalamus although the staining here was to a much lesser extent. MAO-B positive neurones were found in the nucleus raphe dorsalis and nucleus centralis superior regions containing serotonin neurones.

Further evidence for the brain distribution of MAO-B consistent with the findings of Westlund *et al* (1988) is detailed by Yeomanson, 1990. This study also relied on immunohistochemical analysis of frozen sections using 3F12/G10, a specific human MAO-B monoclonal antibody (Billett and Mayer, 1986). The distribution of MAO-B in certain areas of normal human brain was mapped using this antibody. She found that whilst the extraneuronal matrix of the substantia nigra exhibited extensive staining for MAO-B, the cell bodies of the dopaminergic neurones showed no MAO-B immunoreactivity. Glial cells in the substantia nigra contained MAO-B reactivity. Intense MAO-B staining in the serotonergic neurones of the nucleus raphe dorsalis was observed again coupled with a widespread MAO-B immunoreactivity of neuronal fibres and glial cells throughout this region.

These brain regions were also assessed for MAO-B activity in one Parkinsonian brain. Histochemical analysis revealed a degeneration, depigmentation and decrease in number of the neurones in the substantia nigra, a characteristic pathological finding in Parkinsonian brains. MAO-B immunohistochemistry highlighted an absence of the enzyme in the cell bodies of dopaminergic neurones, but, as in the normal brain, MAO-B immunoreactivity was evident in the neuronal fibres and glial cells of the substantia nigra.

Analysis of the nucleus raphe dorsalis in Parkinsonian brain also revealed MAO-B immunoreactivity in the serotonergic neurones. The pigmented noradrenergic neurones of the nucleus locus coeruleus revealed MAO-B staining and MAO-B was seen to co-localise with neuromelanin.

It is clear from these studies that MAO-B positive neurones are localised to areas rich in serotonergic neurones, notably the nucleus raphe dorsalis in the midbrain, a surprising finding as serotonin (5-HT) is preferentially deaminated by MAO-A.

The paradox appears to be that, although the dopaminergic neurones in the pars compacta of the substantia nigra do not contain MAO-B; Parkinson's Disease (PD) is characterised by a massive degeneration of dopaminergic neurones in the midbrain and therapeutic benefits are seen when Parkinson's Disease is treated with a specific MAO-B inhibitor. The question is therefore raised as to the importance of the role of MAO-B in Parkinson's Disease. It is possible that MAO-B in adjacent glial cells may affect dopaminergic neurones in the midbrain through the production of oxygen free radicals which may be the causative agents of nerve cell damage in Parkinson's Disease. MAO-B inhibitors may therefore have a therapeutic effect by reducing the rate of dopamine oxidation or inhibiting formation of toxic compounds. Free radical damage and oxidative stress as a causative agent of Parkinson's Disease is discussed in further detail in section 1.2.2.5.

More recently, Damier *et al*, 1996 have used an anti MAO-B monoclonal antibody in an attempt to elucidate the involvement of MAO-B in Parkinson's Disease by looking at its brain distribution using a double labelling technique. This technique involves the use of two different antibodies staining for MAO-B and tyrosine hydroxylase. The results from this study demonstrate MAO-B immunoreactivity in neuronal cell bodies as well as glial cells and fibres in the human midbrain, which is in contrast to other studies noted earlier which have reported a marked absence of MAO-B in the neuronal cell bodies of the pars compacta of the substantia nigra (Yeomanson, 1990; Westlund *et al*, 1988). Although most of the MAO-B immunoreactivity was located in neurones of the nucleus raphe dorsalis, using the more discriminatory double labelling technique revealed the presence of MAO-B in dopaminergic neurones of both control and Parkinsonian brains.

Interestingly, this report found that the loss of MAO-B containing dopaminergic neurones in Parkinson's Disease was no higher than the MAO-B negative dopaminergic neurones, concluding that the presence of MAO-B in dopamine containing neurones

may not be important in vulnerability to Parkinson's Disease. The importance of MAO-B in Parkinson's Disease is discussed in greater depth in section 1.2.2.3.

It is clear from the evidence that the distribution of MAO in the human brain is complex. However, two main findings are consistent with most reports, i.e. it is MAO-B that is unexpectedly found in serotonergic neurones and not MAO-A and that MAO-B is the enzyme type found in glial cells.

1.2.2 Monoamine oxidase and Parkinson's Disease

1.2.2.1 *Parkinson's Disease*

Parkinson's Disease (PD) is a well characterised movement disorder which affects approximately 1% of the population over 65 years of age. It was first described in 1817 by James Parkinson the London doctor who first recognised its main features and published his "Essay on the shaking Palsy" to that effect. This progressive degeneration of the central nervous system is characterised pathologically by the degeneration of the pigmented brainstem nuclei specifically the pars compacta region of the substantia nigra coupled with the formation of characteristic eosinophilic cytoplasmic inclusions called Lewy bodies. These neuronal inclusions show a central core with a peripheral halo and are situated in the cytoplasm of neurones in almost every case of PD.

The major clinical features of Parkinson's Disease can be divided into four main categories which are used as the basis of diagnosis.

i. Bradykinesia
ii. Muscle stiffness and rigidity
iii. Limb tremor
iv. Gait disturbances and postural instability.

Parkinson's Disease is one of the major neurodegenerative diseases which carries a lifetime risk of development of 1 in 40. The disease can be defined biochemically

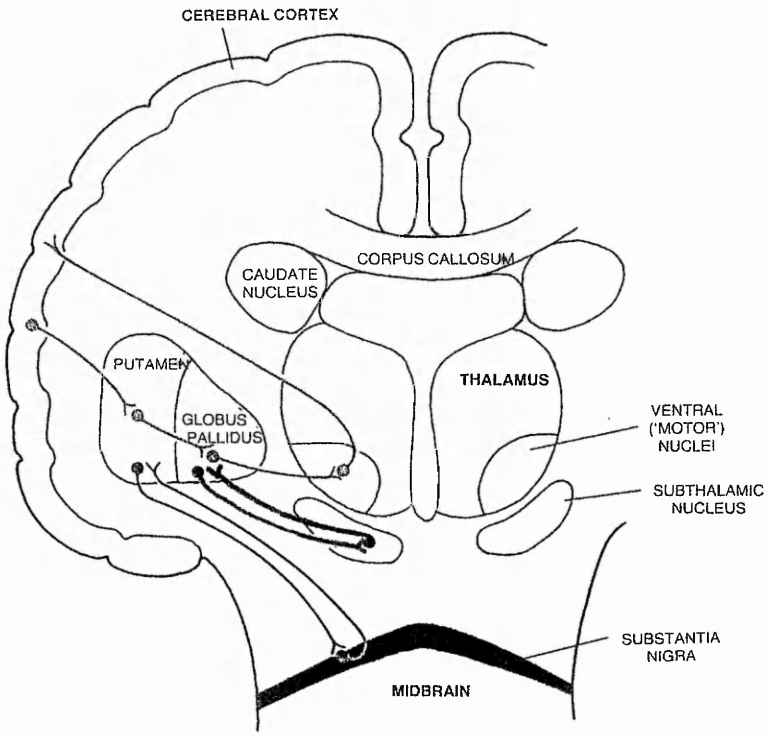


Figure 1.4. Diagram illustrating the neural connections important in movement control. (adapted from England and Wakely, 1991)

primarily as a dopamine deficiency state resulting from the degeneration or injury to the dopamine neurones of the substantia nigra. It is these neurones that produce large amounts of dopamine under normal physiological conditions. The corpus striatum (consisting of the caudate nucleus and the putamen, Figure 1.4) is the site where dopaminergic nigrostriatal neurones terminate and therefore contains the highest concentration of dopamine in the brain. The loss of these neurones therefore results in the severe reduction of striatal dopamine. Under normal conditions the role of dopamine in the corpus striatum is the continuous inhibition of acetylcholine release from the cholinergic neurones of the caudate nucleus. Consequently, a loss of these nigrostriatal dopaminergic neurones results in the uninhibited release of acetylcholine. This in turn results in a neurotransmitter imbalance in the basal ganglia and this increase of acetylcholine causes the characteristic signs and symptoms to become apparent. This dopamine system is involved with the control of locomotor behaviour; consequently, dopamine deficiency in this region contributes to the motor problems evident in Parkinson's Disease. Small dopamine losses can be compensated for and do not produce clinical symptoms and at least 70% of dopaminergic neurones in the nigrostriatal pathway must be lost before clinical signs appear (Bernheimer *et al*, 1973; Hornykiewicz and Kish, 1986)

Treatment of Parkinson's Disease nowadays is mainly achieved by dopamine substitution therapy. The dopamine precursor L- DOPA, is able to cross the blood brain barrier (Friis *et al*, 1981) where it is converted into dopamine by dopa-decarboxylase. It is usually administered in conjunction with a MAO-B specific inhibitor (e.g. deprenyl) to prevent endogenous MAO-B deaminating the administered dopamine.

There are three general hypotheses concerned with the aetiology of PD, but to date the answer has not been resolved. These theories can be listed as genetic factors; ageing of the central nervous system and infections or toxic factors.

1.2.2.2 *MPTP-induced Parkinsonism and its resemblance to idiopathic Parkinson's Disease*

1.2.2.2.1 *MPTP-induced Parkinsonism - its discovery*

In 1979 Davis *et al* reported a Parkinson-like syndrome in a 23 year old student. It was subsequently discovered that this patient had been self administering meperidine (1-methyl-4- propionoxypiperidine), a pethidine analogue and heroin substitute. This student had been synthesising the drug and had been successful on a number of occasions but in an attempt to increase the rate of synthesis he had produced traces of the potentially lethal contaminant MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) which on administration had produced these Parkinson-like symptoms. The symptoms were alleviated using conventional PD treatment strategies. The student later died from a drug overdose and subsequent post-mortem analysis revealed the extensive destruction of the substantia nigra similar to that found in idiopathic PD. Attempts to repeat this discovery by the administration of MPTP to rats failed to produce any significant findings and it was later discovered that rodents were generally found to be insensitive to the pathological effects of MPTP (Chiueh *et al*, 1984).

In 1983 PD research was revolutionised when Langston and co-workers reported four narcotic addicts who had developed a Parkinson-like syndrome clinically indistinguishable from the idiopathic condition after injecting an impure sample of a synthetic narcotic. Subsequent analysis identified this drug as virtually pure MPTP. This MPTP-induced parkinsonism further resembled idiopathic PD by the lack of more extensive damage to the CNS and the successful therapeutic response to treatment with L-DOPA. These similarities between PD and MPTP-induced parkinsonism may suggest that environmental compounds similar to MPTP may be important causative agents of Parkinson's Disease.

1.2.2.3 *Mechanism of action of MPTP-induced Parkinsonism and a possible role for MAO-B in the development of Parkinson's Disease.*

Rats have proved to be unreliable models for MPTP-induced PD due to their insensitivity to the drug, as is the case with most rodents. Mice are more susceptible but require high doses to initiate neurotoxic effects (Heikkila *et al*, 1984 a). The actions of MPTP on cats again exhibit little evidence of neurotoxicity (Schneider *et al*, 1986).

Progress was however made when two groups demonstrated MPTP-induced parkinsonism in primates (Burns *et al*, 1983; Langston *et al*, 1984). Parkinsonian symptoms were observed following a number of intra-venous doses of MPTP and furthermore, these symptoms could be reversed by administration of L-DOPA; post mortem analysis revealed that these symptoms were associated with the selective destruction of the dopaminergic neurones in the nigrostriatal pathway.

MPTP is a fat soluble compound that can be readily absorbed and freely distributed throughout the body including the brain. In 1984 Markey *et al* reported that, after administration and uptake into cells, MPTP is oxidised by MAO- B via an intermediate compound to its quaternary derivative, 1-methyl-4-phenylpyridine (MPP^+) (Figure 1.5), and that this compound was found in the brains of monkeys as long as three weeks after administration of its parent compound MPTP. It was subsequently discovered that blocking the formation of MPP^+ with MAO inhibitors prevented this neurological damage (Heikkila *et al*, 1984 (b); Maret *et al*, 1990). Another important study, also published in 1984 was conducted by Chiba and co-workers which also showed that the formation of MPP^+ was inhibited by MAO-B specific inhibitors but was not inhibited by inhibitors specific to MAO-A, indicating that it is MAO-B which is responsible for the oxidation of MPTP and it has been suggested that MAO-B activity is altered in idiopathic PD.

MPP^+ is selectively accumulated in dopaminergic nerve terminals by the dopamine re-uptake system causing the selective destruction of dopamine neurones seen in both

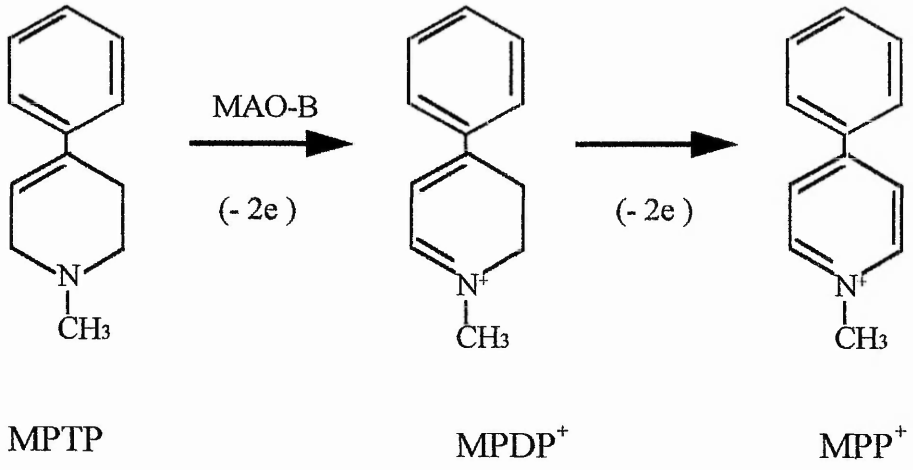


Figure 1.5. The conversion of MPTP into the reactive radical MPP+ by monoamine oxidase B.

idiopathic PD and MPTP-induced Parkinsonism (Javitch *et al*, 1985). Inside the nerve ending MPP^+ is thought to produce free radicals which interfere with mitochondrial respiration. This leads to a depletion of ATP impairing cellular activity and ultimately leading to cell death. The actual mechanisms by which MPP^+ causes cell death have still to be elucidated. The structural resemblance of MPP^+ to the herbicide paraquat indicated that MPP^+ induced cell death may be due to the redox production of free radicals. This concept has been further developed by Singer *et al*, 1987 who suggest that this highly reactive species interferes with mitochondrial energy metabolism specifically inhibiting complex I which in turn generates free radicals. It is therefore possible that other environmental agents may have a similar mechanism of action. The specific site of action of MPTP may be explained by the role of MAO-B which has high activity in the glial cells of the nigrostriatal pathway. However, because MAO-B activity is also high in other brain regions which are resistant to MPTP damage, it is possible that other characteristics of the nigro dopaminergic neurones may increase their chance of free radical damage e.g. reduced glutathione (important in the detoxification of oxidising radicals) concentrations are low in the substantia nigra of PD sufferers. Such a deficiency in glutathione would explain the increased level of lipid peroxidation found in the substantia nigra in Parkinson's Disease (Dexter *et al*, 1989). It could also explain the possibility that the administration of L-DOPA although therapeutically beneficial, may increase the oxidative stress. This would then provide an explanation for the beneficial effects of L-deprenyl as an adjunct to L-DOPA (Birkmayer *et al*, 1983), and may explain the lower incidence of Parkinson's Disease in cigarette smokers, since they have lower MAO activities (Oreland *et al*, 1981) and since the constituents of cigarette smoke inhibit MAO (Yu and Boulton, 1987). Studies of MAO activity in Parkinson's Disease are discussed in depth in chapter 3.1.

1.2.2.4. *Environmental and hereditary aspects*

It is still an open question as to whether environmental agents with a similar mode of action to MPTP are causing PD. Whether or not the disease only manifests itself in predisposed individuals is also not known. Indeed reports describing 'familial clusters' where members of the same family but from different generations developed PD within a few years of one another but at different ages, point to a simultaneous exposure to an

agent of possible environmental origin. Twin studies have also provided some clues as to the involvement of an environmental factor. It has been reported that one member of a monozygotic twin pair has developed PD whilst the other, having lived a separate life has not; this again points towards an environmental factor (Tanner, 1989 for review).

Additional evidence for an environmental cause for Parkinson's Disease was also reported in 1986 when Rajput and co-workers suggested a link between childhood drinking water supplies and development of early onset Parkinson's Disease (< 40 years of age). This and other work prompted a re-evaluation of the twin studies and genetics of Parkinson's Disease. A report by Johnson *et al* (1990), states that it is possible that genetic factors confer to a susceptibility to Parkinson's Disease but it is environmental factors which determine the clinical manifestation of the disease.

A great deal of conflicting literature has been published regarding the possible involvement of genetic factors in the aetiology of Parkinson's Disease. Studies on monozygotic twins have suggested that the appearance of clinical symptoms is not hereditary (Duvoisin *et al*, 1981). However other studies have suggested that a hereditary factor may be important in predisposing an individual to developing Parkinson's Disease (Kurth *et al*, 1993; Ho *et al*, 1994; Hotamisligil *et al*, 1994). Various authors have reviewed evidence suggesting that normal ageing and genetic factors cannot account for most cases of PD (Calne and Langston, 1983). It has also been postulated that one cause of PD may be exposure to environmental agents acting directly or resulting in the formation of endogenous toxins which may result in an acceleration of the normal age related decline of the substantia nigra (Tanner, 1989).

A report in 1991 by Maraganore *et al* studied the significance of Parkinson's Disease occurring in more than one member of the family and concluded that familial Parkinson's Disease exists and it is clinically indistinguishable from sporadic cases of Parkinson's Disease. However, the role of genetic factors in the aetiology of the sporadic cases is unclear.

Later reports have shown conflicting results; Mazzetti and colleagues (1993) suggested that Parkinson's Disease is genetically linked and based on either autosomal recessive or

dominant inheritance. Whereas Wingerchuk *et al* (1993) provided evidence to conclude that in the group of patients they studied, development of Parkinson's Disease was related to the exposure of some environmental agent and there was no evidence of Mendelian inheritance.

A more recent study by Payami *et al*, 1995 has suggested genetic heterogeneity in Parkinson's Disease with at least two different types. Firstly, the involvement of a gene containing an unstable trinucleotide repeat and secondly, a susceptibility gene which, when coupled with exposure to an environmental toxin, manifests the disease phenotype. More recently Polymeropoulos *et al* (1996) describe the identification of a genetic locus for the Parkinson's Disease phenotype. This study is unique as it was able to analyse an extended family pedigree originating from Southern Italy and consisting of 592 members over 11 generations, 60 of which had pathologically confirmed Parkinson's Disease. This group have mapped a gene for Parkinson's Disease to chromosome 4 between position q21-q23 of the long arm. This finding suggests that development of Parkinson's Disease may be due to mutations of a single gene. This conclusion is however not final as the study relates to only one family pedigree.

Further work by Polymeropoulos *et al* (1997) has established that in the same Italian family a mutation has been identified in the α–synuclein gene. α–synuclein is a pre-synaptic protein whose major site of expression is the nerve terminals but whose function has yet to be elucidated. It was originally identified as the pre-cursor to a component present in the characteristic amyloid plaques in the brains of Alzheimer patients. Work published shortly after Polymeropoulos *et al*, 1997 has provided evidence for the presence of α-synuclein in Lewy bodies from non-familial Parkinson's Disease patients (Spillantini *et al*, 1997).

It has been postulated therefore that mutation in this α-synuclein gene in Parkinson's Disease may promote aggregation of α–synuclein into filaments which may result in the formation of characteristic Lewy bodies and that this mutation may be hereditary.

It is therefore still an open question as to how much the aetiology of Parkinson's Disease is dependent on hereditary factors and what role environmental agents play.

1.2.2.5 *Parkinson's Disease caused by free radical damage and oxidative stress*

The initial mechanism behind the degeneration process and causes of cell damage and death in the substantia nigra of patients with Parkinson's Disease remains unsolved. Parkinson's Disease is characterised by the loss of dopaminergic neurones, and as dopamine metabolism results in the production of oxygen free radicals, theories suggesting a link between oxidative stress and cell death in Parkinson's Disease have been postulated (Hirsch, 1993). These theories have been based on a number of different factors all of which are present in the substantia nigra of Parkinson's Disease patients and may generate free radicals. The first major factor is the presence of the neurotransmitter dopamine. The deamination of dopamine to 3,4-dihydroxyphenylacetaldehyde by MAO-B in the substantia nigra results in the formation of a by-product, hydrogen peroxide which can in turn form highly reactive oxygen species and result in cellular damage.

In primates dopamine is transformed into a pigment called neuromelanin by an auto-oxidation reaction that produces oxygen free radicals (Marsden, 1983); neuromelanin is therefore considered to be another important factor in Parkinson's Disease. Indeed a high concentration of neuromelanin exists in the dopaminergic neurones of the substantia nigra i.e. those preferentially affected in Parkinson's Disease. It may be assumed therefore that the free radical concentration would be higher in neurones containing melanin than their non-melanised counterparts.

A link between the presence of neuromelanin in dopaminergic neurones and their vulnerability to oxidative stress in Parkinson's Disease has been reported (Hirsch *et al*, 1988). A later report by Kastner and co-workers, 1992, has provided evidence to substantiate the link between neuromelanin content and nigral cell death in Parkinson's Disease. However, the report does suggest that although this is an important factor it is by no means the only one and there is still some debate as to whether neuromelanin containing cells are more likely to degenerate in Parkinson's Disease.

A third factor in vulnerability of these neurones is their iron content. Iron has been thought to be important due to its ability to generate oxygen free radicals in the presence of dopamine and to selectively bind to neuromelanin resulting in the production of

Fe^{2+}/melanin complexes which may cause oxidative stress (Youdim *et al*, 1990). Free (unbound) iron also catalyses the conversion of hydrogen peroxide to highly reactive hydroxyl radicals. There are a number of reports which have documented the increased iron content in the substantia nigra of Parkinson's Disease patients (Jellinger *et al*, 1990; Hirsch *et al*, 1991). High levels of iron have also been detected in the Lewy bodies of Parkinson's Disease patients which may suggest that an increase in iron content is associated with the disease. More recently, it has been reported by Leveugle and colleagues that a specific iron receptor namely lactoferrin receptor exists at a much higher density in the substantia nigra of Parkinson's Disease patients compared with controls, particularly in areas of abundant iron concentration. It was actually overexpressed in the main dopaminergic areas, especially the pars compacta of the substantia nigra where in Parkinson's Disease iron accumulation is greatest and neuronal loss highest (Leveugle *et al*, 1996). It remains to be seen whether this receptor has an important role in the pathogenesis of Parkinson's Disease.

A dysfunction in the mitochondria has also been implicated in the pathogenesis of Parkinson's Disease. This theory came about from the extensive studies involving MPTP. As previously mentioned MPTP is converted by MAO-B to MPP^+, which is accumulated in dopaminergic neurones and then accumulates in mitochondria. MPP+ is a specific inhibitor of NADH CoQ1 reductase (i.e. complex I), the proximal enzyme of the respiratory chain. This eventually causes a decrease in ATP levels and cells die in the substantia nigra. Indeed, it has been demonstrated that there is a 30-38% deficiency of complex I activity in Parkinson's Disease patients (Schapira *et al*, 1990) which could result in increased free radical production.

To conclude, although many theories exist as to the cause of the pathogenesis of Parkinson's Disease and our understanding of the disease is advancing, the relationships between the different factors and its ultimate cause still remains to be elucidated. It seems reasonable to assume that the disease has no one single cause but may be the result of a number of inter-related factors. The case for a genetic cause is as yet weak, but susceptibility factors may be genetic, and these coupled with the case for oxidative damage, exposure to environmental toxins and the involvement of MAO may give clues to the answer.

1.3 PLATELET MONOAMINE OXIDASE

1.3.1 Formation of platelets

Traditionally blood platelets are thought to be fragments of megakaryocyte cytoplasm, hence they are non-nucleated and are formed in the bone marrow. The idea that platelets are formed as a result of budding of the megakaryocyte cytoplasm was first postulated by Wright, 1910, describing the formation of megakaryocytic buds in the bone marrow but also in the spleen and circulation. Thus providing evidence for a mechanism of platelet formation but not a definite site of production.

A small proportion of bone marrow stem cells are committed to produce platelets. They undergo a series of differentiation stages before forming the mature platelet. The first of these stages is the formation of the megakaryoblast. Once formed this cell ceases to multiply by cell division but matures instead by undergoing a form of mitosis whereby only the nucleus of the cell divides. This process is termed endomitosis. With each nuclear division there is increased membrane formation, increasing cytoplasmic maturation and an increased number of nuclear lobes forming the mature megakaryocyte. The cytoplasmic maturation results in the formation of three distinct cytoplasmic zones. The perinuclear zone containing the golgi apparatus and rough and smooth endoplasmic reticulum which remains attached to the nucleus after platelet formation. The intermediate zone consisting of interconnecting vesicles and tubules which is in contact with the cell membrane, this is the zone of platelet development. Finally the marginal zone containing much of the cytoskeletal constituents of the cell.

Once the megakaryocyte is mature the increased membrane formation is accommodated by invagination of the membrane transforming the cell from a spherical to highly amoeboid irregular shape containing a zone of cytoplasm around the periphery. Fragments of this cytoplasm are pinched off to form individual platelets. Mature megakaryocytes extend pseudopodia through the walls of the marrow sinusoids and either individual platelets or larger fragments of cytoplasm are broken off. These larger pieces of cytoplasm travel to the lungs in the bloodstream where the final breakdown to form platelets occurs mechanically in the pulmonary microvasculature. Reports suggest

that platelets are released from the bone marrow into the circulation at a rate of 3.4 x 10^7 platelets/ml/day. (Penington, 1981). It is also reported that a substantial percentage (20-50%) of mature megakaryoblasts may travel in the circulation to lodge in venous microvasculature of the lungs where they release their cytoplasm (Kaufman *et al*, 1965).

The bone marrow as the traditional site of platelet production is a question of some debate. Martin and Levine (1991) argue that evidence for bone marrow platelet production is lacking and that there is increasing evidence in favour of the lungs as the site of production. They suggest that budding may be associated with the movement of the mature megakaryocyte from the bone marrow into the circulation and that there is little evidence for platelet production in the bone marrow. The site demonstrating the second largest number of mature megakaryocytes is the pulmonary microvasculature. A study conducted by Tinggaard Pederson (1978) found that the numbers of megakaryocytes in the lungs were sufficient to provide normal haemostatic platelet production. The lungs are the first capillary network that mature megakaryocytes encounter and, coupled with the greater forces exerted on them generated by the right ventricle, is plausible to argue that platelet production may occur at this site due to the rapid shape changes that the megakaryocyte must undergo to pass through the lung microcirculation. The site of platelet production is therefore still an open question. The circulatory platelet number is maintained within a narrow range (2 x 10^8/ml) indicating that it is controlled by a regulatory mechanism. Changes in these numbers either by removal or destruction are compensated for restoring the count to the normal range (de Gabriele and Penington, 1967). Their lifespan in the circulation is approximately 10 days after which they are removed by the reticuloendothelial system of the spleen and liver or are incorporated into a haemostatic plug.

1.3.2 Structure of the platelet

Platelets circulate in the blood stream as anucleate discs ranging from 1.5 to 3.5 μm in diameter. A diagrammatic representation of the human platelet is shown in Figure 1.6. The human platelet surface membrane is approximately 7-9 nm in diameter and consists of a bilayer membrane composed of protein, lipid and carbohydrate. The lipid content is mainly phospholipids (75%) and neutral lipid (20%) with a small proportion of glycolipid

present (Shattil and Cooper, 1978). The outer coat or glycocalyx contains receptors important for platelet activation and adhesion and a series of invaginations which connect a system of interconnected intricate canalicular membrane channels with the extracellular fluid. The cytoskeleton of the circulating platelet is responsible for both maintenance of the normal morphology in the unstimulated cell and shape alterations induced by activation. It consists of a ring of microtubules around the periphery of the platelet and actin microfilaments in the cytosol. Tubulin is the major microtubular protein consisting of a heterodimer of two α and β subunit polypeptides each with molecular weight of approximately 55,000. The tubulin subunits polymerise forming bundles which then aggregate to form the tube like tertiary structure of the protein. These microtubules are associated with a number of high molecular weight microtubule associated proteins.

Actin constitutes approximately 20-30% of the total platelet protein (Fox *et al*, 1984). A large number of other structural proteins have also been identified in the human platelet cytoskeleton including actin binding protein, α-actinin, vinculin and tropomyosin.

Platelets contain only a few mitochondria (about 10 per platelet) and numerous glycogen granules for glycolysis, but by far their most numerous organelles are their four types of storage granules which are randomly distributed throughout the cytoplasm. Dense granules (δ-granules) are electron dense and contain high concentrations of 5-hydroxytryptamine which is not synthesised by the platelet but absorbed from the plasma through specific surface membrane receptor systems which operate across the granule membrane and the platelet plasma membrane (Pletscher, 1978). Alpha (α) granules are variable in size and shape and contain important groups of proteins. These proteins include the clotting factors, fibronectin, fibrinogen; platelet derived growth factor which stimulates wound healing; von Willebrand factor important in adhesion and regulating circulatory factor VIII levels; albumin and protease inhibitors. The remaining granules are the membrane bound lysosomes and the peroxisomes which are few in number and have peroxidase activity.

The contents of all these storage granules are specifically released upon platelet activation and pass via the canalicular system into the extracellular fluid where they are

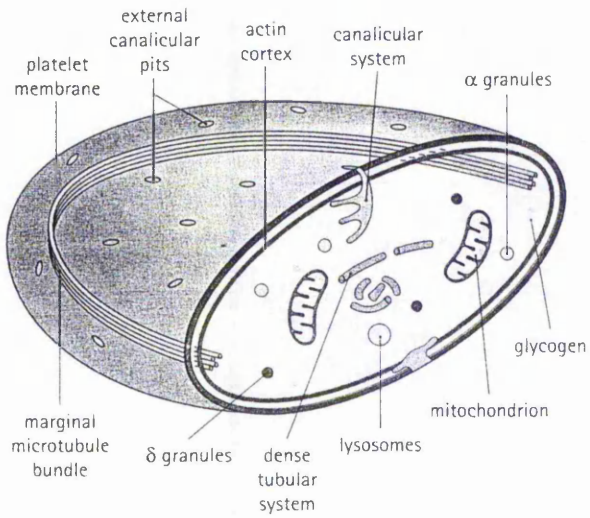


Figure 1.6. Diagrammatic representation of platelet structure (Stevens and Lowe, 1997).

important in maintaining vascular tone, haemostatic plug formation and ultimately vessel repair.

1.3.3 Platelet function

1.3.3.1 *Active transport*

Platelets have specific active transport systems for a number of substances, mainly adenine, adenosine and the vasoactive amines including 5-HT and dopamine. Adenine and adenosine are added to the cytoplasmic store of adenine nucleotides and provide the energy requirements for platelet activation.

By far the major transport system is that for 5-HT which enables the platelet to maintain its 5-HT concentration over a thousand times greater than that in the plasma. Once transported across the platelet plasma membrane 5-HT is taken up into dense δ granules and stored. The other amines, dopamine and noradrenaline are also transported into platelets and stored; however, these amines do not have a separate transport system and are taken up with relative low affinity by the 5-HT system.

1.3.3.2 *Platelet adhesion, aggregation and secretion*

Under normal circumstances platelets do not readily adhere to the surface of blood vessels, a factor important for maintaining blood flow. However, the formation of a haemostatic plug depends almost exclusively on the platelet reactions of adhesion, aggregation and secretion. This contribution is two-fold:- firstly they initiate the formation of a haemostatic plug at the site of injury and secondly they ensure efficient coagulation through the release of their granule contents when stimulated, or factors adsorbed on their surface. After endothelial damage, platelets adhere to the exposed collagen by interacting with specific glycoprotein receptors; this initiates platelet activation. The activated platelets change shape, extend pseudopodia, release the contents of their dense granules (5-HT) via their tubule and external pit system and aggregate with other platelets. They also synthesise thromboxane, which, coupled with ADP and Ca^{2+} ions is released and mediates the adhesion of other passing platelets. Platelet

phospholipids then activate the blood clotting cascade and ultimately form fibrin to plug the injury site. Fibrin strands form around the platelet plug trapping other blood cells, this fibrin mass then retracts into the wound forming a tightly packed plug (Gordon, 1981). The ability of platelets to initiate coagulation in the area of a platelet aggregate is also important for the maintenance of haemostasis.

1.3.4 Platelets as models for monoaminergic neurones

The ability of human platelets to respond to 5-HT, accumulate this vasopressor in dense storage granules via a specific receptor and secrete this agonist, coupled with the fact that they possess the B form of MAO, has prompted a great deal of clinical interest regarding their relevance and use as markers for psychiatric and neurologic disorders. As the MAO form in platelets is the same as the major form of MAO in the human brain, and is selectively inhibited by deprenyl, the platelet enzyme may be a useful model for Parkinson's Disease and a monitor for anti-Parkinson therapy. The importance of this theory and literature relating to it will be discussed in depth in Chapter three.

1.4 MONOAMINE OXIDASE IN OTHER NEUROLOGICAL DISORDERS

Many studies have been conducted to analyse monoamine oxidase activity in other neurological diseases. Most of these studies have targeted the use of platelets as biological markers for MAO-B in other tissues, mainly brain. It has thus been reported that there are correlations between MAO activity levels in platelets and certain personality or neurological disorders. The possibility of an accessible predictive marker for certain of these disease states has given rise to extensive published literature in an effort to ascertain whether or not diagnostic predictions can be made based on the analysis of the platelet enzyme. Published work concerning platelet MAO and Parkinson's Disease will be discussed in chapter 3.1.

Many reports have associated a low platelet MAO activity with a predisposition to the development of psychiatric disorders. However, confusion still exists as to whether or not platelet MAO analysis can indicate these disorders. By far the most studied psychiatric disorder with regard to platelet MAO activity is schizophrenia, but varying

results have been obtained. Indeed Rose *et al*, 1986 found no difference (in activity or concentration) between drug-free schizophrenic female patients and controls, but the concentration of enzyme in male patients was significantly increased. However there was no significant difference in enzyme activity in males as the molecular activity was reduced. Siever and Coursey (1985) reported that low platelet activity was not an adequate marker for schizophrenia but was associated with the characteristics of sensation seeking.

Platelet MAO activity has also been assessed in Alzheimers disease. The use of platelets as markers for this disease state may be of diagnostic if not predictive importance. It has been claimed that high platelet MAO activity may be useful as a diagnostic marker for Alzheimers Disease (Danielczyk *et al*, 1988; Fischer *et al*, 1994).

Some workers have also reported a link between platelet MAO activity and certain personality traits, such as sensation seeking behaviour and anxiety. Indeed a report by Smith, (1994) linked a low level of platelet MAO with the type A personality. This type is commonly called the coronary prone personality and is characterised by impatience, heightened pace of living and easily aroused hostility.

MAO activity has also been studied in groups of women with pre-menstrual syndrome. Ashby *et al* (1988) reported that platelet MAO activity was significantly decreased post-menstrually in patients suffering from pre-menstrual syndrome compared to the pre-menstrual phase. However Rapkin *et al* (1988) found no difference in platelet MAO-B activity throughout the cycle in control populations and in subjects with pre-menstrual syndrome.

It is evident that many psychiatric and neurological diseases and personality disorders are associated with a change in platelet MAO activity, and there is a possibility of a genetic link between brain MAO activity and a predisposition to development of these disorders.

1.5 MONOAMINE OXIDASE IN PERIPHERAL TISSUES

MAO has widespread distribution both within the body and between species. Its presence has been described in all the mammalian species examined and also some non-mammalian species (refer to Weyler *et al*, 1990, for review).

In mammals MAO has been identified in all cell types except erythrocytes. The enzyme is widely distributed and in the majority of tissues both forms of MAO co-localise. Liver and brain are rich in both forms of the enzyme but some other tissues express either one form or the other. Platelets (Donnelly and Murphy, 1977) and lymphocytes (Bond and Cundall, 1977) express only type B MAO whereas it has been reported that placenta expresses only type A (Egashira, 1976). However recent studies (Riley *et al*, 1989) have disputed the results with placenta claiming MAO-B is also present in this tissue (refer to section 1.6.2).

The distribution of MAO is thought to be determined by its physiological roles. Liver, kidney, intestine and placenta all contain either one or both forms of the enzyme in high concentrations where its function is to inactivate potentially harmful endogenous and exogenous amines by oxidising them in the blood or preventing their entry into the circulation.

Early attempts to identify human MAO localisation relied on activity studies involving crude tissue homogenates or mitochondrial preparations (Lewinsohn *et al*, 1980). Localisation of MAO was highlighted further using histochemical analysis of tissue sections to reveal the presence of the MAO-A or B enzymes *in situ*. One of the early reports providing this histochemical evidence of MAO-A distribution involved a coupled peroxidation technique (Ryder *et al*, 1979) employing benzylamine and tyramine as substrates and clorgyline, deprenyl, phenelzine and pargyline as specific inhibitors to determine the distribution of MAO-A in the human placenta. MAO-A was found to be localised in the trophoblastic layer of the chorionic villi.

This early distribution data has been superseded by the advent of immunochemical technology. Immunohistochemical analyses using specific monoclonal antibodies have

been employed to allow the independent detection of both MAO-A and MAO-B *in situ* due to their distinct immunological properties. An extensive study by Thorpe *et al*, 1987 using specific monoclonal antibodies gave an important insight into the localisation of both enzyme forms in human peripheral tissues and brain. This study confirmed that MAO-B alone was found in platelets and was also the form found in lymphocytes whereas MAO-A was observed in the syncytiotrophoblast layer of term placenta. Both forms of enzyme were present in the liver and various neurones of the brain.

An extensive study of the distribution of MAO in the periphery was published in 1996 by Saura and colleagues where MAO localisation was determined in human autopsy cases using specific MAO-A and B radioligands and mapped using autoradiography. This study showed that both forms of MAO were present in heart, lung, liver, kidney, spleen and duodenum. However, some tissue types within these organs showed marked differences. In the duodenum for example, MAO-A was most abundant especially in the mucosa, whereas MAO-B was highest in muscular layers. Both isoenzymes were present in large amounts in the cortex and medulla of the kidney compared to the glomeruli which contained relatively low levels. MAO-A and B were present at high levels in heart myocardium. Both enzymes had homogeneous distribution in liver with MAO-B being noted as being slightly more abundant, lung contained moderate levels of both enzymes.

This study however had been restricted to general tissue distribution and did not allow the determination of the cellular distribution pattern for both isoenzymes. Using monoclonal antibodies produced in our laboratory, 6G11/E1 (MAO-A) and 3F12/G10/2E3 (MAO-B), immunohistochemical studies have been conducted in an attempt to elucidate the cellular distribution of MAO in peripheral tissues. Similar localisation of both isoenzymes was evident in the various cell types of heart, liver, duodenum and blood vessels. However distribution differences were noted in kidney. For example, cells of the glomeruli in the kidney generally showed no staining with either antibody except in the Bowman's capsule and podocytes where only MAO-A immunoreactivity was evident (Rodriguez *et al*, in preparation).The localisation of both enzymes was similar in spleen and lung (Rodriguez *et al*, in press [a]). However differences in distribution were observed in pancreas, thyroid gland and adrenal gland

(Rodriguez *et al*, accepted with modifications [b]) These studies show a comprehensive assessment of peripheral MAO showing widespread distribution and frequent co-localisation which is in contrast to brain MAO where co-localisation is rare.

1.6 MONOAMINE OXIDASE AND THE PLACENTA

1.6.1 Placental physiology

1.6.1.1 *Development of the human placenta*

After fertilisation the newly formed zygote passes through the fallopian tube and undergoes a process of holoblastic cleavage whereby cells divide to form a solid cluster called the morula. Three to four days after ovulation the morula enters the uterus, a fluid filled cavity (blastocele) forms within the morula and the whole structure is now termed the blastocyst which exists in the uterine secretions for approximately three days. At this stage of development, the primary precursor of the foetal placenta is evident as a single layer of cells around the outside of the blastocyst. This layer is termed the trophoblast. The remaining cells are clumped together at one end of the inner surface of the trophoblast forming the inner cell mass. The trophoblast will ultimately develop to form the placenta while the inner cell mass becomes the foetus and amnion. The formation of the placenta is summarised in a flow diagram (Figure 1.7).

After 2-3 days in the uterus the trophoblast becomes sticky, initiating the adherence of the blastocyst to the uterine wall. As trophoblast cells come into contact with uterine epithelium they proliferate and attach to the endometrial wall. This embryonic complex then penetrates further into the highly vascular endometrial connective tissue where embedding takes place; this process is normally complete eleven days after ovulation when the endometrium completely closes over the site. The trophoblast then differentiates into two distinct layers; an inner layer of cytotrophoblast composed of individual cells producing human chorionic gonadotrophin, important for maintaining pregnancy, and a broad outer layer of syncytiotrophoblast which consists of a multinucleated cytoplasmic mass which is capable of breaking down tissue, as in the

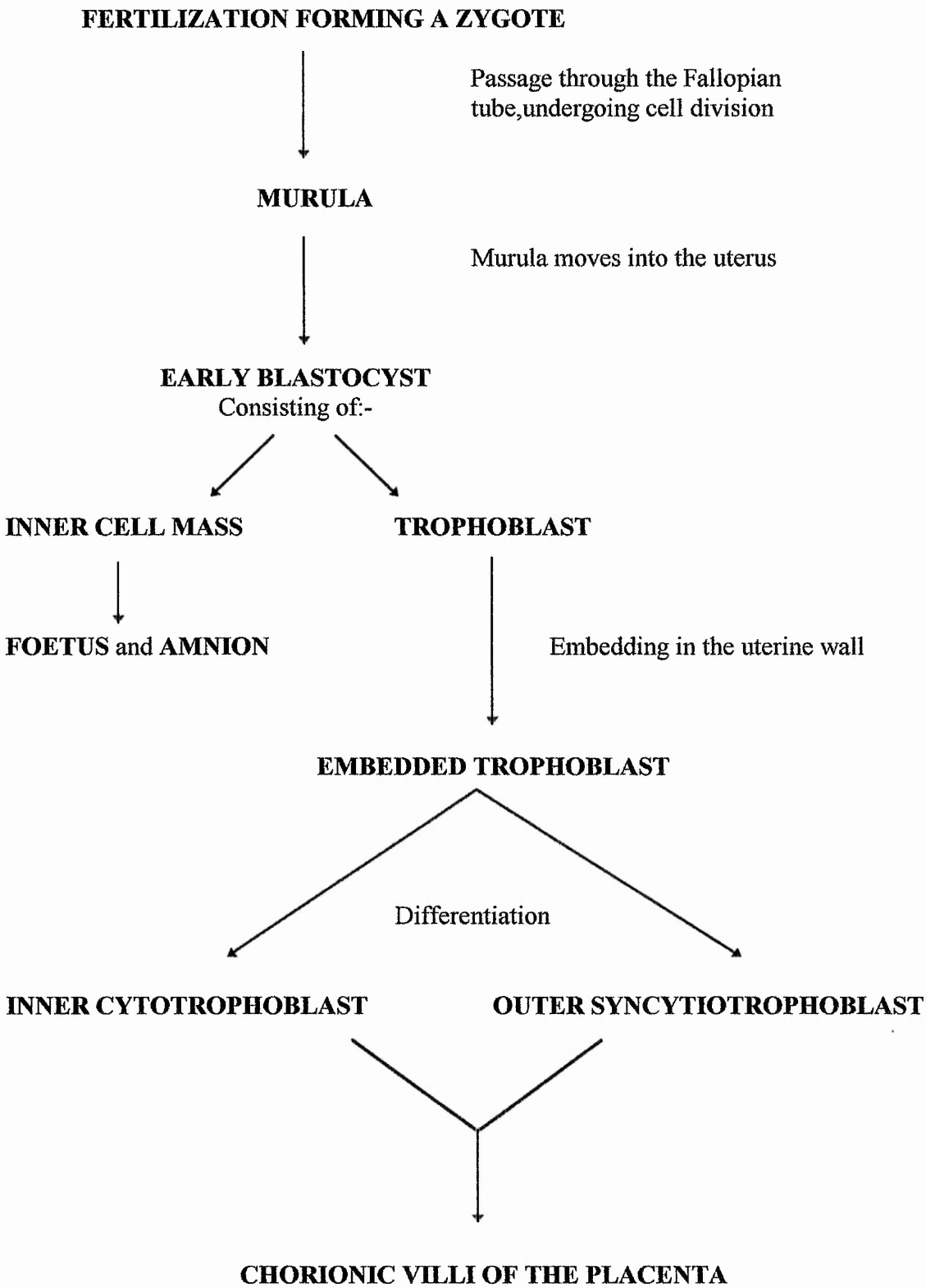


Figure 1.7. Diagramatic representation of the formation of the placenta

process of embedding (Figure 1.8). This layer is important in eroding the walls of the maternal decidua (endometrium during pregnancy) enabling nutritional exchange between maternal and foetal circulation.

The trophoblastic layer continues to proliferate forming projections called the chorionic villi. These villi become most numerous in the basal decidua where the maternal blood supply is the richest. This part of the trophoblast is termed the chorion frondosum which eventually develops into the placenta. The villi under the capsular decidua (covering the basal decidua) encounter a poor nutritional blood supply and gradually degenerate to eventually become the chorionic membrane. The chorionic villi gradually erode the walls of maternal blood vessels as they penetrate the decidua, eventually opening them up into a pool of maternal blood forming blood sinuses and blood spaces (Figure 1.9).

Maternal blood has slow circulation so as to enable efficient absorption of nutrients and oxygen and excretion of waste by the villi. Villi involved in absorption form the nutritive villi and those embedded more deeply into the decidua are the anchoring villi. Each chorionic villus consists of a single stem with many branches (Figure 1.10a). The centre is made up of mesoderm and branches of the umbilical artery and vein. Each villus is covered by a single layer of cytotrophoblast cells and an external layer of syncytiotrophoblast cells. These tissue layers make it impossible for foetal and maternal blood circulations to mix. The placenta is completely formed and functioning from approximately ten weeks after fertilisation, however early in gestation it is a fairly loose structure only becoming more compacted as it matures.

Foetal blood is pumped by the foetal heart along the umbilical arteries via their many branches to the capillaries of the chorionic villi, absorption of oxygen takes place and blood is returned via the umbilical vein. Maternal blood is transported to the placental bed by the spiral arteries where it circulates in the blood spaces surrounding the villi and drains back into branches of the uterine vein (Figure 1.10b).

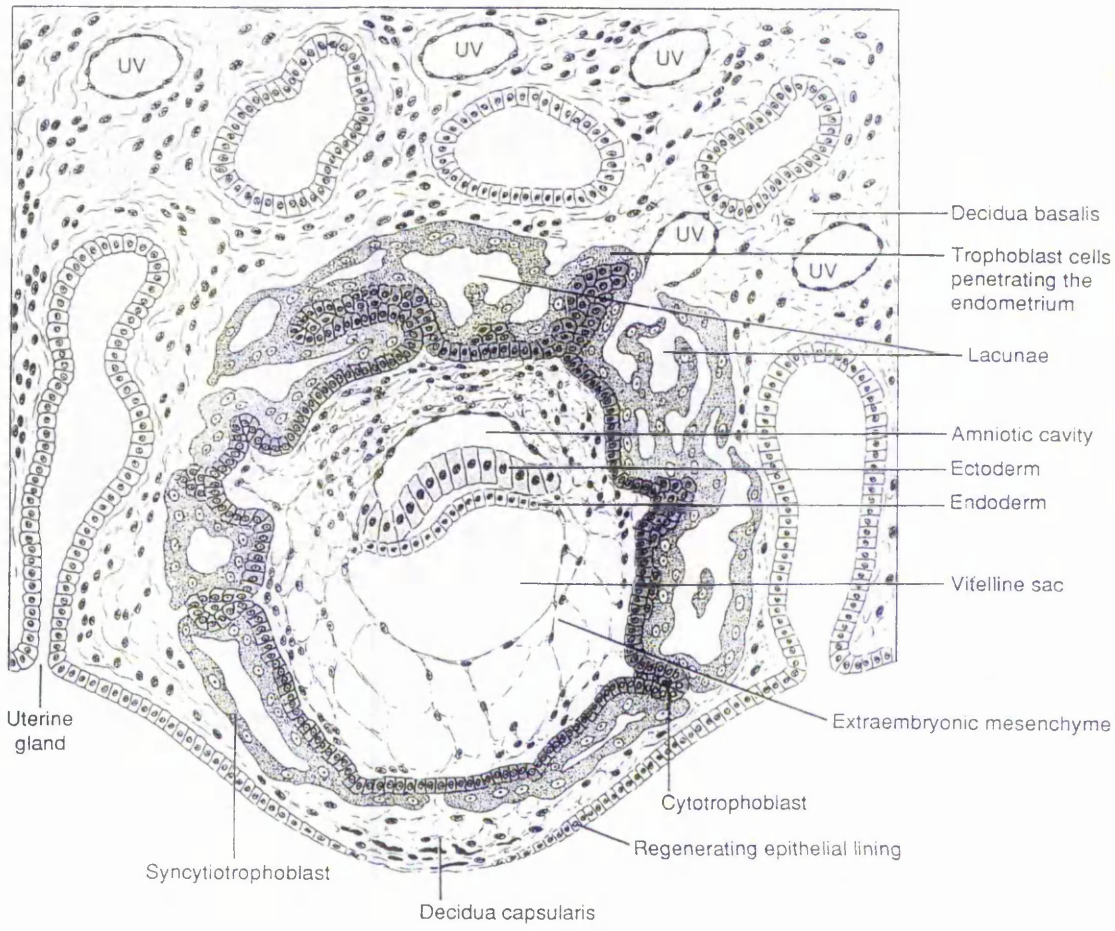


Figure 1.8. Diagram showing a human embryo at the end of implantation. UV = uterine veins. (From : Basic Histology, Junqueira *et al*, 1995)

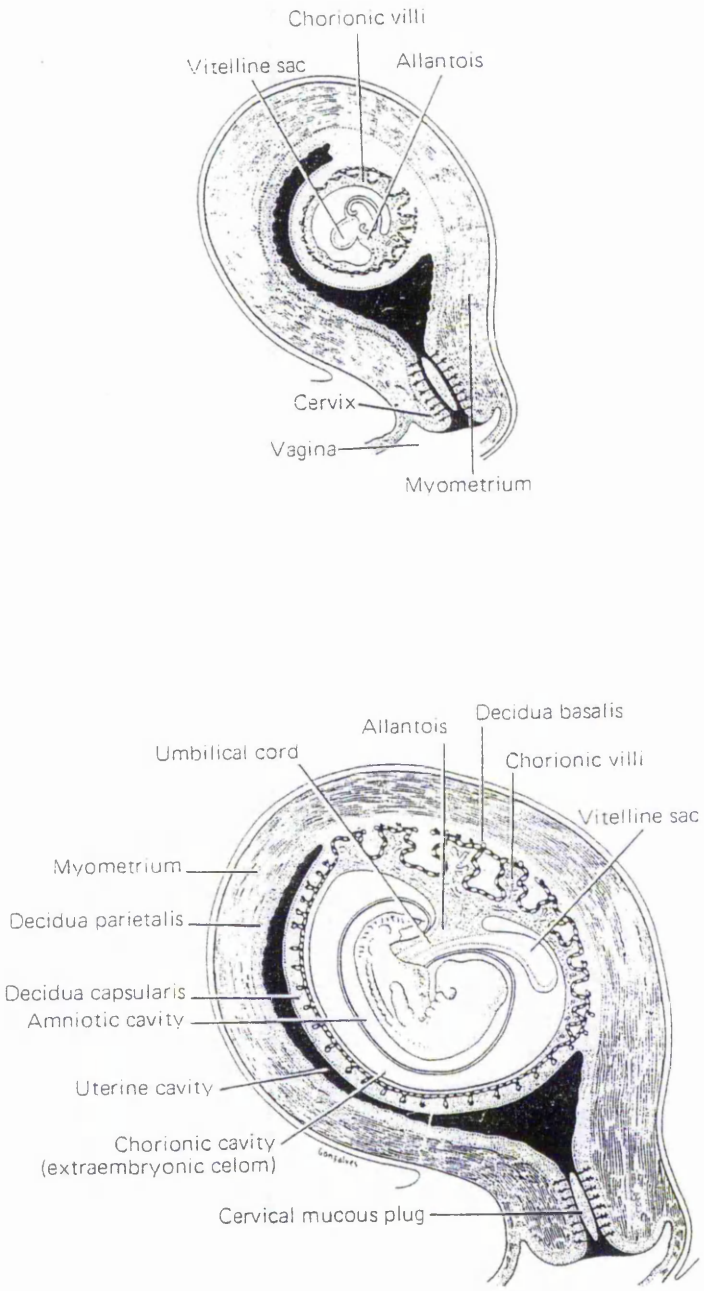
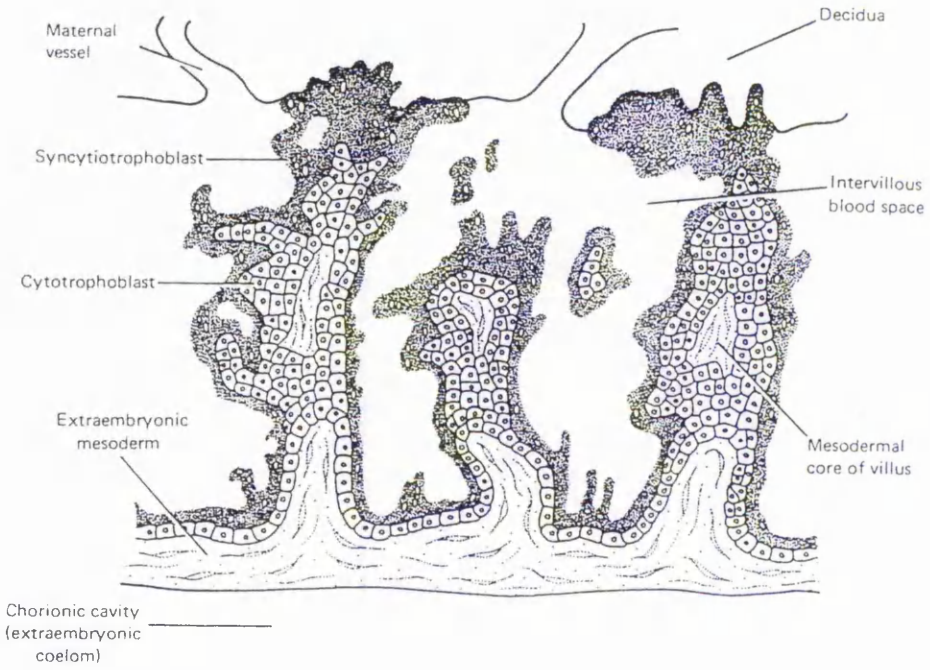


Figure 1.9. Diagram showing the formation of the decidua and the chorionic villi of the placenta. (From : Basic Histology, Junqueira *et al*, 1995)

a



b

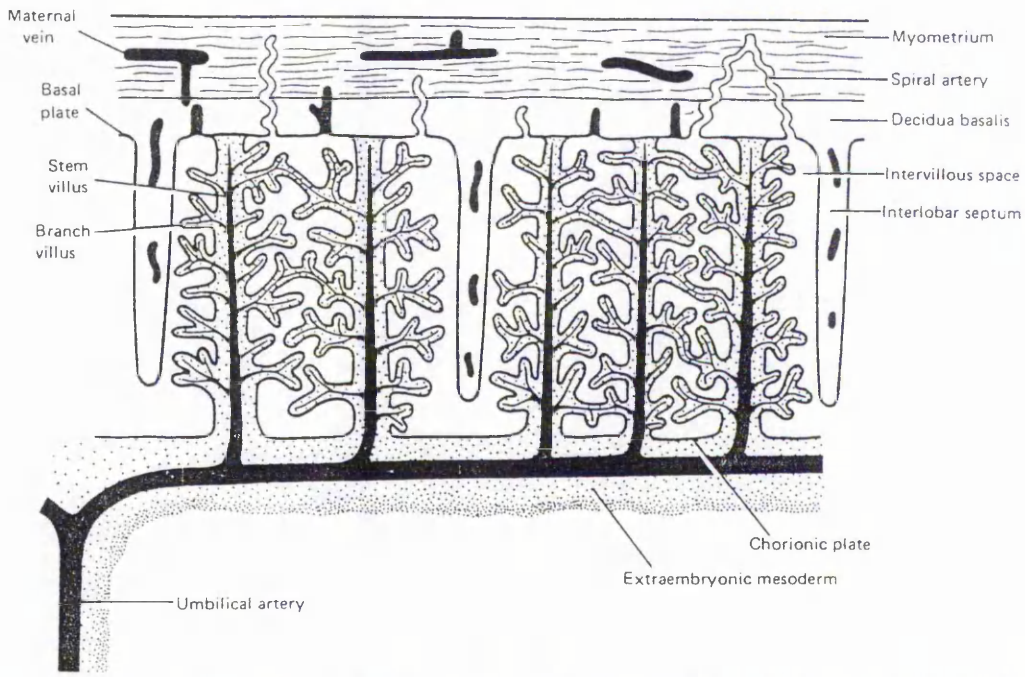


Figure 1.10a. Stem villus stage of the placenta. Chorionic villi have eroded maternal tissue so that maternal blood flows between the villi.
Figure 1.10b. Organisation of term placenta. (From : Human Reproduction and Developmental Biology, Begley *et al*, 1980)

1.6.1.2 *Functions of the mature placenta*

The functions of the placenta are complex but can be divided into three main groups: metabolism, transport of substances and endocrine secretion. The placenta, during early pregnancy synthesises glycogen, cholesterol and fatty acids which are valuable nutritional sources for the growing embryo. As pulmonary gaseous exchange does not take place in the uterus the foetus obtains oxygen and excretes carbon dioxide through the placenta. The placental membrane is almost as efficient as the lungs for gaseous exchange. The quantity of oxygen reaching the foetus is primarily flow limited and exchange takes place by simple diffusion, hence foetal hypoxia results primarily from factors reducing either uterine blood flow or foetal blood flow.

Glucose transport into the placental cells occurs by facilitated diffusion whereas amino acids rely on a specific active transport mechanism. Water is rapidly exchanged by simple diffusion and increases as pregnancy advances. There is little or no transfer of maternal cholesterol, triglycerides or phospholipids. Vitamins cross the placenta and are essential for normal development.

The foetus itself has a poor capacity to produce antibodies until well after birth, hence, some passive immunity is passed to the foetus by placental transfer of maternal antibodies (especially IgG). The placenta provides a limited barrier to infection; whilst few bacteria can penetrate (except the treponema of syphilis and the tuberculosis bacillus), viruses can cross freely increasing the risk of development of congenital abnormalities (eg. rubella virus).

Importantly, the placenta acts as an endocrine organ. The cytotrophoblastic layer of the chorionic villi produces human chorionic gonadotrophin which stimulates the growth and activity of the corpus luteum to prevent the onset of the next menstrual period. As the activity of the corpus luteum declines the placenta takes over the production of oestrogens. Progesterone is synthesised in the syncitial layer from maternal cholesterol.

1.6.2 Placental monoamine oxidase

1.6.2.1 *Localisation of MAO in the placenta*

Many reports have been published regarding the localisation and type of MAO in human placenta. Using histochemical studies involving specific substrates and inhibitors in a coupled peroxidation technique Ryder *et al*, 1979 showed the presence of MAO-A in frozen sections of term human placenta and that the enzyme was specifically localised in the trophoblastic layer of the chorionic villi. This study was further substantiated by work from Yoshimoto *et al* in 1986 who used an enhanced peroxidation method involving diaminobenzidine and nickel chelation to determine the precise location of MAO activity on cryostat sections. Human chorionic and placental tissues were analysed for MAO activity at various gestation times. This study found MAO activity present in the trophoblastic layer of villi as little as six weeks post conception. At higher magnification MAO activity was found to be localised to the syncytiotrophoblast cell layer where the relationship between mother and foetus is highest. No activity was evident in the cytotrophoblast cell layer or the interstitial regions of the villi. The localisation and staining intensity was similar for all gestation times assessed (i.e. 6, 10, 16, 28, 40 weeks) implicating that MAO activity is essential for continued pregnancy and maintenance of foetal life. One other important finding resulting from this study was that only a single MAO type, notably MAO-A was present in all the tissues studied.

MAO activity was also studied in a variety of other placental and foetal tissue types by Saarikoski and colleagues (1983). This study found high activity in placenta and chorio-amniotic membranes, very low activity in the umbilical artery and vein and no activity in the Whartons jelly of the umbilical cord.

The type of MAO in human placenta is still a controversial question, with many groups traditionally believing that placenta contains exclusively MAO-A (eg. Egashira, 1976) while others have found evidence for the presence of small concentrations of MAO-B. In 1989 Riley and co-workers analysed placental mitochondrial homogenates using specific anti- MAO-A and MAO-B monoclonal antibodies. Western blot analysis

provided evidence that, although the major enzyme type in placental preparations was MAO-A, these preparations also contained low but significant amounts of MAO-B. A similar result was obtained using the tritiated selective MAO-B inhibitor (Ro 16-6491) by Cesura *et al*, 1989, who stated that in homogenates of placentae, 3-5% of the total MAO was identified as MAO-B. However they were not clear as to whether this MAO-B was intrinsic to placental cells or contaminating MAO-B from blood platelets or lymphocytes.

More recently, a study by Church *et al*, 1994 has used a MAO-A specific monoclonal antibody, 6G11/E1, prepared in our laboratory to map the immunoreactivity of MAO-A in cryostat sections of the placenta, foetal membranes and umbilical cord. This work has shown the most detailed distribution of MAO-A to date. MAO-A is present in the cytoplasm of syncytiotrophoblastic cells and also the cytoplasm of intermediate trophoblasts in the chorion, as well as in the smooth muscles of the foetal vessels in the chorionic villi. Presence of MAO-A immunoreactivity was evident in the cuboidal epithelium of the foetal membranes and the cytoplasm of the epithelium covering the umbilical cord. In all the tissues studied the staining had a particulate distribution consistent with localisation to the mitochondria.

Another more recent study has looked at MAO-B in placenta (Sekizawa *et al*, 1995); concentrating on the analysis of MAO in foetal membranes and describes evidence for both MAO-A and MAO-B activities. The work also documents the relative concentrations of MAO-A in mitochondrial fractions of various tissues, namely placenta, decidua, chorion and amnion. Results from these findings reveal that MAO-A activity was highest in the placental body with decreasing concentrations in the decidua, chorion and amnion. Significant MAO-B activity was evident in the decidua with small activity observed in the placenta, amnion and chorion.

1.6.2.2 *The role of MAO in human placenta*

The role and type of MAO in placenta is still a matter of debate. However, the general theory centres around the idea that it is MAO-A which is important in limiting placental transfer of vasopressor substances especially 5-HT to the foetus. Hence its position in

the boundary layer of the syncytiotrophoblast, umbilical cord and blood vessel walls and its preferential substrate, 5-HT make MAO-A a key enzyme to study in relation to placental function and pregnancy induced hypertension (pre-eclampsia) pathology. Work relating to the study of MAO in the human placenta and its possible role in pre-eclampsia will be discussed in depth in Chapter 4.

1.7 AIMS OF THIS STUDY

1. To check the specificity of MAO-A and MAO-B monoclonal antibodies prepared in our laboratory so that they can be used to assess the status of MAO in two tissue samples namely platelets and placenta.

2. To assess the value of using platelet MAO as a predictive marker for Parkinson's Disease. Platelet MAO-B activity and concentration will be measured concomitantly in age-matched *de novo* Parkinson's Disease patients and controls from two populations, namely Caucasian and Hong Kong Chinese. This will allow an assessment of the molecular activity of MAO-B in Parkinson's Disease. MAO-B protein will be analysed by a competitive ELISA and activity assays will be conducted using two different substrates (phenylethylamine and dopamine).

3. To assess both the status and identity of MAO in human placenta. This will be achieved using competitive ELISAs, activity assays and immunochemical techniques to assess enzyme identity and its distribution *in situ.*

4. To assess whether MAO is important in pregnancy induced hypertension (Pre-eclampsia). A preliminary study of the status of MAO-A in placentae from patients with pregnancy induced hypertension compared to normotensive controls will be conducted.

CHAPTER TWO

DEVELOPMENT OF REAGENTS AND IMMUNOASSAYS

CHAPTER 2

DEVELOPMENT OF REAGENTS AND ASSAY OPTIMISATION

2.1 INTRODUCTION

This chapter describes the development and characterisation of reagents and assays prior to their use in subsequent sample analysis.

Purification of human MAO

The procedure employed to purify MAO from human liver was as described by Billett and Mayer, 1986 and uses conventional protein purification techniques. The method is multi stepped and involves the preparation of mitochondria by differential centrifugation, mitochondrial membranes (MM) by sucrose density centrifugation followed by purification steps involving a combination of gel filtration, ion-exchange chromatography and ammonium sulphate precipitation. The final product is enriched in MAO and contains both isoforms. Mitochondrial membranes were used as both a coating antigen and a standard in competitive ELISAs for both MAO-A and MAO-B. Purified MAO was also used as a standard in these ELISAs.

Antibody Characterisation

Two murine monoclonal antibodies were used for the specific detection of human MAO-A and MAO-B. Frozen stocks of two hybridoma cell lines secreting MAO specific monoclonal antibody, (3F12/G10, anti human MAO-B and 6G11/E1, anti human MAO-A) were cultured to produce working stocks of the two monoclonal antibodies.

The monoclonal antibody specific for MAO-B was originally prepared and characterised by Billett and Mayer, 1986. A low and steadily declining working concentration (titre) for 3F12/G10 meant that this cell line required re-cloning to attempt to recover titre. Following successful cloning and appropriate screening the cell line 3F12/G10/2E3 was used as a source of antibody specific for MAO-B in subsequent characterisations.

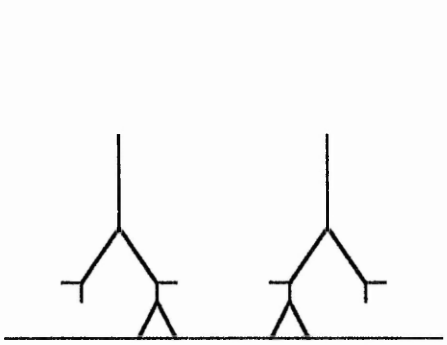
The monoclonal antibody specific for MAO-A had been prepared by Yeomanson, 1990, and preliminary characterisation had been achieved using Western blots. This chapter describes further characterisation of this antibody and determination of its specificity for MAO-A using a number of different techniques.

Immunoassays

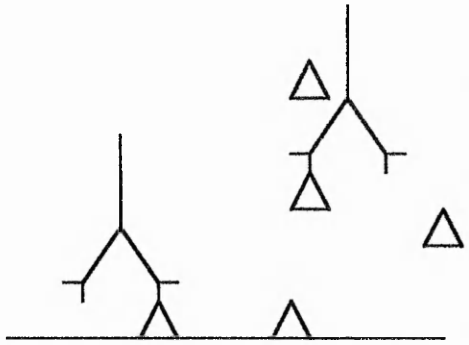
Optimisation of the MAO-B specific competitive ELISA (Yeomanson and Billett, 1992) and development of a MAO-A specific competitive ELISA is also outlined in this chapter. The competitive ELISA used is based on the principle whereby antigen used to coat wells of a microtitre plate (immobile antigen), competes with antigen in solution (free antigen) present in samples (either standards or unknown) for a limited concentration of specific monoclonal antibody (either 3F12/G10/2E3 or 6G11/E1). Consequently the higher the concentration of free antigen the less antibody binds to the immobilised antigen. Primary antibody binding is revealed by a second, enzyme-conjugated polyclonal antibody raised to mouse immunoglobulin, and the amount of bound second antibody is inversely proportional to the amount of free antigen present in the sample (Figure 2.1). Both of these assays were used in the detection and quantification of MAO-B and MAO-A in samples of human platelets and human placentae, described in chapters 3 and 4.

1. Antigen is bound to the solid phase of a microtitre well

2. Incubation with primary antibody

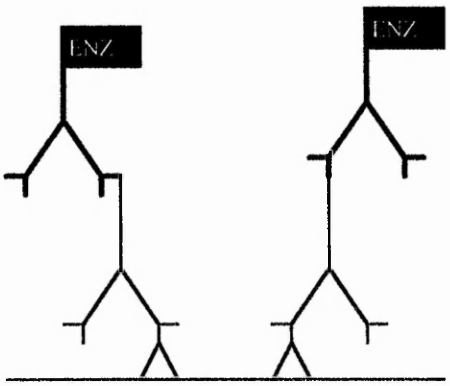


NO FREE ANTIGEN

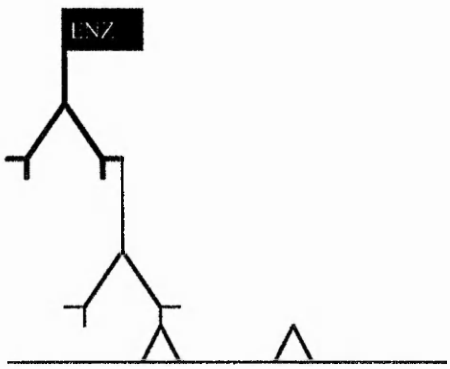


PLUS FREE ANTIGEN

3. Incubation with labelled secondary antibody



NO FREE ANTIGEN



PLUS FREE ANTIGEN

4. The amount of bound enzyme conjugated antibody is inversely proportional to the amount of free antigen present.

Figure 2.1. Schematic diagram of a competitive heterogeneous ELISA

2.2 MATERIALS AND METHODS

All chemicals were of the highest grade available and supplied by Sigma-Aldrich Company Limited unless otherwise stated.

2.2.1 Purification of mitochondrial membranes

2.2.1.1 *Materials*

Class I safety cabinet (Hepaire, BS5726)
Homogeniser (Potter S; B. Braun)
MSE 21 Centrifuge (MSE Scientific Instruments Ltd.)
MSE 24m Centrifuge (MSE Scientific Instruments Ltd.)
Beckman L8-70 Ultracentrifuge (Rotors: 6 x 26 ml 70Ti; 6 x 38.5 ml swingout, SW28)
Sonicator (Soniprep 150, MSE Scientific Instruments)
Liquid scintillation counter (Canberra Packard A 300 CD)
Buffered sucrose solution (see appendix)
Solution 1 (see appendix)
10 mM Tris-Phosphate pH 7.5
1.2 M Sucrose
0.25 M Sucrose
1.0 M Sodium chloride
Phosphate buffer pH 7.6 (see appendix)

2.2.1.2 *Method*

Mitochondrial membranes were isolated from normal human liver at post mortem, less than 24 hours after death (Figure 2.2). All procedures were performed in a class I safety cabinet and at 4 °C unless stated otherwise.

1. 100 g of human liver was cooled on ice following removal at post mortem.

2. Obvious gristle was removed and the liver was minced finely using scissors.

3. 10 g portions were homogenised in five volumes of buffered sucrose solution, pH 7.4 with three passes of a close fitting glass/Teflon homogeniser. This procedure was repeated with a tighter fitting homogeniser.

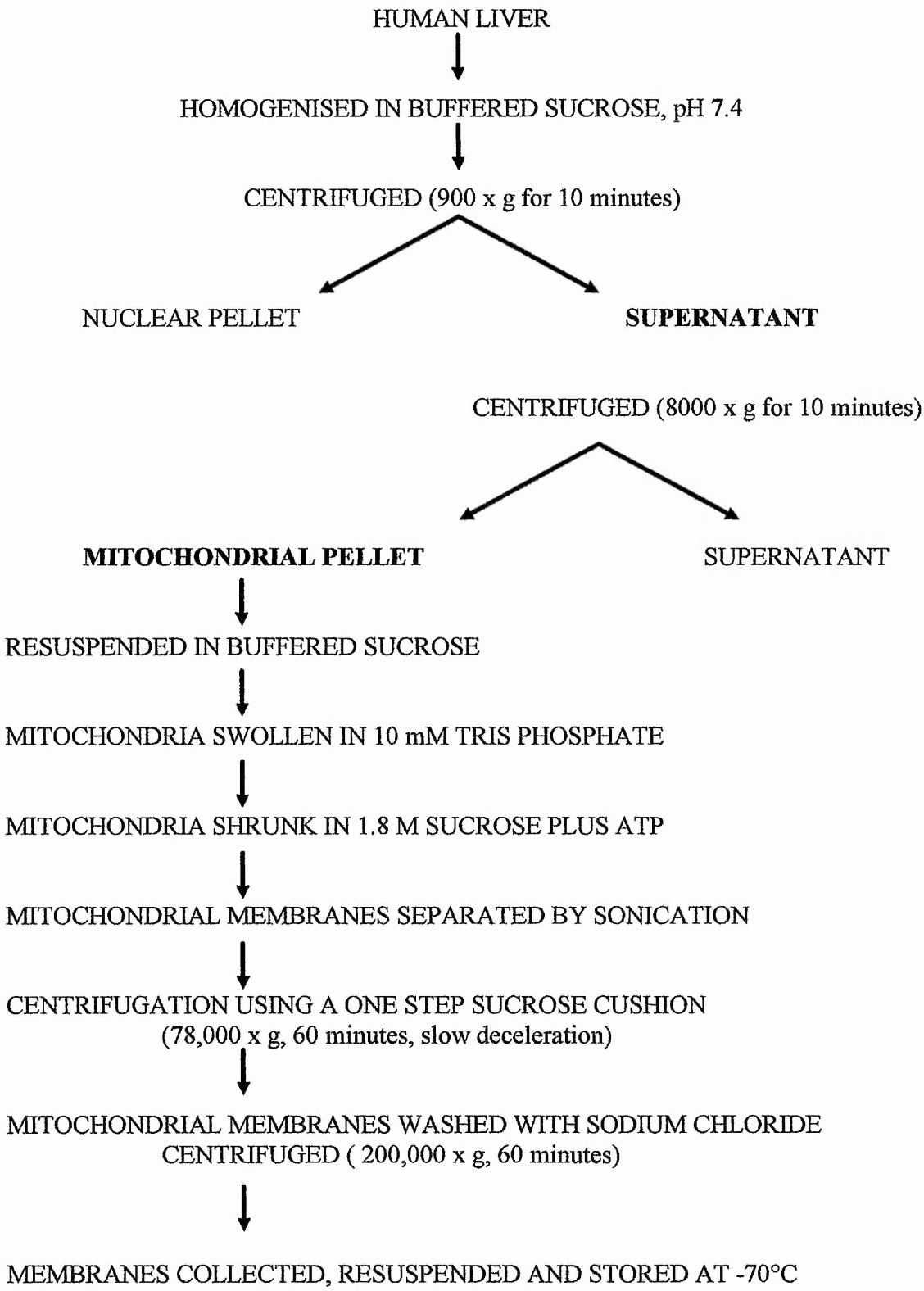


Figure 2.2. Flow diagram describing the production of mitochondrial membranes from human liver

4. The combined homogenate was centrifuged at 900 x g for 10 minutes (MSE 21 centrifuge).

5. The supernatant was collected and centrifuged at 8000 x g for 10 minutes.

6. The resulting supernatant was discarded and the pooled pellet resuspended in 250 ml buffered sucrose solution before centrifugation at 8000 x g for 10 minutes (MSE 21 centrifuge).

7. The final pellet was washed by resuspension in 125 ml buffered sucrose solution and centrifuged at 8000 x g for 10 minutes.

8. The pooled pellet, enriched in mitochondria was resuspended in 67.5 ml 10 mM Tris-Phosphate buffer pH 7.5 to swell the mitochondria and left stirring gently on ice for 10 minutes.

9. 22.5 ml Solution 1 was added and the sample left stirring for a further 10 minutes.

10. The sample was then divided into 6 x 15 ml aliquots and sonicated at 8 kilocycles/second twice for 10 seconds using an immersion probe.

11. Separation of the mitochondrial membranes from the mitoplast was achieved using a one-step sucrose cushion. 15 ml aliquots of the sample were layered onto 15 ml 1.2 M sucrose, taking care not to allow the two layers to mix, and the samples were then overlayed with 0.25 M sucrose to the top of the tube.

12. The tubes were centrifuged (Beckman Ultracentrifuge) at 78,000 x g for 60 minutes with the brake off for slow deceleration.

13. Bands containing the mitochondrial membrane fraction were aspirated with a 0.8 mm bore needle and mixed with at least 2 volumes of cold 1.0 M NaCl to remove any loosely bound protein.

14. The resulting sample was centrifuged (Beckman Ultracentrifuge) at 200,000 x g for 60 minutes.

15. The pooled pellet was resuspended by sonication in 10 ml 100 mM phosphate buffer, pH 7.6 and stored in 0.5 ml aliquots at -70 °C.

2.2.2 Estimation of MAO activity

2.2.2.1 *Materials*

20 mM Potassium phosphate buffer, pH 7.2 (see appendix).

Scintillation fluid (see appendix)

^{14}C tyramine hydrochloride (Amersham)

Liquid scintillation counter (Canberra Packard A 300 CD)

2.2.2.2 *Method*

This assay is based on that of Russell and Mayer, 1983, and relies on the ability of MAO to convert the water soluble substrate ^{14}C tyramine into its corresponding ^{14}C aldehyde product which is soluble in the organic phase.

1. Samples were made to a final volume of 200 µl using 20 mM potassium phosphate buffer, pH 7.4, vortex mixed and incubated for 5 minutes at 37°C.

2. 20 µl (0.02 µCi), 1mM ^{14}C-tyramine hydrochloride (1 mCi/mmol) were added to each sample, vortex mixed and incubated for a further 30 minutes at 37°C.

3. The reaction was stopped by the addition of 200 µl of 0.5 M HCl to each sample and 3.0 ml scintillation fluid added; samples were mixed and counted in a liquid scintillation counter.

2.2.3 Solubilisation of mitochondrial membranes

2.2.3.1 *Materials*

MSE 21 Centrifuge (MSE Scientific Instruments Ltd.)

Triton X-100

Phenylmethylsulphonyl fluoride (PMSF)

2.2.3.2 *Method*

1. Triton X-100 was added to 0.5 ml mitochondrial membranes to produce a final concentration of 1% (v/v).

2. The serine protease inhibitor, PMSF was added to the sample at a final concentration of 0.174 mg/ml (1 mM).

3. The sample was stirred slowly at 4°C for 60 minutes and centrifuged at 100,000 x g for 20 minutes.

4. The protein content of the resulting supernatant was estimated by the method of Lowry *et al,* (1951) (Section 2.2.14) and the solubilised mitochondrial membranes used immediately in the ELISA assay.

2.2.4 Ascites production

Ascites for 3F12/G10/2E3 and 6G11/E1 was produced at The Queens Medical Centre, Nottingham. Hybridoma cells (0.5 x 10^7 cells) resuspended in 0.5 ml sterile RPMI-1640 media were injected into mice injected with pristane 3 weeks - 3 months previously. After at least 8 days ascitic fluid was collected and transferred into another pristane treated mouse. After a further 8 days ascitic fluid was transferred to a non-pristane treated mouse. This process was repeated until the cell line was established. Ascitic fluid was collected and stored at -20 °C.

2.2.5 Ammonium sulphate precipitation of immunoglobulin from murine ascitic fluid

2.2.5.1 *Materials*

MSE 21 Centrifuge (MSE Scientific Instruments Ltd.)
50 % (w/v) saturated ammonium sulphate
Solid ammonium sulphate
20 mM Phosphate buffer, pH 8.0 (see appendix)
10 mM Phosphate buffer, pH 8.0

2.2.5.2 *Method*

1. Ascites samples were thawed from storage at -20 °C, centrifuged (1000 x g, 10 minutes) to remove debris and solid ammonium sulphate was added slowly over a 30 minute period to produce 50 % saturation (2.91 g / 10 ml) whilst constantly stirring on ice.
2. After the final addition the sample was left to stir on ice for a further 30 minutes before centrifuging at 8000 x g for 30 minutes.

3. The supernatant was discarded and the pellet washed in 50 % (w/v) saturated ammonium sulphate to remove any remaining haemoglobin before centrifugation at 8000 x g for 30 minutes. This procedure was repeated until all haemoglobin had been removed.

4. The final pellet was dissolved in half its original volume of 10 mM phosphate buffer, pH 8.0 and dialysed overnight against 2 litres distilled water per sample preparation.

5. The sample was subsequently dialysed again overnight against 2 litres 20 mM phosphate buffer before centrifugation at 8000 x g for 30 minutes to remove any bound lipoprotein and stored in aliquots at -20 °C.

2.2.6 Cell culture

All media for cell culture were made 24 hours prior to use and an aliquot checked for sterility at 37 °C overnight. All procedures were performed in a Class II flow hood (Gelaire BSB 4 Class II Hood, Flow Laboratories) using aseptic techniques

2.2.6.1 *Thawing of hybridoma cell stocks*

2.2.6.1.1 *Materials*

Class II flow hood (Gelaire BSB 4)
Incubator (Jouan IG 150)
Centrifuge (MSE Centaur 2)
24 well sterile tissue culture treated plates (Corning 25820)
RPMI-1640 (Roswell Park Memorial Institute) growth medium
L-glutamine 200 mM (i.e. 100 x concentrated)
Sterile 5 ml pipettes
Sterile plugged Pasteur pipettes

2.2.6.1.2 *Method*

Rapid cell thawing is essential for maximum cell viability.

1. Cells were thawed from storage in liquid nitrogen at -140 °C by gently agitating the freezing vials in a water bath at 37 °C.

2. Immediately after thawing the cells were washed to remove the cryoprotectant (DMSO) by mixing with 20 ml RPMI-1640 containing sterile L-glutamine (final concentration, 4 mM) and centrifuged at 250 x g for 5 minutes.

3. The supernatant was discarded and the cell pellet resuspended in 1 ml of growth medium (varies according to the cell line).

4. The cells were then plated out into 24 - well tissue culture plates, 0.5 ml per well and placed in a humidified incubator at 37 °C, 10 % (v/v) CO_2.

2.2.6.2 *Maintenance of cells in culture*

2.2.6.2.1 *Materials*

Hybridoma medium (see appendix)

24 well sterile tissue culture treated plates (Corning 25820)

2.2.6.2.2 *Method*

After thawing, hybridoma cells were grown as monolayers in culture at 37 °C in a humidified incubator containing 10 % (v/v) CO_2. Cells were observed daily and sub-cultured on approaching confluency.

1. Cells were loosened from the base of tissue culture wells and resuspended by gently squirting the culture medium in and out of the wells using a sterile plugged Pasteur pipette, taking care not to create too much frothing.

2. Half of the medium (containing half of the cells) was transferred to a new well and an equivalent volume of fresh hybridoma medium added to each of the wells to feed the culture. This sub-culturing was repeated when required (commonly at 2 day intervals).

2.2.6.3 *Cryopreservation of cells*

2.2.6.3.1 *Materials*

Centrifuge MSE Centaur 2
Freezing mixture (see appendix)

2.2.6.3.2 *Method*

1. Cells were counted, harvested and collected as a pellet by centrifugation at 250 x g for 5 minutes.
2. Approximately 2 x 10^6 cells were resuspended in 1.0 ml ice-cold freezing mixture, immediately transferred to pre cooled freezing vials and placed at -70 °C for gradual freezing (1 °C per minute) prior to transfer to a liquid nitrogen cooled (-140 °C) cell bank for long term storage.

2.2.6.4 *Preparation of feeder cell layer*

2.2.6.4.1 *Materials*

Haemocytometer
1 % (v/v) Savlon antiseptic in distilled water. (Zyma Healthcare)
70 % (v/v) Ethanol
Freshly killed female rat (approximately 180 g)
Feeder medium (see appendix)
Sterile dissecting equipment
96 well sterile tissue culture treated plates (Corning)

2.2.6.4.2 *Method*

1 A female rat of approximately 180 g was killed by exposure to carbon dioxide and immersed in 1.0 % (v/v) Savlon antiseptic for 5 minutes prior to pinning out onto a dissecting board inside a class II flow hood.
2. The skin was swabbed with 70 % (v/v) ethanol and the skin from the left hand side of the animal removed. The peritoneal wall was also washed with 70 % ethanol.
3. 10 ml of cold (4 °C) feeder medium was injected (taking care not to puncture any internal organs) into the peritoneal cavity using a 10 ml syringe fitted with a 0.8 mm

bore needle and massaged around the cavity by gently agitating the peritoneum on the right side (peritoneal lavage).

4. Medium, containing macrophages, fibroblasts and lymphocytes (feeder cells) was withdrawn from the cavity, the number counted using a haemocytometer and kept on ice until required.

5. The feeder cells were then diluted accordingly with feeder medium before dispensing in 96 well tissue culture plates. A total of 0.5 x 10^6 cells was required for one 96 well plate in a total volume of 12 ml. Using a 5 ml pipette (Volac, blowout, supplied by Merck) 2 drops of the feeder cell suspension was dispensed into each well of a 96 well plate prior to incubating in a humidified incubator at 37 °C with 10 % (v/v) carbon dioxide.

6. Feeder medium was aspirated using sterile apparatus prior to use. Macrophages and lymphocytes remain on the plastic surface of the well.

2.2.6.5 *Cloning of hybridoma cells by limiting dilution*

2.2.6.5.1 *Materials*

Haemocytometer
Hybridoma medium (see appendix)
96 well sterile tissue culture treated plates (Corning)

2.2.6.5.2 *Method*

1. Confluent cells were harvested and cell number estimated using a haemocytometer. The cell number obtained from the haemocytometer count gives a value x 10^4 cells / ml.

2. Cells were diluted in hybridoma medium to give a final cell density of 1000 cells/ml, and then diluted serially to produce final cell concentrations of 4, 2, 1 and 0.5 cell/well to allow cloning by limiting dilution.

3. Plates were left undisturbed in a humidified incubator at 37°C with 10 % (v/v) CO_2 for 14 days.

4. Supernatants from wells containing single colonies were screened accordingly.

2.2.7. SDS polyacrylamide gel electrophoresis

2.2.7.1 *Materials*

All solutions were made with de-ionised water.

Atto mini-slab electrophoresis kit (AE - 6450; Genetic Research Instrumentation Ltd)

Boiling water bath

Tris-glycine electrode buffer, pH 8.3 (see appendix)

Acrylamide stock [30 % (w/v)] [filtered] (see appendix)

Sample buffer- 2x concentrated (see appendix)

Resolving gel (10 %) (see appendix)

Stacking gel (see appendix)

Fixing solution (see appendix)

Water saturated butanol

10 % (w/v) Ammonium persulphate

N, N, N, N' Tetramethylethylenediamine (TEMED)

2.2.7.2 *Method*

Sample Preparation

Samples were precipitated with four volumes of cold acetone prior to resuspension in sample buffer.

Prior to electrophoresis samples including standards were immersed in boiling water for 4 minutes to ensure complete denaturation and dissolution of the proteins.

Electrophoretic procedure

1. Glass gel plates were cleaned with ethanol and assembled in the gel mould apparatus.

2. The resolving gel mix was prepared and degassed for 15 minutes before adding 40 µl 10 % (w/v) Ammonium persulphate and 10 µl TEMED, mixing gently and pouring into the gel apparatus.

3. The gel was overlayed with water saturated butanol to exclude air and allowed to polymerise for 60 minutes at room temperature.

4. Following polymerisation butanol was removed and the gel surface carefully washed with Tris-buffer, pH 8.8.

5. The stacking gel mixture was prepared similarly and the gel comb inserted before pouring stacking gel and allowing to polymerise for 60 minutes.

6. Gels were placed in the tank containing electrode buffer in both the upper and lower reservoirs and samples introduced into individual wells

7. Electrophoresis was allowed to proceed at a constant current of 30 mA per mini-gel for one hour at room temperature or until the tracking dye had reached the bottom of the gel plate.

8. Following electrophoresis gels were fixed in fixing solution, shaking overnight before staining with Silver (section 2.2.8) or electroblotted (section 2.2.9).

2.2.8 Silver stain

2.2.8.1 *Materials*

Fixative solution (see appendix)
Bio-rad Silver stain kit (catalog No. 161-0443)
Stop solution (see appendix)
Orbital shaker
Glass tray

2.2.8.2 *Method*

The following protocol was suitable for a 1.0 mm gel, all steps were performed in a glass tray on an orbital shaker. Manufacturers instructions for the Bio-rad silver stain were followed.

1. The gel was placed in fixative solution overnight while shaking.

2. The oxidiser from the kit with diluted 1 : 10 (v/v) with deionised water and the gel was transferred to 100 ml of diluted oxidiser for 5 minutes.

3. The gel was then washed with two changes of deionised water (10 minutes each).

4. This washing procedure was repeated until all the yellow colour had washed out of the gel.

5. Silver reagent was diluted 1:10 (v/v) with deionised water, the gel was incubated in 100 ml of diluted silver reagent for 20 minutes and then rinsed briefly with deionised water for 1 minute.

6. The gel was then transferred to 100 ml of developer solution (8.0 g in 250 ml deionised water) and allowed to develop until the solution turned a smokey colour, the developer was removed and fresh developer (100 ml) was added. The gel was monitored continually until brown bands had appeared. When the desired intensity had been achieved the gel was fully immersed in stop solution.

2.2.9 Electroblotting

This procedure is based on a modified version of the method of Towbin *et al*, 1979

2.2.9.1 *Materials*

Electrophoretic transfer unit (LKB 2117-005 Multiphor II Novablot, Pharmacia)
Electroblotting buffer (see appendix)
TBS-Tween (see appendix)
Nitrocellulose (Gelman Sciences Inc.; membrane pore size, 0.2 μm)
Ponceau S [0.2 % (w/v)] dissolved in 3 % (w/v) trichloroacetic acid

2.2.9.2 *Method*

1. Gels were washed and incubated in 50-100 ml electroblotting buffer for 20 minutes to partially remove SDS.

2. The graphite electrodes of the transfer unit were soaked with distilled water prior to use and any excess was removed with absorbent paper.

3. A total of eighteen filter papers, slightly larger than the size of the gel were soaked in electroblotting buffer. Nine of these were placed onto the anode on top of one another, forming the first trans unit, taking care to exclude any air bubbles.

4. A sheet of nitrocellulose was cut and similarly soaked in electroblotting buffer and placed on top of the filter papers the gel was then placed on top of the nitrocellulose.

5. The other further nine filter papers were placed on top of the gel and the cathode placed on top. A constant current of 1.0 mA / cm^2 was applied across the electrodes for 60 minutes at room temperature.

6. Blots were reversibly stained with 0.2 % (w/v) Ponceau S dissolved in 3 % (w/v) trichloroacetic acid for 2 minutes to assess electrophoretic transfer and destained in distilled water.

2.2.10 Immunoprobing of electroblots

2.2.10.1 *Materials*

Primary antibody (raised in mouse)

Secondary antibody (goat anti-mouse immunoglobulins, alkaline phosphatase conjugate (Dako code No. D0486).

0.75 M Tris, pH 9.5

5-Bromo-4-chloro-3-indolyl phosphate, p-toluidine salt (BCIP), 50 mg/ml in dimethyl formamide (DMF).

Nitroblue tetrazolium (NBT), 75 mg/ml in 70 % (v/v) DMF

Tris buffered saline [TBS] (see appendix)

Blocking solution (see appendix)

TBS-Tween (see appendix)

Substrate mix (see appendix)

2.2.10.2 *Method*

1. Following electrophoretic transfer excess protein binding sites on the nitrocellulose were blocked with blocking solution for 60 minutes shaking at room temperature, before incubating with primary antibody (containing a final concentration of 0.1 % (v/v) Tween-20.) overnight at 4 °C.

2. The blot was then washed extensively with TBS-Tween for 60 minutes, changing every 20 minutes, before incubation with the secondary antibody, alkaline phosphatase conjugated anti-primary antibody diluted 1:1000 in blocking solution, for two hours at room temperature whilst shaking.

3. Following secondary antibody incubation the blot was washed as previously described and then rinsed with two changes of distilled water, before equilibration for 5 minutes in substrate buffer (0.75 M Tris pH 9.5).

4. Blots were finally developed in the substrate mix for 30 minutes to several hours.

5. Development was stopped by extensive washing with deionised water.

2.2.11 Immunohistochemistry

2.2.11.1 *Slide coating*

2.2.11.1.1 *Materials*

2 % (v/v) Decon 90 (supplied by Merck Ltd.)

2 % (v/v) 3-aminopropyltriethoxysilane

Acetone

Glass slides

2.2.11.1.2 *Method*

1. Slides were immersed in 2 % (v/v) Decon 90 for 30 minutes and washed in running water to remove all traces of detergent.

2. Excess water was drained onto absorbent paper and slides were immersed in acetone for 5 minutes.

3. Excess acetone was drained off and slides were immersed in acetone containing 2 % (v/v) 3-aminopropyltriethoxysilane for 5 minutes.

4. Excess solution was drained off and slides were washed briefly in distilled water before draining and allowing to air dry at room temperature overnight.

2.2.11.2 *Tissue preparation - snap freezing method (post-fixing)*

2.2.11.2.1 *Materials*

Embedding medium (O.C.T compound, code 4583, supplied by Tissue-Tek)

Isopentane

Liquid Nitrogen

2.2.11.2.2 *Method*

1. Post-mortem tissue samples (approximately 0.5 cm square) were surrounded in embedding medium (O.C.T. compound, code 4583, Tissue-Tek), mounted on blocks and snap frozen in liquid nitrogen cooled isopentane (-140 °C) for 1 minute. These blocks were stored at -70 °C until required. Tissue sections were fixed following sectioning.

2.2.11.3 *Tissue preparation - sucrose protection method (pre-fixing)*

2.2.11.3.1 *Materials*

2 % (w/v) Paraformaldehyde solution (see appendix)

15 % (w/v) Sucrose solution (see appendix)

Embedding medium (O.C.T compound, code 4583, supplied by Tissue-Tek)

Dispomoulds (plastic cassettes, code number E/10.8/L15 supplied by Raymond A. Lamb)

Isopentane

Liquid Nitrogen

2.2.11.3.2 *Method*

1. Portions of tissue were cut into 0.5 cm cubes and immediately transferred to 2 % (w/v) paraformaldehyde solution to perfuse for four hours at 4°C.

2. Tissue pieces were then transferred to 15 % (w/v) sucrose solution for 1 hour at 4°C.

3. Tissue samples were transferred to fresh sucrose solution and incubated overnight at 4°C. Sucrose solution was changed again for a further 2 day incubation at 4 °C.

4. 0.5 cm cube pieces were placed in the plastic dispomoulds, covered with embedding medium and frozen by placing the plastic trays on a shallow bath of liquid nitrogen cooled isopentane. Samples were stored at -70°C until required.

2.2.11.4 *Immunohistochemistry for cryostat sections*

2.2.11.4.1 *Materials*

Cryostat (Anglia Scientific cryotome 620)

Paraformaldehyde solution (see appendix).

Normal swine serum (Dako, code No. X0901).

Blocking agent (see appendix).

Primary and secondary antibody diluent (see appendix).

Horse radish peroxidase conjugated Goat anti mouse antibody (Dako code No. P0447)

0.3 % and 3.0 % (v/v) Hydrogen peroxide

3,3' Diaminobenzidine (DAB) peroxidase substrate. Tablet form (Sigma code D4168).

Copper sulphate solution (see appendix).

2.2.11.4.2 *Method*

All steps were carried out at room temperature unless otherwise stated.

1. Sequential frozen sections (either snap frozen or sucrose protected, 12 μm) were cut on a cold microtome (-14 °C), collected onto pre-coated slides and allowed to air dry overnight at room temperature.

2. Dried sections were fixed by immersion into ice-cold 4 % (w/v) PFA for 5 minutes before washing in a PBS bath twice for 5 minutes. Sucrose protected samples had been pre-fixed so did not require post-fixing.

3. They were then transferred to a new PBS bath containing 0.5 % (v/v) Triton X-100 and incubated for a further 5 minutes before washing again in PBS twice for 5 minutes.

4. Endogenous peroxidases were blocked by incubating with H_2O_2 for 10 minutes (0.3 % (v/v) in PBS for liver and 3.0 % (v/v) in PBS for placenta).

5. Excess peroxidase inhibitor was tipped off and sections were washed in PBS for 5 minutes before blocking each section with 200 μl blocking agent for 20 minutes.

6. Excess blocking agent was removed and the sections were incubated with 50 μl per section diluted primary antibody (using appropriate pre-determined dilutions for antibody titre) for 30 minutes.

7. Unbound primary antibody was washed off by incubation in three baths PBS, each for 5 minutes, prior to incubation with the secondary antibody (diluted 1:100 in antibody diluent) for a further 30 minutes.

8. Unbound secondary antibody was washed off in PBS bath 3 changes x 5 minutes.

9. Antibody binding was revealed by the addition of the DAB substrate (prepared according to manufacturers instructions) for at least 10 minutes (observing the sections closely for colour development).

10. Excess DAB was washed away for 15 minutes in running water.

11. All sections were then immersed into copper sulphate solution for 15 minutes and washed well in running tap water before counterstaining with Haematoxylin.

2.2.11.5 *Haematoxylin counterstain*

2.2.11.5.1 *Materials*

Gills Haematoxylin
Scotts solution (see appendix)
Acid alcohol (see appendix)
70 % (v/v) Ethanol
90 % (v/v) Ethanol
Absolute ethanol
DPX mountant (BDH Code 36029 2F)

2.2.11.5.2 *Method*

1. Slides were immersed in Gills Haematoxylin for 20 seconds before washing well in running tap water.

2. Followed by immersion in Scotts solution for a further 20 seconds and washing in running tap water for a further minute.

3. Sections were then dipped in acid alcohol to remove any excess haematoxylin and washed again in running tap water for 1 minute.

4. Dehydration of the stained sections was achieved by immersion in 70 % (v/v) ethanol for 1 minute, 90 % (v/v) ethanol for 1 minute followed by immersion in absolute

ethanol for 2 x 1 minute, and immersion in xylene for 1 minute into fresh xylene for a further minute before permanent mounting under coverslips using DPX xylene based mountant.

2.2.11.6 *Haematoxylin and eosin stain*

2.2.11.6.1 *Materials*

Gills Haematoxlin (No. 3 Sigma code GHS-3-80)
Eosin solution (see appendix)
Scotts solution (see appendix)
Acid alcohol (see appendix)

2.2.11.6.2 *Method*

1. Slides were immersed in Haematoxylin for 2 minutes and rinsed in running tap water.
2. Immersion in Scotts solution for 20 seconds and rinsed in running tap water.
3. Slides were dipped in acid alcohol to remove excess haematoxylin and rinsed in running tap water before immersing in Scotts solution for a further 20 seconds.
4. Slides were then rinsed in running tap water and immersed in eosin for 1 minute, and finally rinsed in running tap water and dehydrated as previously described in section 2.2.11.5.2.

2.2.12 Isolation of human lymphocytes

2.2.12.1 *Materials*

Centrifuge (Beckman, Model GPKR)
10 % (w/v) Ethylenediamine-tetraacetic acid (EDTA)
Ficoll-Paque (Pharmacia code No. 17-0840-02)
RPMI-1640 medium

2.2.12.2 *Method*

1. Whole blood was collected into tubes containing 10 % (w/v) EDTA as an anti coagulant, (100 µl 10 % EDTA per 10 ml blood).

2. Tubes were then centrifuged at 600 x g for 5 minutes to pellet the red blood cells and the buffy coat (containing white cells and platelets) was carefully aspirated using a Pasteur pipette.

3. 4 ml of Ficoll was measured into a fresh tube and carefully overlayed with 2 ml white cells before centrifuging at 450 x g for 30 minutes at 20 °C.

4. Viable lymphocytes (and monocytes) accumulated as a white turbid layer above the Ficoll (dead cells, granulocytes and any remaining erythrocytes sediment to the bottom of the tube). This layer was carefully collected into a clean tube and washed with an equal volume of RPMI-1640 (containing 1.0 mM EDTA) by centrifuging at 250 x g for 5 minutes at room temperature. The supernatant was discarded and the washing procedure repeated.

5. The final lymphocyte pellet was resuspended in 1.0 ml RPMI-1640 containing 1.0 mM EDTA.

2.2.12.3 *Preparation of lymphocyte smears*

Lymphocyte samples were required for immunohistochemistry and prepared as follows :-

1. 50 µl of the lymphocyte preparation was dropped onto a pre-coated slide (see section 2.2.11.1) and allowed to air dry overnight

2. Slides were then dipped into PBS before immediately fixing in cold acetone for 5 minutes.

3. The method used for immunohistochemistry (section 2.2.11.4) was then followed exactly except for the incubation step with PBS containing Triton X-100 which was omitted. Slides were counterstained and mounted as described in section 2.2.11.5.

2.2.13 Heterologous, non-competitive ELISA

2.2.13.1 *Materials*

ELISA reader (Titertec multiskan MCC/340 MK2)

Phosphate buffered saline (PBS), (see appendix).

0.1 M Sodium acetate, adjusted to pH 6.0 with acetic acid.

3,3,5,5-tetramethylbenzidine (TMB) 10 mg/ml in dimethylsulphoxide (DMSO).

2.5 M H_2SO_4

ELISA microtitre plates (Falcon code 3912, Becton Dickenson, supplied by Fahrenheit).

Peroxidase conjugated goat anti-mouse immunoglobulins (Dako code P447).

Substrate solution (see appendix)

Blocking agent (see appendix)

PBS-Tween (see appendix).

2.2.13.2 *Method*

1. Microtitre wells were coated (50 µl / well) with mitochondrial membranes diluted in PBS to give a final protein concentration of 20 µg/ml and incubated overnight at 4 °C.

2. Wells were aspirated, washed 3 times with PBS and dried by inversion onto absorbent paper.

3. Excess protein binding sites were blocked with blocking agent (200 µl / well) for 3 hours at 37 °C, before aspirating and drying onto absorbent paper.

4. 50 µl of primary antibody (diluted in blocking agent if necessary) was added to each well and incubated at 4 °C overnight.

5. Wells were washed 3 times with PBS-Tween followed by 3 washes with distilled water.

6. 50 µl of peroxidase conjugated secondary antibody (diluted in blocking agent 1:1000, v/v) was added to each well and incubated shaking at room temperature for 2 hours.

7. Wells were washed with 3 washes of PBS-Tween, followed by 3 washes with distilled water.

8. 100 µl of freshly prepared substrate solution was added to each well and the reaction allowed to proceed for 15 minutes before stopping with 2.5 M H_2SO_4 (50 µl / well).
9. Absorbance values were read at 450 nm.

2.2.14 Estimation of protein

Based on the method of Lowry *et al.* 1951 with modifications.

2.2.14.1 *Materials*

A set of appropriate protein standards (bovine serum albumin)
Lowry reagent stock solutions (see appendix)
Working Lowry reagent (see appendix)
Folin-Ciocalteau reagent

2.2.14.2 *Method*

1. Each sample (standards and test sample) was made up to a final volume of 0.5 ml. 30 µl of 10 % Sodium dodecyl sulphate was added if required to solubilise membrane proteins.
2. To each 0.5 ml sample 2.5 ml working Lowry reagent was added and each sample vortex mixed.
3. All samples were incubated at 37 °C for 5 minutes.
4. Folin-Ciocalteau reagent was diluted 1:2 with distilled water, 250 µl was added to each sample tube and vortex mixed.
5. Samples were then incubated at 37 °C for 15 minutes and absorbances read at 750 nm.

2.2.15 Competitive ELISA

2.2.15.1 *Materials*

All materials were as used for the non-competitive ELISA

2.2.15.2 *Method*

1. Microtitre wells were coated with 50 µl mitochondrial membranes (20 µg / ml) and blocked as described earlier (section 2.2.13.2).

2. 40 µl solubilised mitochondrial extracts (diluted in blocking buffer where necessary) were incubated in the presence of a limiting amount of primary antibody (10 µl) overnight at 4 °C.

3. Wells were washed as previously described (section 2.2.13.2) and incubated with secondary antibody. The procedure was followed as described in 2.2.13.2.

2.3 RESULTS

2.3.1 Preparation of antigens

2.3.1.1 *Preparation of mitochondrial membranes*

The preparation of mitochondrial membranes from human liver was an integral part of the competitive ELISA optimisation. These membranes contain a rich source of MAO for use as a standard either in the form of mitochondrial membranes or in an enriched form following a number of purification steps. This procedure of mitochondrial membrane preparation was conducted on many occasions from a number of different human livers, all of which were less than 24 hours after death before either direct membrane preparation or freezing.

The method employed for the preparation of mitochondrial membranes was slightly modified from that described by Billett *et al* (1984); mitochondrial outer membranes (MOM) were not separated from mitochondrial inner membranes (MIM) and centrifugation to remove loosly bound protein using ice cold sodium chloride was for 60 minutes. This method is based on the fractionation of mitochondrial membranes from a crude tissue homogenate by a number of differential centrifugation steps. The collection of mitochondria by centrifugation, after first excluding the nuclear pellet and large tissue debris, was initially performed using a one step centrifugation (8000 x g) strategy. However, this method failed to isolate all the mitochondria as a substantial concentration of MAO activity was detected in the resulting supernatant (data not shown). Consequently this supernatant was centrifuged a second time for maximal recovery of the remaining mitochondria. The mitochondrial pellets from both centrifugation steps were pooled and washed using decreasing volumes of buffer before the final mitochondrial pellet collection.

The isolation of membranes from this pellet involved detaching the membranes from the mitoplast through osmotic lysis. Membranes were released from the mitoplast by sonication. Separation and isolation of the membranes was achieved on a one-step sucrose density cushion. This resulted in the separation of a dark brown band on top of the 1.2 M sucrose cushion containing a mixture of MOM and MIM. Intact mitochondria

and the mitoplast formed a pellet. Following aspiration the mitochondrial membrane (MM) band was mixed with ice cold sodium chloride to remove any loosely bound protein and collected by ultracentrifugation. On each occasion 100 g of liver yielded 10 ml of membrane preparation with a protein concentration of between 2 and 5 mg/ml.

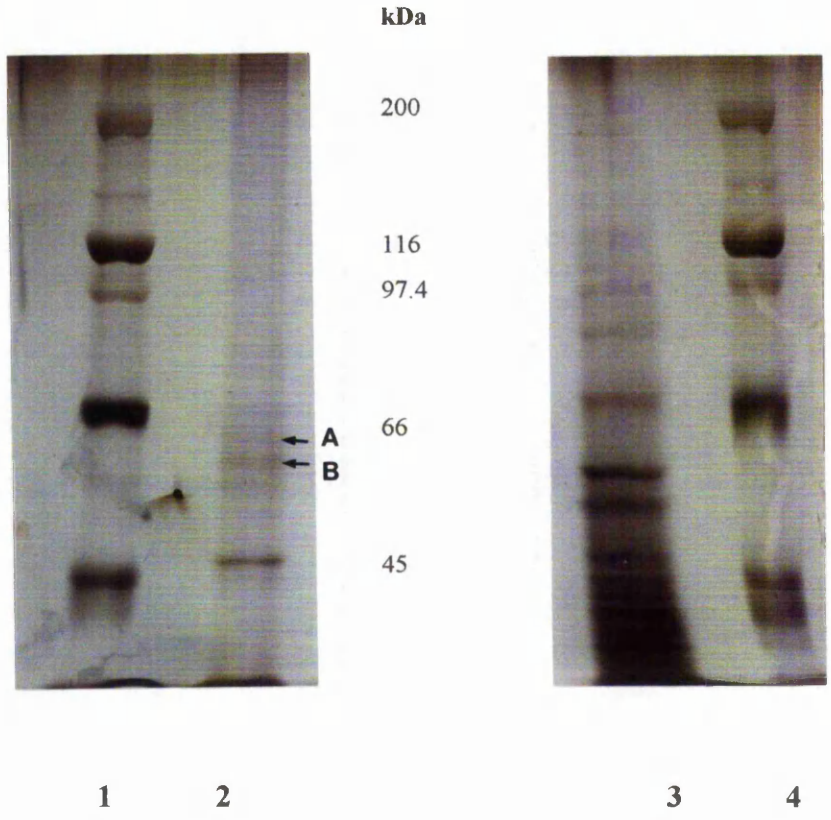
The logistics and difficulties in obtaining human tissue prompted an investigation into the effects of frozen storage. This study revealed that frozen storage of intact tissue for at least six months at -70 °C had no effect on the subsequent recovery of MAO activity. Storage of mitochondrial membranes at -70°C was also investigated and no effects on the recovery of MAO activity as assessed using ^{14}C tyramine were evident (Table 2.1).The lower activity in the fresh sample may be due to substrate accessibility which is improved following freezing (damage) of membranes.

Table 2.1. Effects of storage (-70°C) on MAO activity of Mitochondrial Membranes using tyramine as a substrate.

Storage of MM at -70°C (Months)	Specific Activity (DPM/mg total protein) ± SEM
Fresh Preparation	$0.96 \times 10^5 \pm 0.03$
2	$2.91 \times 10^5 \pm 0.11$
3	$2.57 \times 10^5 \pm 0.14$
5	$2.75 \times 10^5 \pm 0.06$

2.3.1.2 *Preparation of an enriched MAO sample by a combination of gel filtration and ion exchange chromatography*

An enriched MAO sample, required as a standard in the ELISA system, was produced from solubilised mitochondrial membranes by a combination of gel filtration and ion exchange chromatography (Billett and Mayer, 1986). Details of the methods are given in the Appendix.



Lanes 1 and 4 : Standard molecular weight markers.

Lane 2 : Enriched human monoamine oxidases A and B.

Lane 3 : Human liver mitochondrial membranes.

The relative peak areas of the bands are as follows :

BAND	PEAK AREA	% (of the total peak area)
A (MAO-A)	451	19
B (MAO-B)	621	26
C (other)	1329	55

Figure 2.3. SDS-PAGE analysis showing the enrichment of monoamine oxidases A and B from human liver mitochondrial membranes. The gel (15 %) has been stained with Silver.

The MAO enriched final sample contained 2.8 mg protein from a starting tissue weight of 100 g human liver. Figure 2.3 shows a profile of this sample fractionated by SDS-PAGE, stained with silver. The relative contents of MAO-A and MAO-B in this enriched extract were estimated by gel densitometer scanning and computer analysis. Molecular weight values for each of the proteins in the sample were obtained by calculation of their Rf values and comparison of these to the Rf values for molecular weight protein standards. Relative peak areas are also shown on Figure 2.3 and MAO-B contributes 26 % of the total protein in the enriched sample whilst MAO-A constitutes 19 % of the total protein.

2.3.2 Source of antibody, antibody titre and characterisation

2.3.2.1 *Re-cloning of the hybridoma cell line secreting monoclonal antibody specific for MAO-B*

Fifty six single monoclonal colonies resulted from the cloning of the 3F12/G10 hybridoma; antibody secreted from each of these single colonies was assayed against human liver mitochondrial membranes (as a source of MAO-B) using a non-competitive ELISA (section 2.2.13). Twenty three of these clones gave a positive signal of twice the background absorbance (measured at 450 nm). Supernatants from the other thirty three clones were negative, indicating that re-cloning had been necessary to select for secreting colonies and avoid further loss of titre. Further screens were conducted following culture of the clones and results indicated that clone 3F12/G10/2E3 should be selected for future assays as antibody secreted from it gave the highest titre as estimated by non-competitive ELISA. The titre of an antibody is defined as the reciprocal of the dilution required for 50% saturation of solid phase antigen. Figure 2.4 shows the titres of the anti MAO-B antibody before (681) and after (4640) cloning. Ascites fluid was subsequently produced (section 2.2.4) from 3F12/G10/2E3.

2.3.2.2 *Characterisation of antibodies*

The isotype of both antibodies was determined to be IgG_1 using a commercially available kit (MMTRC1 Mouse monoclonal isotyping kit, Serotec UK Ltd.).

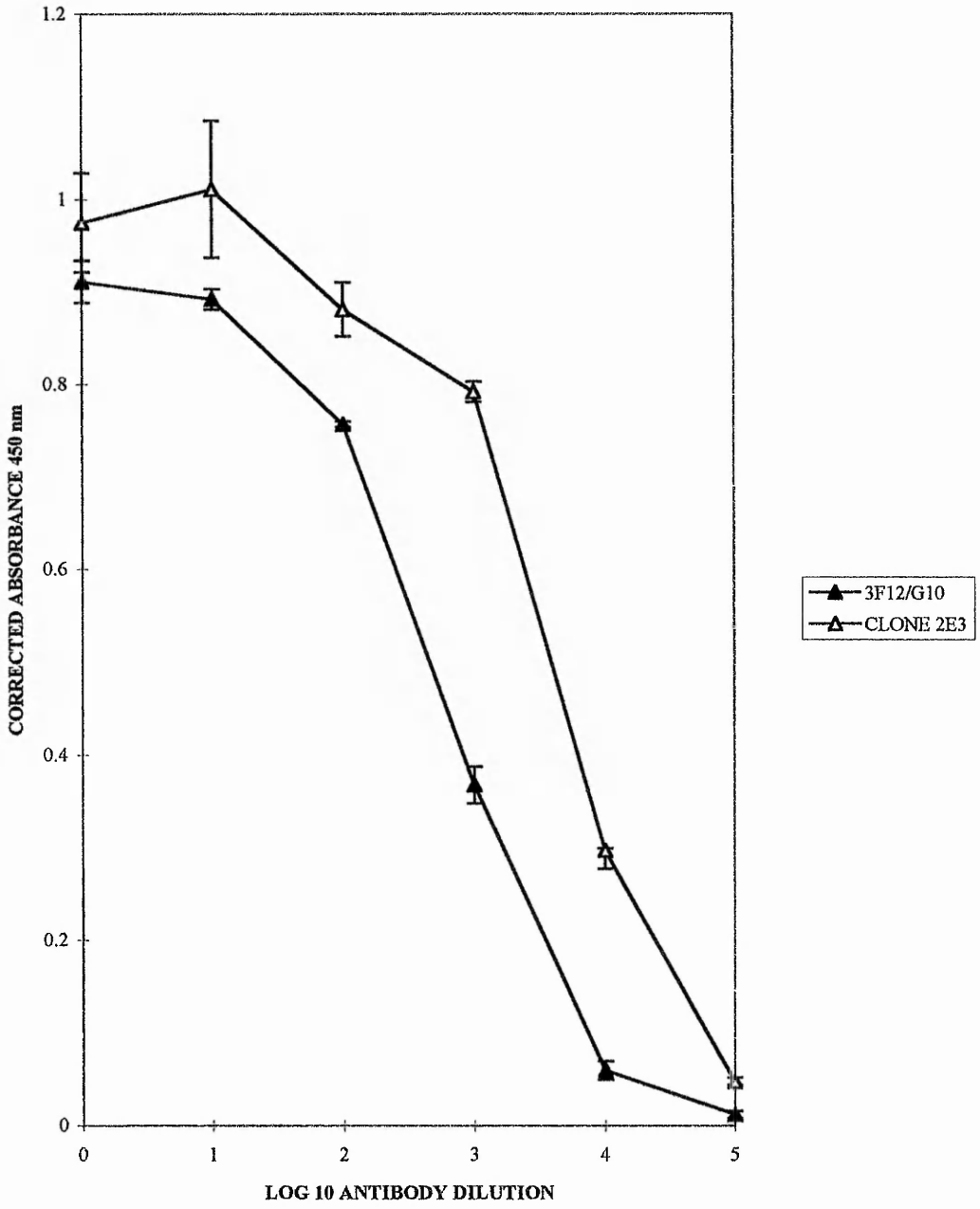


Figure 2.4. Titration of anti-MAO-B antibody 3F12/G10 before and after cloning as assessed using a non-competitive ELISA with human liver mitochondrial membranes as antigen.

The characteristics of 6G11/E1 and 3F12/G10/2E3 were first assessed using human liver mitochondrial membranes in a Western blot system. Further characterisation was achieved immunohistochemically on frozen sections of human liver and human lymphocyte smears.

2.3.2.2.1 *Western blot analysis*

Figure 2.5 shows a Western blot of human liver mitochondrial membranes probed with tissue culture supernatant harvested from 6G11/E1 (lanes 1-4 inclusive) and 3F12/G10/2E3 (lane 5). Lanes 1 - 4 were probed with decreasing concentrations of 6G11/E1 (1:10, 1:100, 1:500 and 1:1000 dilutions respectively). The single band (presumably MAO-A) can still be visualised at a dilution of 1:1000. Unfortunately detection of MAO-B in human liver mitochondrial membranes by 3F12/G10/2E3 (lane 5) in the Western blot system was unsuccessful. Previous attempts by Yeomanson (1990) to visualise MAO-B using this system had also failed. However the specificity of this antibody had already been determined by immunoprecipitaton using radiolabelled pargyline (Billett and Mayer, 1986).

2.3.2.2.2 *Immunohistochemistry*

To further assess the specificity of 3F12/G10/2E3 and 6G11/E1 with respect to MAO protein, a series of immunohistochemical experiments were conducted allowing visualisation of MAO *in situ.* Secondary antibodies were labelled with horse radish peroxidase and the substrate used was 3,3' Diaminobenzidine (DAB) which produces a dark brown stain following oxidation.

A means of checking that 3F12/G10/2E3 remained specific to MAO-B was required. As human lymphocytes only contain the B form of the enzyme, lymphocyte smears were prepared for immunohistochemical analysis using both the MAO antibodies. To assess the success of lymphocyte isolation and locate lymphocyte sub-populations commercially available antibodies (Pan B and Pan T) were employed. The antibodies are specific for cell surface markers on B (CD 22) and T (CD 3) lymphocytes (Pan B and Pan T, Dako). Dark brown positive staining is evident around the surface of both the B and T lymphocytes stained with the appropriate antibodies (figure 2.6 III and IV).

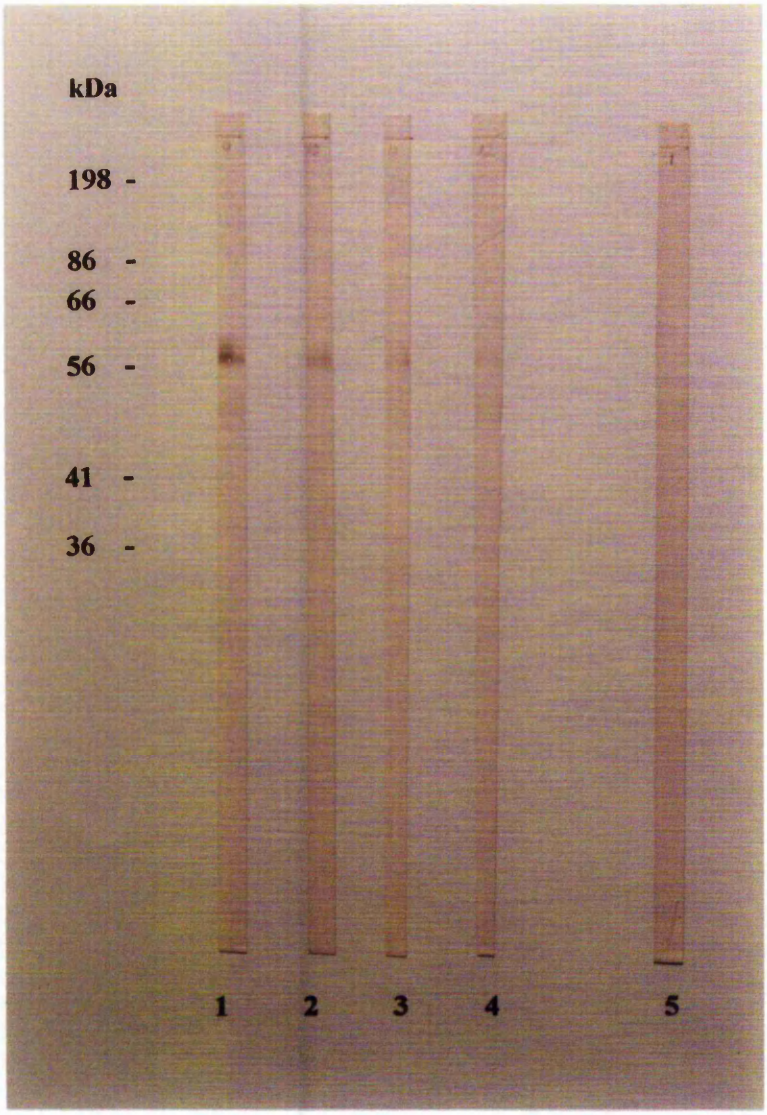


Figure 2.5. Western blot showing human liver mitochondrial membranes probed with tissue culture supernatant from 6G11/E1(anti MAO-A) and 3F12/G10/2E3.

Lane 1	6G11/E1	1:10 dilution
Lane 2	6G11/E1	1:100 dilution
Lane 3	6G11/E1	1:500 dilution
Lane 4	6G11/E1	1:1000 dilution
Lane 5	3F12/G10/2E3	1:10 dilution

This figure also shows the results obtained from staining of lymphocyte smears using the monoclonal antibodies specific for MAO-A and MAO-B. Reactions with 3F12/G10/2E3 (figure 2.6 VI) show positive staining for MAO-B in lymphocytes and MAO-B is also staining positive for platelets. No staining is evident with the MAO-A antibody (figure 2.6 V), indicating the presence of MAO-B but not MAO-A in human lymphocytes and platelets. Figure 2.6 also shows negative controls, namely lymphocytes with no primary antibody (I) and a matched isotype irrelevant antibody, 9H7, anti γ gliadin (II).

The reactivity of the monoclonal antibodies was also characterised on cryosections of human liver. The general morphology of human liver can be visualised in figure 2.7 (I and II) which shows a snap frozen, non sucrose protected post fixed human liver cryosection (12 μm), stained with haematoxylin and eosin.

The effect of sucrose protection subsequent to fixation on antibody staining was studied. This was undertaken because our collaborators, Rodriguez *et al* (submitted for publication), had already successfully used sucrose protection with both antibodies on other human tissues. Sucrose protection appeared to have a detrimental effect on tissue morphology (Figure 2.7 III and IV). It appears that the sinusoidal spaces become much larger and the tissue takes on a more spongy appearance. This may be due to the perfusion of sucrose into the tissue.

Both anti MAO antibodies and 9H7 (a non-immune antibody of the same isotype i.e. IgG_1) were titrated on liver cryosections to establish the optimum working concentrations. These were determined at 1:50 for 6G11/E1 and 1:10 for 3F12/G10/2E3 as higher dilutions produced a reduction in the intensity of staining. A working dilution of 1:10 was chosen for the non-immune control.

Figure 2.8 shows liver cryosections reacted with the non-immune (matched isotype) primary antibody. No staining is observed in either sucrose protected nor non-sucrose protected cryosections. In addition sections incubated in the absence of primary antibody (data not shown) showed the same results as the non-immune control.

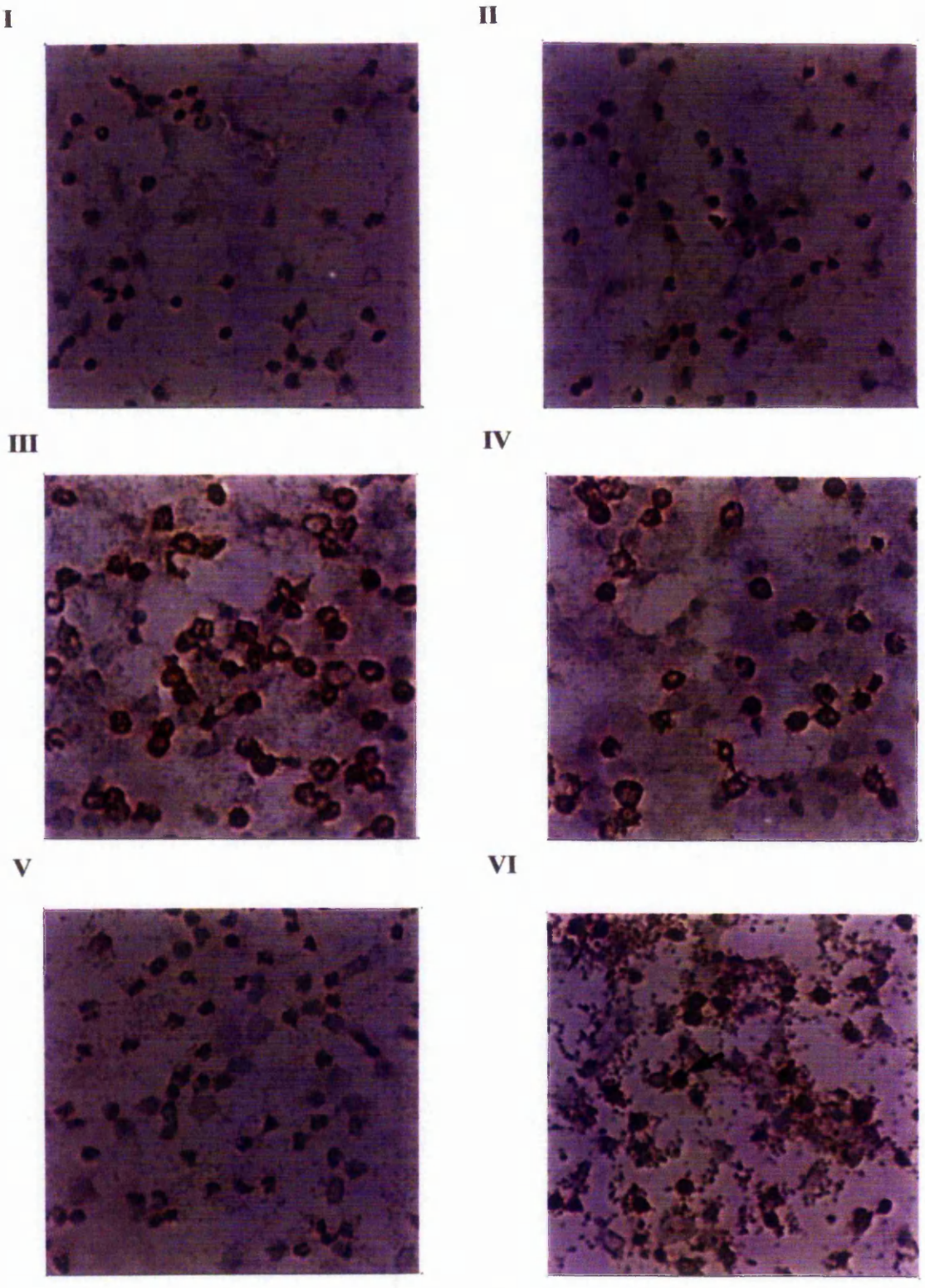


Figure 2.6. Immunohistochemical staining of human lymphocyte smears, (x 400 magnification). **I**. No primary antibody; **II**. Non-immune control; **III**. Dako Pan T antibody; **IV**. Dako Pan B antibody; **V**. 6G11/E1 specific for MAO-A; **VI**. 3F12/G10/2E3 specific for MAO-B.
Note the positive staining of platelets (small arrow) and B lymphocytes (large arrow) for MAO-B.

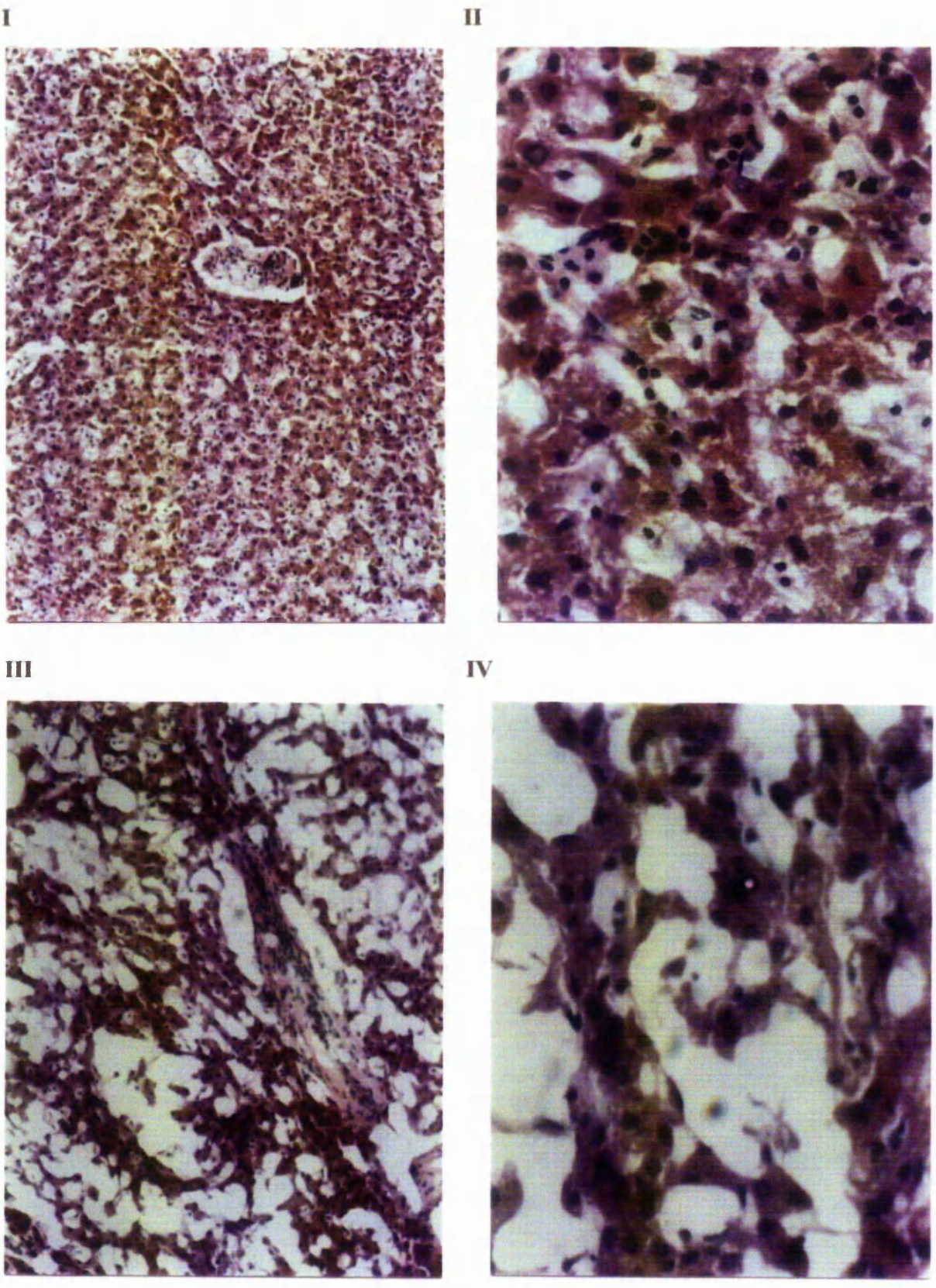


Figure 2.7. Haematoxylin and eosin staining of human liver cryosections (12 μm). **I**. Post fixed following sectioning (x 100 magnification); **II**. Post fixed following sectioning (x 400 magnification); **III**. Pre-fixed plus sucrose protection (x 100 magnification); **IV**. Pre-fixed plus sucrose protection (x 400 magnification).

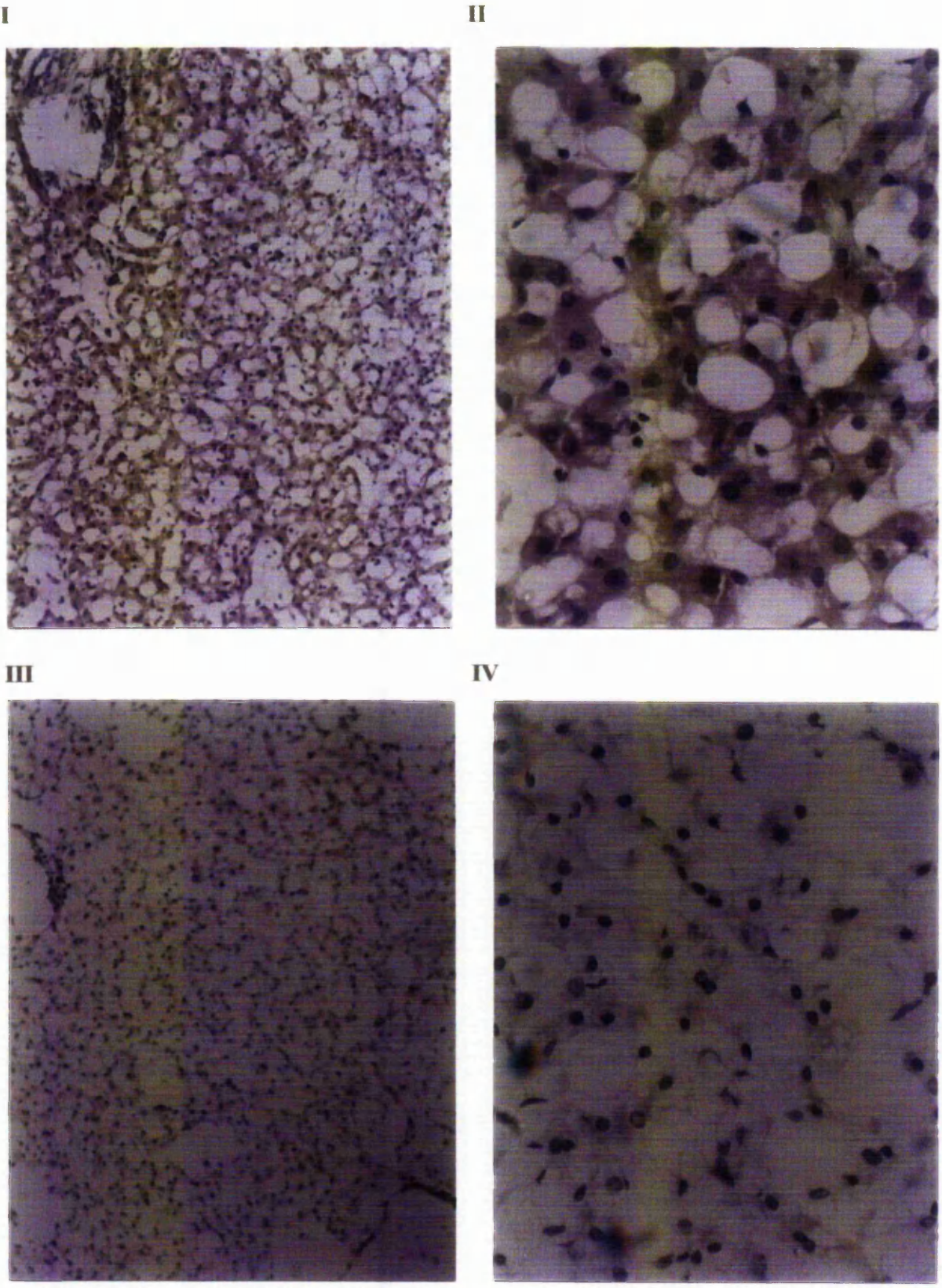


Figure 2.8. Immunohistochemical staining of human liver cryosections (12 μm) with 9H7 a non-immune control (matched isotype) antibody. **I**. Post fixed following sectioning (x 100 magnification); **II**. Post fixed following sectioning (x 400 magnification); **III**. Pre-fixed plus sucrose protection (x 100 magnification); **IV**. Pre-fixed plus sucrose protection (x 400 magnification).

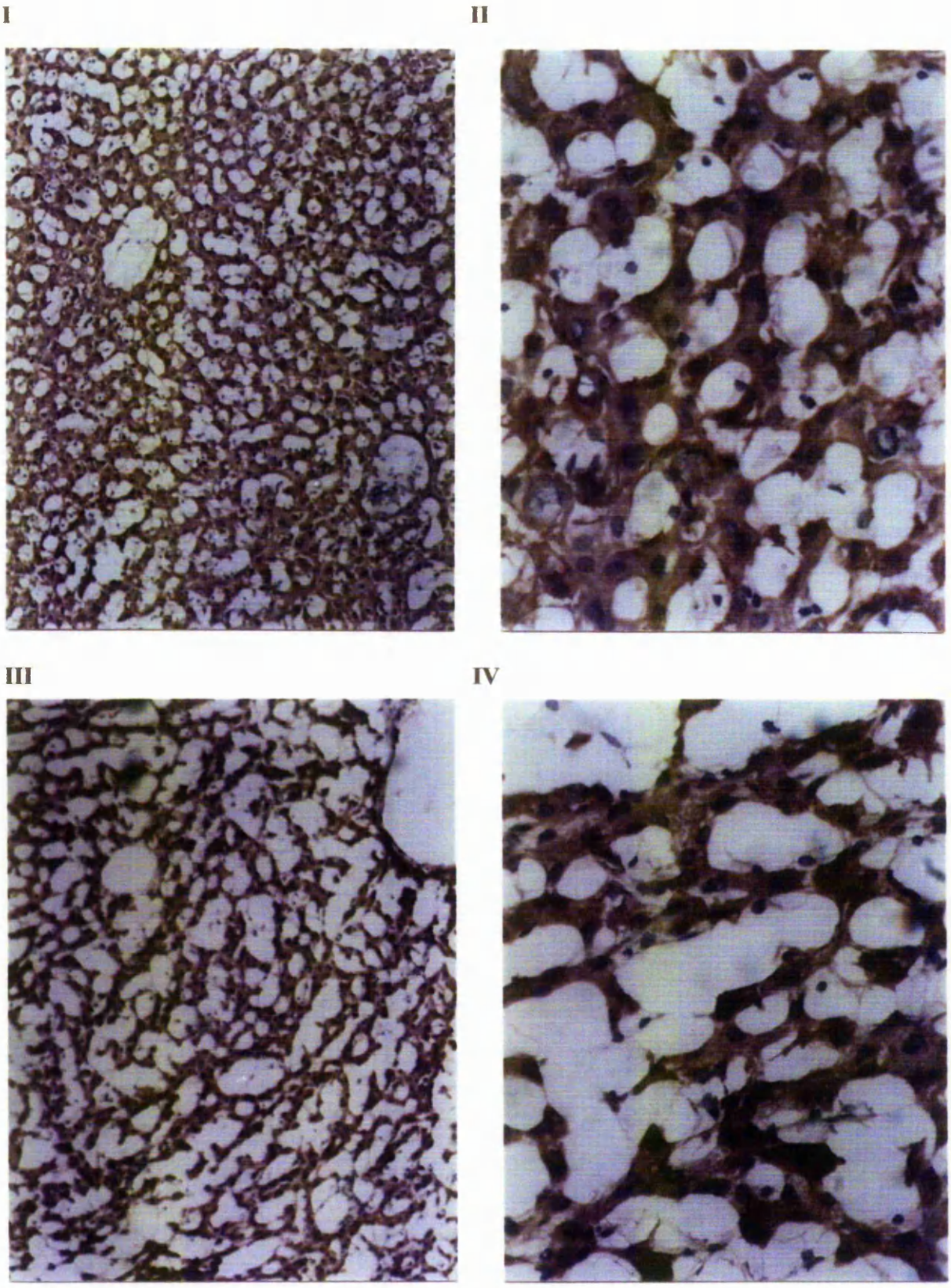


Figure 2.9. Immunohistochemical staining of human liver cryosections (12 μm) with 6G11/E1 specific for monoamine oxidase A. **I**. Post fixed following sectioning (x 100 magnification); **II**. Post fixed following sectioning (x 400 magnification); **III**. Pre-fixed plus sucrose protection (x 100 magnification); **IV**. Pre-fixed plus sucrose protection (x 400 magnification).

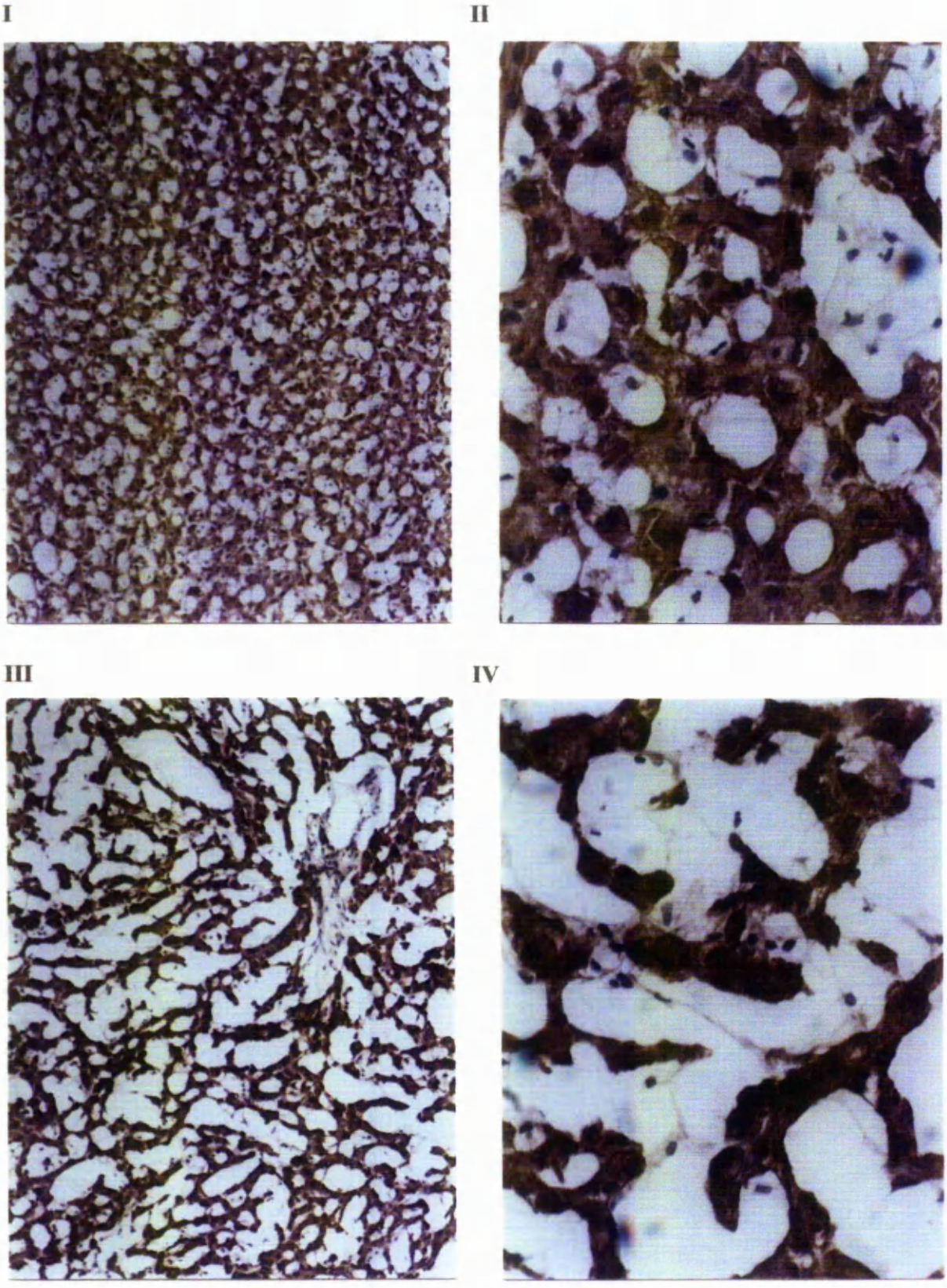


Figure 2.10. Immunohistochemical staining of human liver cryosections (12 μm) with 3F12/G10/2E3 specific for monoamine oxidase B. **I**. Post fixed following sectioning (x 100 magnification); **II**. Post fixed following sectioning (x 400 magnification); **III**. Pre-fixed plus sucrose protection (x 100 magnification); **IV**. Pre-fixed plus sucrose protection (x 400 magnification).

MAO-A and MAO-B staining is found in the cytoplasm of all hepatocytes and shows a punctate appearance, most noticeable at high magnification in figure 2.10 (IV). Sucrose protection appeared to increase the staining with MAO-A, but it is difficult to be confident that this is due to improved epitope quality since the cytoplasm condensed/collapsed following sucrose protection (figure 2.9). A positive effect of sucrose protection on the MAO-B antibody epitope was more evident since the difference between sections with and without sucrose protection was more exaggerated (figure 2.10).

2.3.3 Optimisation of a competitive ELISA to detect MAO-B

2.3.3.1 *The MAO-B competitive ELISA*

The competitive ELISA developed by Yeomanson and Billett (1992) had to be modified to allow the detection of MAO-B in solubilised extracts of human platelets. The concentration of MAO in human platelets is very low so a sensitive assay needed to be employed for its detection. One batch of mitochondrial membranes (MM) was used as a source of solid phase antigen for this ELISA optimisation and designated for that sole purpose throughout the analysis to keep all the subsequent experiments standardised and to limit error. MM were solubilised using Triton X-100 at a final concentration of 1.0 % (v/v) as this concentration had previously been determined to be the optimum as it released greater than 90 % of the total MAO activity into the supernatant (Yeomanson, 1990). The titre of 3F12/G10/2E3 against this specific batch of MM was determined in the presence of 0.1 % Triton X-100 and the dilution (giving half maximum saturation) was utilised throughout the assays. Figure 2.11 shows a typical example of the displacement plot using solubilised MM as the competing antigen.

Since the production of an enriched form of MAO from human liver involves a number of complex time-consuming procedures and results in a relatively low yield, solubilised MM was routinely used as a standard source of MAO for the competitive ELISA. An assay was therefore conducted using both solubilised MM and the enriched MAO sample as competing antigens to determine the MAO content of MM for use as a standard. This and subsequent assays were linearised using a semi-log plot. The

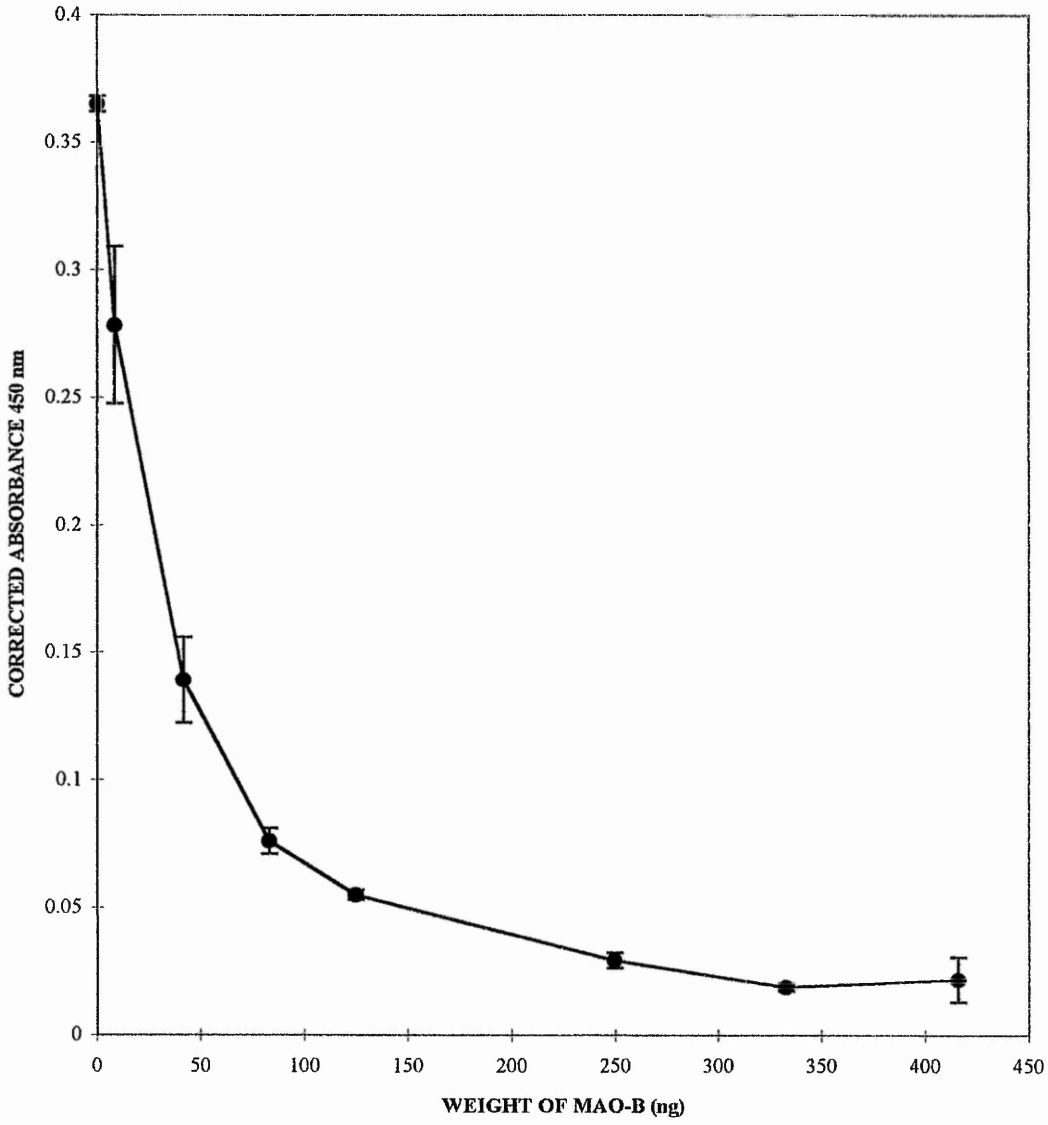


Figure 2.11. An example of a competitive ELISA displacement plot for MAO-B. Microtitre plates were coated with a crude preparation of mitochondrial membranes and solubilised mitochondrial membranes were used as the antigen competing for a limited concentration of MAO-B specific antibody (3F12/G10/2E3), final Triton X-100 concentration was 0.1%,(v/v). Concentration of MM is expressed as MAO-B equivalent.

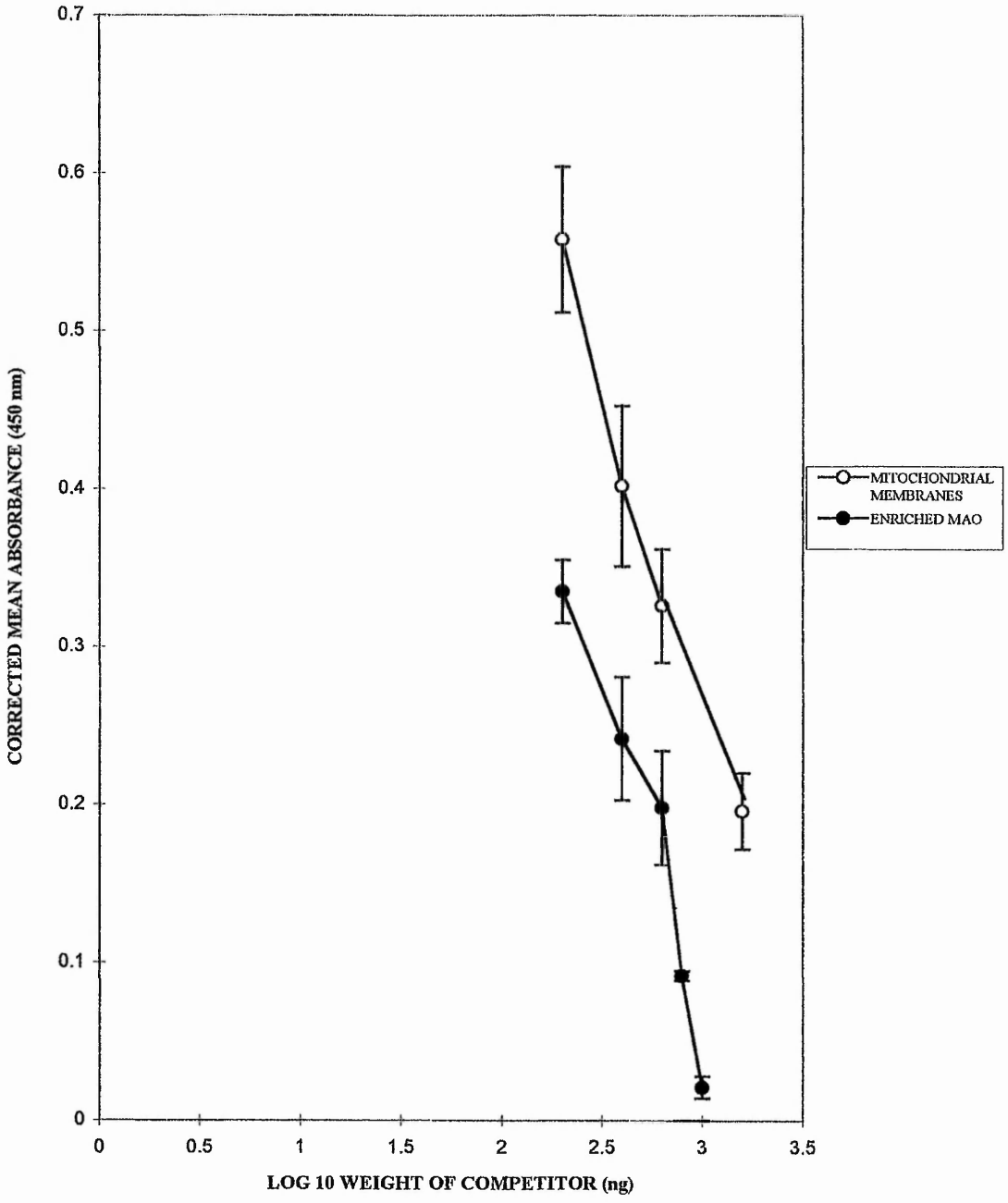


Figure 2.12. Semi-log plot to estimate the MAO-B content of mitochondrial membranes. Enriched MAO was used as a source of MAO-B. The enriched MAO contains a known amount of MAO-B and was used to estimate the amount of MAO-B in MM. Corrected mean absorbances (n = 4) were plotted.

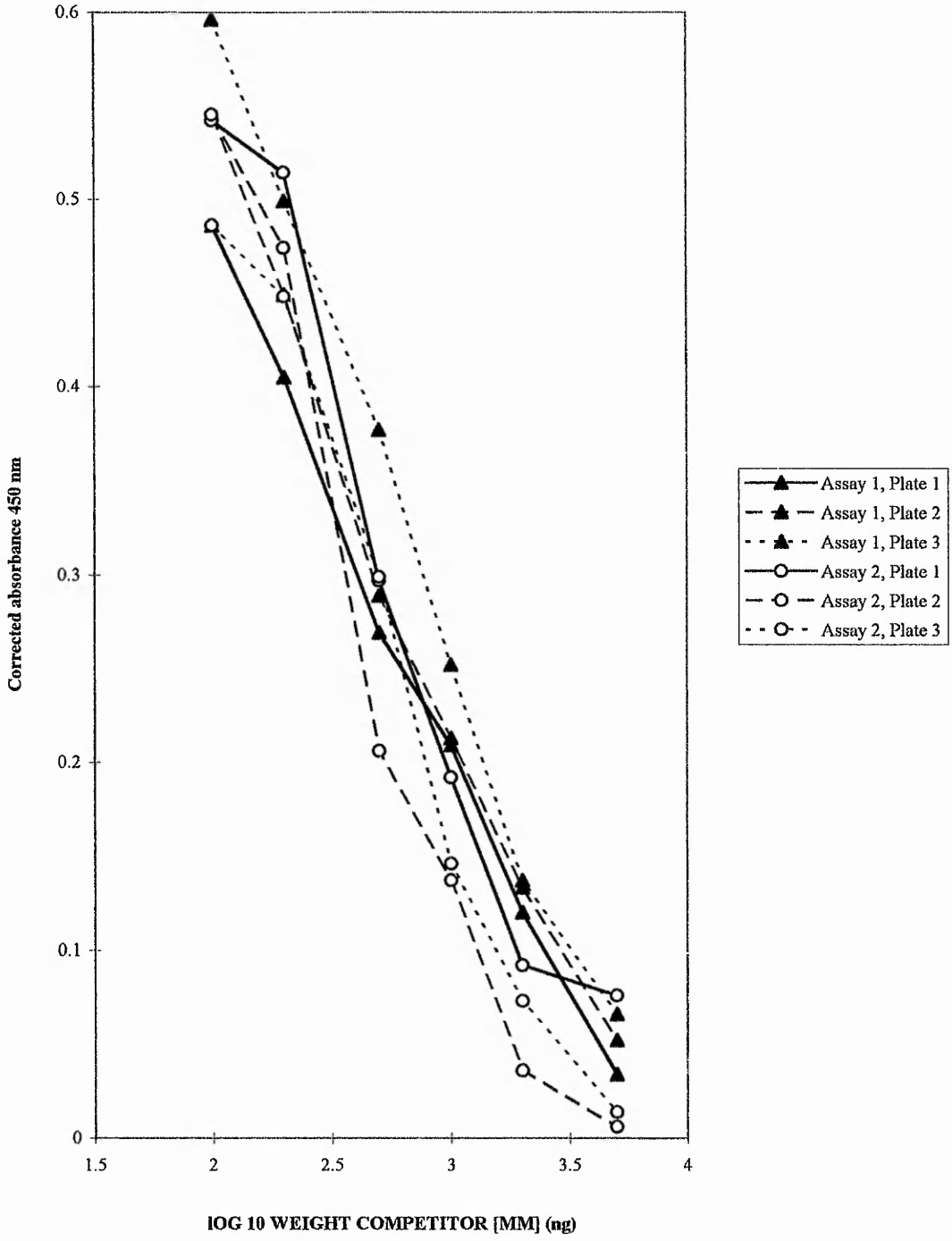


Figure 2.13. Inter assay and between plate variation of solubilised mitochondrial membranes in the MAO-B competitive ELISA.

gradients of the two slopes are equivalent indicating that the antibody is recognising the same epitope in both MM and enriched MAO (figure 2.12). The result indicates that the sample is equivalent to 32 % by weight of protein of MM (e.g. 426 ng enriched MAO gave the same competition level as 1348 ng of solubilised MM). Enriched MAO contains 26 % MAO-B (Figure 2.3) therefore the solubilised MM contains 8.32 % MAO-B by weight of protein. The detection limit for MAO-B protein was determined to be around 8.32 ng or 208 ng/ml.

Inter-assay and inter-plate variations are shown in figure 2.13 using solubilised standard MM as the competing antigen. Even though the plots are similar, indicating that variation is small, standard MM was routinely included on every ELISA plate in every assay.

2.3.3.2 *Effects of Triton X-100*

It has been reported that Triton X-100 has an adverse effect on the activity of MAO solubilised from membranes (Yu, 1981). Previous studies in our laboratory have also indicated that Triton X-100, at concentrations above 0.1% (v/v), has adverse effects on the binding of the 3F12/G10 antibody to MAO protein (Monk, 1992). A study was therefore undertaken to investigate the effects of Triton X-100 on 3F12/G10/2E3 binding to MAO in the competitive ELISA (data not shown). Indeed, Triton X-100 was found to have an inhibitory effect on antibody binding at concentrations of 0.1% (v/v) and greater. Since Triton X-100 was required to solubilise MAO from MM, a compromise between sufficient detergent for solubilisation and minimum effects on antibody binding was required. Hence for standardisation purposes solubilised MAO, irrespective of source was incubated in a final concentration of 0.1% Triton X-100 in all ELISAs. 3F12/G10/2E3 was titrated in the presence of 0.1% Triton X-100 against MM bound to the solid phase of a mictotitre plate in a non-competitive ELISA. The antibody titre was estimated to be 2700 (Figure 2.14) This figure also shows that the titre was greater (6500) in the absence of Triton X-100.

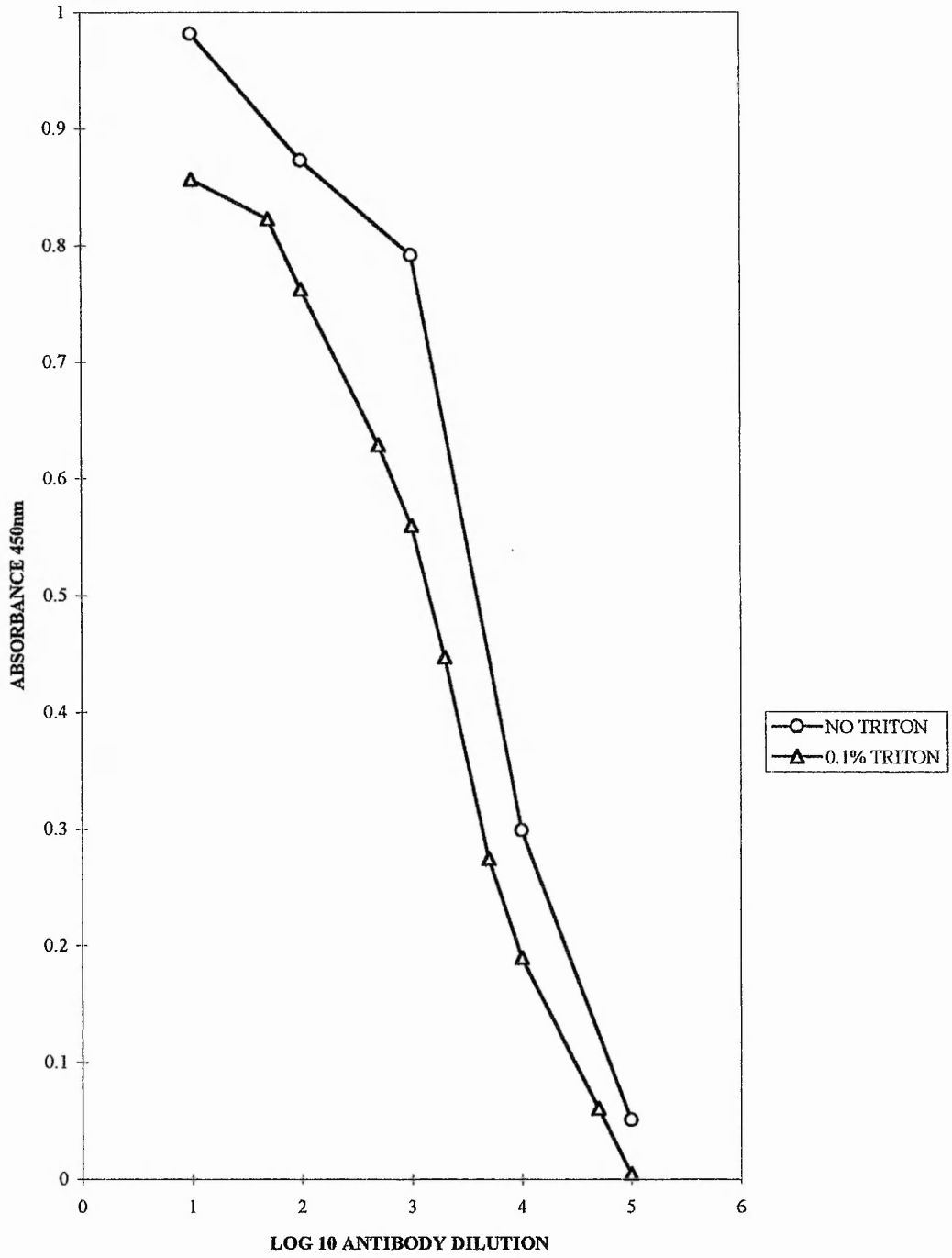


Figure 2.14. Effects of the detergent Triton X-100 on the titration of 3F12/G10/2E3 tissue culture supernatant. Wells were coated with mitochondrial membranes and incubated with serial dilutions of the mouse monoclonal antibody 3F12/G10/2E3 in the presence or absence of Triton X-100 (final concentration 0.1%, v/v) .

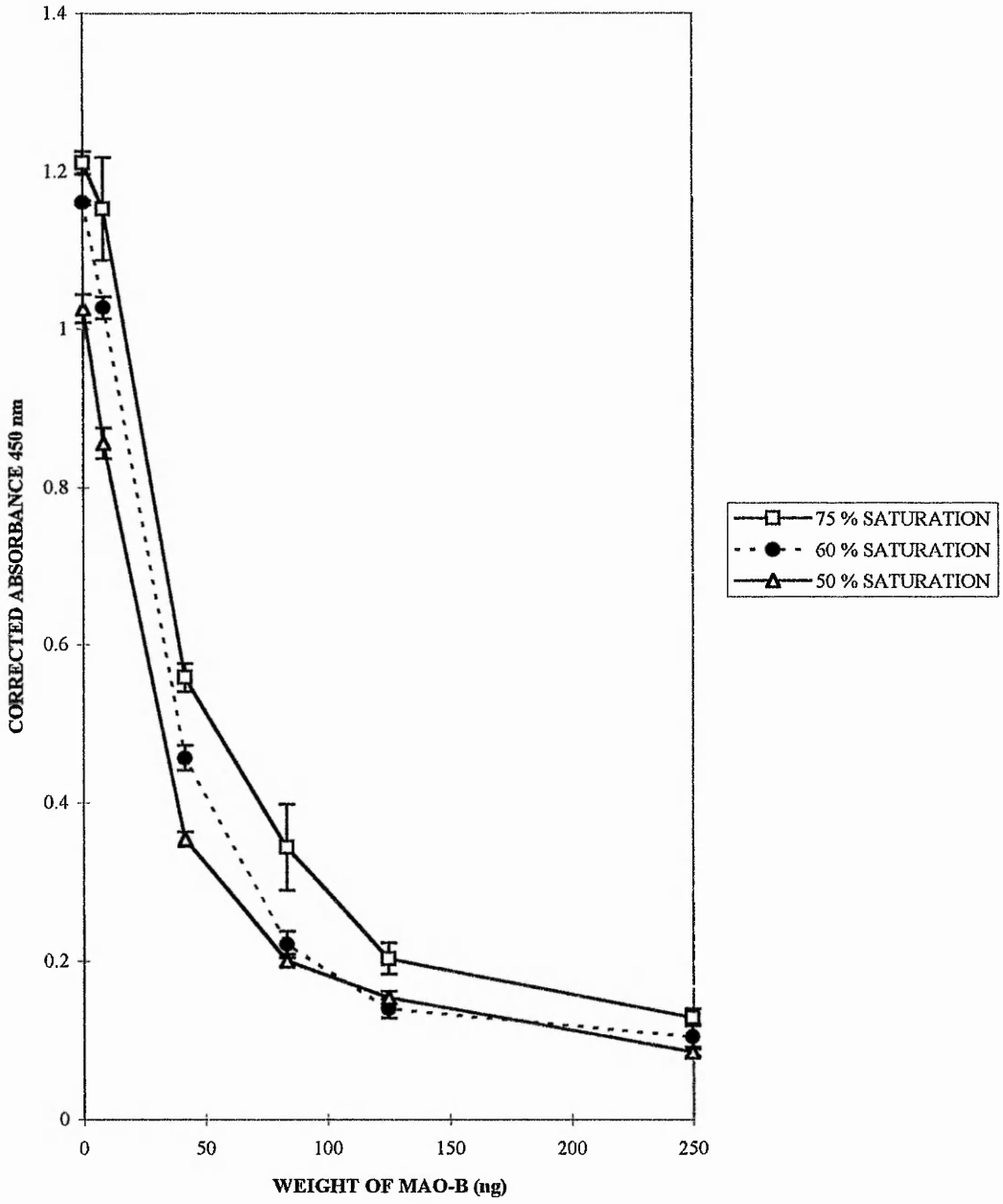


Figure 2.15. The effect of increasing antibody saturation on the competitive ELISA for MAO-B. Wells of a microtitre plate were coated with a crude preparation of human liver mitochondrial membranes (MM). Solubilised MM were used as the antigen competing for varying concentrations of MAO-B specific antibody, 3F12/G10/2E3 (final concentration Triton X-100, 0.1 % v/v). Concentration of MM is expressed as MAO-B equivalent.

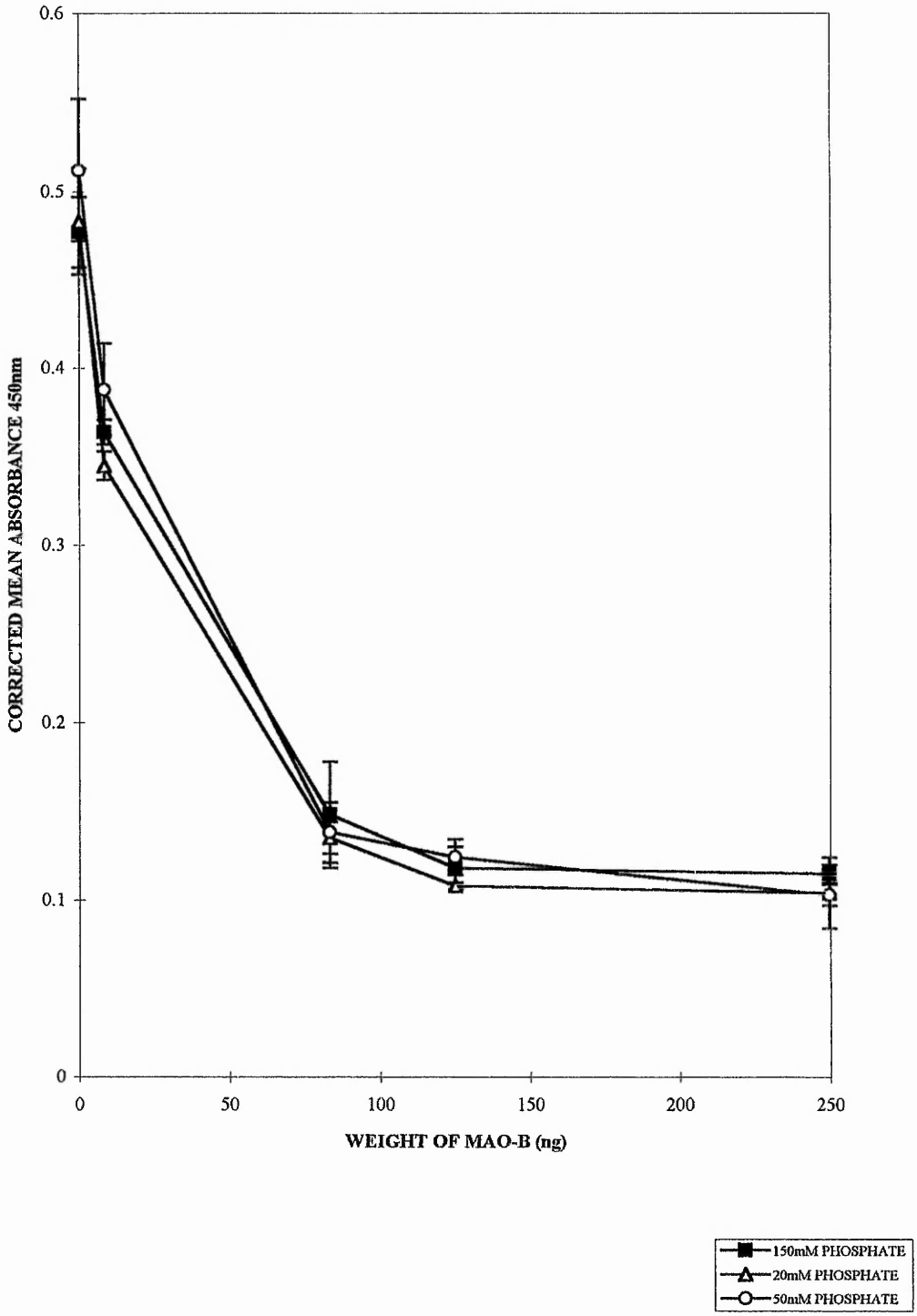


Figure 2.16. The effects of decreasing ionic strength of the incubation buffer on the competitive ELISA for MAO-B. Wells of a microtitre plate were coated with a crude preparation of human liver mitochondrial membranes (MM). Solubilised MM were used as the antigen competing for varying concentrations of MAO-B specific antibody, 3F12/G10/2E3 (final concentration Triton X-100, 0.1 % v/v). Concentration of MM is expressed as MAO-B equivalent.

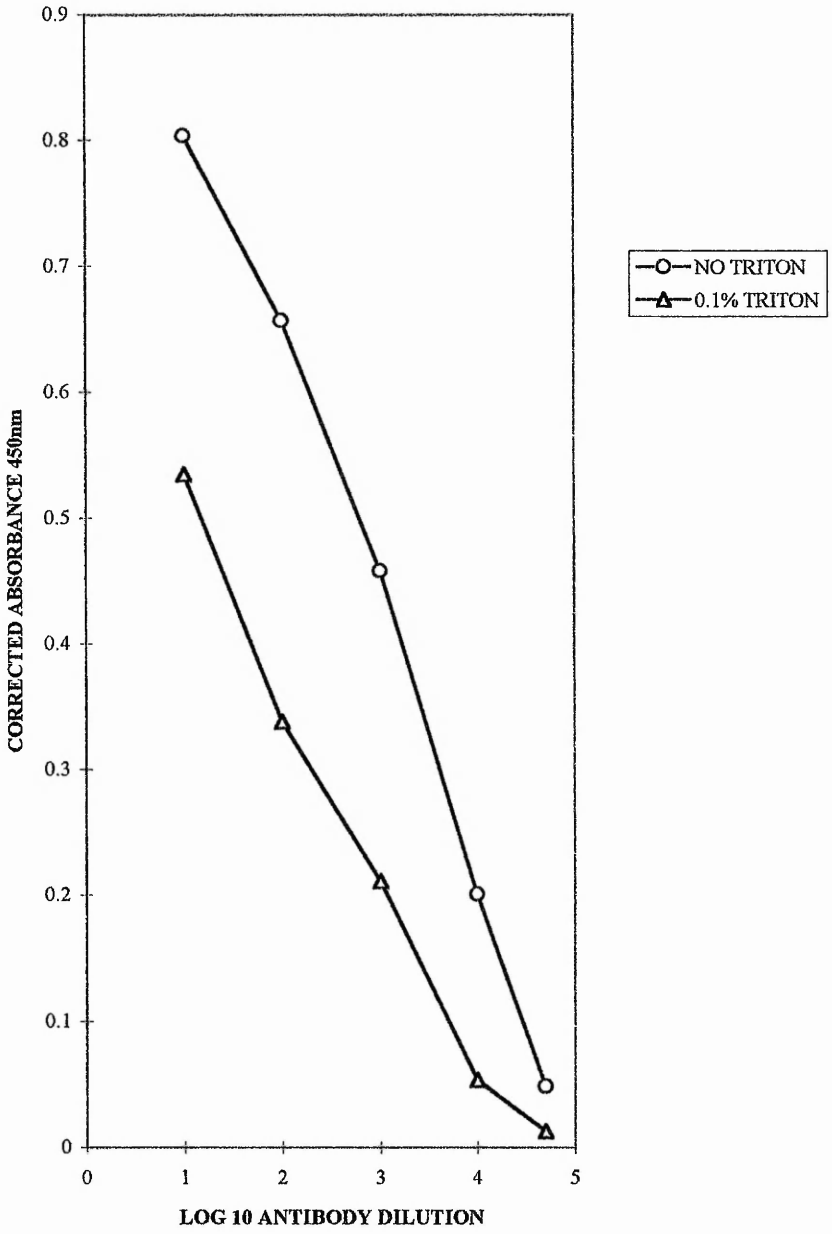


Figure 2.17. Effects of the detergent Triton X-100 on the titration of 6G11/E1 tissue culture supernatant. Wells were coated with mitochondrial membranes and incubated with serial dilutions of the mouse monoclonal antibody 6G11/E1.

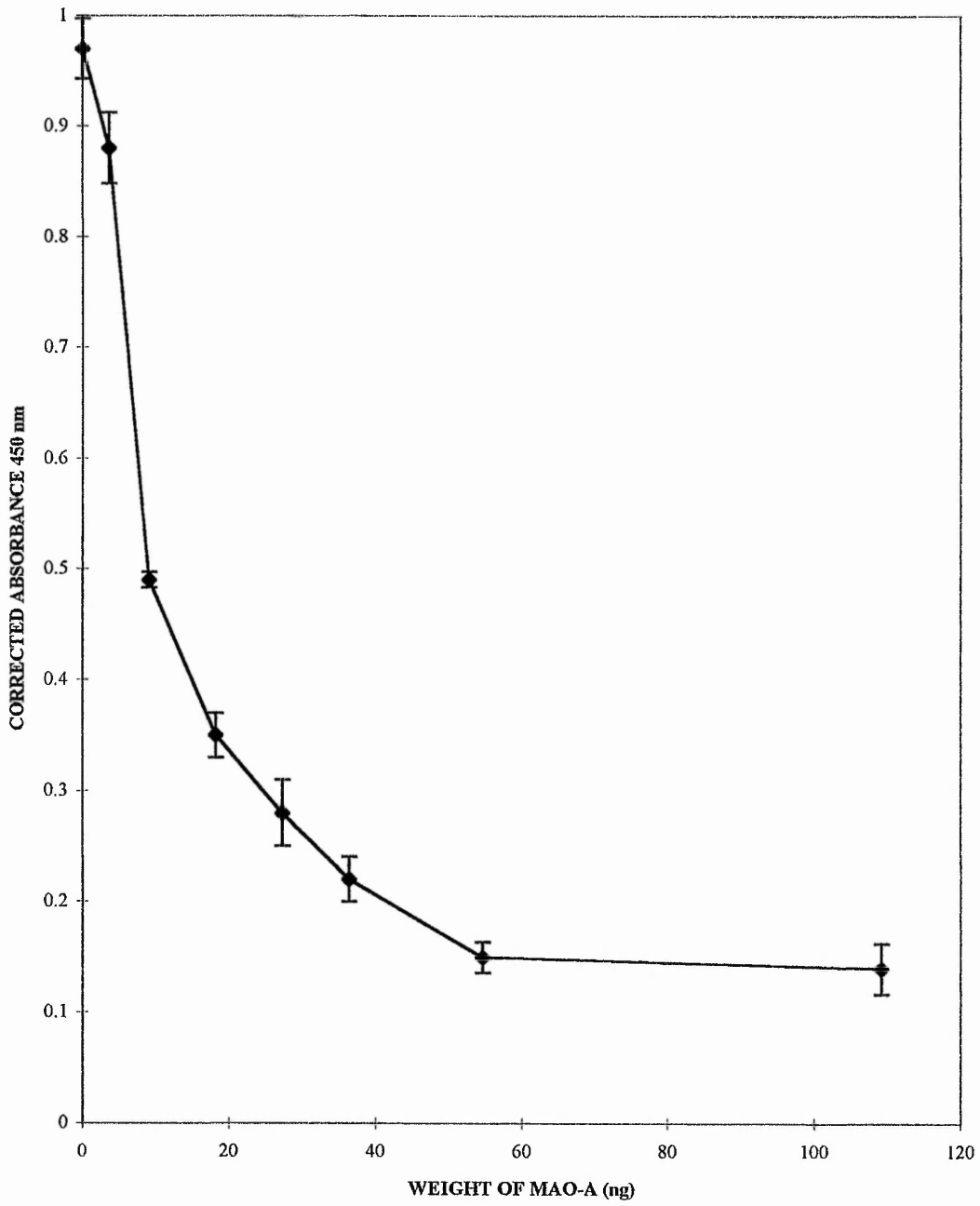


Figure 2.18. An example of a competitive ELISA linear displacement plot for MAO-A. Wells of a microtitre plate were coated with a crude preparation of human liver mitochondrial membranes (MM). Solubilised MM were used as the antigen competing for a limited concentration of MAO-A specific antibody, 6G11/E1 (final concentration Triton X-100, 0.1 % v/v). Concentration of MM was expressed as MAO-A equivalent.

2.3.3.3 *Effects of varying antibody concentrations*

As already indicated antibody concentration in the competitive ELISA needs to be limiting. Antibody concentrations giving 50% saturation (i.e. titre) are often used. Part of the optimisation of this ELISA was to check the effects of varying antibody concentrations. No differences in the detection limit and range of the assay were detected when using the antibody at 50%, 60% saturation (Figure 2.15). The detection limit of 75 % saturation was higher than 50 % and 60 %. Saturation values lower than 50% were not tested as the absorbance signal would have been drastically decreased making result evaluation impossible. An antibody concentration of 50 % was therefore chosen to conserve antibody.

2.3.3.4 *Effect of varying ionic concentration*

The effects of ionic strength on antibody/antigen interactions were also studied. No difference in antibody binding was apparent when ionic strengths ranged from 20 mM to 150 mM phosphate concentrations (Figure 2.16).

2.3.4 Development of a competitive ELISA to detect MAO-A

2.2.4.1 *Effects of Triton X-100 on the titre of 6G11/E1*

Similar results to those obtained with 3F12/G10/2E3 were evident when 6G11/E1 was titrated against human liver mitochondrial membranes in the presence of Triton X-100 (figure 2.17). A reduced titre (287) was obtained compared to the titre of 2253 in the absence of Triton X-100.

A competitive ELISA system similar to that developed for MAO-B was also developed for MAO-A, using the 6G11/E1 antibody, initially to detect the A enzyme in human liver mitochondrial membranes and later using solubilised extracts of human placental tissue. This assay had a detection limit of less than 10 ng MAO-A or 250 ng/ml. Figure 2.18 shows an example of the competitive ELISA for MAO-A.

2.4 DISCUSSION

2.4.1 Preparation of antigens

Mitochondrial membranes were prepared from both fresh and frozen human liver by differential centrifugation and sucrose density gradients. Freezing had no significant effect on the recovery of mitochondrial membranes. Following preparation the final product was assessed for protein concentration, checked for immunoreactivity using 3F12/G10/2E3 and 6G11/E1 in a non-competitive ELISA and assessed for MAO activity. Following these determinations the mitochondrial membranes were solubilised and extracts containing solubilised MAO were used as standards for both the MAO-A and MAO-B competitive ELISAs, standards being run on each microtitre plate to compensate for inter plate variation. One batch of MOM was used both as the coating antigen and (in its solubilised form) as a standard in all the ELISAs.

A combination of gel filtration and ion-exchange chromatography was successful in producing a MAO enriched sample from mitochondrial membranes for use as a standard. SDS-PAGE analysis of the final sample revealed that MAO-B constituted 26 % of the total protein and MAO-A 19 % of the total protein.

Attempts were also made to purify MAO-B by immunoaffinity chromatography using the 3F12/G10/2E3 antibody coupled to an affi-gel support. However although MAO bound to the immunoadsorbant, elution of MAO proved unsuccessful (data not shown).

2.4.2 Source of antibody

Following re-cloning of the anti-MAO-B hybridoma cell line, antibody secreted from a new clone, 3F12/G10/2E3, was selected as having the highest titre against human liver mitochondrial membranes and used in the competitive MAO-B ELISA. Antibody secreted from this recloned hybridoma and hybridoma 6G11/E1 were assessed and characterised using both the Western blot technique and immunohistochemistry.

2.4.2.1 *Western blot analysis*

The Western blot technique successfully showed that 6G11/E1 bound to a single band (MAO-A) of the correct molecular weight, which was still visible at high dilutions of the antibody. Unfortunately 3F12/G10/2E3 failed to reveal MAO-B on a Western blot despite using longer incubation times and the inclusion of Tween-20 in the incubation buffer. However this result was not surprising as previous Western blot analysis by others had also failed to pick up MAO-B (eg Yeomanson, 1990). Although the specificity of 3F12/G10 had been determined by Billett and Mayer (1986), the fact that the hybridoma cell line had been re-cloned necessitated checking its specificity for MAO-B. It was hoped that immunoprecipitation using radiolabelled pargyline could be used but unfortunately a commercial source of the inhibitor was no longer available. An alternative means of determining specificity was therefore sought.

2.4.2.2 *Immunohistochemistry*

It is well documented that lymphocytes contain only the B form of the enzyme (e.g. Balsa *et al*, 1989); they were therefore used to check 3F12/G10/2E3 specificity. Indeed 3F12/G10/2E3 did react with B lymphocytes (and also platelets) isolated from human blood, whilst 6G11/E1 (anti- MAO-A) did not, providing very strong evidence that 3F12/G10/2E3 was specific for MAO-B.

A study of the reactivity of both antibodies on tissue sections was also undertaken to visualise MAO *in situ*. Two methods of tissue preservation were employed to determine the most suitable method for MAO enzyme epitope preservation following freezing. The traditional method of tissue preservation for cryosectioning is to snap freeze in liquid nitrogen cooled isopentane and post fix the tissue after sectioning. However it is often felt that some form of cryoprotection is required to enhance the preservation of specific epitopes. The alternative method used in this study involved fixing the tissue before freezing, to prevent tissue and enzyme degradation; this method was combined with sucrose protection (Hancock and Atkins, 1986). Indeed the method had been successfully employed using both antibodies (Rodriguez *et al*, [a], in press).

A comparison of the two methods of tissue preparation showed that immediate fixing in paraformaldehyde for 4 hours followed by sucrose protection before snap freezing produced an increase in the intensity of staining for both antibodies in human liver cryosections, and that this increase was more pronounced for MAO-B. This suggests that both enzymes are damaged during freezing and/or post fixing and that immediate fixing followed by sucrose perfusion may protect both enzyme forms. As this was more pronounced using the MAO-B antibody it may mean that MAO-B is more susceptible to damage and/or that the epitope against which the MAO-B antibody is directed is less stable. The latter may account for the failure of the antibody to react on a Western blot, knowing that the enzyme will have been denatured following SDS-PAGE.

Overall, this study indicates that the preferred method of tissue preservation for visualisation and determination of the presence of MAO is the sucrose protection technique.

Both the data presented here and previous evidence have demonstrated that 6G11/E1 and 3F12/G10/2E3 are specific for MAO-A and MAO-B respectively. This enables their use in the analysis of MAO enzyme status in two important tissues namely platelets and placenta.

2.4.3 The MAO-B and MAO-A competitive ELISAs

The competitive ELISA previously described was adapted for monitoring MAO-B in solubilised extracts of human platelets. Times and temperatures of incubation of the primary antibody in the presence of the competitor were unaltered as an overnight incubation was convenient and ensured that the reactions reached equilibrium. One of the main problems was the presence of Triton X-100 since it adversely affected the binding of MAO to the antibody, 3F12/G10/2E3. Triton X-100 was found to affect antibody/antigen interactions at final concentrations of 0.1 % (v/v) and greater resulting in a lowering of absorbance signals. However, despite this reduction in binding the detection limit of the MAO-B assay (in 0.1 % [v/v] Triton X-100) was around 8.32 ng (208 ng/ml) MAO-B. This is similar to the detection limit determined by Yeomanson (1990) [1.87 ng/assay] and also to that of Denney *et al*, 1983 (8.5 ng/assay). The latter is based on the assumption that 1 molecule of MAO contains 2 molecules of FAD.

To achieve efficient solubilisation, platelet and liver mitochondrial membrane samples had to be solubilised in a final Triton X-100 concentration of 0.5 % and 1.0% respectively (Yeomanson, 1990); thus these extracts had to be diluted to give a final Triton X-100 concentration of 0.1 %. In a minority of samples this dilution meant that the protein concentration was insufficient to produce competition in the ELISAs.

The effect of antibody saturation on the ELISA was also analysed, 50% and 60 % saturation gave the most sensitive assay over a useful range of MAO concentrations, 50 % (used previously) was used to conserve antibody.

The effect of changes in ionic concentration of the incubation buffer was also checked as previous reports (Browne *et al*, 1978; Yu, 1981) indicated that high ionic strength (0.2 M phosphate buffer) reduced MAO activity and that MAO was much less stable in 0.2 M phosphate buffer, pH 7.5 than in 0.02 M phosphate buffer at the same pH value. All antibody incubations up to this point had been performed in phosphate buffered saline (PBS; 150 mM phosphate). Lowering the ionic strength to 20 mM and 50 mM had no effect on the detection limit and range of the assay so the use of PBS was continued. It therefore appears that the antibody interactions are unaffected by reducing ionic strength of the incubation buffer.

The MAO-B content of both solubilised mitochondrial membranes and solubilised platelet extracts can therefore now be determined in this sensitive competitive ELISA by comparing linearised displacement plots of the specific antigen source with that of the purified MAO. An assessment of the MAO-B content of platelet extracts from Parkinson's Disease patients and controls is thus possible (Chapter 3).

An ELISA system for the specific quantitative analysis of MAO-A was also developed. This assay is identical to that developed for MAO-B in that it relies on the competition of immobilised MAO-A with mobile MAO-A in samples or standards for a limited amount of specific MAO-A antibody (6G11/E1). The assay conditions previously used in the MAO-B assay were used, thus the primary antibody was incubated overnight at 4 °C. 6G11/E1 has also been titrated in the presence of 0.1 % Triton X-100 as this assay will be required to assess the status of solubilised MAO-A in placental samples (chapter 4).

CHAPTER THREE

ANALYSIS OF PLATELET SAMPLES FROM PARKINSON'S DISEASE PATIENTS AND AGE MATCHED CONTROLS

CHAPTER THREE

ANALYSIS OF PLATELET SAMPLES FROM PARKINSON'S DISEASE PATIENTS AND AGE MATCHED CONTROLS

3.1 INTRODUCTION

An *in vivo* marker for Parkinson's Disease which is easily obtainable i.e peripheral and collected by non-invasive means would be helpful for early diagnosis of the disease. Current methods of diagnosis rely on presentation of symptoms which often do not manifest themselves until the disease is well advanced.

In the human brain dopamine is a substrate for MAO-B, the form predominantly found in this tissue (Stahl, 1977; Fowler *et al*, 1980). As already mentioned in chapter 1, the expression of MAO in the brain has been a focus of interest in Parkinson's Disease, (PD) firstly because selective inhibition of MAO-B with L-deprenyl, particularly as an adjunct to L-DOPA, is successfully used therapeutically (Youdim and Finberg, 1986), and secondly, because MAO-B oxidises N-methyl 4-phenyl 1,2,3,6 tetrahydropyridine (MPTP) into the 1-methyl-4-phenylpyridium cation, MPP^+, a potent neurotoxin (Heikkila *et al*, 1984 (b); Maret *et al*, 1990). Thus MAO-B could reasonably be considered as an activating enzyme for other pro-neurotoxins, possibly of environmental origin, and increased MAO-B activities may therefore be expected in PD patients. Indeed it has been suggested that an inherited variant of MAO-B may be involved in a genetic predisposition for Parkinson's Disease (Kurth *et al*, 1993).

Platelets are able to accumulate, store and release the neurotransmitter 5-hydroxytryptamine. This knowledge has fuelled the idea that platelets can serve as models for serotonergic neurones.

As previously described (section 1.3.4) platelet MAO-B has traditionally been used as a model for brain MAO-B (Youdim, 1988); and it is also known that the deduced amino acid sequences of MAO-B derived from human platelets and brain frontal cortex are

identical (Chen *et al*, 1993). This information coupled with the ease of collection of platelet samples by non-invasive means makes platelet MAO-B an ideal candidate as a peripheral PD marker.

However, whether or not platelet MAO-B has a role in predictive diagnosis of PD is still an open question because results obtained to date are conflicting. Some studies have indeed shown relative enzyme excesses compared to controls (Danielczyk *et al*, 1988; Bonuccelli *et al*, 1990; Bongioanni *et al*, 1996) and others indicate either relative deficits (Zeller *et al*, 1976) or no discernible differences (Yong and Perry, 1986).

Data have suggested that measurements of platelet MAO activity are dependent on the substrate used, the activity being slightly higher in untreated PD patients than controls when using phenylethylamine and significantly lower using dopamine (Humfrey *et al*, 1990). Thus it appears that when using non-hydroxylated monoamines as substrates MAO activity in PD patients is greater than in controls while using mono- and di-hydroxylated monoamines as substrates a reduction in activity compared to controls is observed. This may imply that in PD a different MAO-B isoenzyme is being expressed, perhaps mirrored in the CNS. Indeed isoenzymes of MAO-B have been shown to exist in monkey platelets (Obata *et al*, 1990).

One brief study (Jarman *et al*, 1993) has disputed the results with dopamine and another study (Checkoway *et al*, 1992) has suggested that results with PEA are slightly lower in male PD patients compared to controls but slightly higher in female patients compared to controls.

It is possible that conflicting results have arisen because the measurement of MAO activity is unreliable, being influenced by a number of factors including interfering enzymes, inhibitors and the lipid environment. In addition, the variable relative numbers of males and females (often in the menopausal stages) used in the studies may have complicated matters because it is known that MAO activity is affected by hormonal status (Belmaker *et al*, 1974; Poirier *et al*, 1985). It is not known whether the effects seen are due to altered rate of expression, altered MAO protein or the presence

of modulators. This chapter therefore revisits the question of whether platelet MAO-B is a useful marker of pre-disposition to PD. MAO activity assays are complemented by the use of the MAO-B specific murine monoclonal (3F12/G10/2E3) in the competitive ELISA to monitor MAO-B protein in the platelets (Yeomanson and Billett, 1992, chapter 2). This assay measures total MAO-B protein, both active and inactive. The aims of this study were to re-assess the status of platelet MAO in recently diagnosed, drug untreated (*de novo*) patients with clinically defined idiopathic PD compared with age matched controls (healthy volunteers). Two different populations were studied, British Caucasian and Hong Kong Chinese; thus a comparison of two different race populations was also conducted. Few reports have been published investigating Chinese PD. One study (Chia and Liu, 1992) assessed a Taiwanese PD population compared to Western and Japanese patients, and found that the incidence of PD in Chinese males was higher than in Western and Japanese males. However, it is generally accepted that the prevalence of PD is lower in Chinese populations compared to Western populations (Wang *et al*, 1996; Chan *et al*, 1998).

The study was undertaken in collaboration with Professor A.C Williams (Queen Elizabeth Hospital, Birmingham, U.K.) and Dr S. L. Ho (Department of Medicine, University of Hong Kong) who supplied the samples and ensured uniformity with respect to clinical symptoms/history.

To measure the amount of MAO-B protein in human platelet samples, a platelet extraction strategy had to be devised to accommodate the fact that only small volumes of blood would be available. Previous work in the laboratory (Yeomanson, 1990) had revealed that platelet recovery in platelet rich plasma (PRP) was in excess of 80 % of the total number in whole blood. In the current study, platelets were sedimented from the PRP sample and re-suspended in appropriate buffers for analysis; it was assumed that platelet density remains unaltered in PD.

The competitive ELISA was used for the specific measurement of MAO-B protein concentrations and MAO activity assays using dopamine and phenylethylamine (as used

by Humfrey *et al*, 1990) as substrates were performed concomitantly to enable an estimation of the molecular activity of MAO-B.

Most of the Caucasian work has been published in : Progress in Brain Research, 1995, Volume 106, pages 85-90.

3.2 MATERIALS AND METHODS

3.2.1 Patient selection

Parkinson's Disease was diagnosed by the presence of three of the following features : tremor, rigidity, bradykinesia, postural instability, gait disturbances, response to L-DOPA. Patients with atypical features including abnormal eye movements, dementia and autonomic dysfunction were excluded, as was parkinsonism due to any other neurological diseases, chemicals or toxins. None of the subjects used in this study were on medication for PD or drugs known to influence MAO-B activity. All patients and controls were non-smokers. Control samples were kindly donated by age matched healthy volunteers. Caucasian blood sample collection was performed at the Queen Elizabeth Hospital, Birmingham, U.K., while samples from the Hong Kong Chinese population were collected at the Department of Medicine, University of Hong Kong. All samples were collected from fasting subjects.

3.2.1.1 *Sample logging*

Following their receipt, platelet rich plasma (PRP) samples were logged and allocated a specific code number. Each sample was categorised according to age, sex and diagnosis and samples were analysed for MAO-B protein concentration and MAO-B activity in a random order.

3.2.2 Platelet extraction procedure

3.2.2.1 *Materials*

10% EDTA
Washing buffer (see appendix)

3.2.2.2 *Method*

Collection of platelet rich plasma (PRP)

This was a modification of Corash's method, 1980. All procedures were performed at room temperature unless otherwise stated.

Collection of PRP

Venous whole blood (20 ml) was collected into 0.2 ml 10% (w/v) ethylenediamine-tetraacetic acid (EDTA), pH 7.4 and PRP isolated by centrifugation at 600 x g for 5 minutes. PRP was frozen in aliquots at -70°C overnight or until required. This procedure was conducted by staff at Queen Elizabeth Hospital, Birmingham and University of Hong Kong.

Platelet washing

PRP (5 ml) was thawed, mixed well to ensure an even suspension and divided into two portions (3 ml and 2 ml) and each portion centrifuged at 28000 x g, 20 minutes. The resultant pellets were washed by resuspension (3 ml and 2 ml) in washing buffer and centrifuged at 28000 x g for 20 minutes

Solubilisation of platelet MAO for ELISA

1. One platelet pellet (3 ml) was resuspended in 0.3 ml potassium phosphate buffer (0.05 M, pH 7.4, containing Triton X-100, 0.5% w/v) and sonicated for 10 seconds (8 kilocycles/second) to ensure even resuspension. Samples were incubated (37°C, 60 minutes.) stirring at 10 minute intervals. Solubilised platelet protein was collected following centrifugation at 28000 x g, 60 minutes and its total protein content estimated (Bio-rad commercial kit).

2. The second pellet (2 ml) was resuspended in 2 ml potassium phosphate buffer, pH 7.4 (without Triton X-100) and sonicated as previously described, for use in the activity assays.

3.2.3 Assay for MAO activity

3.2.3.1 *Materials*

Phenylethylamine hydrochloride, β-[ethyl-1-^{14}C] - DuPont (NEN) code NEC-502

Dihydroxyphenylethylamine 3,4-[ring-2,5,6-^{3}H] -DuPont (NEN) code NET 673

3.2.3.2 *Method*

Measurement of MAO activity was based on the method of Russell and Mayer, 1983, adapted for the smaller sample volumes. The assay is based on the ability of MAO to convert water soluble substrates (^{14}C phenylethylamine [PEA] and ^{3}H dopamine) to their corresponding aldehyde products which are differentially soluble in an organic phase (Refer to section 2.2.2). Time courses were performed for each substrate in a final assay volume of 200 µl.

3.2.4 Competitive ELISA for measurement of MAO-B protein

Platelet samples for ELISA were prepared as described in section 3.2.2. The final protein concentration of the concentrated platelet extract was estimated using the commercial Bio-rad protein assay kit. Varying concentrations of platelet MAO or standard MAO protein were added to microtitre wells already coated with mitochondrial membranes as a source of competing antigen in the presence of limiting anti MAO-B antibody and allowed to incubate at 4°C overnight. Washing and secondary antibody incubation were as already described in section 2.2.15.

3.2.5 Statistical analysis

Statistical analysis on all data collected from both populations was performed using the Mann Whitney U Test as the sample sizes were small and unmatched.

3.3 RESULTS

3.3.1 Development of a suitable platelet extraction procedure

The main priority was to efficiently collect platelets from a limited volume of platelet rich plasma (PRP). Only a limited volume of whole blood was available (approximately 20 ml) which produced around 6 ml of PRP; this would subsequently be analysed for both MAO-B protein concentration by specific competitive ELISA and MAO activity using two different substrates. Efficient use of the PRP sample was essential to ensure each analysis was performed with the correct number of replicates and controls.

Prior to analysis, platelets need to be collected as a pellet, thus PRP was subjected to different centrifugation times and g forces. Centrifugation at 5000 x g for 20 minutes did not sediment all platelets, since MAO activity was detected in the supernatant. However, further work revealed that centrifugation at 28000 x g for 20 minutes sedimented all the platelets in the sample, (MAO activity detected in the resulting supernatant was negligible, data not shown).

3.3.2 Platelet analysis by ELISA and activity assays

MAO-B was solubilised using Triton X-100 at a final concentration of 0.5 %; this concentration had previously been determined to give a solubilising efficiency of 98 % (Yeomanson, 1990). The MAO-B protein concentration of Triton-X100 solubilised platelet samples was measured using the competitive ELISA system already described. Comparison of the linearised displacement plot (Figure 3.1) of extracted platelet MAO (for each patient) with that of enriched MAO or MM (previously calibrated against enriched MAO, chapter 2) enabled the determination of MAO concentration in each sample. The plots produced for each platelet sample show the displacement of 3F12/G10/2E3 antibody binding to the solid phase antigen by the presence of varying concentrations of standard MAO or serial dilutions of solubilised patient platelet extracts. These semi-log plots obtained from patient sample data are parallel with the

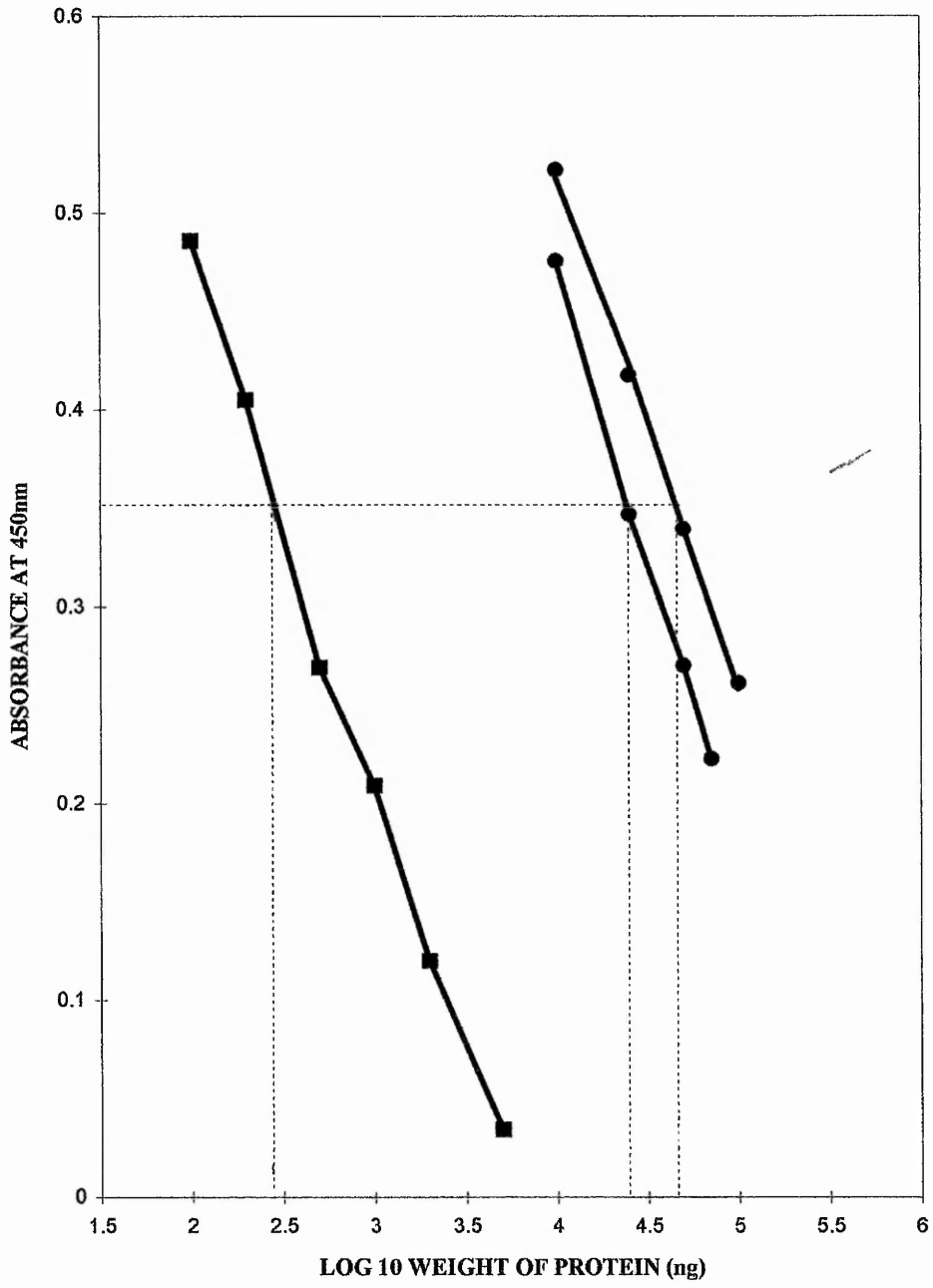


Figure 3.1.Measurement of MAO-B concentration in human platelets using the competitive ELISA. Semi-log plot of competitive data for standard MAO (■) and two platelet samples (●) as examples; n = 3. In the absence of competitor the mean absorbance was 0.54±0.03.

standard MAO plots indicating that the 3F12/G10/2E3 antibody recognises the same epitope in patient platelet extracts and in the standard MAO.

Time course studies for MAO activity with ^{3}H dopamine (Figure 3.2A) and ^{14}C PEA (Figure 3.2B) were conducted using a final concentration of 50 μM and 20 μM respectively. An end point (60 minutes for both substrates) within the linear phase of the reaction was chosen.

The reproducibility of both assays was assessed using platelets extracted from 5 normal healthy volunteers. Standard deviation was always within 10 % of the mean values for both ELISA and activity assays (data not shown). Platelet samples were assessed on more than one occasion each giving reproducible results.

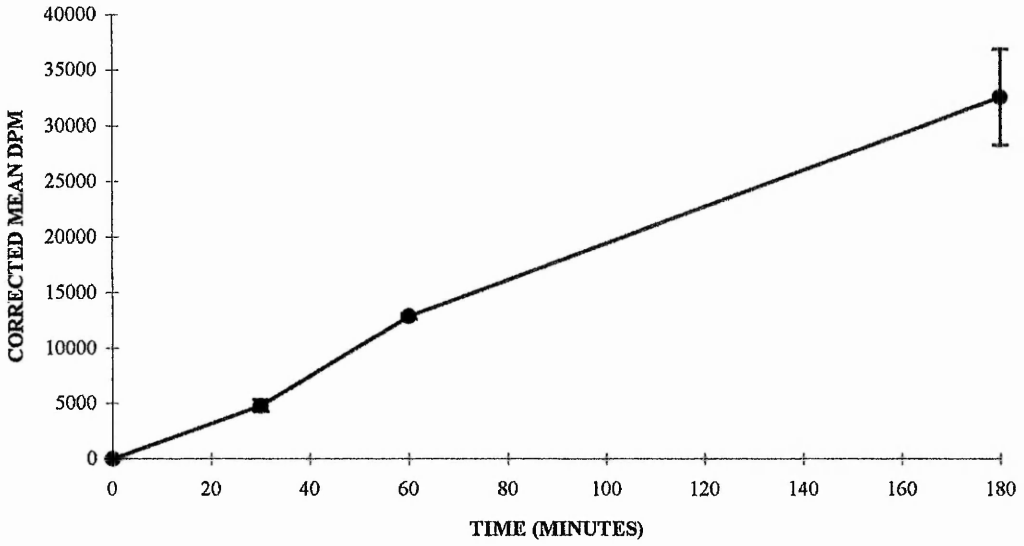
3.3.3 Analysis of platelet extracts from Parkinson's Disease patients and controls

In the ELISA, displacement plots were obtained for the majority of patient samples analysed. However a few sample extracts failed to show any antigen competition as the solubilised protein concentrations were too low. Semi-log plots were obtained from each sample and calibrated against standard MAO or MM. MAO activity using both PEA and dopamine as substrates was calculated for each sample. Once a profile of results had been obtained statistical analyses using the Mann-Whitney U test were performed.

3.3.3.1 *Caucasian study*

Male and female PD patients and matched controls were analysed separately. In the male population the mean concentration of MAO-B protein per milligram of total platelet protein was approximately 40% greater in the PD patients compared with the control group (Table 3.1). However this difference was not significant as analysed by the Mann-Whitney U test. MAO activity in males was analysed and expressed as a measure of total platelet protein and MAO protein. Indeed MAO activity per milligram total platelet protein and MAO molecular activity (i.e. nmol substrate deaminated hr^{-1}

A



B

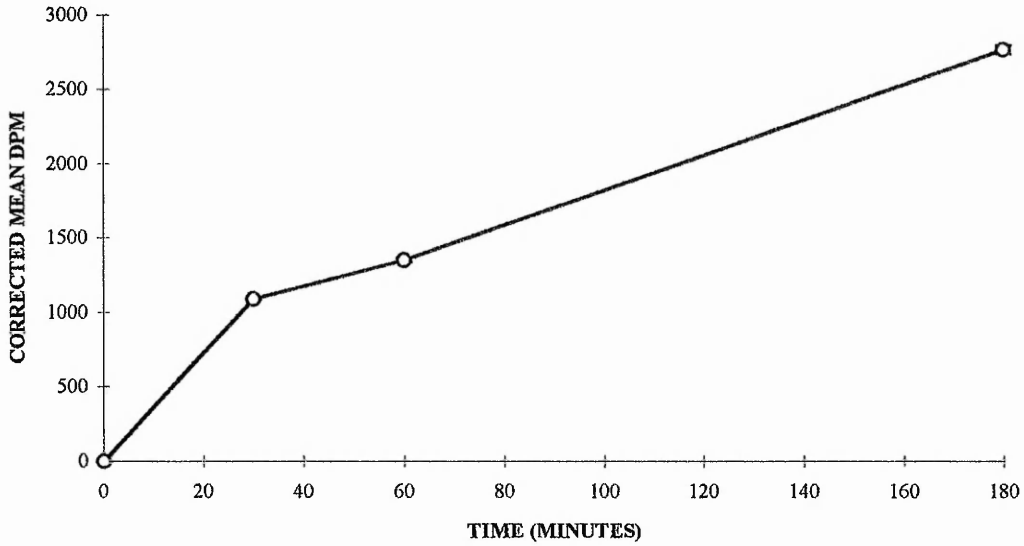


Figure 3.2. Measurement of platelet MAO activity over time using, **A**, Dopamine, **B**, phenylethylamine as substrates. Values have been corrected using an appropriate sample blank and background count.

TABLE 3.1

Platelet MAO-B activity and MAO-B protein concentration in male Caucasian Parkinson's Disease patients and age matched controls.

	CONTROLS	**PARKINSON'S DISEASE**
NUMBER	7	12
AGE	56 +/- 12	62 +/- 12
TOTAL PROTEIN CONCENTRATION (mg/ml)	6.42 +/- 2.24	6.80 +/-4.39
MAO CONCENTRATION (µg MAO-B/mg platelet protein)	2.33 +/- 0.55	3.28 +/- 1.93
PEA DEAMINATION a) nmol hr^{-1} mg^{-1} total protein b) nmol hr^{-1} mg^{-1} MAO-B protein	3.02 +/- 1.53 1291 +/- 637	2.79 +/- 2.28 1285 +/- 1319
DOPAMINE DEAMINATION a) nmol hr^{-1} mg^{-1} total protein b) nmol hr^{-1} mg^{-1} MAO-B protein	0.96 +/- 0.42 407 +/- 169	0.95 +/- 0.53 476 +/- 430

All values are expressed as the mean +/- standard deviation.
None of the above differences are significant analysed using the Mann-Whitney U-Test, chosen because the sample size is small and unmatched.

TABLE 3.2

Platelet MAO-B activity and MAO-B protein concentration in female Caucasian Parkinson's Disease patients and age matched controls.

	CONTROLS	**PARKINSON'S DISEASE**
NUMBER	6	7
AGE	55 +/- 8	59 +/- 9
TOTAL PROTEIN CONCENTRATION (mg/ml)	6.04 +/-1.30	5.91 +/-2.67
MAO-B CONCENTRATION (μg MAO-B/mg platelet protein)	4.25 +/- 2.78	4.04 +/- 3.30
PEA DEAMINATION a) nmol hr^{-1} mg^{-1} total protein b) nmol hr^{-1} mg^{-1} MAO-B protein	3.51 +/- 1.53 1358 +/- 1233	4.31 +/- 3.33 2545 +/- 2488
DOPAMINE DEAMINATION a) nmol hr^{-1} mg^{-1} total protein b) nmol hr^{-1} mg^{-1} MAO-B protein	1.13 +/- 0.35 360 +/- 223	1.12 +/- 0.42 583 +/- 489

All values are expressed as the mean +/- standard deviation.
None of the above differences are significant analysed using the Mann-Whitney U test, chosen because the sample size is small and unmatched.

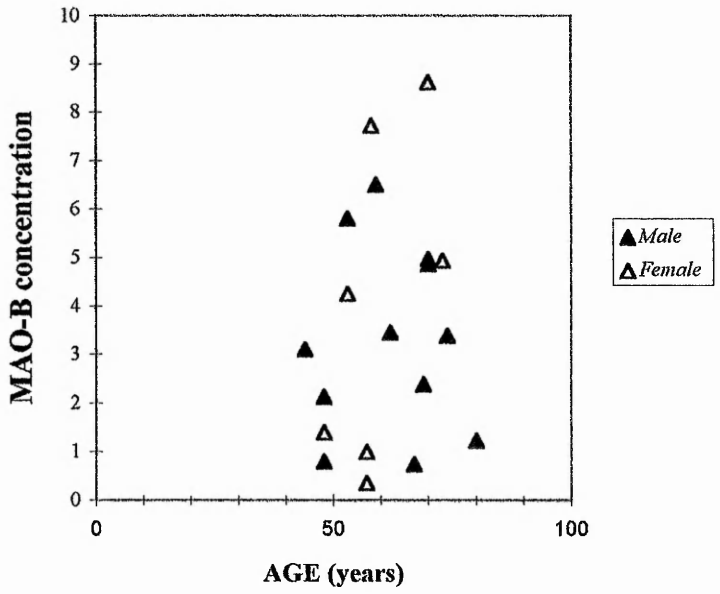
TABLE 3.3

Platelet MAO activity and MAO-B protein concentration in a mixed population of Caucasian Parkinson's Disease patients and age matched controls.

	CONTROLS	**PARKINSON'S DISEASE**
NUMBER (M/F)	13 (7/6)	19 (12/7)
AGE	55 +/- 11	61 +/- 11
TOTAL PROTEIN CONCENTRATION (mg/ml)	6.25 +/-1.80	6.47 +/-3.79
MAO CONCENTRATION (μg MAO-B/mg platelet protein)	3.22 +/- 2.10	3.56 +/- 2.46
PEA DEAMINATION a) nmol hr^{-1} mg^{-1} total protein b) nmol hr^{-1} mg^{-1} MAO-B protein	 3.25 +/- 1.50 1322 +/- 915	 3.50 +/- 2.68 1749 +/- 1875
DOPAMINE DEAMINATION a) nmol hr^{-1} mg^{-1} total protein b) nmol hr^{-1} mg^{-1} MAO-B protein	 1.04 +/- 0.38 385 +/- 189	 1.01 +/- 0.49 515 +/- 442

All values are expressed as the mean +/- standard deviation.
None of the above differences are significant analysed using the Mann-Whitney U test, chosen because the sample size is small and unmatched.

PARKINSON'S DISEASE



AGE MATCHED CONTROLS

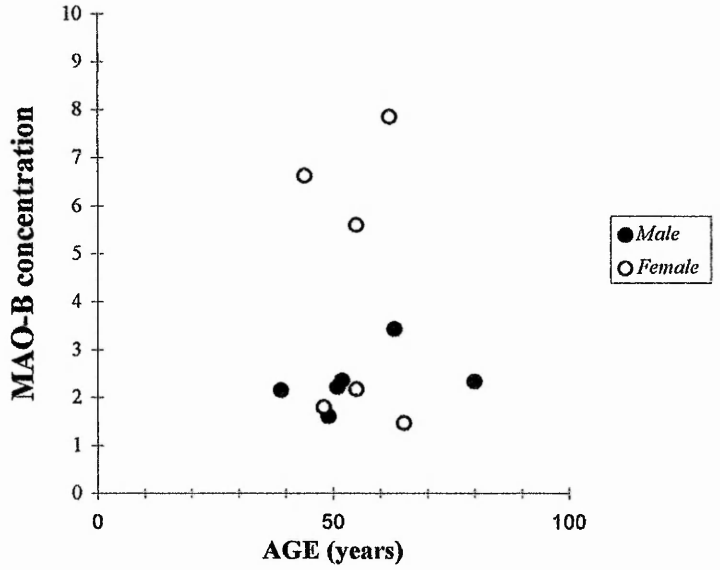
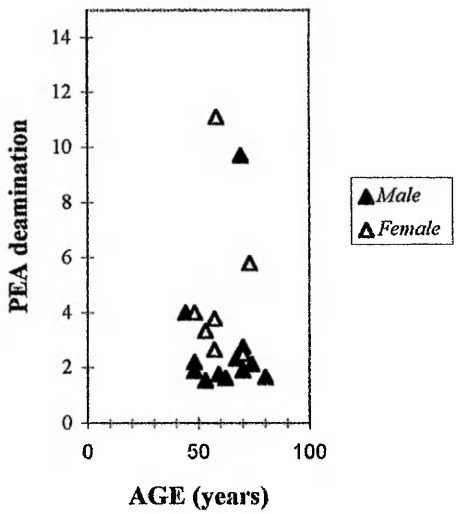


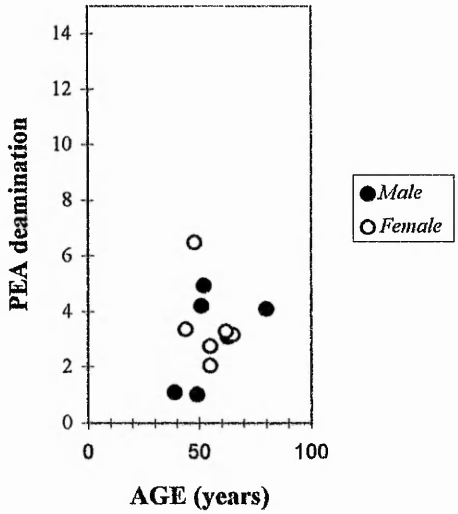
Figure 3.3. Concentration of platelet MAO-B in Parkinson's Disease patients (Δ), n = 19 and age matched controls (o), n = 13. MAO-B concentration is expressed as μg MAO-B per mg total platelet protein.

a) PHENYLETHYLAMINE

Parkinson's Disease

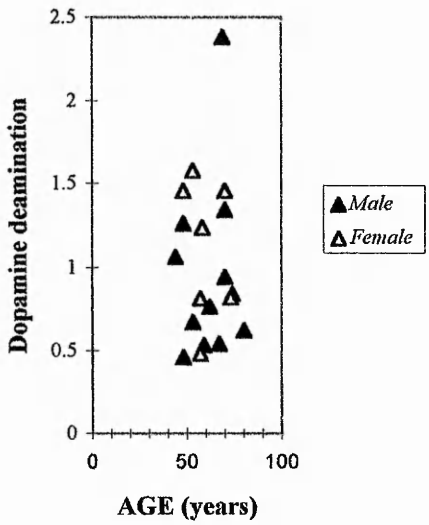


Age matched controls



b) DOPAMINE

Parkinson's Disease



Age matched controls

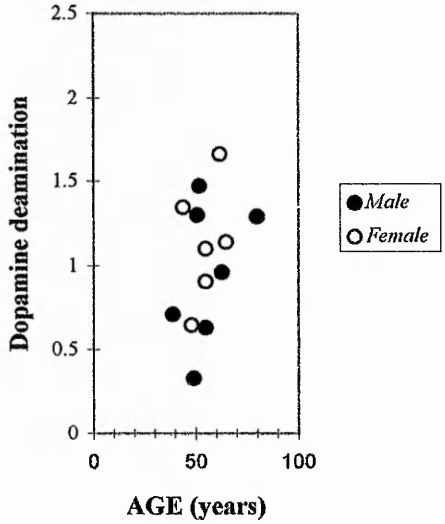
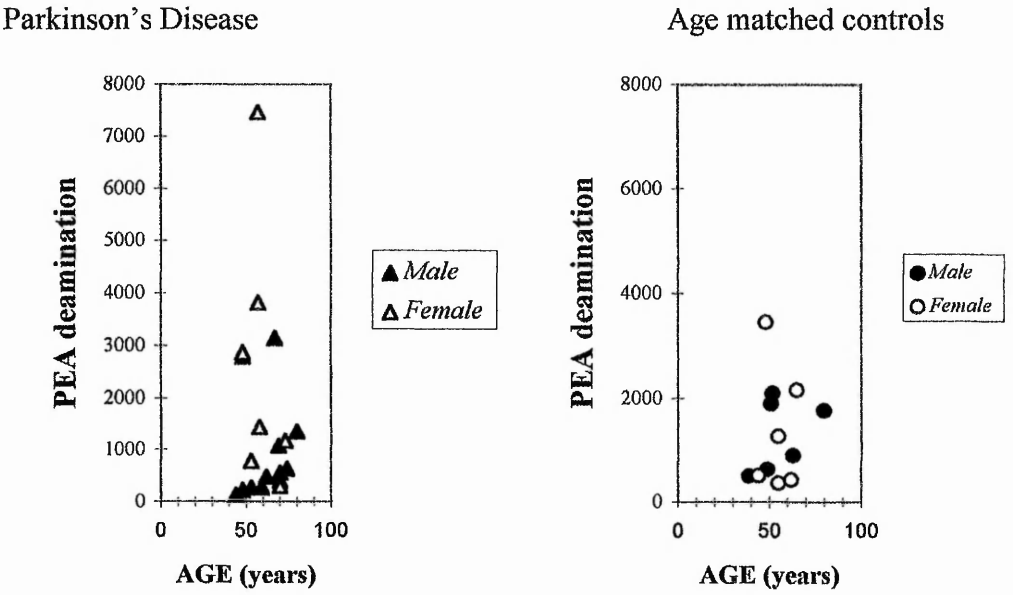


Figure 3.4. Platelet MAO-B activity in Parkinson's Disease patients (Δ), n = 19 and age matched controls (o), n = 13. MAO activity is expressed per milligram total platelet protein (nmol. substrate deaminated $hour^{-1}$ mg^{-1} total platelet protein).

a) PHENYLETHYLAMINE



b) DOPAMINE

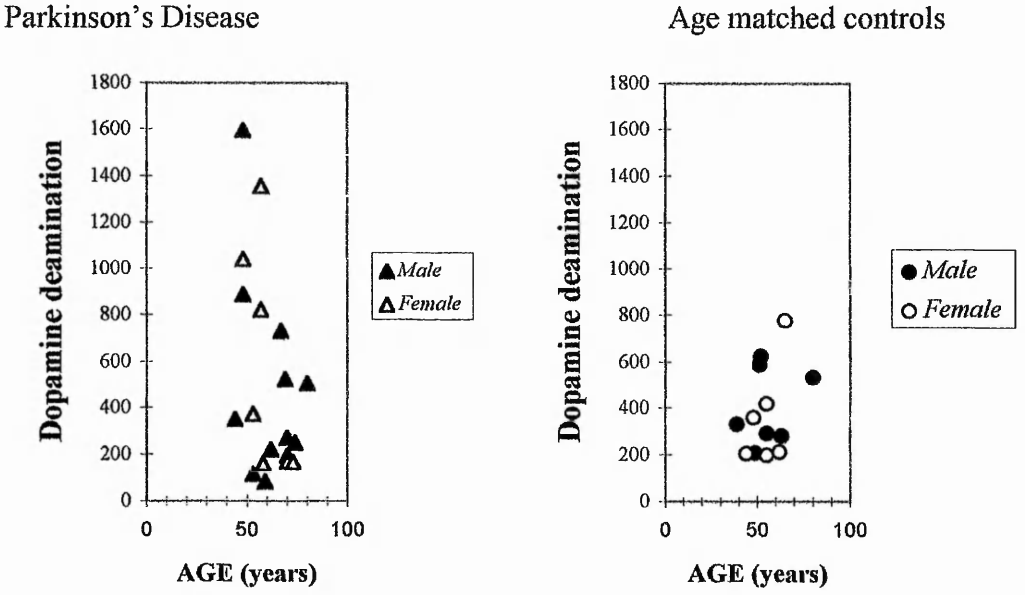


Figure 3.5. Molecular activity of platelet MAO-B in Parkinson's Disease patients (Δ), n = 19 and age matched controls (o), n = 13. MAO activity is expressed per milligram MAO-B (nmol. substrate deaminated $hour^{-1}$ mg^{-1} MAO-B).

mg^{-1} MAO-B) were similar in the two groups for both substrates. Scatter plots of MAO concentration (Figure 3.3) revealed that the male PD group was more heterogeneous than the controls.

Considering the female population, the concentration of MAO-B per milligram of total platelet protein (Table 3.2) was very similar within the two groups (PD and controls), differing from the male population study. MAO activity (based on both total platelet protein and MAO protein i.e molecular activity) was also similar in both groups for both substrates and a little more variable within the PD group (Figures 3.4 and 3.5). Again, the scatter plots reveal more heterogeneity in the PD group (also see table values for standard deviations). Activity and MAO concentration per mg platelet protein is generally higher in females (both controls and PD) than males. Scatter plot (Figure 3.3) draws attention to this in the control population.

The male and female populations were combined and statistically analysed as a mixed population (Table 3.3). Scatter plots (Figures 3.3, 3.4 and 3.5) do not reveal sub-populations in the PD group and again no statistical differences between the PD group and controls were evident.

3.3.3.2 *Hong Kong Chinese population study*

This study also involved the analysis of both male and female groups separately. The concentration of MAO-B per mg total platelet protein in the control group was higher (approximately 14% for males and 62% for females) than in the PD group, although again these differences were not statistically significant (Tables 3.4 and 3.5). In both the control and PD groups MAO concentration was greater in males than in females whilst MAO activity was greater in females compared with males irrespective of substrate; again these differences were not significant. A scatter plot for MAO concentration did not show greater heterogeneity in the PD group (Figure 3.6).

This population was also statistically analysed as a mixed population (Table 3.6) and again the concentration of MAO was greater in controls compared to the PD group

TABLE 3.4

Platelet MAO-B activity and MAO-B protein concentration in male Hong Kong chinese Parkinson's Disease patients and age matched controls.

	CONTROLS	**PARKINSON'S DISEASE**
NUMBER	10	8
AGE	65 +/- 2	66 +/- 7
TOTAL PROTEIN CONCENTRATION (mg/ml)	2.92 +/-1.54	5.38 +/-2.64
MAO CONCENTRATION (µg MAO-B/mg platelet protein	25.4 +/- 13.69	21.99 +/- 7.98
PEA DEAMINATION a) nmol hr^{-1} mg^{-1} total protein b) nmol hr^{-1} mg^{-1} MAO-B protein	2.97 +/- 1.71 163 +/- 178	2.86 +/- 0.65 147 +/- 67
DOPAMINE DEAMINATION a) nmol hr^{-1} mg^{-1} total protein b) nmol hr^{-1} mg^{-1} MAO-B protein	3.31 +/- 1.97 213 +/- 201	3.39 +/- 2.17 162 +/- 80

All values are expressed as the mean +/- standard deviation.
None of the above differences are significant analysed using the Mann-Whitney U test, chosen because the sample size is small and unmatched.

TABLE 3.5

Platelet MAO-B activity and MAO-B protein concentration in female Hong Kong chinese Parkinson's Disease patients and age matched controls.

	CONTROLS	**PARKINSON'S DISEASE**
NUMBER	8	7
AGE	68 +/- 5	63 +/- 11
TOTAL PROTEIN CONCENTRATION (mg/ml)	3.55 +/-1.88	3.47 +/-3.07
MAO CONCENTRATION (μg MAO-B/mg platelet protein)	15.82 +/- 5.74	6.09 +/- 2.99
PEA DEAMINATION a) nmol hr^{-1} mg^{-1} total protein b) nmol hr^{-1} mg^{-1} MAO-B protein	 4.84 +/- 2.81 340 +/- 197	 3.63 +/- 2.61 783 +/- 955
DOPAMINE DEAMINATION a) nmol hr^{-1} mg^{-1} total protein b) nmol hr^{-1} mg^{-1} MAO-B protein	 4.43 +/- 1.41 287 +/- 129	 2.76 +/- 2.43 571 +/- 754

All values are expressed as the mean +/- standard deviation.
None of the above differences are significant analysed using the Mann-Whitney U test, chosen because the sample size is small and unmatched.

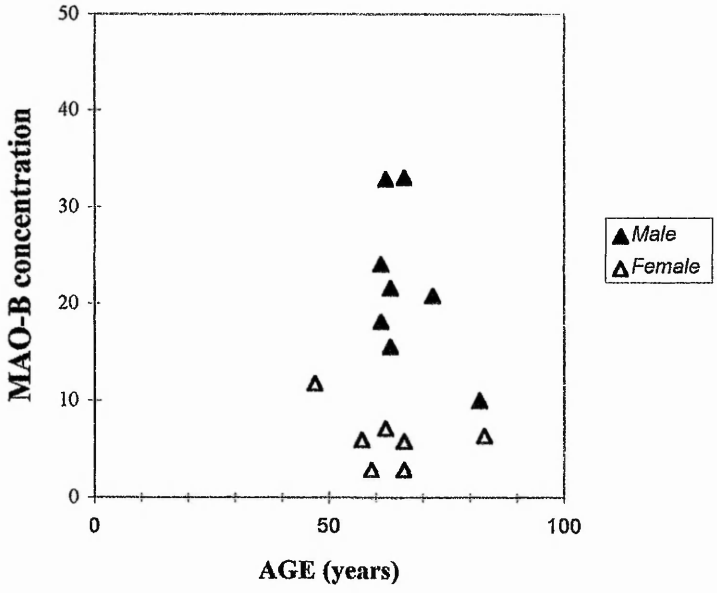
TABLE 3.6

Platelet MAO-B activity and MAO-B protein concentration in a mixed population of Hong Kong chinese Parkinson's Disease patients and age matched controls.

	CONTROLS	**PARKINSON'S DISEASE**
NUMBER (M/F)	18 (10/8)	15 (8/7)
AGE	66 +/- 4	65 +/- 9
TOTAL PROTEIN CONCENTRATION (mg/ml)	3.20 +/-1.68	4.49 +/-2.92
MAO CONCENTRATION (μg MAO-B/mg platelet protein)	21.14 +/- 11.70	14.57 +/- 10.15
PEA DEAMINATION a) nmol hr^{-1} mg^{-1} total protein b) nmol hr^{-1} mg^{-1} MAO-B protein	 3.80 +/- 2.39 242 +/- 202	 3.22 +/- 1.81 444 +/- 708
DOPAMINE DEAMINATION a) nmol hr^{-1} mg^{-1} total protein b) nmol hr^{-1} mg^{-1} MAO-B protein	 3.80 +/- 1.79 246 +/- 172	 3.10 +/- 2.24 353 +/- 540

All values are expressed as the mean +/- standard deviation.
None of the above differences are significant as analysed by the Mann-Whitney U test, chosen because the sample size is small and unmatched.

PARKINSON'S DISEASE



AGE MATCHED CONTROLS

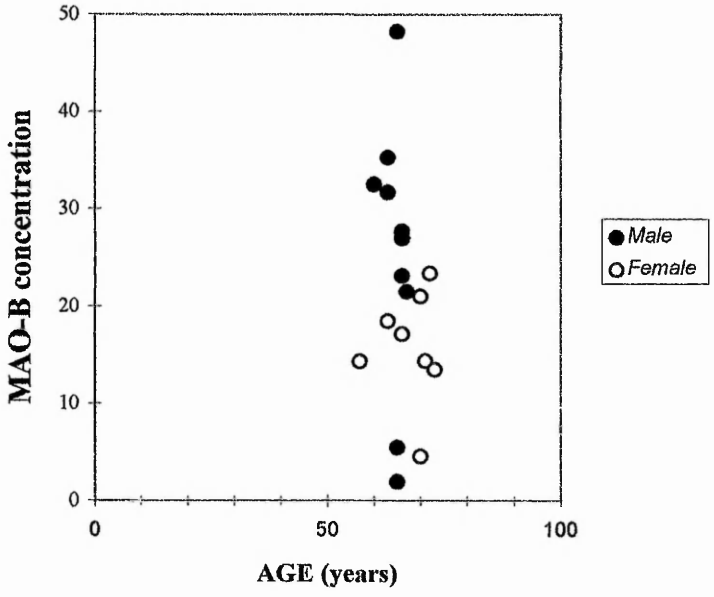
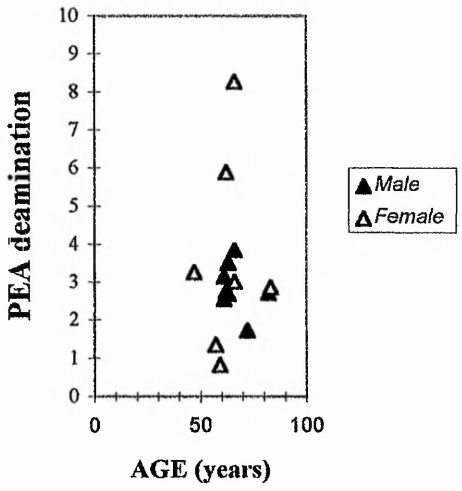


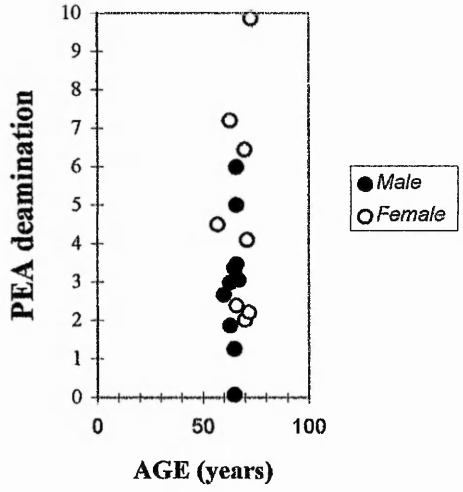
Figure 3.6. Concentration of platelet MAO-B in Hong Kong Chinese Parkinson's Disease patients (Δ), n = 15 and age matched controls (o), n = 18. MAO-B concentration is expressed as μg MAO-B per mg total platelet protein.

a) PHENYLETHYLAMINE

Parkinson's Disease

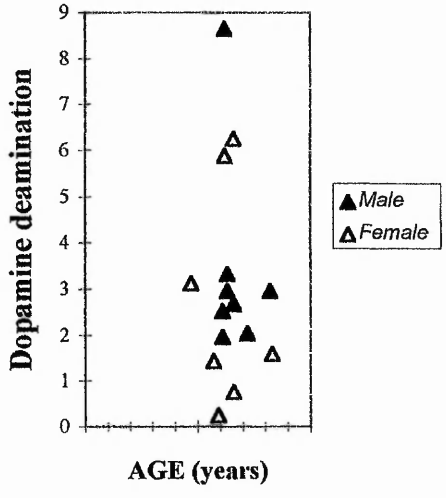


Age matched controls



b) DOPAMINE

Parkinson's Disease



Age matched controls

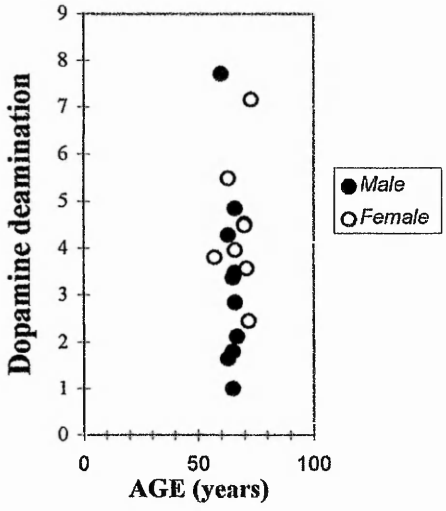
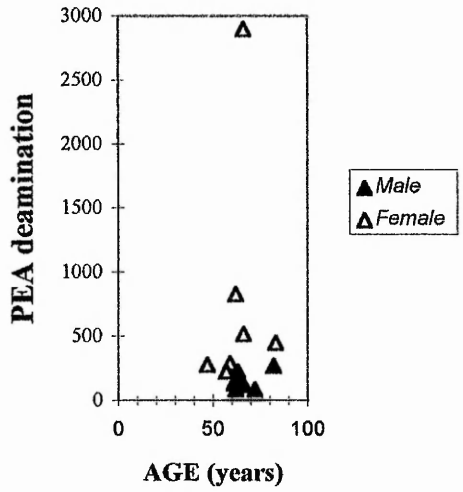


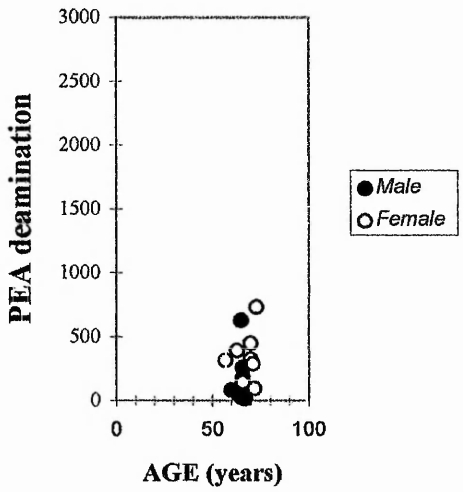
Figure 3.7. Platelet MAO-B activity in Hong Kong Chinese Parkinson's Disease patients (Δ), n = 15 and age matched controls (o), n = 18. MAO activity is expressed per milligram total platelet protein (nmol. substrate deaminated $hour^{-1}$ mg^{-1} total platelet protein).

a) PHENYLETHYLAMINE

Parkinson's Disease

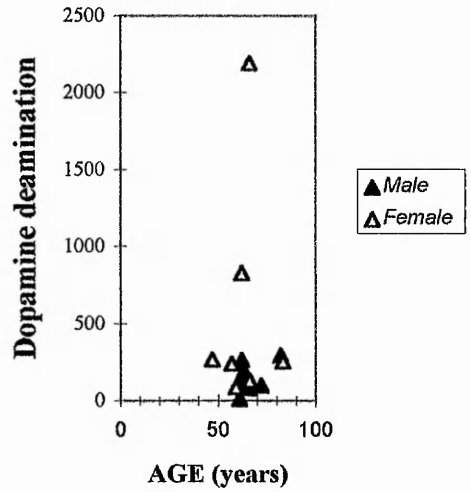


Age matched controls



b) DOPAMINE

Parkinson's Disease



Age matched controls

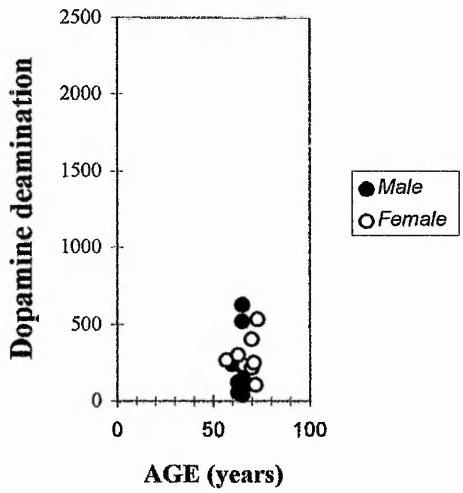


Figure 3.8. Molecular activity of platelet MAO-B in Hong Kong Chinese Parkinson's Disease patients (Δ), n = 15 and age matched controls (o), n = 18. MAO activity is expressed per milligram MAO-B (nmol. substrate deaminated $hour^{-1}$ mg^{-1} MAO-B).

although statistical significance was not proved as large variations within both groups were observed (Figure 3.6). MAO activity for both substrates was similar in PDs and controls for both males and females (Figure 3.7). MAO molecular activity is also similar between PDs and controls for both sexes (Figure 3.8), but an erroneous female PD sample, which is much higher than the rest, increased the mean value for the PD group (Table 3.6).

The general trends evident from analysis of the whole population of Hong Kong Chinese PD patients and controls indicate that platelet MAO activity (with PEA and dopamine as substrates) is slightly (but not significantly) reduced in PD patients compared with age matched controls.

A small number of samples from patients receiving treatment (Sinemet CR BD, carbidopa and L-DOPA) were analysed for MAO activity. As only a limited PRP volume was available these samples could not be analysed by ELISA. The effect of treatment was most evident in the female population, with all subjects tested showing a decrease in MAO activity for both PEA and dopamine (Table 3.7). Indeed mean MAO activity with PEA as a substrate was 93 % lower in female patients after treatment. These results however are not statistically significant as sample numbers were very small. Only two male samples were analysed, one showing reduction, the other an increase. A larger sample size is therefore required to assess the effects of Sinemet CR BD treatment on platelet MAO activity, but 4 out of 5 patients showed a reduction in their platelet MAO activity following Sinemet treatment.

3.3.3.3 *Comparison of controls in the Caucasian and Chinese populations*

A comparison of the control populations from each race was conducted to assess ethnic differences in normal populations. The results are summarised in Table 3.8.

MAO-B protein concentration in the Chinese control population was significantly higher ($p < 0.05$) than in the Caucasian controls; this was true for both males and females (Table 3.8). With dopamine (but not PEA) as a substrate MAO activity per total

TABLE 3.7

Effect of treatment (Sinemet CR BD) on platelet MAO-B activity in Hong Kong Chinese Parkinson's Disease patients using different substrates.

a) PHENYLETHYLAMINE

Patient ID	Sex	Age	MAO activity **before** treatment (nmol hr^{-1} mg^{-1} total platelet protein)	MAO activity **after** treatment (nmol hr^{-1} mg^{-1} total platelet protein)
1	F	70	6.40	0.66
3	F	69	6.72	0.68
11	F	66	8.26	0.08
27	M	66	3.84	4.56
31	M	82	2.70	1.36

b) DOPAMINE

Patient ID	Sex	Age	MAO activity **before** treatment (nmol hr^{-1} mg^{-1} total platelet protein)	MAO activity **after** treatment (nmol hr^{-1} mg^{-1} total platelet protein)
1	F	70	6.24	0.52
3	F	69	5.32	2.40
11	F	66	6.24	0.84
27	M	66	2.68	3.60
31	M	82	2.96	1.64

TABLE 3.8

Platelet MAO-B activity and MAO-B protein concentration in Caucasian and Hong Kong Chinese age matched controls.

	MALE CAUCASIAN	MALE CHINESE	FEMALE CAUCASIAN	FEMALE CHINESE
NUMBER	7	10	6	8
AGE	56 ± 12	65 ± 2	55 ± 8	68 ± 5
PROTEIN CONCENTRATION (mg/ml)	6.42 ± 2.24	2.92 ± 1.54	6.04 ± 1.30	3.55 ± 1.88
MAO CONCENTRATION (μg MAO-B/mg platelet protein	2.33 ± 0.55	25.4 ± 13.69	4.25 ± 2.78	15.82 ± 5.74
PEA DEAMINATION				
a) nmol hr^{-1} mg^{-1} total protein	3.02 ± 1.53	2.97 ± 1.71	3.51 ± 1.53	4.84 ± 2.81
b) nmol hr^{-1} mg^{-1} MAO-B protein	1291 ± 637	163 ± 178	1358 ± 1233	340 ± 197
DOPAMINE DEAMINATION				
a) nmol hr^{-1} mg^{-1} total protein	0.96 ± 0.42	3.31 ± 1.97	1.13 ± 0.35	4.43 ± 1.41
b) nmol hr^{-1} mg^{-1} MAO-B protein	407 ± 169	213 ± 201	360 ± 223	287 ± 129

All values are expressed as the mean +/- standard deviation.

platelet protein was also significantly higher ($p < 0.05$) in Chinese males than in Caucasians. However this difference was reversed when expressed as MAO molecular activity, i.e. Caucasian males (and females) had a higher MAO molecular activity for dopamine compared with Chinese males; this difference was significant ($p < 0.05$) for the male group.

Again using PEA as a substrate, MAO molecular activity in Caucasian males and females is much greater than in the Chinese population. These differences were statistically significant ($p < 0.05$).

3.4 DISCUSSION

3.4.1 Development of a platelet extraction procedure

A simple approach to collecting a representative and reproducible population of platelet rich plasma (PRP) was used; based on the method of Corash (1980) as checked out by Yeomanson (1990). PRP samples were kindly prepared at the Queen Elizabeth Hospital and the University of Hong Kong. PRP was immediately frozen in aliquots at -70 ° C where it remained until analysis; each PRP sample underwent only one thaw cycle and platelet collection was achieved through a one step centrifugation and washing step.

3.4.2 Initial studies

Test time course profiles for MAO activity using ^{14}C PEA and ^{3}H dopamine as substrates allowed the choice of an end point (60 minutes) within the linear phase of the reaction. To allow a comparison with our collaborating establishment final substrate concentrations of 20 μM for PEA and 50 μM for dopamine were chosen (Humfrey *et al*, 1990); these concentrations are commonly used and 20 μM PEA ensures that substrate inhibition is avoided.

Platelet MAO analysis was achieved using the specific competitive ELISA; this system was adapted to accommodate the limited sample volumes. Following solubilisation of platelet samples, protein analysis was conducted allowing a serial range of protein concentrations to be used as competing antigen for each patient and control sample. Platelet samples had to be diluted five fold to ensure that the final concentration of Triton X-100 was reduced to 0.1% (v/v). This concentration had previously been shown to be the compromise between minimal effects on antibody/antigen interactions and efficiency of solubilisation (section 2.3.3.2). Complete displacement of antibody binding was achieved by adding competing antigen at its highest concentration.

3.4.3 Analysis of platelet extracts from Parkinson's Disease patients and controls

3.4.3.1 *Caucasian population study*

The main aims of this study were (a) to measure total MAO-B protein (active and inactive) by ELISA and (b) using the most common substrates used previously by other workers, to assess MAO activity in the same samples. This study represents the first time both platelet MAO-B protein (active and inactive) and MAO activity have been assessed concomitantly in PD patients and controls.

The results obtained from this study were firstly split into categories depending on sex. Attempts were made to age match the control and PD samples.

In general these analyses showed higher MAO activities in females than in males irrespective of substrate, agreeing with other published data. Indeed studies by Robinson *et al*, (1971) revealed that females had a significantly higher mean platelet MAO-B activity than males over a wide age range. These studies also suggested that MAO activity increased with age. A more recent study has also confirmed that the platelet MAO activity increases with age and that this increase is more prominent in females (Veral *et al*, 1997), thus highlighting the importance of both age and sex matching.

A study in 1974 by Belmaker *et al* reported variation in platelet MAO activity during the menstrual cycle. Hence, the majority of women controls and patients analysed in the present study were post-menopausal, thereby eliminating this variation. Indeed, PD does not often manifest itself until post menopause.

Efforts were therefore made to eliminate the possible sources of error within specific populations. Both males and females were statistically analysed separately although experimental analysis was performed at random. The results obtained from the separate studies were also analysed together as a mixed population revealing similar results as the individual analyses.

Another important consideration when choosing patients/volunteers for this study was the subject of cigarette smoking. PD has been reported to occur more commonly in non-smokers than smokers (Bauman *et al*, 1980). PD may be caused by one or more neurotoxins (maybe resembling MPTP, already discussed) which are converted by MAO-B into toxic moieties which result in the destructive damage of the nigrostriatum. MAO-B activity has been reported to be significantly lower in platelets of heavy cigarette smokers compared with none smokers (Yong and Perry, 1986). It has therefore been postulated that hydrazine, present in tobacco smoke may have a protective effect and may therefore explain why non-smokers are more likely to develop PD. The patients and controls used in this study were therefore non-smokers. This however poses another important question. By choosing non-smokers were we preferentially selecting for PD and a high MAO activity?

The first analysis assessed Caucasian patients and controls. To eliminate potential complications in females resulting from hormonal status, each sex was analysed separately. Despite this separate analysis no significant differences were evident between PDs and controls in both sexes.

A trend was observed when analysing molecular activity in the female group. For both dopamine and PEA (but PEA in particular) molecular activity in the PD population was greater than that of the controls but again this difference failed to reach statistical significance even for PEA. These results agree with data published by Jarman *et al* (1993), for a mixed but mainly female population, showing that MAO activity (expressed per mg platelet protein) using PEA as a substrate was statistically significantly higher in PD patients (1 male; 5 females), compared with controls (3 males; 7 females); however, when using dopamine as a substrate, no significant difference was observed (Table 3.9).

Similar results were obtained when the analyses were combined and expressed as a mixed population of males and females (Table 3.3). In general PD MAO-B concentration and activity (with both substrates) was higher than controls but none of these results were statistically significant.

These findings are not supported by Humfrey *et al* (1990) who, using a different method of analysis (HPLC), found that control MAO-B activity was higher than in the PD population using dopamine as a substrate, but lower when using PEA as a substrate. Other workers have also produced conflicting results. Bonuccelli *et al*, 1990 found an increase in MAO activity in platelets from PD patients compared to controls when using benzylamine as a substrate although there were large variations in the PD group, as found in my study. Checkoway *et al*, 1992 found sex differences when assaying platelet MAO activity with PEA as a substrate. They observed a decrease in platelet MAO-B activity in male PDs compared with controls but a slight increase in MAO-B activity in female PDs.

Bongioanni *et al* (1996) assessed platelet MAO molecular activity in PD patients using benzylamine as a substrate. They reported significantly higher platelet MAO molecular activity in PD patients than in controls. However no effect on the number of MAO enzyme molecules was observed. The study by Bongioanni *et al* differed in a number of important respects from the work presented in this thesis. Firstly, benzylamine was used as a substrate for MAO-B activity assays; this may not be a good choice as endogenous benzylamine oxidase is present in blood and residual contaminants may remain thereby competing with MAO for the substrate. Secondly, in the competitive radioimmunoassay only two protein concentrations were used as competitor. Finally the most significant difference is in patient selection. Bongioanni's patients/controls may have smoked up to 5 cigarettes a day and some patients may have been receiving previous deprenyl treatment one month before the study commenced. Both of these factors may potentially affect MAO activity and may therefore introduce erroneous differences. Patients selected for the study presented in this thesis were non-smokers and were newly diagnosed with PD i.e no PD treatment had been administered before blood samples were collected. PD was later confirmed by a positive response to L-DOPA.

The general findings from this Caucasian study indicate that the status of platelet MAO-B in PD is unchanged and that the MAO activity and protein concentration was very variable in the PD group. Indeed Fitzgerald *et al*, (1996) using the same substrates (PEA and dopamine) also found no significant difference in platelet MAO activity between

PD and controls. A recent paper (Kuhn *et al*, 1998) has also reported no significant differences in platelet MAO-B activity using PEA between mixed populations of PD (9 females, 8 males) and controls (9 females, 8 males).

As population sizes in the study presented here were small and statistical errors were large, any differences observed were not significant. This may however not be the final conclusion; large populations need to be assessed to reduce statistical error and reach a significant conclusion as to whether analysis of platelet MAO-B can be used as a diagnostic test for PD. Nevertheless, evidence to date would suggest that platelet MAO-B is not a useful peripheral marker. This conclusion is supported by Mann *et al* (1992) who have reported that mitochondrial complex I deficiency in PD is localised in the substantia nigra and may be directly involved in cell death in PD. More importantly, they have analysed platelet mitochondrial function from PD patients and found it to be normal. This may suggest that analysis of a platelet enzyme (such as MAO) cannot be used as a diagnostic test for PD. In contrast however, a recent study by Blandini *et al*, (1998) reported a decrease in mitochondrial complex I in platelets as well as the substantia nigra of PD patients, indicating that platelet mitochondrial function is altered, and that changes in platelets may be representative of similar changes in the brain.

3.4.3.2 *Hong Kong Chinese population study*

Similar analyses conducted for the Caucasian study were employed to assess the status of platelet MAO in a Hong Kong Chinese population. Each sex was statistically analysed separately and as a mixed population. Measurements of the concentration of MAO-B showed a similar result for both sexes, namely that the concentration of platelet MAO-B in PD patients was lower than in controls but that this decrease was not statistically significant. MAO molecular activity and MAO activity per platelet protein was similar in male controls and PD patients for both substrates (Table 3.4). However in the female population (Table 3.5) MAO activity per platelet protein was less in PDs, whilst molecular activity was higher in the PD group. The opposite was true for the male populations; MAO molecular activity was higher in controls than in the PD

population; again this increase was not statistically significant due to the large standard errors.

The results for both males and females were pooled and analysed as a mixed population (Table 3.6). PD MAO concentration was lower compared with controls and MAO total activity for both substrates was also decreased in the PD group. However, MAO molecular activity for both substrates showed an increase in the PD group compared to the controls indicating that although MAO concentration was lower in the PD group the actual activity per MAO enzyme was greater, although again these differences were only trends as statistical significance was not reached. This result poses the question, that although the concentration of MAO in PD is lower, is the enzyme becoming more active resulting in manifestation of the disease?

The Hong Kong Chinese study was undertaken as part of a larger study involving PRP samples of insufficient volume for analysis by ELISA. The study included a small number of samples taken after treatment with Sinemet CR (L-DOPA and carbidopa) for 3 months. Again each sex was treated separately initially and then as a whole population.

This preliminary analysis of the effects of treatment indicated that MAO activity (4 out of 5 samples) was reduced after administration of Sinemet, an effect most evident in the female population using both PEA and dopamine as substrates. Unfortunately as subject numbers were so small it is unwise to draw any final conclusions from this. However a reduction in MAO activity following L-DOPA treatment has been reported previously by a number of workers (Zeller *et al*, 1976, Zeller and Davis, 1980). Glover *et al*, 1983 also reported a reduction in platelet MAO activity in PD with respect to controls and a further reduction of platelet MAO activity in L-DOPA treated PD. In contrast, a recent publication by Kuhn *et al* (1998) stated that peripheral platelet MAO-B is unaltered in L-DOPA therapy.

PD is generally characterised by a lack of dopamine in the nigrostriatal pathways in the brain. A common treatment is the administration of L-DOPA, the pre-cursor of

dopamine. L-DOPA is able to cross the blood- brain barrier more easily than dopamine and is therefore able to replenish brain dopamine. L-DOPA is often administered with carbidopa (α methyldophydrazine) which is an inhibitor of L aromatic amino acid decarboxylase. This decarboxylase is responsible for converting L-DOPA into dopamine. This conversion is not required in the periphery and as 95% of L-DOPA is normally converted to dopamine in the periphery, carbidopa is administered with L-DOPA to inhibit this peripheral dopamine formation. As carbidopa does not penetrate the blood-brain barrier this conversion is not inhibited in the brain. Combined with L-DOPA carbidopa enables the dose of L-DOPA to be reduced 4-8 fold, thus reducing peripheral side effects including involuntary movements, nausea and hypotension.

These preliminary results obtained from the treatment data indicate that the MAO enzyme may have been down regulated in the presence of L-DOPA. Whether this effect is due to a decrease in enzyme concentration or molecular activity is not known as the limited sample volume prevented sample analysis by ELISA. It is clear from this very small study that with L-DOPA treatment platelet MAO activity is reduced. Zeller *et al*, 1976 postulated that this decrease was due to the enhanced release of gonadotrophins and subsequently an increase in sex hormones which are capable of reducing MAO activity. This reduction in MAO activity would lead to a longer half life for the dopamine formed from L-DOPA, leading to an enhanced therapeutic result. Thus MAO activity may play an important role in the therapeutic effects of L-DOPA.

It is evident from my study that platelet MAO-B activity is very variable, reflected in the large standard deviations. This variability is seen in both controls and to a greater extent in PD populations in both ethnic groups. As both assays were reproducible when checked with normal human platelets it is probable that the variation seen in this study is due to general population heterogeneity and not assay variation. Indeed this variability is not unique as wide heterogeneity is also noted in other published studies (detailed in Table 3.9). This variability suggests that measurement of platelet MAO activity is difficult and that the increased heterogeneity in PD may reflect the fact that PD is a multi-factorial and multi-symptomatic disease.

TABLE 3.9. Variation in platelet MAO activity in Parkinson's Disease and controls

Reference	Substrate (activity units)	PD Number (M/F)	PD Age ± SD	PD Platelet MAO-B activiy ± SD	CONTROL Number (M/F)	CONTROL Age ± SD	CONTROL Platelet MAO-B activiy
Zeller *et al*, 1976	**Tyramine** (nmol/10^9 platelets/h)	15 (5/10)		31.0 ± 4.2	18 (10/8)		39 ± 3.7
Danielczyk *et al*, 1988	**PEA** (nmol/mg/min)	18 (4/14)	73.9 ± 6.2	0.275 ± 0.101	18 (4/14)	73.9 ± 10.0	0.210 ± 0.056
Humfrey *et al*, 1990	**Dopamine** (nmol/mg/h)	20 (10/10)	62.5 ± 11.8	230.6 ± 29.4	27 (14/13)	52.2 ± 23.4	476.2 ± 107.3
Bonuccelli *et al*, 1990	**Benzylamine** (nmol/mg prot./h)	18 (11/7)	61.3 ± 10.6	39.48 ± 15.22	20 (9/11)	65.4 ± 4.8	26.28 ± 4.26
Jarman *et al*, 1993	**Dopamine** (nmol.oxidised/mg protein/30 min)	6 (1/5)	65 ± 8	6.7 ± 5.1	10 (3/7)	57 ± 10	4.1 ± 0.9
	PEA			15.3 ± 7.3			7.8 ± 3.3
Bongioanni *et al*, 1996	**Benzylamine** (nmol/mg prot./h)	30 (16/14)	64.6 ± 11.6	41.8 ± 10.8	34 (18/16)	65.3 ± 12.6	25.1 ± 8.8
Kuhn *et al*, 1998	**PEA** (pmol/mg/min)	17 (8/9)	63.9 ± 10.5	362.4 ± 76.5	17 (8/9)	63.9 ± 10.5	384.7 ± 111.0

The results obtained from studies of both the Caucasian and the Hong Kong Chinese populations were variable. The differences discussed in most cases unfortunately did not reach statistical significance. However, this study involved relatively small sample numbers, a fact which would have contributed to increases in standard errors. The results generally reveal that in this small study the status of MAO in PD is unchanged with respect to the control populations, however it is unwise to conclude that these observations are necessarily final as a more extensive study involving larger sample numbers would provide a clearer insight.

An increase in platelet MAO-B or an up-regulation of activity may have answered the question of reduced brain dopamine and the development of PD, however, the status of MAO is only a small factor in a number of theories relating to the development and pre-disposition to development of PD and the question as to whether changes in platelet MAO could be used as a peripheral marker for MAO status remains unanswered. In addition, analysis of platelet MAO activity is a useful monitor of the effectiveness of deprenyl therapy in inhibiting MAO (Lee *et al*, 1989).

3.4.3.3 *Comparison of controls in the Caucasian and Chinese populations*

MAO status in control subjects from each study were statistically compared to assess race differences. This is particularly interesting since some evidence suggests that the prevalence of PD is lower in Chinese countries (Wang *et al*, 1991) whilst other evidence suggests that the prevalence of PD in Chinese males is higher than Western males (Chia and Liu, 1992). It was therefore important to assess the control populations with respect to MAO activity and concentration to identify any differences which may explain PD epidemiology in Chinese populations.

One of the most striking observations was the much higher concentration of platelet MAO-B (both active and inactive species) in both male and female individuals from the Chinese population. This was also manifested in MAO activity per total platelet protein in females using both PEA and dopamine as substrate and in males with dopamine as substrate. However, molecular activity (MAO activity per MAO-B protein) was larger

in Caucasians for both males and females using both substrates, but most evident using PEA as a substrate. This result indicates that MAO in the Chinese population is less efficient than MAO in Caucasians.

The reason for this may be multifactorial and may involve diet, environment and genetics. Indeed genetic and environmental risks have been identified for PD in a Chinese population (Chan *et al*, 1998) and tea drinking and pesticide exposure have been implicated.

CHAPTER FOUR

A STUDY OF THE STATUS OF MAO IN HUMAN PLACENTA

CHAPTER FOUR

A STUDY OF THE STATUS OF MAO-A IN HUMAN PLACENTA

4.1 INTRODUCTION

It has been suggested that the placenta plays an important role in protecting the foetus from the effects of biogenic amines during pregnancy or delivery. Placental MAO-A has been shown to exist in the boundary between the foetal and maternal circulation (Church *et al*, 1994) and its presence is thought to limit the transfer of MAO-A substrates across the placental barrier. For a detailed account of MAO-A in placenta, refer to chapter 1, section 1.6. Whether or not MAO-B is involved in limiting transfer of vasopressor substances to the foetus is still an open question.

This chapter describes: firstly the development of an extraction procedure suitable for an assessment of MAO in human placenta; secondly, the utilisation of the competitive ELISAs developed for MAO-A and B (described in Chapter 2) and immunohistochemistry to assess the relative levels of MAO-A and MAO-B in normal human placenta and, thirdly a preliminary assessment of the role of MAO-A in placentae in patients suffering from pregnancy induced hypertension compared to normotensive controls.

4.1.1 Pregnancy induced hypertension (pre-eclampsia)

4.1.1.1 *Symptoms and pathology*

Pre-eclampsia is a disease specific to pregnancy and most commonly occurs after the twenty sixth week. It is characterised by hypertension, proteinuria and oedema and is the precursor to eclampsia, a serious condition which causes generalised convulsions, cerebral haemorrhage and often results in both maternal and foetal death. This condition is one of the most common antenatal complications in the United Kingdom affecting between 5-8% of all pregnancies. The incidence is more frequent in young primigravidae, in first pregnancies with a new partner and in mothers over 35 years of

age. Pre-eclampsia can be life threatening to both mother and foetus often causing foetal growth retardation and even foetal death.

It is generally accepted that pre-eclampsia originates in the placenta. Generalised vasoconstriction occurs which affects most of the physiological activities of the tissues within the body. Capillary permeability increases and the fluid which escapes contributes to the general oedema within the tissues. The presence of excess fluid within the cells interferes with oxygenation and tissue hypoxia occurs which can ultimately lead to necrosis of vital organs. The effects on the uterus are devastating. Vessels supplying the placental bed are constricted which reduces uterine blood flow causing vascular lesions within the placental bed. Reduction in blood flow to the choriodecidual blood spaces decreases the oxygen supply which diffuses through the cells of the syncytiotrophoblast and the cytotrophoblast into the foetal circulation within the placenta; this results in thrombosis of the chorionic villi capillaries and generalised placental ischaemia. This ultimately leads to severely impaired placental function, foetal growth retardation and even foetal death.

4.1.1.2 *The role of 5-hydroxytryptamine in pre-eclampsia*

The role of vasopressor substances in the pathogenesis of pre-eclampsia has been widely studied. Pre-eclampsia has been characterised by an imbalance between prostacyclin and thromboxane production (Bussolino, *et al*, 1980), and substances affecting platelet function have been used to treat the condition e.g. aspirin (Benigni *et al*, 1989; Schiff *et al*, 1989). However, a multiregional report has been published questioning the effectiveness of aspirin treatment for pre-eclampsia (CLASP, 1994).

Attention therefore has been turned to other vasopressor substances, primarily 5-hydroxytryptamine (5-HT). 5-HT is present in highest concentration in blood platelets and in the gastrointestinal tract where it is found in the enterochromaffin cells of the mucosa of the small intestine. Lower amounts are found in the brain and the retina.

5-HT is formed in the body by hydroxylation and decarboxylation of the amino acid tryptophan. After its release from serotonergic neurones much of the released 5-HT is

recaptured by an active reuptake mechanism and inactivated by monoamine oxidase (primarily MAO-A) to form 5-hydroxyindoleacetic acid (5-HIAA). This substance is the principal urinary metabolite of 5-HT; consequently the urinary output of 5-HIAA is used as an index of the rate of 5-HT metabolism in the body (Filshie *et al*, 1992). Circulatory 5-HT is very difficult to measure directly, as 5-HT concentration increases during patient stress (eg. at the sight of a needle) and platelets will release stored 5-HT when handled. Hence, urinary metabolite measurements are commonly used.

There have been a number of reports published supporting the involvement of 5-HT in pre-eclampsia, notably an interesting report from Horn *et al* (1990) describing the successful treatment of an eclamptic patient with nimodipine, a 5-HT antagonist. This result led to a further study from this group (Filshie *et al*, 1992) involving the analysis of 5-HIAA concentration in the urine of patients with pre-eclampsia. This study provided evidence that the excretion of urinary 5-HIAA was significantly higher in patients with pre-eclampsia compared to normotensive controls, matched for age and gestation time. This increase was interpreted to reflect increased circulatory levels of 5-HT and it has been suggested that it may be related to the release of trophoblast fragments into the maternal circulation. Trophoblast embolisation into the peripheral maternal circulation is a well documented phenomenon unique to pregnancy (e.g. Mueller *et al,* 1987; Gänshirt *et al*, 1995). It has also been shown that an increase in trophoblastic embolisation is observed in pre-eclampsia (Chua *et al*, 1991) and may be a contributory factor to the pathogenesis of the disease. The theory proposed suggests that the increase in 5-HT results from platelet release in response to the presence of these trophoblastic fragments and that this increase results in hypertension.

Whether or not the inferred increase in circulatory 5-HT is due only to increased release of 5-HT is unknown. It is also possible that MAO activity is reduced in pre-eclampsia. The increased vasoconstriction resulting from the increase in 5-HT levels would further exacerbate the problem as highly anoxic areas will in turn result in poor MAO activity. Indeed, the involvement of MAO in the development of pre-eclampsia has been implicated for many years. A study conducted by Sandler and Coveney in 1962 found a small but significant decrease in placental MAO activity from pre-eclamptic patients compared with healthy controls. A similar finding was reported by De Maria and See

(1965) and later by Barnea *et al*, (1986). Important questions which need answering include : (1) Is there always a decrease in MAO activity? (2) If MAO activity is reduced, is this the cause of the condition or merely a result of vasoconstriction causing anoxic conditions, thus reducing the activity of the enzyme? (3) Is the enzyme damaged in pre-eclamptic patients? To help answer these questions studies need to be conducted to assess the concentration of MAO protein in the placenta concomitantly with MAO activity.

It has been a matter of debate as to the presence or absence of MAO-B in human placenta. Traditionally the majority of literature (see chapter 1) has accepted that human placenta contains only MAO-A, and it is without doubt that MAO-A is present in human placenta in very large amounts, although its exact function there is still unknown. The presence of MAO-B in human placenta still remains to be elucidated.

4.2 MATERIALS AND METHODS

Placental samples were provided by Mr G. M. Filshie and his team, Department of Obstetrics and Gynaecology, University Hospital, Queens Medical Centre, Nottingham.

4.2.1 Final placental extraction procedure

The extraction procedure described here is the final method developed for analysis of MAO in human placenta (Figure 4.1).

4.2.1.1 *Materials*

Buffered sucrose solution (see appendix)
Extraction buffer (see appendix)
Liquidiser and coffee mill attachment (Philips HR2845/AM)
MSE 24M centrifuge (MSE Scientific Instruments Ltd)

4.2.1.2 *Method*

1. A portion of placenta (approximately 100 g) was washed with cold buffered sucrose to remove blood, allowed to drain then was blotted dry.
2. This was then transferred to the coffee mill attachment of the blender and homogenised using 3 x 5 second pulses.
3. Approximately 5 g of the homogenised tissue was removed into pre-weighed tubes. Tubes were capped and weighed again to calculate the exact weight of placental tissue.
4. 5 volumes of extraction buffer was added to each tube and tubes were inverted to ensure thorough mixing.
5. Tubes were then incubated for 14 hours at 4°C with shaking to ensure thorough contact between tissue and buffer and to allow release of membrane proteins by Triton X-100 extraction.
6. Samples were centrifuged 27,000 x g for 30 minutes to produce a cell free extract of solubilised protein.

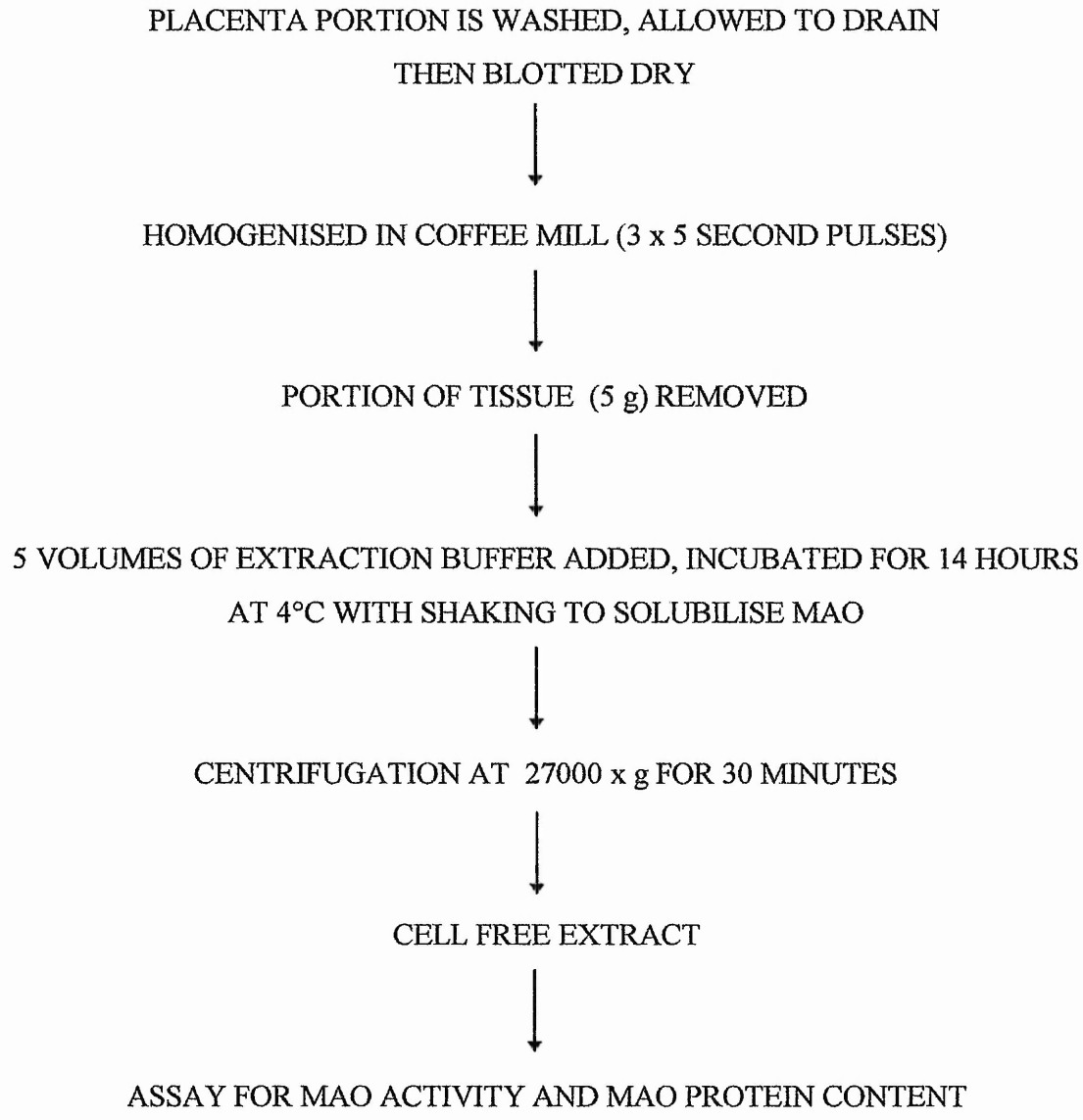


Figure 4.1. Flow diagram describing the final placental extraction procedure.

7. This extract was used to measure total protein, MAO activity and MAO protein concentration.

4.2.2 Competitive ELISA

The competitive ELISA is as already described in section 2.2.15

4.2.3 MAO activity using 5-HT as a substrate

The method used for placental activity measurements is described in section 2.2.2 but using ^{3}H 5-HT as a substrate. Hydroxytryptamine creatinine sulfate, 5-[1,2-^{3}H(N)] (serotonin) was supplied by DuPont, NEN Division, code NET 498.

4.2.4 Immunohistochemistry

The methods used were as described in section 2.2.11. The monoclonal antibodies 6G11/E1 and 3F12/G10/2E3 were used to probe cryosections of human placental tissue.

4.3 RESULTS

4.3.1 Development of a suitable extraction procedure to assess MAO-A in placenta

A reliable extraction procedure was established suitable for both an evaluation of MAO activity and MAO concentration.

The initial extraction method was based upon that previously used for extracting mitochondria from human liver (section 2.2.1). Tissue (5g) was cut into a fine mince, buffered sucrose solution added (1:10, w/v) and the mixture homogenised using three passes of a close fitting glass/teflon homogeniser, prior to being subjected to differential centrifugation and washing to produce the final mitochondrial enriched pellet. Aliquots were taken from each step and assayed for MAO activity using tyramine as a substrate to assess the distribution of total MAO activity. The results revealed that large amounts of MAO activity were evident in all fractions (table 4.1). This could be due to mitochondrial damage and/or the presence of MAO-B originating from blood platelets or lymphocytes. The nuclear pellet contains approximately 50 % of the total MAO activity from the first centrifugation step which may be due to the presence of platelets and lymphocytes sedimenting with the nuclei.

Table 4.1. Initial extraction method - MAO activity using tyramine as a substrate. Values have been corrected using an appropriate sample blank and background count.

FRACTION	MAO Activity (DPM/mg total protein)	Total MAO Activity (DPM/fraction)
Crude Homogenate	1.06×10^5	4.24×10^7
Nuclear Pellet	1.25×10^5	3.11×10^7
Post nuclear supernatant	1.70×10^5	3.91×10^7
Mitochondrial Pellet	0.39×10^5	0.55×10^7
Post mitochondrial supernatant	1.22×10^5	2.72×10^7

The results from the first extraction indicated that MAO activity was being lost during centrifugation steps. An alternative extraction method was therefore sought which eliminated differential centrifugation and made efforts to minimise MAO-B contamination from blood. Placental tissue (5g) was first cut into pieces before being extensively washed with copious volumes of ice cold buffered sucrose solution in an effort to remove the majority of blood from the blood spaces within the tissue. The tissue pieces were then minced finely using sharp scissors before homogenising in 10 volumes of buffered sucrose with the wide fitting glass mortar and teflon pestle and repeating with a closer fitting mortar and pestle. In order to optimise MAO-A detection, 5-hydroxy tryptamine was used as a substrate (final concentration 100 μM, as used by Riley *et al*, 1989) as this is preferentially deaminated by MAO-A. This crude homogenate was monitored for total protein concentration and MAO activity using the MAO inhibitors, deprenyl (inhibits MAO-B) and clorgyline (inhibits MAO-A), table 4.2. The concentrations of the inhibitors were selected following titration using 10 fold serial dilutions and 5-HT as a substrate. A concentration of 10^{-7} M for both inhibitors completely inhibited MAO-A or B activity (data not shown).

The MAO activity in this crude homogenate was found to be 95% type A using 5-HT as a substrate.

Table 4.2. MAO activity in a crude placental homogenate using MAO inhibitors deprenyl and clorgyline to determine the identity of MAO. Values have been corrected using an appropriate sample blank and background count.

	MAO activity (DPM/mg total protein)
No inhibitor	1.169×10^5
+ Deprenyl	1.128×10^5 **(95 %)**
+ Clorgyline	0.055×10^5 **(5 %)**

All the extractions described so far had used only one small piece of tissue. In order to assess whether this was representative of the whole placenta, five sites were sampled at random and homogenised as described previously. The homogenates were assayed for total protein content and MAO activity.

Table 4.3. MAO activity for placental extracts from 5 random sites using 5-HT as a substrate. Results are expressed as DPM per mg of total protein obtained from each of the 5 sites. Values have been corrected using an appropriate sample blank and background count.

SITE NUMBER	TOTAL PROTEIN CONCENTRATION (mg/ml)	DPM mg^{-1} TOTAL PROTEIN
1	3.65	4.02×10^5
2	4.70	4.14×10^5
3	4.85	5.56×10^5
4	4.05	3.74×10^5
5	4.25	4.48×10^5

Mean for sites 1-5 expressed as $\pm$ standard deviation : $4.388 \times 10^5 \pm 0.707 \times 10^5$.

Table 4.3 shows that MAO activity across 5 random sites within the placenta ranges from 3.74×10^5 to 5.56×10^5 DPM per mg of total protein. This indicates that MAO activity varies from the mean in one human placenta.

In an attempt to eliminate this variation the final extraction procedure involved the use of a large piece of placenta, (approximate weight 100g). This piece of tissue was cut into smaller pieces and each was washed with copious amounts of buffered sucrose and blotted dry. Instead of homogenising using a mortar and pestle system all the tissue was placed into the coffee mill attachment of a Philips liquidiser and given 3 x 5 second

pulses to produce an homogeneous mixture, then a 5g representative portion was removed into pre-weighed tubes for extraction.

4.3.2 The solubilisation of placental MAO for use in the competitive ELISA.

Triton X-100, previously used to solubilise both MAO-A and MAO-B from human liver mitochondrial membranes and MAO-B from human platelets, was assessed for its efficiency in solubilising MAO-A from placenta. As Triton X-100 has an inhibitory effect on the binding of 6G11/E1 to MAO-A (chapter 2), it was important to use a Triton X-100 concentration which maximally released MAO-A from the tissue whilst not interfering with binding of the antibody in the competitive ELISA.

Following homogenisation the homogenate was divided into aliquots which were incubated with Triton X-100 (final concentrations of 0.5%, 0.75% or 1.0%) for 14 hours at 4°C whilst stirring. The samples were then centrifuged producing a MAO containing solution and pellet of cell debris. The supernatants were assessed for total protein and MAO activity using 5-HT. The pellets were resuspended in the original volume of buffer (containing the appropriate concentration of Triton X-100) and also assayed for total protein concentration and MAO activity.

Table 4.4 (a). Solubilising efficiency of Triton X-100 for human placenta based on total protein estimations and MAO activity. Triton X-100 was added to a crude placental homogenate and incubated at 4°C for 14 hours whilst shaking. The sample was centrifuged at 100,000 x g. MAO-A activity was assessed using 5 HT as a substrate.

	0.5 % (v/v) Triton X-100	0.75 % (v/v) Triton X-100	1.0 % (v/v) Triton X-100
Total MAO activity (DPM/ml)			
PELLET	2.856×10^5	1.258×10^5	1.066×10^5
SUPERNATANT	17.945×10^5	7.040×10^5	4.120×10^5
TOTAL	20.801×10^5	8.295×10^5	5.186×10^5
% DISTRIBUTION			
PELLET	13.73	15.16	20.56
SUPERNATANT	86.27	84.84	79.44

Crude homogenate (no Triton X-100) : Protein concentration = 4.00 mg/ml

MAO-A activity = 16.36×10^5 DPM/ml

Table 4.4 (a) shows the solubilising efficiency of varying concentrations of Triton X-100. All concentrations of Triton X-100 solubilised around 80 % of MAO activity. The recovery of total MAO activity was low with Triton X-100 concentrations of 0.75 % and 1.0 %. The total activity with 0.5 % was similar to the total activity of crude homogenate without Triton X-100. Thus 0.5 % Triton X-100 was chosen for future extractions.

This experiment also incorporated use of the MAO inhibitors clorgyline and deprenyl to ensure that MAO-B activity was being inhibited and that MAO-A alone was contributing to the activity measurements. Table 4.4 (b) shows the results obtained using the MAO inhibitors.

Table 4.4 (b). Effect of MAO inhibitors clorgyline and deprenyl on solubilised/non-solubilised MAO. A concentration of 10^{-7} M was used for both inhibitors.

		Total (x 10^5 DPM/mg protein)	**Plus Deprenyl** (x 10^5 DPM/mg protein)	**Plus Clorgyline** (x 10^5 DPM/mg protein)
Crude Homogenate		4.09	**4.47** (97 %)	**0.13** (3 %)
0.5 % Triton	Supernatant	4.85	**5.20** (99.96 %)	**0.002** (0.04 %)
	Pellet	2.38	**2.27** (90.8 %)	**0.23** (9.2 %)
0.75 % Triton	Supernatant	1.60	**1.49** (99.53 %)	**0.007** (0.47 %)
	Pellet	1.31	**0.58** (100 %)	**0.00** (0.00 %)
1.0 % Triton	Supernatant	1.03	**0.94** (94.76 %)	**0.052** (5.24 %)
	Pellet	0.74	**0.53** (61 %)	**0.34** (39 %)

The results from Table 4.4 (b) again show that the majority of MAO in human placenta is of the A type as clorgyline inhibits most of the activity (i.e. MAO-A). It must be remembered however that the substrate used is 5-HT which is preferentially deaminated by MAO-A. Nevertheless a small amount of MAO-B activity is detected, as shown earlier.

The final method of placental extraction and assessment of MAO-A in human placenta is detailed in the materials and methods section of this chapter (section 4.2). This method has been used to assess the identity of MAO in human placenta and in the preliminary study to compare the status of MAO-A in pregnancy induced hypertension and normotensive controls.

4.3.3 Preliminary study of the status of placental MAO-A in pregnancy induced hypertension compared to normotensive controls

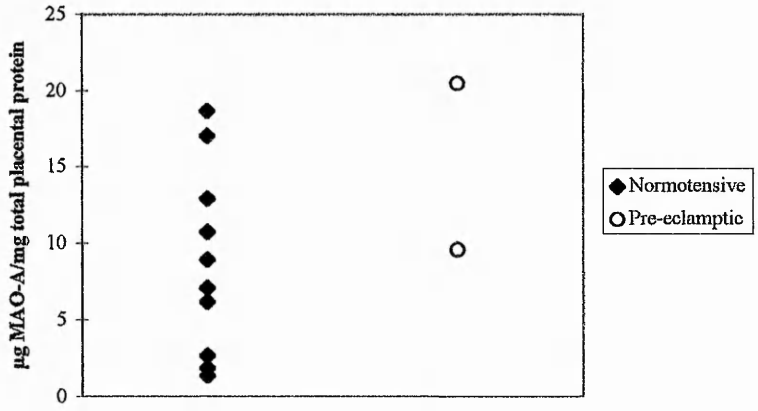
The competitive ELISA and activity assays already described were employed in a preliminary study to assess the status of MAO-A in pre-eclampsia. A 100 g portion of placenta was removed immediately after delivery and frozen at -70°C. Placental extracts were prepared using the final extraction procedure described in figure 4.1.

Figure 4.2 (A-C) shows the results obtained from the analysis of 10 normotensive and 2 pre-eclamptic placentae. Each placenta was analysed for MAO-A concentration using the MAO-A specific competitive ELISA and MAO-A activity using 5-HT as a substrate. Plot A shows the concentration of MAO-A expressed per milligram of total placental protein. There is a large variation in the normotensive group ranging from 1.38 μg to 18.66 μg MAO-A/mg total placental protein. The two pre-eclamptic placentae are within the higher portion of the normal range at 9.57 and 20.47 μg/mg total protein.

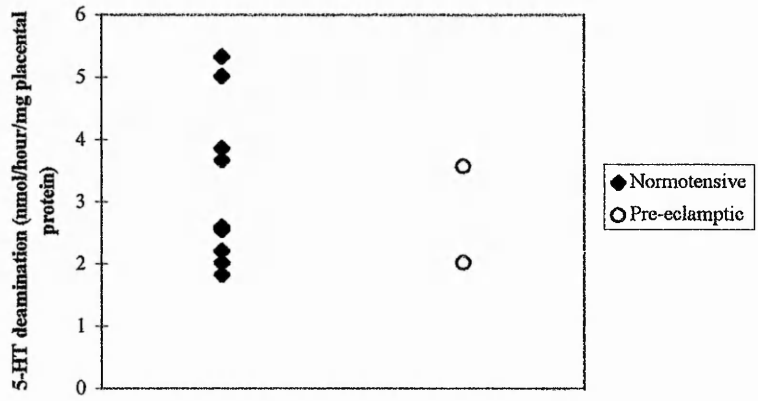
Plot B details the enzyme activity of MAO-A expressed as deamination of 5-HT as a function of total placental protein. Again this plot shows heterogeneity within the normotensive group (1.82 - 5.32 nmol 5-HT deaminated/hour/mg total placental protein). The pre-eclamptic placentae fall within this range (2.02, 3.58).

Plot C shows the molecular activity of MAO-A, i.e. the amount of 5-HT deaminated per mg of MAO-A. This again shows large variation in the normotensive group (118 - 1945 nmol 5-HT deaminated/hour/mg MAO-A) but interestingly the pre-eclamptic group are at the very bottom end of this range (211, 175).

A



B



C

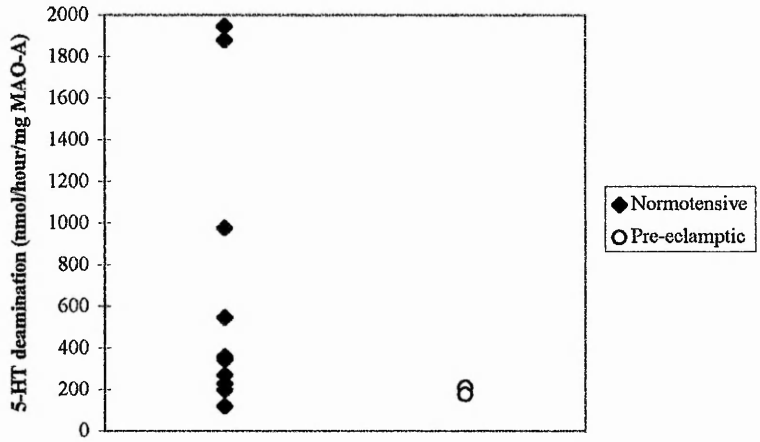


Figure 4.2. Analysis of MAO in normotensive (n = 10) and pre-eclamptic (n = 2) placentae. Plot A, Concentration of MAO-A.
Plot B, MAO-A activity expressed per milligram total protein.
Plot C, MAO-A activity expressed per milligram MAO protein.

4.3.4 MAO isoenzymes in human placenta

Using the techniques already described for assessing MAO-A protein concentration and activity, attempts were made to determine whether or not MAO-B was present in human placenta. A healthy placenta was washed and homogenised as described in figure 4.1 and incubated in a final concentration of 0.5% Triton X-100 before centrifugation to produce solubilised MAO.

4.3.4.1 *MAO protein estimation by competitive ELISA*

Wells of a microtitre plate were coated as described previously (section 2.2.15) and solubilised placental MAO was used as the competing antigen in ELISAs for MAO-A and MAO-B Solubilised liver MM were used as a standard. Figures 4.3 and 4.4 show examples of the displacement plots from these ELISA systems. These plots and table 4.5 show that both MAO-A and MAO-B are detected in human placental extracts and that MAO-B contributes 10 % of the total MAO protein.

4.3.4.2 *MAO activity assay*

MAO activity was also assessed in the same extract using 5-HT as substrate; it must be noted that this substrate is not the ideal choice for MAO-B estimations as the isoenzyme will only deaminate 5-HT if the concentration is high enough. These results indicate that MAO-B activity is present in placental extracts although at a very low level (3 % of the total).

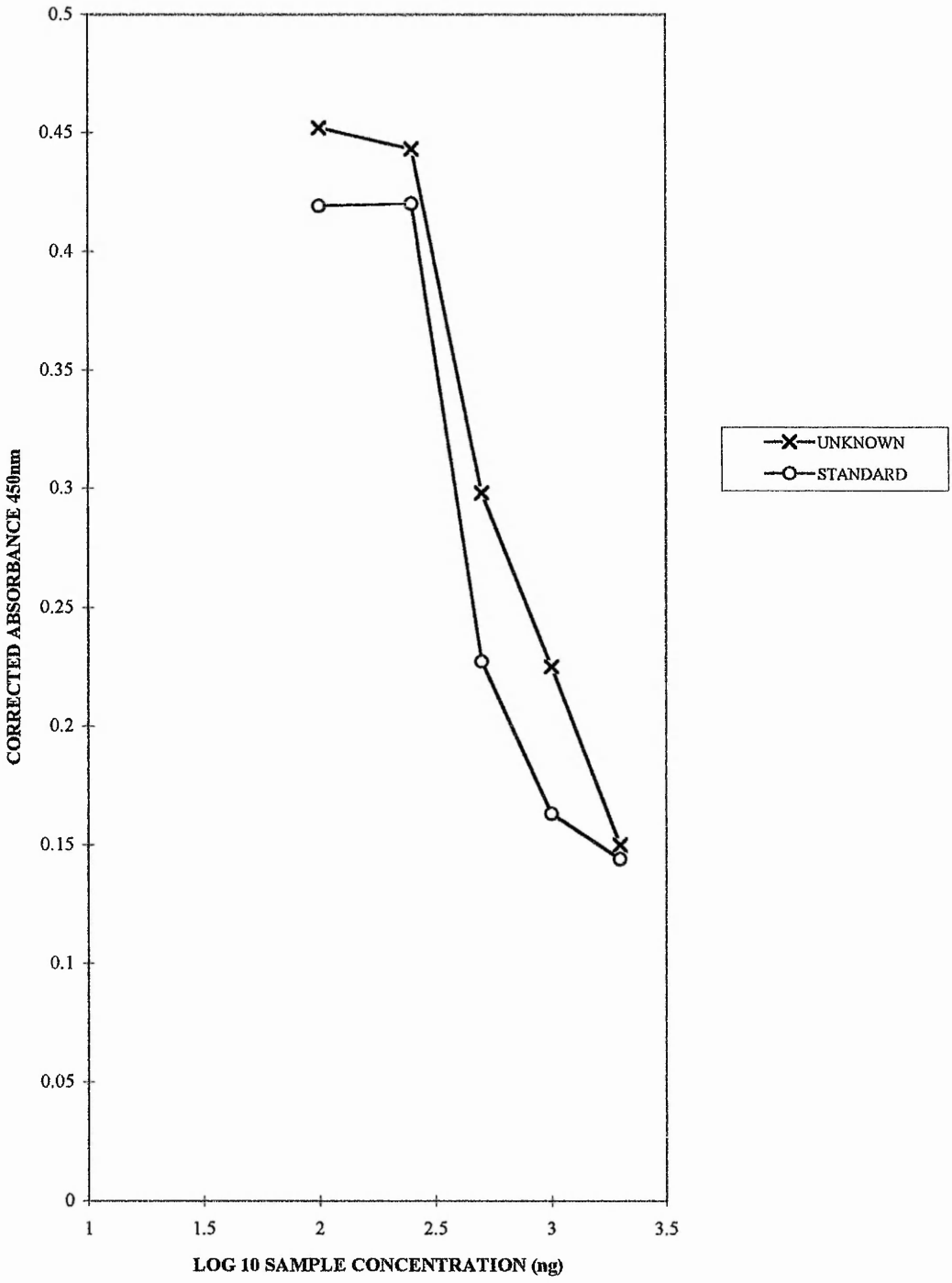


Figure 4.3 Measurement of MAO-A concentration in human placenta using the competitive ELISA. Semi-log plot for liver MM (o) and placenta (x). In the absence of competitor the mean absorbance was 0.452.

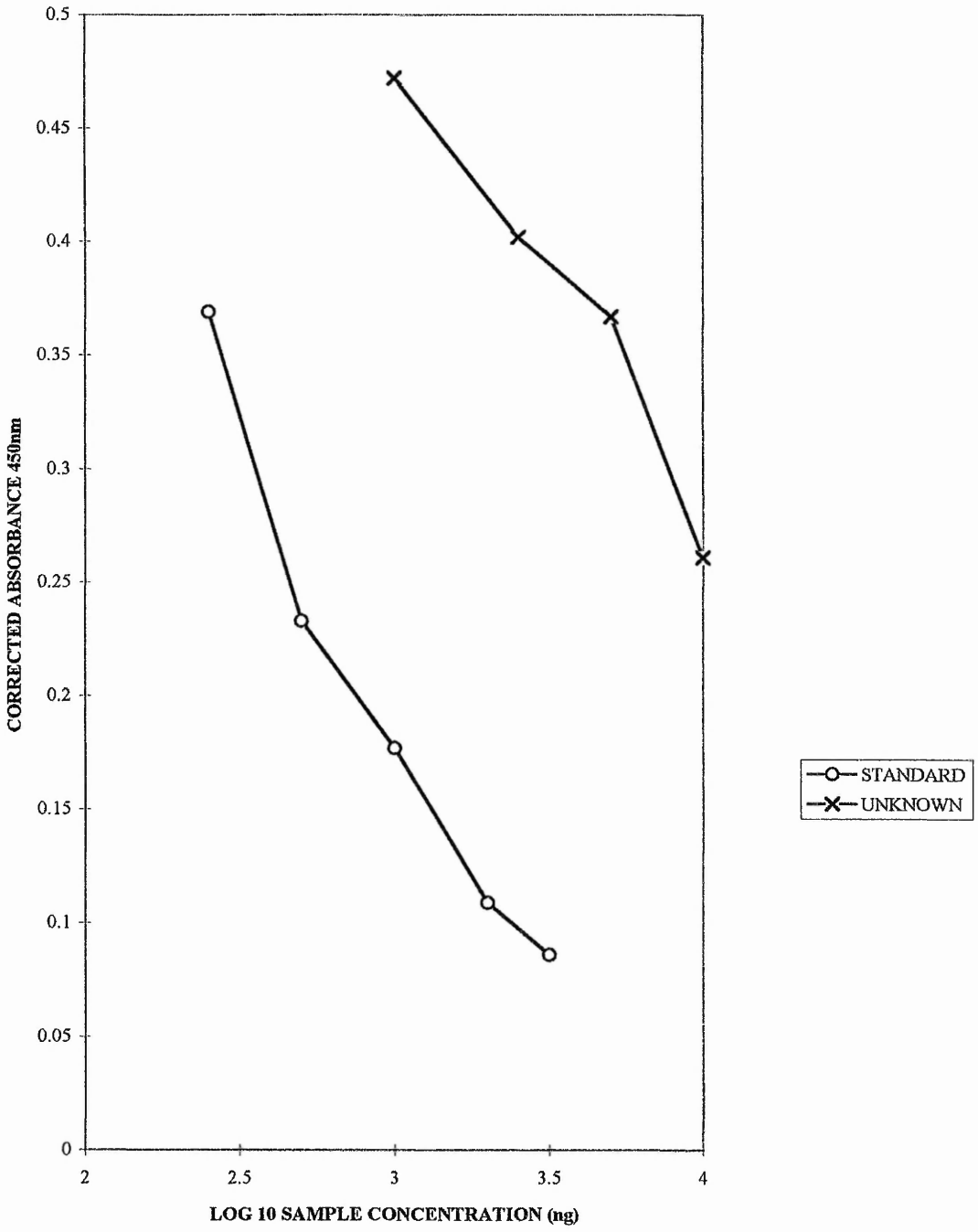


Figure 4.4 Measurement of MAO-B concentration in human placenta using the MAO-B competitive ELISA. Semi-log plot for liver MM (o) and placenta (x). In the absence of competitor the mean absorbance was 0.48.

Table 4.5. MAO isoenzymes in solubilised placental extracts. MAO concentration was determined by competitive ELISAs for MAO-A and MAO-B. Placental MAO activity was determined using 5-HT as a substrate. Values have been corrected using an appropriate sample blank and background count.

MAO Isoenzyme	**Concentration** (μg/mg total protein)	**Activity** (nmol 5-HT deaminated /hr/mg **MAO-A or B**)
A	96.52 (90 %)	12.52 (97 %)
B	11.10 (10 %)	0.44 (3 %)

4.3.4.3 *Immunohistochemistry*

The competitive ELISA had detected a substantial amount of MAO-B in placental extracts. To unequivocally prove whether MAO-B is intrinsic to human placental tissue or is of blood origin (i.e. platelets and lymphocytes), the MAO-A and MAO-B antibodies were used to immunostain placental sections.

Snap frozen cryosections (12 μm) were first stained with Haematoxylin and Eosin to visualise placental morphology (Figure 4.5 I and II). This figure clearly shows the multi-nucleate syncytiotrophoblastic layer (S) around the chorionic villus.

Previous work (chapter 2) had suggested that fixing in 2 % (w/v) paraformaldehyde followed by sucrose perfusion prior to snap freezing enhanced the detection of the MAO-B (and possibly MAO-A) isoform in human liver. Therefore to maximise MAO-B detection in human placenta, tissue processing by the sucrose protection method was employed. Figure 4.5 III and IV shows Haematoxylin and Eosin staining of sucrose protected placenta. Tissue culture supernatants of 3F12/G10/2E3 (1:10 dilution) and 6G11/E1 (1:50 dilution) (chapter 2, section 2.3.2.2.2) were used to visualise MAO-B and

MAO-A respectively on sucrose protected placental cryosections. These conditions were considered optimal for the visualisation of MAO-A and determining the presence/absence of MAO-B. Tissue culture supernatant from an irrelevant monoclonal antibody (9H7 anti γ gliadin; matched isotype) was used at a dilution of 1:10 as a negative control. Primary antibody was also omitted to assess non specific binding.

The results from this study are shown in figures 4.6 and 4.7. Figure 4.6 (I and II) shows sections of placenta stained with 3F12/G10/2E3, the anti-MAO-B antibody; there is no evident staining, indicating the absence of MAO-B protein in human placenta. Figure 4.6 (III and IV) shows a sequential placental section using the matched isotype irrelevant monoclonal antibody (9H7) as a control; identical results were obtained with a no primary antibody control (data not shown). Figure 4.7 shows both low and high power magnifications of sections of the same human placenta incubated with 6G11/E1 the anti-MAO-A antibody. Very strong positive granular staining is evident in the syncytiotrophoblast layers of the chorionic villi, indicating that MAO-A protein is present in substantial amounts.

Identical results using both MAO antibodies and appropriate controls have been obtained from both sucrose protected and snap frozen samples from four normotensive human placentae.

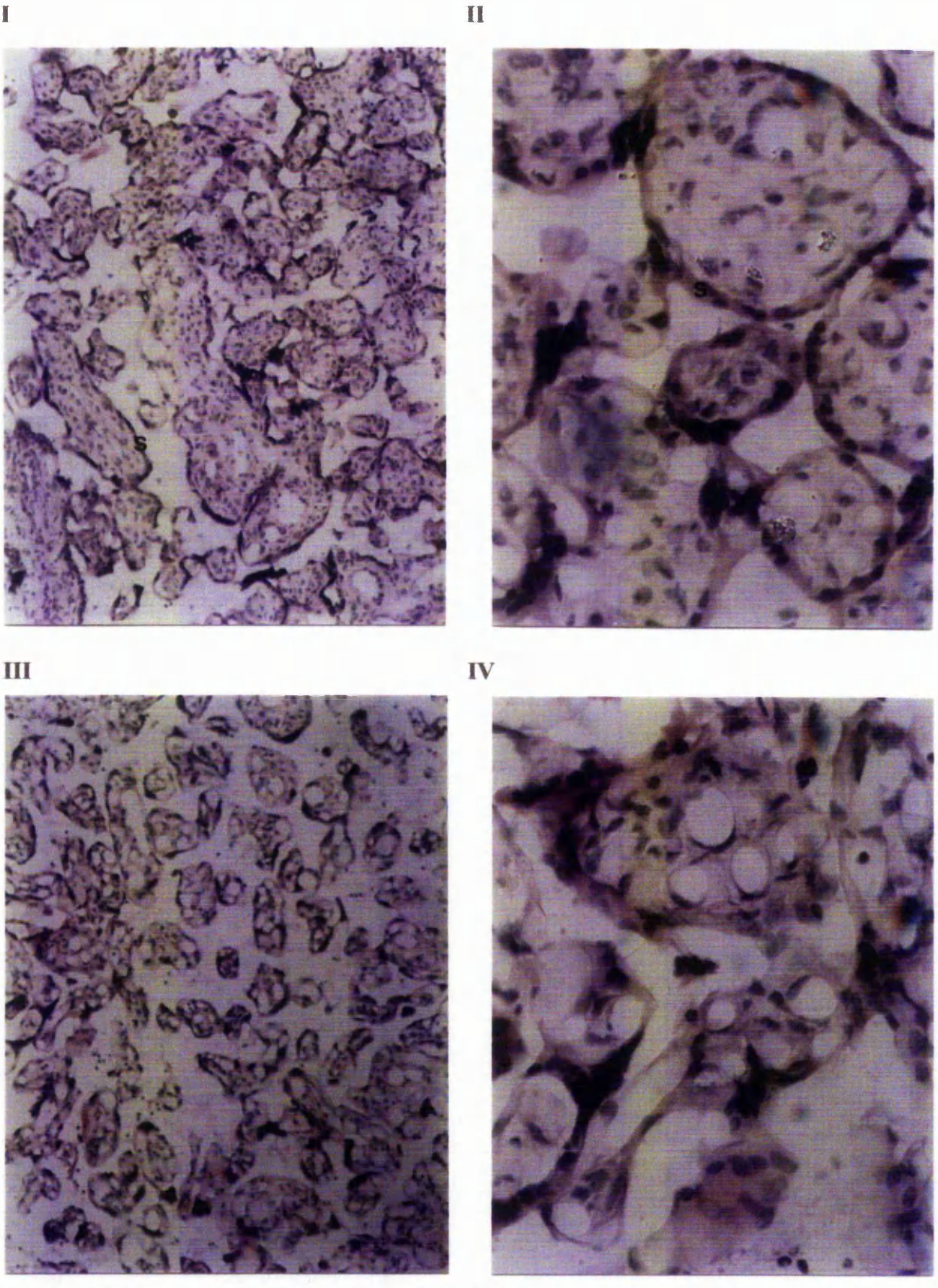


Figure 4.5. Haematoxylin and eosin staining of human placenta cryosections (12 μm). **I**. Post fixed following sectioning (x 100 magnification); **II**. Post fixed following sectioning (x 400 magnification); **III**. Pre-fixed plus sucrose protection (x 100 magnification); **IV**. Pre-fixed plus sucrose protection (x 400 magnification). S = syncytiotrophoblast layer.

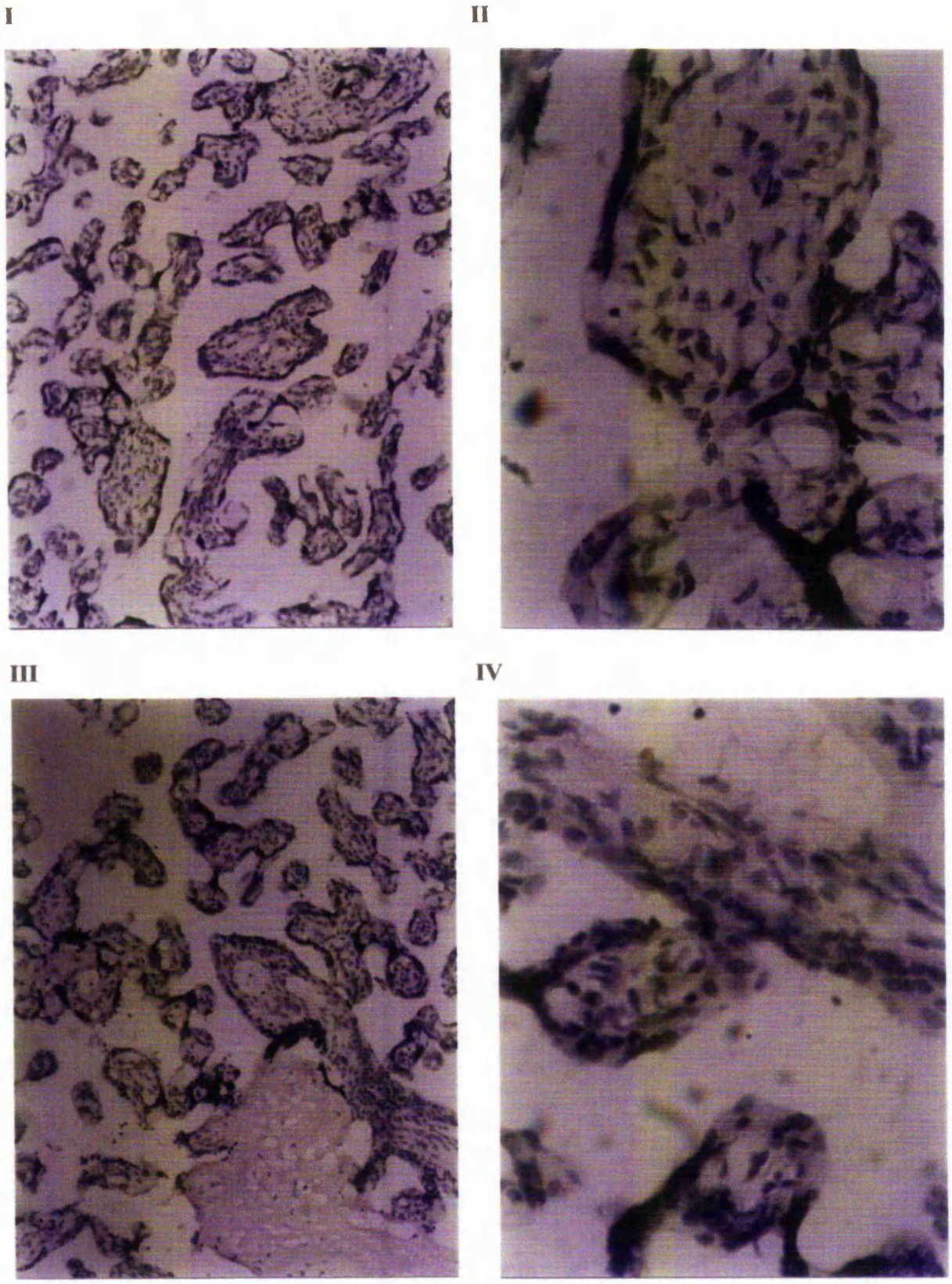
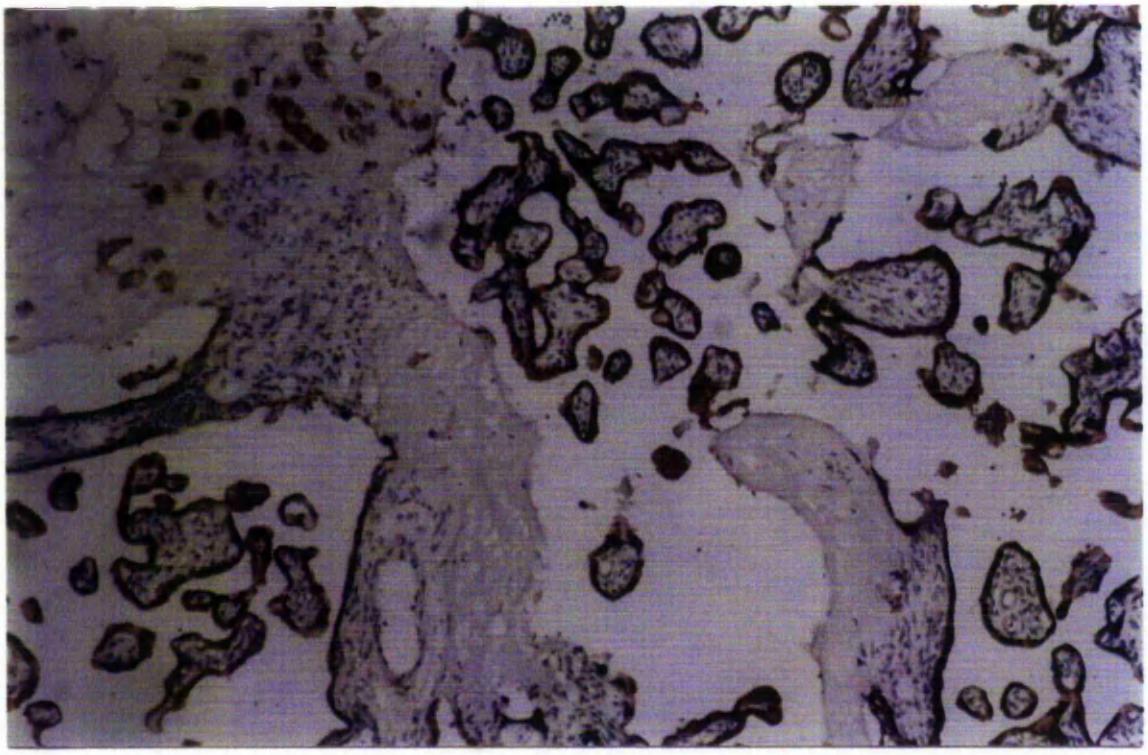


Figure 4.6. Immunohistochemical staining of sucrose protected cryosections (12 μm) of human placenta. **I**. 3F12/G10/2E3 specific for MAO-B (x 100 magnification); **II**. 3F12/G10/2E3 (x 400 magnification); **III**. Non-immune control (matched isotype) antibody (x 100 magnification); **IV**. Non-immune control (x 400 magnification). Sections have been counterstained with haematoxylin.

I



II

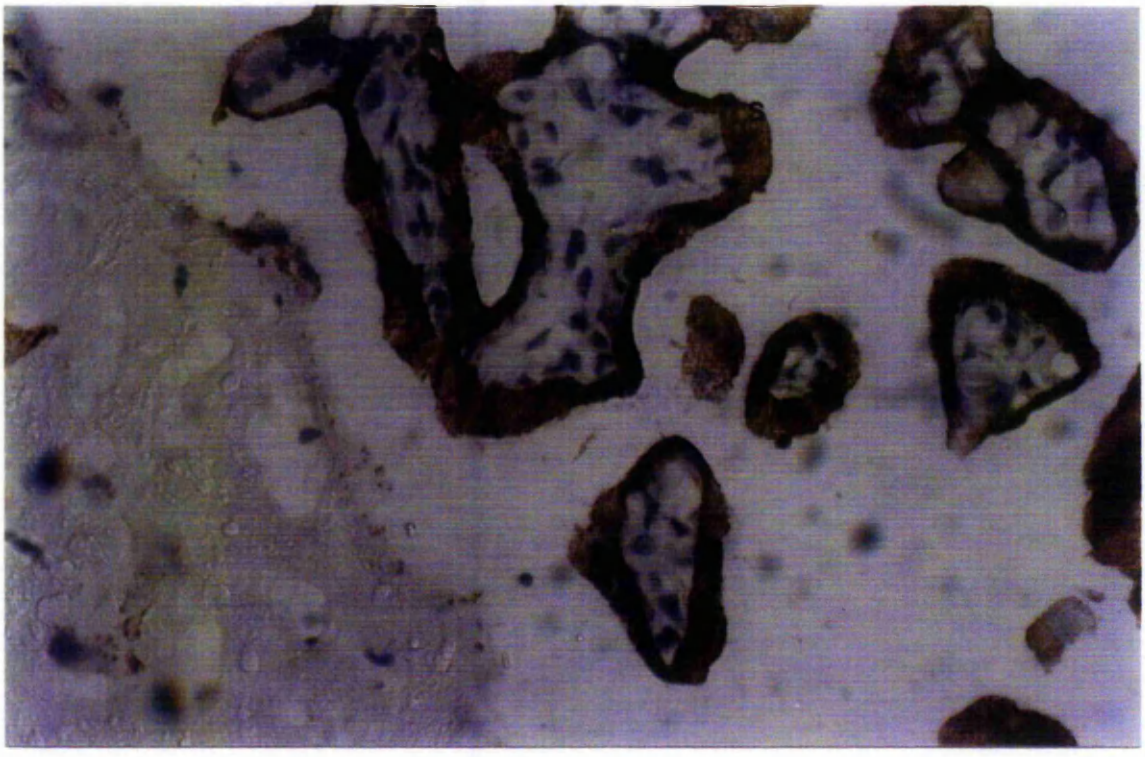


Figure 4.7. Immunohistochemical staining of sucrose protected cryosections (12 μm) of human placenta. **I**. 6G11/E1 specific for MAO-A, showing staining in the syncytiotrophoblastic layer (S) and intermediate trophoblasts (T) (x 100 magnification) **II**. 6G11/E1, showing granular MAO-A staining in the syncytiotrophoblastic layer (x 400 magnification). Sections have been counterstained with haematoxylin.

4.4 DISCUSSION

4.4.1 Development of a placental extraction procedure to assess MAO-A

A procedure suitable for the recovery of MAO from placenta was developed. When extracts were subjected to differential centrifugation, large amounts of MAO activity were detected in all fractions. It is possible that some of this activity was due to MAO-B from lymphocytes and platelets, especially since tyramine was used as a substrate, but it was felt that a simpler procedure was needed to avoid losses.

In an attempt to reduce MAO-B contamination from blood, careful washing in ice cold buffered sucrose solution was employed to rinse the tissue. Analysis using 5-HT and MAO-A and B inhibitors revealed that MAO activity was mainly type A.

Initial experiments had relied on a small weight (5 g) of tissue for extraction. When a larger piece was sampled (20 g at five different sites), MAO-A activity ranged from 3.74 to 5.56 x 10^5 DPM mg^{-1} total protein. To eliminate this variation a larger portion of placenta (100 g) was homogenised (without buffer) using a coffee mill grinder instead of a mortar and pestle homogeniser. Smaller representative 5 g samples of this homogeneous paste were then extracted.

Triton X-100 was used to solubilise MAO from human placenta for use in the MAO-A competitive ELISA and activity assay. A Triton concentration of 0.5 % (v/v) was employed as 86 % of the total MAO was extracted and minimal effects on MAO activity measurements and antibody binding were evident.

As there is variation in MAO activity within a portion of placenta, the whole organ should ideally be homogenised; unfortunately obtaining the whole placenta was not possible for the pre-eclamptic study (section 4.4.2). Instead, a 100 g sample was taken from healthy areas of the placenta washed free from blood and homogenised and extracted as described in figure 4.1

4.4.2 Preliminary study of the status of placental MAO-A in pregnancy induced hypertension compared with normotensive placentae

The MAO-A competitive ELISA and activity assays were employed to assess the status of MAO-A in pre-eclampsia. This data is preliminary as only two pre-eclamptic and ten normotensive placentae were available for analysis. Data show that the normotensive group is very heterogeneous showing wide variation. The two pre-eclamptic placentae are at the top half of the normal range for MAO-A protein concentration, indicating a possible increase in MAO-A protein in pre-eclamptic placentae. However analysis of MAO-A activity shows the pre-eclamptic placentae to lie in the middle to the bottom of the normal range.

MAO-A molecular activity in pre-eclamptic placentae is at the bottom of the normal range, which may indicate that the enzyme concentration may be within the wide normal range, but its activity is in some way impaired. This may not be surprising as in pre-eclampsia the increase in circulating 5-HT causes vasoconstriction resulting in local anoxic conditions which may have a lasting inhibitory effect on MAO activity. As the assays were conducted *in vitro* and oxygen was freely available, these results suggest that there has been some permanent damage to the enzyme before delivery. If the MAO-A function is impaired in pre-eclampsia the foetus may be at risk from maternal monoamines. Indeed Ishikawa *et al* (1998) suggest that MAO activity in foetal membranes may have a role in the protection of the foetus during uterine contraction during labour.

These data are by no means complete and it is unwise to draw any firm conclusions from them. There is an indication, however, that MAO is damaged in pre-eclampsia but more samples will require analysis before statistical significance can be reached.

4.4.3 MAO isoenzymes in human placenta

The presence of MAO-B in extracts of human placenta is still a matter of controversy, with many still believing that any MAO-B activity is due to contaminating lymphocytes or platelets. As it is extremely difficult to ensure that all blood contamination is removed

at least some of the MAO-B may be of blood origin. As indicated in Chapter 1 small levels of MAO-B have previously been reported in placental extracts using specific antibodies (Riley *et al*, 1989) and MAO-B mRNA has also been detected in placental extracts (Bach *et al*, 1988).

The competitive ELISAs for MAO-A and B were employed to analyse the type of MAO found in placental extracts. Indeed substantial amounts of MAO-B protein were detected in placental extracts (10 % of the total MAO protein). There is still a possibility that the MAO-B is derived from blood origin (lymphocytes, platelets) but it is recognised that this is a very high percentage to have originated from blood alone. My results agree with data published by Riley *et al*, 1989, which suggested that MAO-B is intrinsic to placental tissue. The latter study detected both MAO-B activity and MAO-B protein (using specific antibodies) in placental mitochondrial extracts. They estimated that MAO-B contributes between 5 and 14 % of the total placental MAO concentration. They also suggested that MAO-B is unlikely to have come from blood contamination alone, and, estimated that for all MAO-B to have originated from platelet contamination the placenta needed to contain 7900 ml of blood and that all circulating platelets were lodged in the placenta, which is unlikely. However they overlooked the contribution of MAO-B from lymphocytes and granulocytes in this calculation.

The MAO activity assays detected low levels of MAO-B, even when using the preferential MAO-A substrate 5- HT. However differential MAO activity assays are problematic as inhibitors and substrates are not exclusive to one isotype.

To categorically check whether or not placental tissue contains MAO-B protein immunohistochemical methods were used. Thus pre-fixed, sucrose protected placental cryosections were incubated in the presence of the MAO-A and MAO-B antibodies. 6G11/E1 (MAO-A) produced exceptionally strong positive staining especially within the syncytiotrophoblast layer around each chorionic villus. Intermediate trophoblasts seen in some sections also stained strongly positive for MAO-A. This indicated that there was a high concentration of MAO-A in normal placenta. The MAO-B antibody on the other hand showed no staining and in fact showed a very similar staining profile to the negative control antibody (9H7) and sections incubated in the absence of primary antibody.

I believe that this is conclusive evidence that MAO-A is the only MAO isoform present in normal human term placenta. This may not, of course, be the whole story as this work was completed using normal term placentae. There are a number of abnormalities which may occur during pregnancy and the involvement of MAO-A or even MAO-B cannot be discounted. Indeed a recent paper published by Auda *et al* (1998) has assessed the localisation of MAO specific mRNA in normal human placenta by *in situ* hybridisation, and found large levels of MAO-B and MAO-A mRNA present in the syncytiotrophoblast layers. Thus it seems as if MAO-B specific mRNA is expressed but not the MAO-B protein. Why this should be is not known and requires further study.

There may of course be some abnormal conditions of pregnancy in which MAO-B protein is expressed. Riley *et al*, 1989 did not provide information about the condition of the placentae used in the study nor whether pregnancy and delivery were normal. They did however look at more than one placenta and found quite large differences in MAO-B activity. It may be that abnormalities were present in the placentae containing the most MAO-B and that those placentae containing little MAO-B could be due to blood contamination. It would therefore be interesting to conduct an analysis of abnormal placentae/pregnancies to attempt to explain the presence of MAO-B mRNA in normal placentae.

CHAPTER FIVE

GENERAL DISCUSSION

GENERAL DISCUSSION

Antibody characteristics and ELISA development

The principal aims of this study were to assess the status of monoamine oxidase in extracts of human tissues, namely platelets and placenta. These were achieved using competitive ELISAs using monoclonal antibodies specific for MAO-A and MAO-B. The results from these ELISAs were combined with enzyme activity studies to give an overall picture of MAO status i.e. its molecular activity.

The MAO-B monoclonal antibody (3F12/G10) was developed by Billett *et al*, 1986 and had been previously used in a competitive ELISA to estimate MAO-B content in human liver and a preliminary study of MAO-B content in normal female human platelets (Yeomanson, 1990; Yeomanson and Billett, 1992). 6G11/E1 had been produced by Yeomanson, 1990 and used to detect MAO-A in cryosections of various human peripheral tissues (Church *et al*, 1994; Rodriguez *et al*, in preparation).

This study describes the use of both of these antibodies in a competitive ELISA format for the estimation of MAO-A and B in human placenta (chapter 4) and platelets (chapter 3) and represents the first time that MAO molecular activity has been determined in human placental extracts.

The specificity of both antibodies was checked (chapter 2) using various techniques. The hybridoma cell line secreting the MAO-B monoclonal antibody was found to be unstable so re-cloning was necessary to improve titre and ensure a continuous supply of antibody. Antibody secreted by the re-cloned cell line (3F12/G10/2E3) required analysis to ensure its specificity had been retained.

One of the problems faced with the competitive ELISA technique was the need to use the detergent Triton X-100 to solubilise MAO from mitochondrial membranes. Triton X-100 has been found to have an adverse effect on the binding of both 6G11/E1 and 3F12/G10/2E3 to their respective epitopes. The competitive ELISA relies on competition for antibody by solid phase and Triton X-100 solubilised mobile phase

antigen; in consequence the antibody is in contact with the detergent during antibody binding. Hence it was important to establish the lowest useful detergent concentration to minimise these problems.

One answer to this problem could be to use a two site or sandwich ELISA. This type of technique involves immobilising a capture antibody to the solid phase, followed by incubation with the solubilised antigen, which would then be washed off before addition of labelled revealing antibody ie 3F12/G10/2E3 or 6G11/E1. Indeed in the early part of my study I tried to develop this assay using another monoclonal antibody, 5A11/2F4, which reacts with both forms of MAO. The epitope to which 5A11/2F4 is directed is different from the epitopes to which 3F12/G10/2E3 and 6G11/E1 are directed, so in principle a two site assay should work provided that the revealing antibodies could be successfully labelled. Labelling was achieved by biotinylation.

Commercially available 'avid' plates were used, which optimally orientate the capture antibody (5A11/2F4) by immobilising it to the solid phase through the Fc region; I introduced solubilised extracts of liver mitochondrial membranes and tried to reveal MAO binding using the biotin labelled MAO-A and B specific monoclonals. The method produced promising results with 6G11/E1 but failed to produce signals with 3F12/G10/2E3. This failure may have been due to the labelling procedure i.e. biotinylation, which may have prevented 3F12/G10/2E3 from recognising its epitope. Although this two site approach does not completely eliminate the presence of Triton X-100 with antibody, 5A11/2F4 (or the epitope to which it binds) is more robust and binding appears not to be affected by the detergent. It may be possible to use an alternative antibody label for 3F12/G10/2E3 e.g. fluorochromes, enzymes, which may increase sensitivity. Further development of the 2 - site assay for MAO-A is warranted.

Studies on platelet MAO

Since platelet MAO has been postulated to be an accessible peripheral marker of MAO-B status in the brain, and MAO-B has been implicated in PD, it has been proposed that the peripheral enzyme may have a role in predictive testing for PD. A large proportion of this study therefore has focused on the measurement of platelet MAO-B activity and

concentration in an attempt to discover whether or not changes in the platelet enzyme where evident in PD.

Most of the previous work looking at PD has concentrated on activity measurements only and has produced conflicting results; indeed a literature review revealed that similar numbers of studies reported increases or decreases in platelet MAO-B activity in PD. It also revealed that other workers had found large population variations in platelet MAO activity measurements for both control and PD groups. In an attempt to determine the status of MAO enzyme (active and inactive species) an assessment of MAO concentration coupled with activity measurements was conducted.

This assessment of molecular activity revealed that there was no significant difference between platelet MAO from Caucasian PD patients compared with controls. Heterogeneity was observed in both control and PD populations although MAO in the PD population was more heterogeneous than in the control population; this may be explained by the fact that PD is not one disease entity but is multi symptomatic. The patients used in this study were newly diagnosed i.e. had received no PD drug treatment, an important difference to previous studies looking at MAO activity. Indeed a positive response to L-DOPA later confirmed the diagnosis of PD. Heterogeniety in platelet MAO activity measurements is also evident in other studies (Table 3.9) and it may be that large variations do exist in normal control populations.

The fact that no differences between disease and controls were evident does not preclude the possibility that brain MAO-B is different in the PD group. Ideally a parallel study of platelet and brain MAO-B would be conducted; but of course subjects are likely to have received treatment before death.

Assessment of platelet MAO molecular activity was also conducted in a population of Hong Kong Chinese PD patients and matched controls. As in the Caucasian study, no significant difference was noted between PD and controls.

The most interesting finding was the difference in platelet MAO concentrations between Caucasian and Hong Kong Chinese control populations; this represents the

first study of this kind. MAO concentration in the Hong Kong Chinese population was significantly higher than in the Caucasians. However MAO molecular activity for both substrates was lower (significant for males with dopamine and both sexes with PEA) in the Hong Kong Chinese, as total activities were similar in the two groups. This would suggest ethnic differences in MAO concentration and MAO molecular activity. On the other hand, as total activities are similar, it is unlikely that toxic monoamines would accumulate in the Hong Kong Chinese population. However, as this represents the first study of its kind, and since population numbers were low, the work needs to be repeated.

It has been reported that the prevalence of PD is much lower in Chinese populations (Wang *et al*, 1995) compared to other races. Whether or not this is linked to the lower MAO molecular activity in the Chinese population needs further investigation. As PD is characterised by a lack of dopamine an increase in active MAO enzyme may be expected. It appears from my work that the Chinese enzyme, although present in significantly higher concentrations compared with the Caucasian enzyme may be less active which may contribute to a lower prevalence of PD in Chinese populations.

In summary in this small study, platelet MAO-B is unchanged in PD compared with controls, irrespective of ethnic origin, which indicates that the peripheral form of the enzyme is not a useful marker, or this may mean that MAO-B is not changed in PD. To check this out further, an actual assessment of the brain enzyme is important, specifically the areas of the brain damaged in PD and the neighbouring support cells (astrocytes, glial cells). It would also be useful to look at sections of PD brains using the 3F12/G10/2E3 antibody. The antibody has been used on brain cryosections successfully by Yeomanson (1990). PD brain banks are available, however all tissue is preserved in paraffin and the MAO-B antibody will not work using conventional immunohistochemical techniques on paraffin embedded sections. It is thought that the dehydration process damages the MAO-B epitope. More work should be done to either allow the MAO-B monoclonal to work by epitope retrieval methods e.g. microwaving/autoclaving, protease digestion or develop a new MAO-B specific monoclonal antibody suitable for use on paraffin sections.

As detection of brain MAO-B in paraffin brain banks may be difficult using antibodies an alternative approach would be to use MAO-A and B specific mRNA probes for an assessment of mRNA using in situ hybridisation. A major drawback of this technique is that mRNA is being assessed which may not necessarily mean that the protein is expressed. Indeed Auda et al, (1998), have found that in human placenta MAO-B mRNA is expressed but not MAO-B protein.

The fact that platelet MAO-B is not a useful peripheral marker for PD does not preclude the usefulness of other platelet enzymes. Indeed, platelet mitochondrial complex I has been studied in PD (eg. Blandini *et al*, 1998).

Studies on placental MAO

The second tissue targeted in this study was human placenta. The MAO-A and B competitive ELISAs were used together with immunohistochemistry to assess the enzyme status and identity in human placenta. Traditionally the placenta is thought to contain type A only, however a few reports do exist arguing that small but significant amounts of MAO-B are intrinsic to human placenta (e.g. Riley et al, 1989). In order to establish whether MAO-B was intrinsic to placenta or from merely blood contamination, the competitive ELISAs, activity assays and immunohistochemisty techniques were employed.

Activity assays revealed that 2-5 % of MAO activity was due to MAO-B however activity measurements are difficult to estimate due to substrates and inhibitors not being entirely exclusive to one isoenzyme. Competitive ELISAs using the MAO-A and MAO-B specific monoclonal antibodies estimated that MAO-B contributes 10 % of total MAO in placental extracts; this of course could still be due to blood contamination. Immunohistochemical techniques using the same antibodies revealed no MAO-B protein intrinsic to placental tissue. I believe this provides the most convincing evidence for the absence of MAO-B in human placenta.

A preliminary study of MAO-A in pre-eclampsia was conducted, although it is unwise to draw any firm conclusions from this study as sample numbers were very small. The

general trends indicate that placental MAO-A molecular activity in pre-eclamptics is lower than in normotensive controls; this may contribute to the increase in circulatory 5 HT in pre-eclampsia (Filshie *et al*, 1992).

The proposed increase in 5-HT may of course not be due to defective MAO, but another mechanism namely trophoblastic embolisation, whereby trophoblasts are released into the maternal circulation. These trophoblasts may cause platelet release of 5-HT, resulting in an increase in circulatory 5 HT. A reduced MAO activity may not be able to cope with this increase in 5-HT thereby further exacerbating the problem. It may therefore be a combination of the two factors.

It has been reported that the presence of trophoblasts in the maternal circulation increases in pre-eclampsia (Chua *et al*, 1991). Trophoblastic embolisation has been studied using a trophoblast specific monoclonal antibody (Mueller *et al*, 1987). It would be interesting to compare 6G11/E1 with this other antibody in the detection of trophoblastic embolisation. It would also therefore be interesting to study MAO-A in pre-eclamptic trophoblastic embolisation compared with normotensive controls, and assess the numbers of MAO-A positive trophoblasts compared with the molecular activity of the enzyme in the placenta. Finally, it may also be possible to purify and concentrate trophoblasts using immunoaffinity chromatography as an alternative non-invasive method of pre-natal diagnosis.

REFERENCES

Ashby, C. R. (jnr.), Carr, L. A., Cook, C. L., Steptoe, M. M. and Franks, D. D. 1988. Alteration of platelet serotonergic mechanisms and monoamine oxidase actitivy in premenstrual syndrome. Biological Psychiatry, **24**, 225-233.

Auda, G. R., Kirk, S. H., Billett, M. A. and Billett, E. E. 1998. Localization of monoamine oxidase mRNA in human placenta. The Journal of Histochemistry and Cytochemistry, **46(12)**, 1393-1400.

Bach, A. W. J., Lan, N. C., Johnson, D. L., Abell, C. W., Bembenek, M. E., Kwan, S. W., Seeburg, P. H. and Shih, J. C. 1988. cDNA cloning of human liver monoamine oxidase A and B : molecular basis of differences in enzymatic properties. Proceedings of the National Academy of Science. USA, **85,** 4934-4938.

Balsa, M. D., Gómez, N. and Unzeta, M. 1989. Characterisation of monoamine oxidase activity present in human granulocytes and lymphocytes. Biochimica et Biophysica Acta, **992**, 140-144.

Barnea, E. R., Maclusky, N. J., Decherney, A. H. and Naftolin, F. 1986. Monoamine oxidase activity in the term human placenta. American Journal of Perinatology, **3(3)**, 219-224.

Baumann, R. J., Jameson, H. D., Mckean, H. E., Haack, D. G. and Weisberg, L. M. 1980. Cigarette smoking and Parkinson's Disease: I. A comparison of cases with matched neighbors. Neurology, **30**, 839-843.

Begley, D. J., Firth, J. A. and Hoult, J. R. S. Human reproduction and developmental biology. The Macmillan Press Limited, London and Basingstoke, 1980.

Belmaker, R. H., Murphy, D. L., Wyatt, R. J. and Loriaux, D. L. 1974. Human platelet monoamine oxidase changes during the menstrual cycle. Archives of General Psychiatry, **31**, 553-556.

Benigni, A., Gregorini, G., Frusca, T., Chiabrando, C., Ballerini, S., Valcamonico, A., Orisio, S., Piccinelli, A., Pinciroli, V., Fanelli, R., Gastaldi, A. and Remuzzi, G. 1989. Effect of low dose aspirin on fetal and maternal generation of thromboxane by platelets in women at risk for pregnancy -induced hypertension. The New England Journal of Medicine, **321(6)**, 357-362.

Bernheimer, H., Birkmayer, W., Hornykiewicz, O., Jellinger, K. and Seitelberger, F. 1973. Brain dopamine and the syndromes of Parkinson and Huntington : Clinical, Morphological and Neurochemical Correlations. Journal of the Neurological Sciences, **20**, 415-455.

Billett, E. E. and Mayer, R. J. 1986. Monoclonal antibodies to monoamine oxidase B and another mitochondrial protein from human liver. Biochemical Journal, **235**, 257-263.

Billett, E. E., Gunn, B. and Mayer, R. J. 1984. Characterisation of two monoclonal antibodies obtained after immunisation with human liver mitochondrial membrane preparations. Biochemical Journal, **221**, 765-776.

Birkmayer, W., Knoll, J., Riederer, P. and Youdim, M. B. H. 1983. (-)-Deprenyl leads to prolongation of L-Dopa efficacy in Parkinson's Disease. Modern Problems of Pharmacopsychiatry, **19**, 170-176.

Blandini, F., Nappi, G. and Greenamyre, J. T. 1998. Quantitative study of mitochondrial complex I in platelets of Parkinsonian patients. Movement Disorders, **13(1)**, 11-15.

Blaschko, H., Richter, D. and Schlossmann, H. 1937. The oxidation of adrenaline and other amines. Biochemistry, **31**, 2187-2196.

Bond, P. A. and Cundall, R. L. 1977. Properties of monoamine oxidase (MAO) in human blood platelets, plasma, lymphocytes and granulocytes. Clinica Chimica Acta, **80**, 317-326.

Bongioanni, P., Mondino, C., Boccardi, B., Borgna, M. and Castagna, M. 1996. Monoamine oxidase molecular activity in platelets of Parkinsonian and demented patients. Neurodegeneration, **5**, 351-357.

Bonuccelli, U., Piccini, P., Del Dotto, P., Pacifici, G. M., Corsini, G. U. and Muratorio, A. 1990. Platelet monoamine oxidase B activity in Parkinsonian patients. Journal of Neurology, Neurosurgery and Psychiatry, **53**, 854-855.

Browne, B. J., Fowler, C. J. and Callingham, B. A. 1978. The effects of homogenization of rat liver in different buffer solutions on the yield and kinetic properties of monoamine oxidase. Journal of Pharmacy and Pharmacology, **30**, 573-575.

Brown, G. K., Powell, J. F. and Craig, I. W. 1982. Immunological studies of human monoamine oxidases. Journal of Neurochemistry, **39(5)**, 1266-1270.

Burns, R. S., Chiueh, C. C., Markey, S. P., Ebert, M. H., Jacobowitz, D. M. and Kopin, I. J. 1983. A primate model of parkinsonism : selective destruction of dopaminergic neurones in the pars compacta of the substantia nigra by N-methyl- 4- phenyl, 1,2,3,6 tetrahydropyridine. Proceedings of the National Academy of Sciences. USA, **80**, 4546-4550.

Bussolino, F., Benedetto, C., Massobrio, M. and Camussi, G. 1980. Maternal vascular prostacyclin activity in Pre-eclampsia. The Lancet, ii (Sept), 702.

Calne, D. B. and Langston, J. W. 1983. Aetiology of Parkinson's Disease. The Lancet, **2(8365)**, 1457-1459.

Cawthon, R. M. and Breakefield, X. O. 1979. Differences in A and B forms of monoamine oxidase revealed by limited proteolysis and peptide mapping. Nature, **281**, 692-694.

Cawthon, R. M., Pintar, J. E., Haseltine, F. P. and Breakefield, X. O. 1981. Differences in the structure of A and B forms of human monoamine oxidase. Journal of Neurochemistry, **37(2)**, 363-372.

Cesura, A. M., Bös, M., Galva, M. D., Imnof, R and Da Prada, M. 1989. Characterisation of the binding site of [^{3}H] Ro41-1049 to the active site of human monoamine oxidase A. Molecular Pharmacology, **37**, 358-366.

Chan, D. K. Y., Woo, J., Ho, S. C., Pang, C. P., Law, L. K., Ng, P. W., Hung, W. T., Kwok, T., Hui, E., Orr, K., Leung, M. F. and Kay, R. 1998. Genetic and environmental risk factors for Parkinson's Disease in a Chinese population. Journal of Neurology, Neurosurgery and Psychiatry, **65**, 781-784.

Checkoway, H., Costa, L. G., Woods, J. S., Castoldi, A. F., Lund, B. O. and Swanson, P. D. 1992. Peripheral blood cell activities of monoamine oxidase B and superoxide dismutase in Parkinson's Disease. Journal of Neural Transmission (P. D. Section), **4(4)**, 283-290.

Chen, K., Wu, H-F. and Shih, J.C. 1993. The deduced amino acid sequences of human platelet and frontal cortex monoamine oxidase B are identical. Journal of Neurochemistry, **61(1)**, 187-190.

Chia, L-G. and Liu, L-H. 1992. Parkinson's Disease in Taiwan : An analysis of 215 patients. Neuroepidemiology, **11**, 113-120.

Chiba, K., Trevor, A. and Castagnoli, N. (jnr.). 1984. Metabolism of the neurotoxic tertiary amine, MPTP, by brain monoamine oxidase. Biochemical and Biophysical Research Communications, **120(2)**, 574-578.

Chiueh, C. C., Markey, S. P., Burns, R. S., Johannessen, J. N., Pert, A. and Kopin, I. J. 1984. Neurochemical and behavioural effects of systemic and intranigral administration of N-methyl-4-phenyl- 1,2,3,6 tetrahydropyridine in the rat. European Journal of Pharmacology, **100**, 189-194.

Chua, S., Wilkins, T., Sargent, I. and Redman, C. 1991. Trophoblast deportation in pre-eclamptic pregnancy. British Journal of Obstetrics and Gynaecology, **98**, 973-979.

Church, R. J., Robinson, G. and Billett, E. E. 1994. The localisation of monoamine oxidase in human placenta using a new specific monoclonal antibody to monoamine oidase A. Royal Microscopical Society Proceedings, **29(4)**, 243.

CLASP (Collaborative Low-dose Aspirin Study in Pregnancy). 1994. CLASP : a randomised trial of low-dose aspirin for the prevention and treatment of pre-eclampsia among 9364 pregnant women. The Lancet, **343**, 619-629.

Corash, L. 1980. Platelet Heterogeneity: Relevance to the use of platelets to study psychiatric disorders. Schizophrenia Bulletin, **6(2)**, 254-258.

Craig, I. W., Powell, J. F., Brown, G. K. and Summers, K. M. 1982. Studies on human monoamine oxidase. In Monoamine oxidase : Basic and clinical frontiers. Edited by Kamijo, K., Usdin, E. and Nagatsu, T. Excerpta Medica, Princeton, 18-27.

Damier, P., Kastner, A., Agid, Y. and Hirsch, E. C. 1996. Does monoamine oxidase type B play a role in dopaminergic nerve cell death in Parkinson's Disease. Neurology, **46**, 1262-1269.

Danielczyk, W., Streifler, M., Konradi, C., Riederer, P. and Moll, G. 1988. Platelet MAO-B activity and the psychopathology of Parkinson's Disease, senile dementia and multi-infarct dementia. Acta Psychiatrica Scandinavica, **78**, 730-736.

Davis, G. C., Williams, A. C., Markey, S. P., Ebert, M. H., Caine, E. D., Reichert, C. M. and Kopin, I. J. 1979. Chronic Parkinsonism secondary to intravenous injection of meperidine analogues. Psychiatry Research, **1**, 249-254.

De Gabriele, G. and Penington, D. G. 1967. Physiology of the regulation of platelet production. British Journal of Haematology, **13**, 202-209.

De Maria, F. J. and See, H. Y. C. 1965. Role of the placenta in pre-eclampsia. American Journal of Obstetrics and Gynecology, **94(4)**, 471-476.

Denney, R. M., Fritz, R. R., Patel, N. T. and Abell, C. W. 1982. Human liver MAO-A and MAO-B separated by immunoaffinity chromatography with a MAO-B specific monoclonal antibody. Science, **215**, 1400-1403. (a).

Denney, R. M., Patel, N. T., Fritz, R. R. and Abell, C.W. 1982. A monoclonal antibody elicited to human platelet monoamine oxidase - isolation and specificity for human monoamine oxidase B but not A. Molecular Pharmacology, **22**, 500-508. (b).

Denney, R. M., Fritz, R. R., Patel, N. T., Widen, S. G. and Abell, C. W. 1983. Use of a monoclonal antibody for comparative studies of monoamine oxidase B in mitochondrial extracts of human brain and peripheral tissues. Molecular Pharmacology, **24**, 60-68.

Dennick, R. G. and Mayer, R. J. 1977. Purification and immunochemical characterisation of monoamine oxidase from rat and human liver. Biochemical Journal, **161**, 167-174.

Dexter, D. T., Carter, C. J., Wells, F. R., Javoy-Agid, F., Agid, Y., Lees, A., Jenner, P. and Marsden, C. D. 1989. Basal lipid peroxidation in substantia nigra is increased in Parkinson's Disease. Journal of Neurochemistry, **52(2)**, 381-389.

Donnelly, C. H. and Murphy, D. L. 1977. Substrate and inhibitor related characteristics of human platelet monoamine oxidase. Biochemical Pharmacology, **26**, 853-858.

Duvoisin, R. C., Eldridge, R., Williams, A., Nutt, J. and Calne, D. 1981. Twin study of Parkinson's Disease. Neurology, **31**, 77-79.

Egashira, T. 1976. Studies on monoamine oxidase. XVIII Enzymatic properties of placental monoamine oxidase. Japan Journal of Pharmacology, **26**, 493-500.

Filshie, G. M., Maynard, P., Hutter, C., Cooper, J. C., Robinson, G. and Rubin, P. 1992. Urinary 5-hydroxyindole acetate concentration in pregnancy induced hypertension. British Medical Journal, **304**, 1223.

Finch, C. C., Ho, S. L., Williams, A.C. and Billett, E. E. 1995. Platelet MAO activities and MAO-B protein concentrations in Parkinson's Disease and controls. Progress in Brain Research, **106**, 85-90.

Fischer, P., Götz, M. E., Ellinger, B., Streifler, M., Riederer, P and Danielczyk W. 1994. Platelet monoamine oxidase B activity and vitamin B12 an dementia. Biological Psychiatry, **35**, 772-774.

Fitzgerald, D. H., Tipton, K. F., Anderson, M. C. and Lawlor, B. 1996. Substrate specificity of human platelet monoamine oxidase B activity in Parkinson's Disease and Alzheimers disease. Biochemical Society Transactions, **24**, 63S

Fowler, C. J., Wiberg, A., Oreland, L. and Winblad, B. 1980. Titration of human brain type B monoamine oxidase. Neurochemical Research, **5(7)**, 697-708.

Fox, J. E. B., Boyles, J. K., Reynolds, C. C. and Philips, D. R. 1984. Actin filament content and organisation in unstimulated platelets. The Journal of Cell Biology, **98**, 1985-1991.

Friis, M. L., Paulson, O. B., Hertz, M. M. and Bolwig, T. G. 1981. Blood-brain barrier permeability of L-DOPA in man. European Journal of Clinical Investigation, **11**, 231-234.

Gänshirt, D., Garritsen, H. S. P. and Holzgreve, W. 1995. Fetal cells in maternal blood. Current Opinion in Obstetrics and Gynecology, **7(2)**, 103-108.

Glover, V., Lees, A. J., Ward, C., Stern, G. M. and Sandler, M. 1983. Platelet phenolsulphotransferase activity in Parkinson's Disease. Journal of Neural Transmission, **57**, 95-102.

Gordon, J. L. 1981. Platelets in perspective. From:- Platelets in Biology and Pathology-2, 1-17, Edited by Gordon. Elsevier/North Holland Biomedical Press.

Gorkin, V. Z. 1963. Partial separation of rat liver mitochondrial amine oxidases. Nature, **4901**, 77.

Grimsby, J., Chen, K., Wang, L., Lan, N. C. and Shih, J. C. 1991. Human monoamine oxidase A and B genes exhibit identical exon-intron organisation. Proceedings of the National Academy of Science. USA, **88**, 3637-3641.

Hancock, W. W and Atkins, R. C. 1986. Immunohistological studies with monoclonal antibodies. Methods in Enzymology, Part 1 Hybridoma technology and monoclonal antibodies, **121**, 828-848.

Hare, M. L. C. 1928. Tyramine oxidase I. A new enzyme system in liver. Biochemical Journal, **22**, 968-979.

Heikkila, R. E., Hess, A. and Duvoisin, R. C. 1984. Dopaminergic neurotoxicity of 1-methyl-4-phenyl- 1,2,5,6 tetrahydropyridine in mice. 1984. Science, **224**, 1451-1453. (a).

Heikkila, R. E., Manzino, L., Cabbat, F. S. and Duvoisin, R. C. 1984. Protection against the dopaminergic neurotoxicity of 1-methyl-4-phenyl 1,2,5,6 tetrahydropyridine by monoamine oxidase inhibitors. Nature, **311**, 467-469. (b).

Hirsch, E. C. 1993. Does oxidative stress participate in nerve cell death in Parkinson's Disease? European Neurology, **33(1)**, 52-59.

Hirsch, E. C., Brandel, J-P., Galle, P., Javoy-Agid, F. and Agid, Y. 1991. Iron and aluminium increase in substantia nigra of patients with Parkinson's Disease: An X-ray microanalysis. Journal of Neurochemistry, **56(2)**, 446-451.

Hirsch, E. C., Graybiel, A. M. and Agid, Y. A. 1988. Melanized dopaminergic neurons are differentially susceptible to degeneration in Parkinson's Disease. Nature, **334**, 345-348.

Ho, S. L., Kapadi, A. L., Ramsden, D. B. and Williams, A. C. 1994. Sequence of the 5' flanking region of the monoamine oxidase-B (MAO-B) gene : differences with an earlier report. Biogenic Amines, **10(6)**, 575-578.

Horn, E. H., Filshie, M., Kerslake, R. W., Jaspan, T., Worthington, B. S. and Rubin, P. C. 1990. Widespread cerebral ischaemia treated with nimodipine in a patient with eclampsia. British Medical Journal, **301**, 794.

Hornykiewicz O. and Kish, S. J. 1986. Biochemical Pathophysiology of Parkinson's Disease. Advances in Neurology, **45**, 19-34.

Hotamisligal, G. S. and Breakefield, X. O. 1991. Human monoamine oxidase A gene determines levels of enzyme activity. American Journal of Human Genetics, **49**, 383-392.

Hotamisligil, G. S., Girmen, A. S., Fink, J. S., Tivol, E., Shalish, C., Trofatter, J., Baenziger, J., Diamond, S., Markham, C., Sullivan, J., Growdon, J. and Breakefield, X. O. 1994. Hereditary variations in monoamine oxidase as a risk factor for Parkinson's Disease. Movement Disorders, **9(3)**, 305-310.

Houslay, M. D. and Tipton, K. F. 1974. The Nature of the electrophoretically separable forms of monamine oxidase. Biochemical Pharmacology, Suppl. 1, 98-100.

Humfrey, C. D. N., Steventon, G. B., Sturman, S. G., Waring, R. H., Griffiths, B. and Williams, A. C. 1990. Monoamine oxidase substrates in Parkinson's Disease. Biochemical Pharmacology, **40(11)**, 2562-2564.

Ishikawa, H., Sekizawa, A., Suzuki, A., Saito, H., Yanaihara, T., Arai, Y. and Yasuharah, H. 1998. Transference of monoamines in amniotic fluid across the fetal membrane. Biogenic Amines, **14(3)**, 203-216.

Jarman, J., Glover, V., Sandler, M., Turjanski, N. and Stern, G. 1993. Platelet monoamine oxidase B activity in Parkinson's Disease : a re-evaluation. Journal of Neural Transmission (P.D. Section), **5**, 1-4.

Javitch, J. A. and Snyder, S. H. 1985. Uptake of MPP^+ by dopamine neurones explains selectivity of Parkinsonism inducing neurotoxin, MPTP. European Journal of Pharmacology, **106**, 455-456.

Jellinger, K., Paulus, W., Grundke-Iqbal, I., Riederer, P. and Youdim, M. B. H. 1990. Brain iron and ferritin in Parkinson's and Alzheimers diseases. Journal of Neural Transmission (PD section), **2**, 327-340.

Johnston, J. P. 1968. Some observations upon a new inhibitor of monoamine oxidase in brain tissue. Biochemical Pharmacology, **17**, 1285-1297.

Johnson, W. G., Hodge, S. E. and Duvoisin, R. 1990. Twin studies and the genetics of Parkinson's Disease - a reappraisal. Movement Disorders, **5(3)**, 187-194.

Jossan, S. S., Gillberg, P. G., d'Argy, R., Aquilonius, S. M., Långström, B., Halldin, C. and Oreland, L. 1991. Quantitative localisation of human brain monoamine oxidase B by large section autoradiography using L-[^{3}H] deprenyl. Brain Research, **547**, 69-76.

Junqueira, L. C., Carneiro, J. and Kelly, R. O. Basic Histology, 8th Edition. Prentice Hall International (UK) Limited, London, 1995.

Kastner, A., Hirsch, E. C., Lejeune, O., Javoy-Agid, F., Rascol, O. and Agid, Y. 1992. Is the vulnerability of neurones in the substantia nigra of patients with Parkinson's Disease related to their neuromelanin content? Journal of Neurochemistry, **59(3)**, 1080-1089.

Kaufman, R. M., Airo, R., Pollack, S., Crosby, W. H. and Doberneck, R. 1965. Origin of pulmonary megakaryocytes. Blood, **25(5)**, 767-775.

Knoll, J. and Magyar, K. 1972. Some puzzling pharmacological effects of monoamine oxidase inhibitors. Advances in Biochemical Psychopharmacology, **5**, 393-408.

Kochersperger, L. M., Waguespack, A., Patterson, J. C., Hsieh, C. C. W., Weyler, W., Salach, J. I. and Denney, R. M. 1985. Immunological uniqueness of human monoamine oxidases A and B : New evidence from studies with monoclonal antibodies to human monoamine oxidase A. Journal of Neuroscience, **5(11)**, 2874-2881.

Kochersperger, L. M., Parker, E. L., Siciliano, M., Darlington, G. J. and Denney, R. M. 1986. Assignment of genes for human monoamine oxidases A and B to the X chromosome. Journal of Neuroscience Research, **16**, 601-616.

Kopin, I. P. 1964. Storage and metabolism of catecholamines : the role of monoamine oxidase. Pharmacology Review, **16**, 179-191.

Kuhn, W., Müller, T., Gerstner, A., Winkel, R. and Goetz, M. E. 1998. Platelet monoamine oxidase B activity in 'de novo' and L-Dopa treated Parkinsonian patients and controls. The Canadian Journal of Neurological Sciences, **25**, 249-251.

Kurth, J. H., Kurth, M. C., Poduslo, S. E. and Schwankhaus, J. D. 1993. Association of a monoamine oxidase B allele with Parkinson's Disease. Annals of Neurology, **33(4)**, 368-372.

Lan, N. C., Chen, C. and Shih, J. C. 1989. Expression of functional human monoamine oxidase A and B cDNA's in mammalian cells. Journal of Neurochemistry, **52(5)**, 1652-1654.

Langston, J. W., Ballard, P., Tetrud, J. W. and Irwin, I. 1983. Chronic Parkinsonism in humans due to a product of meperidine - analog synthesis. Science, **219**, 979-980.

Langston, J. W., Irwin, I., Langston, E. B. and Forno, L. S. 1984. Pargyline prevents MPTP-induced Parkinsonism in primates. Science, **225**, 1480-1482.

Lee, D. H., Mendoza, M., Dvoroznik, M. T., Chung, E., Vanwoert, M. H. and Yahr, M. D. 1989. Platelet monoamine oxidase in Parkinson patients: effect of L-deprenyl therapy. Journal of Neural Transmission (Parkinson's Disease section), **1**, 189-194.

Leveugle, B., Faucheux, B. A., Bouras, C., Nillesse, N., Spik, G., Hirsch, E. C., Agid, Y. and Hof, P. R. 1996. Cellular distribution of the iron-binding protein lactotransferrin in the mesencephalon of Parkinson's Disease cases. Acta Neuropathologica, **91**, 56-572.

Lewinsohn, R., Glover, V. and Sandler, M. 1980. Development of benzylamine oxidase and monoamine oxidase A and B in man. Biochemical Pharmacology, **29**, 1221-1230.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. 1951. Protein measurement with the Folin Phenol Reagent. Journal of Biological Chemistry, **193**, 265-275.

Mann, V. M., Cooper, J. M., Krige, D., Daniel, S. E., Schapira, A. H. V. and Marsden, C. D. 1992. Brain, skeletal muscle and platelet homogenate mitochondrial function in Parkinson's Disease. Brain, **115**, 333-342.

Maraganore, D. M., Harding, A. E. and Marsden, C. D. 1991. A clinical and genetic study of familial Parkinson's Disease. Movement Disorders, **6(3)**, 205-211.

Maret, G., Testa, B., Jenner, P., El-Tayer, N. and Carrupt, P. A. 1990. The MPTP story : MAO activates tetrahydropyridine derivatives to toxins causing Parkinsonism. Drug Metabolism Reviews, **22(4)**, 291-332.

Markey, S. P., Johannessen, J. N., Chiueh, C. C., Burns, R. S. and Herkenham, M. A. 1984. Intraneuronal generation of a pyridinium metabolite may cause drug-induced Parkinsonism. Nature, **311**, 464-467.

Marsden, C. D. 1983. Neuromelanin and Parkinson's Disease. Journal of Neural Transmission (suppl.), **19**, 121-141.

Martin, J. F. and Levine, R. F. 1991. Evidence in favour of the lungs and against the bone marrow as the site of platelet production. In:- The Platelet in health and disease, 1-9. Edited by Page C.P. Blackwell Scientific Publications Ltd, Oxford, London.

Mazzetti, P., Bonnet, A. M., Vidailhet, M., Broucker, M., Haguenau, M., Destee, A., Brice, A. and Agid, Y. 1993. Clinical and genetic study of familial Parkinson's Disease. Neurology, **43**, A238.

McCauley, R. and Racker, E. 1973. Separation of two monoamine oxidases from bovine brain. Molecular Cell Biochemistry, **1**, 73-81.

Monk, N. J. 1992. Immunological assays on the enzyme monoamine oxidase. Honours project, The Nottingham Trent University.

Mueller, U. W., Hawes, C. S. and Jones, W. R. 1987. Identification of extra-villous trophoblast cells in human decidua using an apparently unique murine monoclonal antibody to trophoblast. Histochemistry Journal, **19**, 288-296.

Obata, T., Egashira, T. and Yamanaka, Y. 1990. Isoelectric focusing of isoenzymes of monkey platelet monoamine oxisdase. Biochemical Pharmacology, **40(8)**, 1689-1693.

Oreland, L., Fowler, C. J. and Schalling, D. 1981. Low platelet monoamine oxidase activity in cigarette smokers. Life Sciences, **29(24)**, 2511-2518.

Ozelius, L., Hsu, Y-P., Bruns, G., Powell, J. F., Chen, S., Weyler, W., Utterback, M., Zucker, D., Haines, J., Trofatter, J. A., Conneally, P. M., Gusella, J. F. and Breakefield, X. O. 1988. Human monoamine oxidase gene (MAO-A): Chromosome position (xp21-p11) and DNA polymorphism. Genomics, **3**, 53-58.

Parkinson, J. 1817. An essay on the shaking palsy.

Payami, H., Bernard, S., Larsen, K., Kaye, J. and Nutt, J. 1995. Genetic anticipation in Parkinson's Disease. Neurology, **45**, 135-138.

Penington, D. G. 1981. Formation of Platelets. From:- Platelets in Biology and Pathology 2, Chapter 2, 19-41. Edited by Gorden. Elsevier/North-Holland Biomedical Press.

Pintar, J. E., Levitt, P., Salach, J. I., Weyler, W., Rosenberg, M. B. and Breakefield, X. O. 1983. Specificity of antisera prepared against pure bovine MAO-B. Brain Research, **276**, 127-139.

Pletcher, A. 1978. Platelets as models for monaminergic neurons. From:- Essays in Neurochemistry and Neuropharmacology, Chapter 3, 49-102.

Poirier, M-F., Lôo, H., Dennis, T., LeFur, G. and Scatton, B. 1985. Platelet monoamine oxidase activity and plasma 3,4-Dihydroxyphenylethylene glycol levels during the menstrual cycle. Neuropsychobiology, **14**, 165-169.

Polymeropoulos, M. H., Higgins, J. J., Golbe, L. I., Johnson, W. G., Ide, S. E., Di Iorio, G., Sanges, G., Stenroos, E. S., Pho, L. T., Schaffer, A. A., Lazzarini, A. M., Nussbaum, R. L. and Duvoisin, R. C. 1996. Mapping of a gene for Parkinson's Disease to chromosome 4q21-q23. Science, **274**, 1197-1199.

Polymeropuolos, M. H., Lavedan, C., Leroy, E., Ide, S. E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E. S., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W. G., Lazzarini, A. M., Duvoisin, R. C., Di Iorio, G., Golbe, L. I. and Nussbaum, R. L. 1997. Mutation in the α- synuclein gene identified in families with Parkinson's Disease. Science, **276**, 2045-2047.

Powell, J. F. and Craig, I. W. 1977. Biochemical and immunological studies of the monoamine oxidising activities of cultured human cells. Biochemical Society Transactions, **5**, 180-182.

Powell, J. F., Hsu, Y-P., Weyler, W., Chen, S., Salach, J., Andrikopoulos, K., Mallet, J. and Breakefield, X. O. 1989. The primary structure of bovine monoamine oxidase type A. Biochemical Journal, **259**, 407-413.

Rajput, A. H., Uitti, R. J., Stern, W. and Laverty, W. 1986. Early onset Parkinson's Disease and childhood environment. Advances in Neurology, **45**, 295-297.

Rapkin, A. J., Buckman, T. D., Sutphin, M. S., Chang, L. C. and Reading, A. E. 1988. Platelet monoamine oxidase B activity in women with premenstrual syndrome. American Journal of Obstetrics and Gynecology, **159(6)**, 1536-1540.

Richards, J. G., Saura, J., Ulrich, J. and Da Prada, M. 1992. Molecular neuroanatomy of monoamine oxidases in human brainstem. Psychopharmacology, **106**, S21-23.

Riley, L. A., Waguespack, M. A. and Denney, R. M. 1989. Characterisation and quantitation of monoamine oxidases A and B in mitochondria from human placenta. Molecular Pharmacology, **36**, 54-60.

Robinson, D. S., Davis, J. M. and Nies, A. 1971. Relation of sex and aging to monoamine oxidase activity of human brain, plasma and platelets. Archives of General Psychiatry, **24**, 536-539.

Rodriguez, M. J., Saura, J., Billett, E. E., Finch, C. C. and Mahy, N. Analysis of cellular localisation of monoamine oxidase A and B protein in human peripheral (Non CNS) tissues. (in preparation).

Rodriguez, M. J., Saura, J., Billett, E. E., Finch, C. C. and Mahy, N. Monoamine oxidase A and Monoamine oxidase B localisation in human lung and spleen [a] (in press, Neurobiology)

Rodriguez, M. J., Saura, J., Finch, C. C., Mahy, N. and Billett, E. E. Localisation of monoamine oxidase A and B in human pancreas, thyroid and adrenal gland.[b] (Accepted with modifications, The Journal of Histochemistry and Cytochemistry)

Rose, R. M., Castellani, S., Boeringa, J. A., Malek-Ahmadi, P., Lankford, D. A., Bessman, J. P., Fritz, R. R., Denney, C. B., Denney, R. M. and Abell, C. W. 1986. Platelet MAO concentration and molecular activity: II Comparison of normal and schizophrenic populations. Psychiatry Research, **17**, 141-151.

Russell, S. M., Davey, J. and Mayer, R. J. 1979. Immunochemical characterisation of monoamine oxidase from human liver, placenta, platelets and brain cortex. Biochemical Journal, 181, 15-20.

Russell, S. M. and Mayer, R. J. 1983. Degradation of transplanted rat liver mitochondrial outer membrane proteins in hepatoma cells. Biochemical Journal, **216**, 163-175.

Ryder, T. A., Mackenzie, M. L., Pryse-Davies, J., Glover, V., Lewinsohn, R. and Sandler, M. 1979. A coupled peroxidatic oxidation technique for the histochemical localisation of monoamine oxidase A and B and benzylamine oxidase. Histochemistry, **62**, 93-100.

Saarikoski, S. 1983. Metabolic inactivation of noradrenaline in human placenta, umbilical cord and fetal membranes. British Journal of Obstetrics and Gynaecology, **90(6)**, 525-527.

Sandler, M. and Coveney, J. 1962. Placental monoamine oxidase activity in toxaemia of pregnancy. The Lancet, May 26, 1096-1098.

Saura, J., Nadal, E., Van den Berg, B., Vila, M., Bombi, J. A. and Mahy, N. 1996. Localisation of monoamine oxidases in human peripheral tissues. Life Sciences, **59(16)**, 1341-1349.

Schapira, A. H. V., Cooper, J. M., Dexter, D., Clark, J. B., Jenner, P. and Marsden, C. D. 1990. Mitochondrial complex I deficiency in Parkinson's Disease. Journal of Neurochemistry, **54(3)**, 823-827.

Schiff, E., Peleg, E., Goldenberg, M., Rosenthal, T., Ruppin, E., Tamarkin, M., Barkai, G., Ben-Baruch, G., Yahal, I., Blankstein, J., Goldman, B. and Mashiach, S. 1989. The use of aspirin to prevent pregnancy-induced hypertension and lower the ratio of thromboxane A_2 to prostacyclin in relatively high risk pregnancies. The New England Journal of Medicine, **321(6)**, 351-356.

Schnaitman, C., Erwin, V. G. and Greenawalt, J. W. 1967. The submitochondrial localisation of monoamine oxidase. Journal of Cell Biology, **32**, 719-735.

Schneider, J. S., Yuwiler, A. and Markham, C. H. 1986. Production of a Parkinson-like syndrome in the cat with N- methyl-4-phenyl- 1,2,3,6 tetrahydropyridine (MPTP) : behaviour, histology and biochemistry. Experimental Neurology, **91**, 293-307.

Sekizawa, A., Ishikawa, H., Morimoto, T., Hirose, K., Suzuki, A., Saio, H., Yanaihara, T., Arai, Y. and Yasuhara, H. 1995. The possible role of monoamine oxidase in fetal membranes. Biogenic Amines, **11(6)**, 443-451.

Shattil, S. J. and Cooper, R. A. 1978. Role of membrane lipid composition organisation and fluidity in human platelet function. Progress in Hemostasis and Thrombosis **4**, 59-86.

Shih, J. C., Zhu, Q-S., Grimsby, J. and Chen, K. 1994. Identification of human monoamine oxidase (MAO) A and B gene promoters. Journal of Neural Transmission (suppl.) **41**, 27-33.

Siever, L. J. and Coursey, R. D. 1985. Biological markers for schizophrenia and the biological high-risk approach. Journal of Nervous and Mental Disease, **173(1)**, 4-16.

Singer, T. P., Castagnoli, N. (jnr.), Ramsay, R. R. and Trevor, A. J. 1987. Biochemical events in the development of Parkinsonism induced by 1-methyl-4-phenyl- 1,2,3,6 tetrahydropyridine. Journal of Neurochemistry, **49(1)**, 1-8.

Smith, D. F. 1994. Type A personalities tend to have low platelet monoamine activity. Acta Psychiatrica Scandinavica, **89**, 88-91.

Smith, D., Filipowicz, C. and McCauley, R. 1985. Monoamine oxidase A and Monoamine oxidase B activities are catalysed by different proteins. Biochimica et Biophysica Acta, **831**, 1-7.

Spillantini, M. G., Schmidt, M. L., Lee, V. M-Y, Trojanowski, J. Q., Jakes, R. and Goedert, M. 1997. α-synuclein in Lewy bodies. Nature, **388**, 839-840.

Stahl, S. M. 1977. The human platelet. Archives of General Psychiatry, **34**, 509-516.

Stevens, A. and Lowe, J. Human Histology. Mosby (Time Mirror International Publishers Limited) London, 1997.

Tanner, C. M. 1989. The role of environmental toxins in the etiology of Parkinson's Disease. Trends in Neurosciences, **12(2)**, 49-54.

Thorpe, L. W., Westlund, K. N., Kochersperger, L. M., Abell, C. W. and Denney, R. M. 1987. Immunological localisation of monoamine oxidases A and B in human peripheral tissues and brain. The Journal of Histochemistry and Cytochemistry, **35(1)**, 23-32.

Tinggaard-Pedersen, N. 1978. Occurance of megakaryocytes in various vessels and their retention in the pulmonary capillaries in man. Scandinavian Journal of Haemaology, **21**, 369-375.

Tipton, K. F., Fowler, C. J. and Houslay, M. D. 1982. Specificities of the two forms of monoamine oxidase. In Monoamine oxidase, basic and clinical frontiers. Edited by : Kamijo, K., Usdin, E. and Nagatsu, T., Excerpta Medica, Amsterdam, 87-99.

Towbin, H., Staehelin, T. and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proceedings of the National Acadamy of Science (USA), **76(9)**, 4350-4354.

Tsugeno, Y. and Ito, A. 1997. A key amino acid responsible for substrate selectivity of monoamine oxidase A and B. The Journal of Biological Chemistry, **272(22)**, 14033-14036.

Veral, A., Alper, G., Mentes, G. and Ersöz, B. 1997. Age and sex related alterations in serum and platelet monoamine oxidase. European Journal of Clinical Chemistry and Clinical Biochemistry, **35(4)**, 265-268.

Wang, Y-S., Shi, Y-M., Wu, Z-Y., He, Y-X. and Zhang, B-Z. 1991. Parkinson's Disease in China. Chinese Medical Journal, **104(11)**, 960-964.

Westlund, K. N., Denney, R. M., Rose, R. M. and Abell, C. W. 1988. Localisation of distinct monoamine oxidase A and monoamine oxidase B cell populations in human brainstem. Neuroscience, **25(2)**, 439-456.

Weyler, W. 1989. Monoamine oxidase A from human placenta and monoamine oxidase B from bovine liver both have one FAD per subunit. Biochemical Journal, **260**, 725-729.

Weyler, W., Hsu, Y-P. P. and Breakefield, X. O. 1990. Biochemistry and genetics of monoamine oxidase. Pharmacology and Therapeutics, **47**, 391-417.

Wingerchuk, D. M., Rajput, A. H. and Meleth, R. 1993. Shared environmental exposure and Parkinson's Disease. Neurology, **43**, A238.

Wright, J. H. 1910. The histogenesis of the blood platelets. Journal of Morphology, **21**, 263-278.

Yeomanson, K. B. 1990. An immunochemical analysis of monoamine oxidase profiles. PhD Thesis. The Nottingham Trent University.

Yeomanson, K. B. and Billett, E. E. 1992. An enzyme immunoassay for the measurement of human monoamine oxidase B protein. Biochimica et Biophysica Acta, **1116**, 261-268.

Yong, V. W. and Perry, T. L. 1986. Monoamine oxidase B, smoking and Parkinson's Disease. Journal of the Neurological Sciences, **72**, 265-272.

Yoshimoto, Y., Sakamoto, T., Aono, T., Kimura, H., Maeda, T. and Tanizawa, O. 1986. Histochemical studies on human placental and chorionic monoamine oxidase. Obstetrics and Gynecology, **67(3)**, 344-348.

Youdim, M. B. H., Ben-Shachar, D. and Riederer, P. 1990. The role of monoamine oxidase, iron-melanin interaction and intracellular calcium in Parkinson's Disease. Journal of Neural Transmission (suppl.) **32**, 239-248.

Youdim, M. B. H. and Finberg, J. P. M. 1986. MAO type B inhibitors as adjunct to L-DOPA therapy. Advances in Neurology, **45**, 127-136.

Yu, P. H. 1981. Some effects of non-ionic detergent Triton X-100 on rat liver monoamine oxidase. Biochemical Pharmacology, **30(11)**, 1283-1287.

Yu, P. H. and Boulton, A. A. 1987. Irreversible inhibition of monoamine oxidase by some components of cigarette smoke. Life Sciences, **41**, 675-682.

Zeller, E. A., Boshes, B., Arbit, J., Bieber, M., Blonsky, E. R., Dolkart, M. and Huprikar, S. V. 1976. Molecular biology of neurological and psychiatric disorders. I Effect of Parkinsonism, age, sex and L-DOPA on platelet monoamine oxidase. Journal of Neural Transmission, **39**, 63-77.

Zeller, E. A. and Davis, J. M. 1980. Protein content and monoamine oxidase activity in platelets. Schizophrenia Bulletin, **6(2)**, 267-274.

Zhu, Q., Grimsby, J., Chen, K. and Shih, J. C. 1992. Promoter organisation and activity of human monoamine oxidase A and B genes. The Journal of Neuroscience, **12(11)**, 4437-4446.

APPENDICES

APPENDIX I

CHAPTER 2

Solutions and Recipes

2.2.1 PURIFICATION OF MITOCHONDRIAL MEMBRANES

Buffered sucrose solution

250 mM Sucrose

3 mM Imidazole

Solution 1

1.8 M Sucrose

4m M ATP

4 mM $MgSO_4$

Phosphate Buffer pH 7.6

100 mM Phosphate	g/100 ml
Na_2HPO_4	1.242
KH_2PO_4	0.170

2.2.2 ESTIMATION OF MAO ACTIVITY

20 mM Potassium Phosphate buffer, pH 7.2

	g/L
K_2HPO_4	3.857
KH_2PO_4	0.422

Scintillation Fluid

1.0 % (w/v) 2,5, Diphenyloxazole in Toluene : Ethyl acetate (mixed in a ratio 1:1)

2.2.5 AMMONIUM SULPHATE PRECIPITATION OF IMMUNOGLOBULIN FROM MURINE ASCITIC FLUID

20 mM Phosphate Buffer, pH 8.0

10.6 ml of 0.2 M KH_2PO_4

189.9 ml of 0.2 M Na_2HPO_4

pH to 8.0 and make up to 2 litres with distilled water

2.2.6 CELL CULTURE

2.2.6.2 MAINTAINANCE OF CELLS IN CULTURE

Hybridoma medium

RPMI-1640 containing L- glutamine (10 ml 200 mM L-glutamine added to 500 ml RPMI-1640)

2 % (v/v) Penicillin and streptomycin (Sigma, P0781 : 10,000 units Penicillin and 10 mg streptomycin in 0.9 % NaCl).

15 % (v/v) Foetal calf serum

2.2.6.3 CRYOPRESERVATION OF CELLS

Freezing mixture

95 % (v/v) Sterile Foetal calf serum

5 % (v/v) Sterile DMSO

2.2.6.4 PREPARATION OF FEEDER CELL LAYER

Feeder Medium

RPMI-1640

2 % (v/v) Penicillin and streptomycin

10 % Newborn calf serum

2.2.7. SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

Tris-glycine electrode buffer, pH 8.3

	g/l
Tris-base	3.1
Glycine	14.4
Sodium dodecyl sulphate	1.0

Acrylamide stock [30 % (w/v)] (filtered)

29.2 % (w/v)Acrylamide

0.8 % (w/v) Bis-acrylamide

Sample buffer 2x concentrated

0.5 M Tris-HCl pH 6.8

20 % (v/v) Glycerol

3 % (w/v) SDS

50 mM Dithiothreotol

0.01 % (w/v) Bromophenol blue

Resolving gel (10%)

4.4 ml deionised water

2.5 ml 1.5M Tris-HCl, pH 8.8

100 µl 10 % (w/v) SDS

3.3 ml Acrylamide stock

40 µl 10 % (w/v) Ammonium persulphate

10 µl TEMED (N, N, N, N'-Tetramethylethylenediamine)

Resolving gel (15%)

2.4 ml deionised water

2.5 ml 1.5 M Tris-HCl, pH 8.8

100 µl 10 % (w/v) SDS

5.0 ml Acrylamide stock

40 µl 10 % (w/v) Ammonium persulphate

10 µl TEMED (N, N, N, N'-Tetramethylethylenediamine)

Stacking Gel

6.1 ml deionised water

2.5 ml 0.5 M Tris-HCl, pH 6.8

100 µl 10 % (w/v) SDS

1.3 ml stock acrylamide

50 µl 10 % (w/v) Ammonium persulphate

10 µl TEMED

Fixing Solution

25 % (v/v) Ethanol

10 % (v/v) Glacial acetic acid

in deionised water

2.2.8 SILVER STAIN

Fixative solution

25 % (v/v) Ethanol

10 % (v/v) Acetic acid

Stop solution

5 % (v/v) Acetic acid

2.2.9 ELECTROBLOTTING

Electroblotting Buffer

39 mM Glycine

48 mM Tris

0.0375 % (w/v) SDS

20 % (w/v) Methanol

TBS-Tween

50 mM Tris

200 mM NaCl

pH to 7.4 with HCl

Add 0.1 % (v/v) Tween-20

2.2.10 IMMUNOPROBING OF ELECTROBLOTS

Tris buffered saline (TBS)

50 mM Tris

200 mM NaCl

pH to 7.4 with HCl

Blocking Solution

3 % (w/v) Marvel skimmed milk powder dissolved in TBS

TBS-Tween

TBS with 0.1 % (v/v) Tween-20

Substrate Mix

20 ml 0.75 M Tris, pH 9.5

33 µl BCIP

44 µl NBT

2.2.11 IMMUNOHISTOCHEMISTRY

2.2.11.3 TISSUE PREPARATION - SUCROSE PROTECTION METHOD

2 % (w/v) Paraformaldehyde solution

2 % (w/v) Paraformaldehyde (PFA) made up in PBS. For every 2 g of PFA add 3 µl 10 M NaOH. Warm to 60 °C to dissolve. Cool on ice.

15 % (w/v) Sucrose solution

15 % (w/v) sucrose in PBS

2.2.11.4 IMMUNOHISTOCHEMISTRY FOR CRYOSTAT SECTIONS

Paraformaldehyde solution

4 % (w/v) Paraformaldehyde (PFA) made up in PBS. For every 4 g of PFA add 6 µl 10 M NaOH. Warm to 60 °C to dissolve. Cool on ice.

Blocking agent

20 % (v/v) Normal Swine serum in PBS

Primary and secondary antibody diluent

5 % (v/v) Normal swine serum in PBS

Copper sulphate solution

0.5 % (w/v) $CuSO_4$. $5H_2O$

0.9 % (w/v) NaCl

2.2.11.5 HAEMATOXYLIN COUNTERSTAIN

Scotts Solution

0.2 % (w/v) $KHCO_3$

2 % (w/v) $MgSO_4$

Acid Alcohol

750 ml Absolute ethanol

9.75 ml 1.0 M HCl

225 ml Distilled water

2.2.11.6 HAEMATOXYLIN AND EOSIN STAIN

Eosin solution

10 g Eosin

200 µl glacial acetic acid

into 1000 ml distilled water

Scotts Solution

0.2 % (w/v) $KHCO_3$

2 % (w/v) $MgSO_4$

Acid Alcohol

750 ml Absolute ethanol

9.75 ml 1.0 M HCl

225 ml Distilled water

2.2.13 HETEROLOGOUS, NON-COMPETITIVE ELISA

Phosphate Buffered Saline (PBS)	g/L
NaCl	8.0
KCl	0.2
Na_2HPO_4 (anhydrous)	1.15
KH_2PO_4	0.2

Substrate solution (made up just prior to use)

20 ml 0.1 M Sodium acetate buffer

150 µl TMB solution

8 µl 30 % (v/v) Hydrogen peroxide

Blocking agent

3 % (w/v) Marvel skimmed milk powder diluted in PBS.

PBS-Tween

PBS containing 0.05 % (v/v) Tween-20.

2.2.14 ESTIMATION OF PROTEIN

Lowry reagent stock solutions

Solution A : 2 % (w/v) Na_2CO_3 in 0.1 M NaOH made up just prior to use

Solution B : 0.5 % (w/v) Cu_2SO_4

Solution C : 1.0 % (w/v) NaK- tartrate

Working Lowry Reagent

100 ml Solution A

2 ml Solution B

2 ml Solution C

PURIFICATION OF MAO FROM SOLUBILISED EXTRACTS OF MITOCHONDRIAL MEMBRANES

MATERIALS

Running buffer (20 mM Potassium Phosphate Buffer, pH 7.2)

	g/2 L
K_2HPO_4 (20 mM)	6.664
KH_2PO_4 (20 mM)	0.147

Triton X-100

2-mercaptoethanol

Sepharose 6B-100 (Sigma code 6B-100)

Blue Dextran 2000

Potassium dichromate

Biobeads SM-2 (20-50 mesh; Bio-rad No. 152-3920)

DEAE cellulose (Sigma D6418, Lot 32H0698)

Column for gel filtration (Pharmacia XK16, 2 cm internal diameter x 90 cm long)

Column for ion-exchange (2.8 cm internal diameter x 6 cm long)

METHODS

All procedures were performed at 4 °C unless otherwise stated.

Gel Filtration Chromatography

Solubilised extracts were first fractionated on Sepharose 6B-100

1. Sepharose 6B was supplied pre-swollen in ethanol as a thick slurry. The required amount of gel slurry (300 ml) was removed from the stock and allowed to settle, excess ethanol was decanted and the gel slurry was mixed with an equal volume of running buffer containing 0.1 mM 2-mercaptoethanol, allowed to settle and again equilibrated with an equal volume of running buffer, the fine particles were removed and the gel slurry mixed again with running buffer. When the gel had been equilibrated it was diluted in running buffer containing 0.1 mM 2-mercaptoethanol and 0.1 % (v/v) Triton X-100.

2. The gel slurry was degassed on ice and the gel allowed to settle in the column under gravity overnight. The column was then packed in running buffer with a flow rate of 0.8 ml/minute and equilibrated with 2 volumes of running buffer at constant pressure under ascending flow with a flow rate of 0.3 ml/minute.

3. The V_O and V_T of the column were determined by fractionating 2 ml each of blue dextran 2000 and potassium dichromate as a mixture (0.1 % ,w/v diluted in running buffer).

4. A Triton X-100 solubilised extract of mitochondrial membranes (10 ml) was loaded onto the column and 4 ml fractions were collected, flow rate 0.3 ml/minute.

5. Alternate fractions falling within the range V_O -V_T were assayed for MAO activity with ^{14}C labelled tyramine.

6. Fractions demonstrating an activity level of greater than twice that of the background were pooled.

7. Triton X-100 was removed by adsorption to Biobeads.

Activation of Biobeads SM-2

1. 50 g of Biobeads were swollen in 450 ml methanol for 15 minutes at room temperature, collected on a glass sinter filter and washed with 750 ml methanol followed by 3 litres de-ionised water before storage under sterile de-ionised water at 4 °C.

2. Prior to use, the Biobeads were equilibrated with 20 mM potassium phosphate buffer, pH 7.2 for 60 minutes at 4 °C.

Removal of Triton X-100 using Biobeads SM-2

1. Pooled fractions from the gel filtration fractionation were stirred at 4 °C and 5 g (wet weight) Biobeads were added every 30 minutes.

2. Triton X-100 removal was monitored at 30 minutes intervals in a 60 µl aliquot of the sample, diluted 1:10 with potassium phosphate buffer, pH 7.2 and its absorbance measured at 260 nm and 280 nm.

3. Once the 280 nm : 260 nm ratio had stabilised, it was assumed that the detergent had been removed, as Triton X-100 has an absorbance maximum at 260 nm.

4. Following the removal of Triton X-100, the sample was filtered through a double thickness of Whatman Number 1 filter paper and the Biobeads washed and reactivated for future use.

Ammonium sulphate precipitation

1. Following removal of the detergent, solid ammonium sulphate was added in aliquots to the sample over a 30 minute time period whilst stirring slowly at 4 °C, to give a final saturation of 60 % (3.61 g/ 10 ml).

2. After the final addition, the sample was left stirring for a further 15 minutes before centrifugation at 200,000 x g for 60 minutes.

3. The resultant floating pellet was collected by filtering under vacuum through a glass microfibre filter.

4. The sample was resuspended in 10 ml 20 mM potassium phosphate buffer (pH 7.2) containing 0.1 mM 2-mercaptoethanol and dialysed overnight against 3 x 2 litres of the same buffer.

Ion-exchange chromatography

Ion-exchange chromatography was performed using the weak anion exchanger, DEAE-cellulose.

1. 2 g of DEAE-cellulose was pre-swollen overnight at 4 °C in 20 mM potassium phosphate buffer (pH 7.2), yielding a gel volume of approximately 15 ml.

2. The dialysed sample was added and the gel slurry/sample mix left stirring slowly for 60 minutes.

3. The gel slurry/sample mix was packed into a column and allowed to settle under gravity.

4. The column was then washed with 2 column volumes of 20 mM potassium phosphate buffer (pH 7.2) and allowed to equilibrate in the same buffer.

5. The sample was then eluted with a 200 ml linear 0 % - 1.5 % (w/v) Triton X-100 gradient in 20 mM potassium phosphate buffer (pH 7.2) containing 0.2 M potassium chloride.

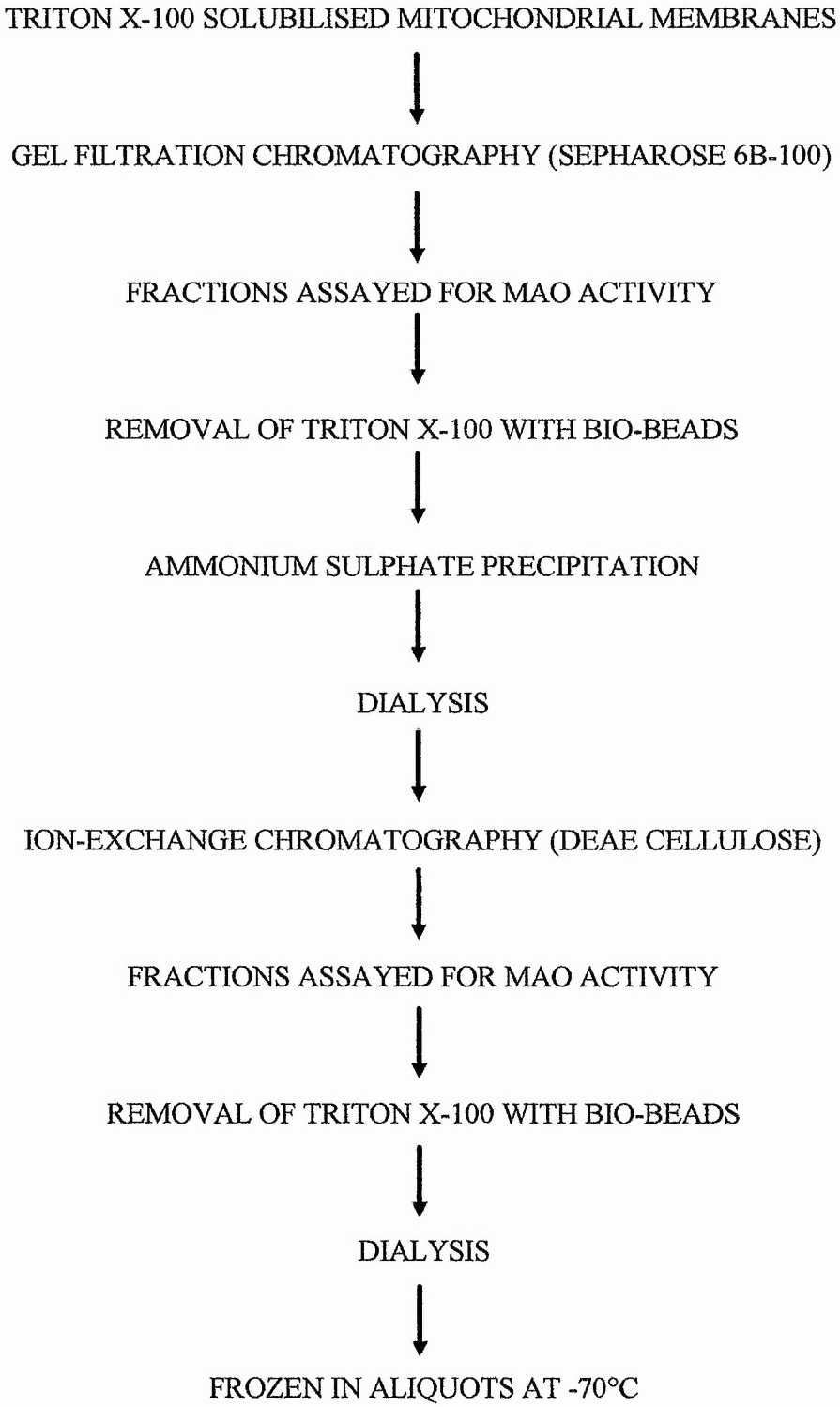
6. 3 ml fractions were collected with a flow rate of 1ml/minute.

7. Alternate fractions were assayed for MAO activity and those fractions demonstrating a level of MAO activity greater than twice that of the background were pooled.

8. Triton X-100 was removed by adsorption to Biobeads SM-2 and protein was precipitated using solid ammonium sulphate as described earlier.

9. The floating pellet was resuspended into approximately 3 ml 20 mM potassium phosphate (pH 7.2) and dialysed as described earlier. The dialysed sample was then

sonicated to aid resuspension, protein content estimated and stored in aliquots at -70 °C until required.



Flow diagram describing the purification of monoamine oxidase from solubilised human liver mitochondrial membranes.

APPENDIX II

CHAPTER 3

Solutions and recipes

3.2.2 PLATELET EXTRACTION PROCEDURE

Washing Buffer

5 mM EDTA

0.154 M NaCl, pH to 7.2

APPENDIX III

CHAPTER 4

Solutions and recipes

4.2.1 PLACENTAL EXTRACTION PROCEDURE

Buffered sucrose solution

250 mM Sucrose

3 mM Imidazole

Extraction buffer

Buffered sucrose solution containing 0.5 % (v/v) Triton X-100 and 1.0 mM PMSF

APPENDIX IV

Data for Parkinson's Disease analysis

MALE CAUCASIAN

	Age years	Concentration of MAO-B (μg/mg platelet protein)	PEA Deamination nmol hr^{-1} mg^{-1} **total** protein	PEA Deamination nmol hr^{-1} mg^{-1} **MAO-B** protein	DOPAMINE Deamination nmol hr^{-1} mg^{-1} **total** protein	DOPAMINE Deamination nmol hr^{-1} mg^{-1} **MAO-B** protein
PD n=12	48	2.13	1.88	217	0.46	887
	62	3.45	1.61	467	0.76	220
	67	0.74	2.32	3135	0.54	730
	48	0.79	2.20	2785	1.26	1595
	70	4.97	2.76	555	1.34	270
	80	1.23	1.66	1350	0.62	504
	70	4.86	1.90	391	0.94	193
	44	3.10	4.00	1290	1.06	342
	59	6.50	1.74	268	0.53	82
	53	5.80	1.52	262	0.67	116
	74	3.38	2.12	627	0.84	249
	69	2.38	9.70	4076	2.38	521
C n=7	63	3.44	3.08	895	0.96	279
	51	2.22	4.20	1892	1.30	586
	52	2.36	4.94	2093	1.47	623
	80	2.33	4.10	1760	1.29	534
	55	2.17	2.74	1263	0.63	290
	39	2.15	1.08	502	0.71	330
	49	1.61	1.02	634	0.33	205

FEMALE CAUCASIAN

	Age years	Concentration of MAO-B (μg/mg platelet protein)	PEA Deamination nmol hr^{-1} mg^{-1} **total** protein	PEA Deamination nmol hr^{-1} mg^{-1} **MAO-B** protein	DOPAMINE Deamination nmol hr^{-1} mg^{-1} **total** protein	DOPAMINE Deamination nmol hr^{-1} mg^{-1} **MAO-B** protein
PD n=7	48	1.40	4.00	2857	1.46	1040
	57	0.35	2.64	7458	0.48	1356
	57	0.99	3.78	3818	0.81	820
	70	8.62	2.50	290	1.46	169
	53	4.25	3.32	781	1.58	371
	73	4.94	5.80	1174	0.82	165
C n=6	55	2.17	2.74	1263	0.90	417
	65	1.47	3.16	2450	1.14	776
	44	6.63	3.36	507	1.34	203
	55	5.60	2.04	364	1.10	196
	62	7.85	3.28	418	1.66	211

MALE HONG KONG CHINESE

	Age years	Concentration of MAO-B (μg/mg platelet protein)	PEA Deamination nmol hr^{-1} mg^{-1} **total** protein	PEA Deamination nmol hr^{-1} mg^{-1} **MAO-B** protein	DOPAMINE Deamination nmol hr^{-1} mg^{-1} **total** protein	DOPAMINE Deamination nmol hr^{-1} mg^{-1} **MAO-B** protein
PD n=8	63	15.48	3.5	226	2.96	191
	66	32.99	3.84	116	2.68	81
	61	18.12	2.54	140	1.96	10
	82	9.98	2.70	270	2.96	293
	63	21.60	2.68	124	3.32	154
	62	32.84	2.74	83	8.64	263
	72	20.82	1.72	83	2.04	98
	61	24.05	3.14	131	2.52	105
C n=10	65	1.90	0.07	37	1.00	518
	63	31.66	1.86	59	1.64	52
	65	5.37	3.36	626	3.36	626
	67	21.46	3.04	14	2.12	99
	60	32.46	2.66	82	7.72	236
	66	26.99	3.46	128	2.84	105
	63	35.2	2.98	85	4.28	122
	66	27.69	5.00	181	3.48	126
	66	23.10	5.98	259	4.84	210
	65	48.22	1.26	26	1.80	37

FEMALE HONG KONG CHINESE

	Age years	Concentration of MAO-B (μg/mg platelet protein)	PEA Deamination nmol hr^{-1} mg^{-1} **total** protein	PEA Deamination nmol hr^{-1} mg^{-1} **MAO-B** protein	DOPAMINE Deamination nmol hr^{-1} mg^{-1} **total** protein	DOPAMINE Deamination nmol hr^{-1} mg^{-1} **MAO-B** protein
PD	66	5.80	3.00	517	0.76	131
n=7	66	2.85	8.26	2898	6.24	2189
	59	2.85	0.82	288	0.25	88
	62	7.10	5.88	828	5.88	828
	57	5.97	1.34	224	1.44	214
	47	11.71	3.26	278	3.12	266
	83	6.35	2.86	450	1.60	252
C	73	13.49	9.86	731	7.16	531
n=8	66	17.12	2.38	139	3.96	231
	57	14.33	4.50	314	3.80	265
	63	18.42	7.20	391	5.48	298
	70	21.00	6.44	322	4.52	215
	71	14.36	4.10	286	3.56	248
	70	4.48	2.00	446	4.48	402
	72	23.35	2.20	94	2.44	104

APPENDIX V

LIST OF SUPPLIERS

Amersham Pharmacia Biotech
23 Grosvenor Road
St Albans
Hertfordshire
AL1 3AW

Tel : 01727 814000
Fax :01727 814001
www.apbiotech.com

Bio-Rad Laboratories Limited
Bio-Rad House
Maylands Avenue
Hemel Hempstead
Hertfordshire
HP2 7TD

Tel : 01442 232552
Fax : 01442 259118

Dako Limited
Denmark House
Angel Drove
Ely
Cambridge
CB7 4ET

Tel : 01353 669911
Fax : 01353 668989
www.dakoltd.co.uk

Du Pont de Nemours (Deutschland) GmbH
NEN Division
D-6072 Dreiech
W. Germany
Postfach 401240

Fahrenheit
Northfield Road
Rotherham
South Yorkshire
S60 1RR

Genetic Research Instumentation Limited
Gene House
Dunmow Road
Felsted
Dunmow
Essex
CM6 3LD

Tel : 01371 821082
Fax : 01371 820131
www.gri.co.uk/gri

ICN Biomedicals Limited
Unit 18
Thame Park Business Centre
Wenman Road
Thame
Oxfordshire
OX9 3XA

Tel : 0800 282474
Fax : 0800 614735

Raymond A. Lamb
6 Sunbeam Road
London
NW10 6JL

Tel : 0181 965 1834

Merck Limited
Merck House
Poole
Dorset
BH15 1TD

Pall Gelman Sciences
Brackmills Business Park
Caswell Road
Northampton
NN4 7EZ

Tel : 01604 704704
Fax : 01604 704724
www.gelman.com

Serotec Limited
22 Bankside
Station Approach
Kidlington
Oxford

Tel : 01865 852700
Fax : 01865 373899
www.serotec.co.uk

Sigma-Aldrich Company Limited
Fancy Road
Poole
Dorset
BH12 4QH